

# Nitrosative Stress in Aging – Its Importance and Biological Implications in NF- $\kappa$ B Signaling

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**Abstract** The free radical theory of aging is supported by the findings that overexpression of CuZn superoxide dismutase (SOD) and catalase or the enhancement of reductive capacity by overexpression of glucose-6-phosphate dehydrogenase extends the life span of *Drosophila melanogaster*. Furthermore, treatment with small-molecule antioxidant SOD mimetics, which successfully increases the life span in multicellular models of aging such as *Drosophila melanogaster* and *Caenorhabditis elegans*, gave additional needed credibility to the free radical theory of aging. Also, nitration of proteins by reactive nitrogen species and 4-hydroxy-2-nonenal production by lipid peroxidation were found to increase with age, lending additional support to the oxidative stress hypothesis of aging. A number of research groups have reported alterations in transcriptional activity of nuclear factor kappa B (NF- $\kappa$ B) outside the immune system that was characterized by striking increases in constitutive NF- $\kappa$ B activity in different tissues of aging humans and animals. On the other hand, reactive nitrogen species are signaling molecules that modulate NF- $\kappa$ B activity. In this way, the concept of nitrosative stress has emerged from an understanding that interactions between nitrosants and oxidants may produce products that are more toxic than either reactants alone. Thus, the proinflammatory peroxynitrite molecule presents such a unifying link between reactive oxygen species and reactive nitrogen species by virtue of its generation from the reaction between superoxide, a reactive oxygen species, and nitric oxide, a reactive nitrogen species. Importantly, peroxynitrite effects on NF- $\kappa$ B signaling can be considered in light of the relation that exists between molecular inflammation and nitrosative stress and with the aging process itself. This will reflect on the pro-aging signaling of redox-sensitive NF- $\kappa$ B and on the development of age-related diseases.

**Keywords** Aging · Peroxynitrite · Nitrosative stress · Nuclear factor kappa B · Age-related diseases

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## 1 Introduction

### 1.1 The Oxidative Stress Hypothesis of Aging – Historical Account

In the mid-1950s, Denham Harman, M.D., Ph.D., scientist from the Donner Laboratory of Biophysics and Medical Physics, University of California, Berkeley, proposed in his insightful article “Aging: A Theory Based on Free Radical and Radiation Chemistry” that the damaging effects of reactive oxygen species (ROS) such as superoxide radical ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) may play a key role in the mechanism of aging [1]. He wrote “it seems possible that one factor in aging may be related to deleterious side attacks of free radicals (which are normally produced in the course of cellular metabolism) on cell constituents” [1]. He proposed that oxygen free radicals, specifically hydroxyl and hydroperoxyl radicals, are formed endogenously from normal oxygen-utilizing metabolic processes and play an essential role in the aging process. He concluded, “Aging and the degenerative diseases associated with it are attributed basically to the deleterious side attacks of free radicals on cell constituents and on the connective tissues. The free radicals probably arise largely through reactions involving molecular oxygen catalyzed in the cell by the oxidative enzymes and in the connective tissues by traces of metals such as iron, cobalt, and manganese” [1]. At first incomplete, this theory became more attractive in concert with the discoveries of free radicals by Commoner and co-workers [2, 3] and findings that erythrocuprein is an antioxidant superoxide-radical dismutating CuZn superoxide dismutase (CuZn-SOD) enzyme, which is a protector of oxygen-metabolizing organisms in concert with Mn-SOD [4]. Also, the in vivo presence of hydrogen peroxide was documented [5]. Furthermore, the fact that all aerobic organisms containing cytochrome systems have both SOD and catalase [6, 7] and that their overexpression or treatment with small-molecule antioxidant SOD mimetics successfully increases life span in standard multicellular models of aging such as *Drosophila melanogaster* [8, 9] and *Caenorhabditis elegans* [10] or murine life-span [11] strongly contributed to the validity of the free radical theory of aging. Additionally, the finding that enhancement of reductive capacity by overexpression of glucose-6-phosphate dehydrogenase (G6PD), a key cytosolic enzyme for NADPH biosynthesis that is susceptible to oxidative damage, could protect against oxidative stress and extend the life span of transgenic *D. melanogaster* [12] strongly supported the oxidative stress hypothesis of aging and gave the free radical theory of aging a needed credibility. In addition, Harman later proposed his modified mitochondrial theory of aging [13], which was based on the fact that the mitochondria generate large amounts of ROS in cells. Generally, the results from the more correlative genetic studies of distantly related species such as *C. elegans*, *D. melanogaster*, and mice and studies “from yeast to men” support the free radical hypothesis of aging. The evidence included claims that:

1. Variation in species life span is correlated with metabolic rate and protective antioxidant activity.
2. Enhanced expression of antioxidative enzymes in experimental animals can produce a significant increase in longevity.

3. Cellular levels of free radical damage increase with age.
4. Reduced calorie intake leads to a decline in the production of reactive oxygen species and an increase in life span [14–20]. Also, the free radical theory may also be used to explain many of the structural features that develop with aging including protein oxidation, the lipid peroxidation of membranes, formation of age pigments, and cross-linkage of proteins, DNA damage, and decline of mitochondrial function.

Generally, it is agreed that there is a correlation between aging and the accumulation of oxidatively damaged proteins, lipids, and nucleic acids. Protein carbonyls, 8-oxo-2'-deoxyguanosine, acrolein, or 4-hydroxy-2-nonenal (4-HNE), are well established biomarkers of protein, DNA, and lipid oxidation, respectively. Basic principles that govern the oxidation of proteins by ROS were established in several pioneering studies that characterized reaction products formed by performing studies in which proteins were exposed to ionizing radiation under conditions where only hydroxyl radical ( $\text{OH}^\bullet$ ), superoxide anion ( $\text{O}_2^-$ ), or a mixture of both was made available [21–24].

Oxidatively modified proteins have been shown to increase as a function of age, demonstrated by an age-related increase in the level of protein carbonyl content, oxidized methionine, protein hydrophobicity, and cross-linked and glycated proteins, as well as the accumulation of less active enzymes that are more susceptible to heat inactivation and proteolytic degradation [25]. For the most part, oxidatively modified proteins are not repaired and must be removed by proteolytic degradation, and a decrease in the efficiency of proteolysis will cause an increase in the cellular content of oxidatively modified proteins. The carbonyl content of proteins increases almost exponentially in widely different animal species and tissues as a function of animal age. Within the protein molecule, both the peptide bond or side chain may be targeted by free radicals in a site-specific fashion, and these reactions are often influenced by redox metal cycling cations, such as iron copper [26]. Protein carbonylation may occur due to direct oxidation of amino acid side chains (e.g., proline and arginine to  $\gamma$ -glutamyl semialdehyde, lysine to amino adipic semialdehyde, and threonine to aminoketobutyrate) [27]. It can also be induced through the interaction of proteins with oxidative by-products of other molecules, such as lipid peroxidation derivatives like 4-HNE and malondialdehyde (MDA) [26, 28]. Collectively, oxidation of amino acid residue side chains, formation of protein–protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation can be observed.

It should also be noted that specific proteins may be particularly susceptible to oxidative damage exemplified by key mitochondrial enzymes adenine nucleotide translocase and aconitase [29]. The oxidative modifications of key metabolic enzymes such as aconitase and its role in the citric acid cycle may have a profound effect on the cellular burden of oxidatively modified proteins. Of particular interest is oxidation of methionine residues by ROS to methionine sulfoxide (MetO). The oxidation of Met residues is readily reversed by the action of methionine sulfoxide reductase (Msr), which catalyzes the thioredoxin-dependent reduction of MetO residues of proteins back to Met. This cyclic interconversion of Met and

MetO residues of proteins is an important antioxidant mechanism for the scavenging of ROS and is likely involved in the regulation of enzyme activities and cell signaling and can target proteins for proteolytic degradation [30]. Furthermore, a loss in the ability to catalyze the reduction of protein MetO to Met residues leads to a decrease in the maximum life span, whereas overexpression of this activity leads to an increase in the life span of animals. In addition, a decrease in Msr activities in brain tissues is associated with the development of Alzheimer's disease [30].

The levels of oxidatively modified proteins increases with age, not only in whole cell but also in mitochondrial fractions, and this change correlates with a decline in the intracellular ATP level [31]. Also, age-related decline in the ATP level reduces the cell capacity to induce apoptosis and promotes necrotic inflammation. This switch may trigger a number of age-dependent disorders. A number of age-related diseases and pathologic disorders have been shown to be associated with elevated levels of oxidatively modified proteins including amyotrophic lateral sclerosis, respiratory distress syndrome, muscular dystrophy, cataractogenesis, rheumatoid arthritis, progeria, and Werner's syndrome [24]. Also, oxidative modification of proteins has been implicated in atherosclerosis, diabetes, Parkinson's disease, essential hypertension, cystic fibrosis, cancer, arteriosclerosis, rheumatoid arthritis, lupus erythematosus, chronic inflammatory diseases of the gastrointestinal tract, cataract, diabetes, diabetic retinopathy, Parkinson's disease, Alzheimer's disease, as well as aging itself [32, 33].

Oxidation of proteins can be induced by direct oxidation of proteins by ROS as well as by indirect modification of proteins by the secondary by-products of oxidative stress. The secondary modifications include oxidatively modified carbohydrates and lipids, which may react with the proteins by cross-linking. The most abundant lipid peroxidation products are MDA and HNE, which when cross-linking with proteins may have inactivation effects on these molecules [34, 35]. Proteins can also be damaged by glycation, also called Maillard reaction, or nonenzymatic glycosylation resulting in reducing sugars becoming chemically attached to proteins. The reaction occurs through the formation of Schiff base (i.e., an imine double bond between the aldehyde group of glucose and the epsilon amino group of lysine residues in proteins). The imine can quickly rearrange to form a ketoamine and is called an Amadori product. The Amadori products can be oxidized to form advanced glycation end products, and the formation of advanced glycation end products is irreversible [36]. Both reactive carbonyl compounds (RCCs) formed during lipid peroxidation and sugar glycoxidation, namely advanced lipid peroxidation end products (ALEs) and advanced glycation end products (AGEs), accumulate with aging and oxidative stress-related diseases, such as atherosclerosis, diabetes, or neurodegenerative diseases [37]. RCCs induce the "carbonyl stress" characterized by the formation of adducts and cross-links on proteins, which progressively leads to impaired protein function and damage in all tissues, and pathologic consequences including cell dysfunction, inflammatory response, and apoptosis. Because lipids are a major component of living organisms and probably the first easy target of free radicals once they are produced, lipid peroxidation

might play an important role in initiating and/or mediating some aspects of the aging process. It has been widely demonstrated that there is an age-associated increase in the steady-state concentrations of lipid peroxidation products [38]. In that way, proteomic techniques prove that many serum proteins are modified by HNE as well as by the reactive proinflammatory reactive nitrogen species (RNS) peroxynitrite ( $\text{ONOO}^-$ ). Importantly, nitration and HNE adduction were found to increase with age, lending additional support to the oxidative stress hypothesis of aging [39].

To summarize, the link between oxidative stress and aging has been indicated by the fact that aging is associated with accumulation of oxidized forms of protein [40], nucleic acids [41], and lipids [42], and also by the fact that there is an inverse relationship between the maximum life span of organisms and the age-related accumulation of oxidative damage [40, 43, 44]. Protein carbonyls, 8-oxo-2'-deoxyguanosine, acrolein, or 4-HNE, are well established biomarkers of protein, DNA, and lipid oxidation, respectively. The intracellular level of protein carbonyl has become one of the most widely accepted measurements of oxidative stress-dependent cellular damage [45].

## *1.2 The Concept of Nitrosative Stress*

As previously mentioned, oxidative stress can also be caused by RNS. The concept of nitrosative stress has emerged from an understanding that interactions between nitrosants and oxidants may produce products that are more toxic than either reactant alone. In other instances, nitrosative mechanisms of cellular injury may predominate [46, 47]. Under such conditions, nitrosylation may directly inhibit critical protein functions [47, 48] and/or promote deleterious oxidative modifications [46]. At the cellular level, nitric oxide (NO) has been widely implicated in nitrosative stress, which was linked to inhibition of cell growth and apoptosis [49].

Higher concentrations of oxidative species promote the conversion of NO to higher oxide forms, such as nitrogen dioxide and peroxynitrite. One consequence of the production of such species is the formation of nitrotyrosine [50]. Peroxynitrite is a highly reactive molecule that induces many changes in proteins by oxidizing the sulfhydryl groups of cysteine and methionine as well as tryptophan residues and selectively nitrating tyrosine residues [51, 52]. The detection of nitrotyrosine ( $\text{NO}_2\text{-Tyr}$ ) formation in various inflamed tissues and during the process of aging is recognized as a peroxynitrite-triggered mechanism of nitrosative injury [53–58].

Peroxynitrite can cause cell necrosis as well. This process is mediated by a complex process involving activation of the DNA repair enzyme poly(ADP-ribose) polymerase-1 (PARP-1). Activated PARP consumes NAD to build up poly(ADP-ribose) (PAR) polymers, which are themselves rapidly metabolized by the activity of poly(ADP-ribose) glycohydrolase (PARG). Some free PAR may exit the nucleus and travel to the mitochondria, where they amplify the mitochondrial efflux of

apoptosis-inducing factor (AIF) – nuclear to mitochondria cross-talk. Mild damage to DNA activates the DNA repair machinery, whereas excessive oxidative and nitrosative stress-induced DNA damage ends in apoptosis in case of moderate permeability transition pore (PTP) opening and PARP activation with preservation of cellular ATP or in necrosis in the case of widespread PTP opening and PARP over-activation, leading to massive NAD consumption and collapse of cellular ATP [59]. The peroxynitrite-PARP pathway is relevant in a wide variety of disparate diseases, ranging from myocardial ischemia/reperfusion injury, myocarditis, heart failure, circulatory shock, and diabetic complications to atherosclerosis, arthritis, colitis, and neurodegenerative disorders [60].

Consequently, besides presenting the historical background to the development of the free radical theory of aging and the effects of the oxidative stress caused by the ROS, we aim to present here the role of oxidative stress caused by RNS, particularly the role of proinflammatory peroxynitrite. By virtue of its origin, which reflects a change in redox state homeostasis and which results in its generation from the reaction between superoxide, ROS, and nitric oxide, the RNS peroxynitrite molecule presents a unifying link between ROS and RNS. We will also discuss its effects in light of the relation that exists between molecular inflammation and oxidative stress in aging and pro-aging signaling of redox-sensitive nuclear factor kappa B (NF- $\kappa$ B). We will also relate NF- $\kappa$ B signaling to different age-related diseases with emphasis on loss of muscle mass in old age. We will discuss the importance and clinical implications of NF- $\kappa$ B signaling in muscle loss having in mind that muscle mass comprises about 40% of total body weight in an average adult person. Conclusions and perspectives will also be presented.

## 2 Reactive Nitrogen Species and Nitrosative Stress

### 2.1 *First Come Definitions*

**Nitration** – Attachment of an  $\text{-NO}_2$  (nitro) group to a compound. Usually, it is not easily reversible.

**Nitrosation** – A process converting compounds into nitroso derivatives containing R-NO (nitroso) functionality. We can recognize C, N, O, and S nitrosations in biological systems.

**Nitrosylation** – The attachment of an NO (nitroso, nitrosyl) group to a thiol (S-nitrosylation) or a metal. It is usually reversible.

#### 2.1.1 “Nitrosation” and “Nitrosylation” – It Obviously Needs Some Clarification

From the chemical point of view, “nitrosation” means the addition of a nitroso group (i.e., the NO diatomic group). “Nitrosylation” means the addition of a nitrosyl group NO, stressing the concept of the addition of a chemical group that, if it were free,

would be a radical (in analogy to other chemical additions containing the “-yl-” particle: acetylation, phosphorylation, etc.).

The fact is that in the nitric oxide species, both atomic groups and the radicals are the same, as the radicals themselves are relatively stable. Even so, some authors prefer to distinguish the incorporation of the NO radical to a metal by a complex (or coordinating bond) as “nitrosylation” and the covalent incorporation of an NO diatomic group to another chemical group (regardless of the reaction mechanism) as “nitrosation” (in accordance with the nomenclature, which uses the term “nitroso” when defining the names of the resulting compounds). Also, the increasing recognition of the functional importance of this posttranslational modification and the widespread inclusion of the “-yl-” particle in terms describing other posttranslational modifications (glycosylation, phosphorylation) has led pioneer investigators in the field to make a case for the use of nitrosylation both for thiols and metals [61, 62]. In any case, incorporation to a thiol can be clearly distinguished because of the prefix “S-,” referring to the incorporation of the NO moiety to a sulfur atom to form the S–NO bond: “S-nitrosation” or “S-nitrosylation.”

## ***2.2 Reactive Nitrogen Species – Chemistry and Availability***

The family of nitric oxide synthase (NOS) isoenzymes [endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS)] [63, 64] catalyze the five-electron oxidation of one N $\omega$ -atom of the guanidino L-arginine to produce NO and L-citrulline through cofactors including NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH4) [65]. The NOS isoforms are denoted by descriptive terms, based on the requirement of intracellular calcium transients for full activity. Constitutive NOS enzymes, such as eNOS and nNOS, are activated by a transitory increase generally in cytosolic calcium, which promotes the release of NO over several minutes. A cytokine-inducible NOS isoform is expressed in many cells including macrophages and hepatocytes after the stimulation of immunologic or inflammatory reactions. This produces large amounts of NO for several days [66, 67].

The physiologic actions of NO at its nanomolar concentrations include regulation of vascular tone and blood pressure, prevention of platelet aggregation, and inhibition of vascular smooth muscle proliferation. They are a result of the activation by NO of the soluble guanylate cyclase and consequent generation of cyclic guanosine monophosphate (cGMP). In contrast, inflammatory cells such as macrophages produce local concentrations of nitrogen monoxide that are two or three orders of magnitude higher than its nanomolar concentrations [68]. The biological lifetime of nitrogen monoxide is close to 5 s, and during this time it can diffuse over several cell diameters and thereby carry out its function as an intracellular as well as extracellular messenger [69].

When produced in the presence of appropriate reactive targets, NO can be readily converted into other nitrogen oxide moieties. For example, one of the reactive



targets of NO is the cytochrome c oxidase, the terminal enzyme in the electron transport chain, which is inhibited by NO in a manner that is reversible and competitive with oxygen. The consequent reduction of cytochrome c oxidase activity leads to the release of superoxide anion, which under certain circumstances may react with NO to form another RNS species, the peroxynitrite (ONOO) [70]. In that way, the concept of “reactive exposure” was introduced by Joseph Beckman with his consideration of the formation of peroxynitrite from the reaction of NO with superoxide. According to the proposed principle [71], the relative amount of a compound (e.g., NO) that reacts with its respective targets is determined by the relative concentrations of those targets and the reaction rates of the compound with each of them. For example, the reaction of NO with superoxide is high ( $\sim 10^{10}$   $\mu\text{M/s}$ ), but the physiologic concentration is low ( $\sim 0.1\text{--}1$  nM) [71]. However, because the reaction rate for NO with superoxide is high, even a small increase in the concentration of this reactant will result in a large increase in reactive exposure; a 1 nM increase would lead to a 10-fold increase in reactive exposure [72]. Given that the rate constant for reaction of NO with superoxide is higher than that for reaction of superoxide with any of the three SOD isoforms, peroxynitrite will be formed in any cell or tissue where both radicals exist simultaneously [73].

## ***2.3 Nitrosylation and Nitration Are Mediators of Cell Signaling***

### **2.3.1 Nitrosylation**

In addition to the mediation of cytotoxicity, the RNS serve as important mediators and intracellular signaling molecules [74, 75]. NO can affect the cellular functions through posttranslational modifications of proteins directly (i.e., nitrosylation and nitration) and indirectly (i.e. methylation and ribosylation). The list of cGMP-independent effects of NO is growing at a rapid rate in relation to the importance and relevance of nitrotyrosine formation [76, 77]. For example, the inhibition of mitochondrial complex I-mediated respiration was demonstrated in cells after incubation with activated macrophages [78]. This inhibition of complex I activity was found to be due to NO [79]. S-Nitrosation of the reactive thiols on the surface of proton-translocating NADH:ubiquinone oxidoreductase (mitochondrial complex I), which is responsible for oxidation of matrix NADH and presents the major entry point for electrons to the respiratory chain, has been proposed to play a significant role in disorders such as Parkinson's disease and sepsis [80]. Also, S-nitrosation of insulin receptor has been proposed as a mechanism of insulin resistance in diabetes [81]. Within mammalian tissues, the concentration of S-nitrosothiols can vary from nanomolar to micromolar levels [82, 83], and thiol S-nitrosylation and NO transfer reactions (transnitrosation reactions) are involved in virtually all classes of cell signaling, including apoptotic cell death pathway. The work of Moran Benhar and Jonathan S. Stamler [84] revealed a key signal transduction pathway through which nitric oxide regulates apoptosis induced by disparate cellular stresses: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is S-nitrosylated



by NO, which initiates an interaction with the E3 ligase Siah1, leading to nuclear translocation and ubiquitin-mediated degradation of nuclear target proteins [84]. In another example, S-nitrosylation of two key apoptosis-regulatory proteins of the intrinsic and extrinsic death pathways, namely B-cell lymphoma-2 (Bcl-2) and FLICE-inhibitory protein (FLIP), has also been described [85]. These proteins have been shown to be upregulated in a variety of tumors and have been implicated in cancer chemoresistance through dysregulation of apoptosis. S-Nitrosylation of these proteins precludes their ubiquitination and subsequent degradation by the proteasome, thus accentuating their antiapoptotic effect, which is critical in the context of tumorigenic potential and cancer progression. Such posttranslational modifications of proteins by NO may be a general mechanism that tumor cells exploit to tilt the scales toward survival and proliferation by evading cell death.

### 2.3.2 Nitration

A balanced analysis of existing evidence indicates that (a) different nitration pathways can contribute to tyrosine nitration in vivo and (b) most, if not all, nitration pathways involve free radical biochemistry with carbonate radicals ( $\text{CO}_3^{\bullet-}$ ) and/or oxo-metal complexes oxidizing tyrosine to tyrosyl radical followed by the diffusion-controlled reaction with  $\bullet\text{NO}_2$  to yield 3-nitrotyrosine [86]. Peroxynitrite-mediated tyrosine nitration plays a key role in inflammation and pain. Nitration can be focused on specific tyrosine residues in proteins and potentially results in modification, loss, or gain of function.

Protein tyrosine nitration has three major effects: It may affect protein function, modulate phosphorylation cascades, and induce an immunologic response. The nitration of tyrosine residues was considered already 10 years ago as being of particular importance as nitration precludes the ability of tyrosine residues to undergo cyclic introversions between phosphorylated and unphosphorylated forms [87] or between nucleotidylated and unmodified forms [88]. To be considered a cellular signaling mechanism, protein nitration must meet four basic criteria: (1) controlled rates of formation, (2) specificity, (3) modification of target protein and cell function, and (4) reversibility. The specificity of protein nitration and modification of protein and cell functions by protein nitration have been demonstrated; it has also been suggested that protein nitration by 3-nitrotyrosine (3-NT) can be a reversible process [89, 90], and 3-NT may disappear without the need for a proteolytic pathway similar to what is observed for phosphorylation–dephosphorylation or methionine oxidation enzymatic repair. Koeck et al. [91] have shown that rat liver mitochondria are capable of completely eliminating 3-nitrotyrosyl adducts during 20 min of hypoxia–anoxia and of a selective partial reduction after only 5 min. They identified the modified proteins, having verified that all main nitrated proteins before hypoxia–anoxia and after reoxygenation are identical, thus markedly increasing the possibility that nitrotyrosine clearance is indeed a protein denitration process involved in a nitrative-signaling mechanism. Recently, it has been shown that a macrophage lipopolysaccharide (LPS)-inducible denitrase activity is capable

of specifically acting on nitrated calmodulin, a calcium signaling protein, forming native tyrosine calmodulin without the formation of any aminotyrosine [92].

Usually, nitrated proteins are recognized and degraded by the proteasome system. However, tyrosine nitration and dimerization may promote assembly of protein filaments or protein aggregates that become poor proteasome substrates and can accumulate as intracellular or extracellular amyloids. In this way, metabolism of nitrated proteins includes the potential reduction by yet-to-be-established biological reductants or the removal of the nitro group by putative denitrase activities. Although the concept of denitrase activity is attractive, the gene(s) or the specific protein(s) have not yet been identified, and more work is needed to more solidly prove its existence [93].

### 3 NF- $\kappa$ B, Aging, and Nitrosative Stress

#### 3.1 NF- $\kappa$ B Signaling

The activities of a variety of nuclear regulatory proteins are affected by proinflammatory signals, RNS, S-nitrosylation, and tyrosine nitration chemistry. One of these RNS-sensitive transcription factors is NF- $\kappa$ B [94–96]. Although the transcription factor NF- $\kappa$ B was originally recognized in regulating gene expression in B-cell lymphocytes [97], subsequent investigations have demonstrated that it is one member of a ubiquitously expressed family of Rel-related transcription factors that serve as critical regulators of many genes, including those of proinflammatory cytokines.

In the classic (canonical) pathway of activation, which is the main NF- $\kappa$ B signaling pathway, stimulating cells with an agonist such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) or interleukin-1 $\beta$  (IL-1 $\beta$ ) activates the I kappa B Kinase (IKK) complex, which is composed of two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and a regulatory subunit, NEMO (IKK $\gamma$ ). The IKK $\beta$ , but not IKK $\alpha$ , phosphorylates I $\kappa$ B proteins at two amino-terminal serine residues (Ser-32 and Ser-36) *in vivo*. This signal-induced phosphorylation targets I $\kappa$ B for ubiquitination and subsequent degradation by the proteasome [98].

After the release from I $\kappa$ B, NF- $\kappa$ B is transported to the nucleus where it binds specifically to  $\kappa$ B enhancer elements of DNA and promotes gene transcription by binding to responsive elements in the DNA, called  $\kappa$ B motifs [76]. The binding of specific  $\kappa$ B moieties sets off target gene transcription that encode cytokines such as interleukin-1 (IL-1), IL-2, IL-6, IL-8, IL-12, and interferon- $\beta$  and - $\gamma$  (IFN- $\beta$ , IFN- $\gamma$ ), TNF $\alpha$ , growth factors, membrane proteins such as major histocompatibility complex classes I and II, intracellular adhesion molecule 1 (ICAM-1), E-selectin, inhibitors of apoptosis (e.g., c-FLIP and Bcl-XL), and iNOS [16, 17]. The iNOS enzyme isoform is usually associated with malignant tissue transformation [99].

A hallmark of the noncanonical (nonclassic) NF- $\kappa$ B pathway is inducible p100 processing, leading to liberation of the mature transcription factor p52 in complex with RelB [100]. Also, one of the manifest characteristics of the noncanonical pathway is the slow kinetics of p100 processing, lasting several hours, which stands

in apparent contrast with that of the canonical pathway in which the process of I $\kappa$ B $\alpha$  degradation occurs within minutes. In addition, the IKK-independent pathway of NF- $\kappa$ B activation has also been recognized; it is based on casein kinase-2 (CK2) activation in response to DNA damage [101, 102]. The operating mechanism includes CK2-induced I $\kappa$ B $\alpha$  degradation through I $\kappa$ B $\alpha$  phosphorylation at a cluster of serine residues contained in its C-terminus [103]. Finally, it is worthwhile to mention that the localization of NF- $\kappa$ B within mitochondria has been reported [104–106].

### 3.2 NF- $\kappa$ B Signaling Increases in Aging

The NF- $\kappa$ B signaling pathway is a key molecular link between inflammation and tumor initiation and progression and provides a mechanistic link between inflammation and cancer [107, 108]. In addition, NF- $\kappa$ B is implicated in promoting proliferation [109–111], survival [112–116], and angiogenesis [117–119] of a variety of tumors. Importantly, NF- $\kappa$ B is considered the master regulator of innate immunity, which is activated during aging [120]. Accordingly, the NF- $\kappa$ B system induces a proinflammatory condition called “inflamm-aging” [121–123]. Recent studies have revealed that SIRT1 (Sir2 homolog) and FoxO (DAF-16), the key regulators of aging in budding yeast and *C. elegans* models, regulate the efficiency of NF- $\kappa$ B signaling and the level of inflammatory responses [124]. FoxOs are transcription factors, and Sirtuins are the protein deacetylases that regulate the activity of FoxOs and NF- $\kappa$ B [125].

Aging-associated signaling may represent life-span extending, longevity regulation or age-related degenerative, pro-aging signaling. Accordingly, it is proposed that the signaling cascades mediated via Sirtuins and FoxO represent the life-span extending, anti-aging type of regulation. Conversely, NF- $\kappa$ B signaling enhances tissue atrophy and inflammation and supports inflamm-aging [124]. In addition, imposed calorie restriction (CR) is shown to result in the most reproducible end point of life-span extension in all animal models tested [126]. Calorie restriction results in the suppression of NF- $\kappa$ B signaling [127], thus providing evidence that NF- $\kappa$ B signaling may be involved in regulation of aging and longevity [128].

Kumar et al. [129] have listed the genes whose expression is regulated by NF- $\kappa$ B, as well as the major diseases associated with functional changes in the NF- $\kappa$ B system. Most of the diseases are chronic and age-associated degenerative diseases such as atherosclerosis, dementia (both vascular and Alzheimer's types), and cancer. Several serum proinflammatory markers have been shown in relation to dementia and cognitive decline, and inflammatory responses are hypothesized to modulate the pathogenic processes associated with Alzheimer's disease (AD) [130, 131]. In this way, pathology evidence indicates that major, chronic age-related diseases are inflammation-related, and a proposal for the molecular inflammation hypothesis of aging is based on the observation that the redox derangements that occur during aging are the major factor for increased risk for age-related inflammation [132]. Accordingly, continuous (chronic) upregulation of proinflammatory

mediators is induced during the aging process due to an age-related redox imbalance that activates many proinflammatory signaling pathways, including the NF- $\kappa$ B signaling pathway [133, 134]. However, this position of inflamm-aging should not be viewed as being contradictory to the free radical oxidative theories of aging because redox-sensitive NF- $\kappa$ B, the main proinflammatory signaling pathway, is activated by different ROS and RNS, including proinflammatory peroxynitrite, which disturb redox balance. Thus, inflamm-aging and free radical theories of aging should rather be viewed as complementary.

The age-related “constitutive activation of NF- $\kappa$ B” has been verified in various tissues during aging [135–139]. In mice and rats, NF- $\kappa$ B activity has consistently been shown to be increased with age and in a variety of tissues, including heart, liver, kidney, and brain [140]. In humans, NF- $\kappa$ B protein concentrations were found to be fourfold higher in elderly human muscles compared with those of young people [141].

In addition, it was observed in studies performed in rodents that the levels of NF- $\kappa$ B components p52 and p65 were prominently increased in nuclear but not in cytoplasmic fractions in the tissues of old rodents [142]. Such observation may imply that during aging, the retention of NF- $\kappa$ B proteins into the nuclei is increased. It also implies that the efficiency of the NF- $\kappa$ B system is not solely based on the protein levels of the NF- $\kappa$ B components in cytoplasm but rather on the translocation of protein components to nuclei and the efficiency of transcriptional regulation [142]; as well, efficacy of the process of regulatory nuclear polyubiquitination of NF- $\kappa$ B subunits may be changed with aging (see later).

### ***3.3 Nitration of Proteins Increases with Aging – Tyrosine Nitration***

Increased nitration of proteins in aging is a well-documented phenomenon, such as observation of increased nitration of serum proteins with age [143] or peroxynitrite-induced senescence and apoptosis of human red blood cells [144]. Importantly, increased nitration is often linked to development of age-related diseases. It is argued that peroxynitrite excess will lead to the disruption of modulation of mitochondrial respiration providing a platform for development of prevalent neurodegenerative and metabolic diseases [145]. For example, it has been revealed that the Lewy bodies, a pathologic hallmark of Parkinson’s disease, contain nitrated alpha-synuclein, which is prone to oligomerization. Studies have shown that oxidation and nitration of alpha-synuclein lead to the formation of stable dimers and oligomers through dityrosine cross-linking [146]. In addition, greater nitration of alpha-synuclein was shown to occur in the substantia nigra of 16-month-old rats versus 3-month-old rats, which is accompanied by a higher expression level of inducible nitric oxide synthase [147]. Also, elevated levels of 3-nitrotyrosine were found in brain from subjects with amnesic mild cognitive impairment (MCI), suggesting that nitrosative damage occurs early in the course of cognitive impairment

and that protein nitration may be important for conversion of MCI to AD [148]. Moreover, age-related changes in dopamine transporters and accumulation of 3-nitrotyrosine in rhesus monkey midbrain dopamine neurons were found; these findings are consistent with a role for age-related accumulation of nitrative damage and vulnerability of dopaminergic neurons in Parkinson's disease [149].

Also, there is the appearance of the 3-nitrotyrosine-modified isoform of the endoplasmic/sarcoplasmic reticulum  $\text{Ca}(2^+)\text{-Mg}(2^+)\text{-adenosine triphosphatase}$  (SERCA2) of the sarcoplasmic/endoplasmic reticulum  $\text{Ca}(2^+)\text{-ATPase}$  in aging and disease in both striated and smooth muscle of humans and rodent models [150]. Structure–function studies of nitrated SERCA2 in aging heart and skeletal muscle demonstrated stoichiometric nitration of vicinal tyrosines, Tyr-294 and Tyr-295, on the luminal side of the membrane-spanning helix, M4, which correlates with partial inhibition of  $\text{Ca}(2^+)\text{-ATPase}$  activity [150]. Nitration of protein tyrosine residues of Mn-SOD and SERCA is implicated in cardiovascular disease and aging; SOD and SERCA immunostaining for 3-NT were found positive in aging rat skeletal muscle as well as in atherosclerotic aorta and cardiac atrium from human diabetic patients [151]. Moreover, nitration of a critical tyrosine residue in the allosteric inhibitor site of muscle glycogen phosphorylase has been found to impair its catalytic activity, suggesting that glycogen phosphorylase functions may be regulated by tyrosine nitration [152]. Notably, significant levels of nitrotyrosine-modified proteins were present at an earlier age in the semimembranosus muscle in comparison with those in the soleus muscle [153].

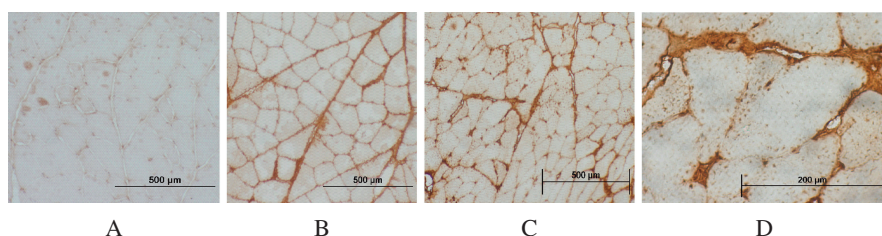
### ***3.4 NF- $\kappa$ B Signaling and Skeletal Muscle Atrophy***

Aging is characterized by a gradual loss of muscle mass and strength (sarcopenia of old age) and presents a major health problem in elderly people. Age-related loss of muscle mass occurs through a reduction in the rate of protein synthesis, an increase in protein degradation, or a combination of both. However, the underlying mechanisms are still poorly understood, although muscle atrophy in many conditions shares a common mechanism in the upregulation of the muscle-specific ubiquitin E3-ligases atrophy gene-1/muscle atrophy F-box (Atrogin-1/MAFbx) and muscle ring-finger protein 1 (MuRF1) [154].

Sarcopenia is specifically restricted to age-related muscle wasting [155, 156]. Also, disuse of skeletal muscles (e.g., due to inactivity or denervation) also induces muscle atrophy [157]. Different approaches have provided evidence that the activation of NF- $\kappa$ B signaling may be the major signaling mechanism involved in inducing tissue atrophy [157–160]. It should be noted that most of the animal studies on skeletal muscle atrophy involved use of young and mature animals. Documented studies that investigate the exact mechanism by which NF- $\kappa$ B acts in the disuse atrophy of aging sarcopenic muscle are still lacking. Accordingly, the effects of nitrosative stress and the role for peroxynitrite in NF- $\kappa$ B signaling in skeletal muscle atrophy in old age have not yet been fully elucidated.

### 3.5 Loss of Skeletal Muscle in Old Age Is Associated with Increased Nitration of Muscle Proteins and NF- $\kappa$ B Activation

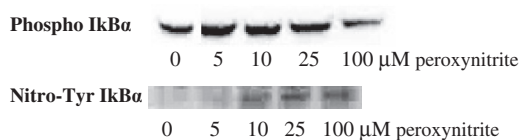
Aging is characterized by a gradual loss of skeletal muscle mass and strength (sarcopenia of old age) [161] and increased nitration of muscle proteins [162, 163]. Tyrosine nitration is particularly evident inside the fibers of aged muscle in disuse conditions (Fig. 1).



**Fig. 1** Immunohistochemistry (using anti-nitrotyrosine antibody) showing level of tyrosine nitration. Cross section of gastrocnemius muscles of (a) 6-month-old unimmobilized rat, (b) 24-month-old rat, and (c, d) 24-month-old rat immobilized for 4 weeks. (c, d) In the immobilized old muscle, positive granulation of staining was observed inside the muscle fibers. Negative control studies, where primary anti-nitrotyrosine antibody was omitted, were also performed (figure not shown). Magnification: (a, b, c) 10 $\times$ ; (d) 40 $\times$

Peroxynitrite (ONOO<sup>-</sup>) mediates nitration *in vivo* through free radical biochemistry, which results in formation of 3-nitrotyrosine [59, 86, 164, 165]. Tyrosine nitration represents an important posttranslational modification *in vivo* and could serve as a signaling pathway by itself [166] or by effecting phosphorylation [167, 168]. As seen in Fig. 2, with increasing concentration of peroxynitrite above 10  $\mu$ M, there is an increase in nitro-Tyr I $\kappa$ B $\alpha$  with concomitant decrease of P-I $\kappa$ B $\alpha$  (Fig. 2). Accordingly, peroxynitrite molecule is considered a key mediator of the interplay between tyrosine nitration and phosphorylation [169, 170].

Loss of skeletal muscle is associated with activation of NF- $\kappa$ B signaling pathway [157–160, 171, 172] and all the NF- $\kappa$ B family members, c-Rel, p65 (Rel A), Rel B, p50, and p52, are expressed at different levels in mammal skeletal muscle [157]. Kandarian and co-workers found that association of p50 with a B-cell



**Fig. 2** Western immunoblot of I $\kappa$ B $\alpha$  in HT-29 cells after 20-min exposure to increasing concentrations of peroxynitrite. At 25  $\mu$ M peroxynitrite, both P-I $\kappa$ B $\alpha$  and nitro-Tyr I $\kappa$ B $\alpha$  were presented. Consequently, it may be assumed that the pathways of NF- $\kappa$ B activation and posttranslational modifications may be triggered by peroxynitrite through nitration and/or phosphorylation



lymphoma-3 (Bcl-3) family member, which functions as a transcriptional coactivator, presented an alternative pathway of NF- $\kappa$ B activation during skeletal muscle atrophy in young healthy rodents [157, 159]. They also established that I $\kappa$ B $\alpha$  mediates the atrophy process because super-repressor I $\kappa$ B $\alpha$  $\Delta$ N expression inhibited genes known to be upregulated with atrophy during muscle unloading [173]. By performing muscle atrophy studies on old animals, work from our laboratory showed that the classic p65/p50 pathway, which was actually downregulated in the first 2 weeks of atrophy due to hind-limb immobilization, becomes activated in weeks 3 and 4 [161]. In fact, macrophages penetration and acid phosphatase activity were noticeable in weeks 2 and 3 after onset of hind-limb immobilization, whereas in young animals acid phosphatase activity and ubiquitin degradation systems were activated mostly in weeks 3 and 4 [161]. Thus, it was suggested that NF- $\kappa$ B may be activated by different mechanisms in young and old muscles during the atrophy process [161, 174, 175].

### ***3.6 Peroxynitrite-Induced Tyrosine Nitration of I $\kappa$ B $\alpha$ Causes NF- $\kappa$ B Activation***

In the past decade, it was shown that RNS are inducers of NF- $\kappa$ B activation and expression of genes involved in inflammation [176–178]. In our recent *in vitro* studies, we have demonstrated the role of peroxynitrite in the activation of NF- $\kappa$ B–dependent protein degradation systems in skeletal muscle cells [179]. Also, we were first to show that peroxynitrite caused tyrosine nitration of I $\kappa$ B $\alpha$  and its dissociation from NF- $\kappa$ B, which accounted for the prolonged NF- $\kappa$ B activation and high levels of iNOS expression in cultured skeletal muscle cells in the absence of typical I $\kappa$ B $\alpha$  serine phosphorylation and proteosomal degradation [96]. In short, the NO donors 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPANONOate) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) as well as the authentic peroxynitrite and its donor 3-morpholiniosydnonimine (SIN-1) were able to activate NF- $\kappa$ B in cultured muscle cells as measured by p65 nuclear translocations and luciferase expression. However, NO donor-induced NF- $\kappa$ B activation was transient, dependent on I $\kappa$ B $\alpha$  degradation, and was decreased in the presence of I $\kappa$ B $\alpha$  super-repressor. Conversely, peroxynitrite donors induced NF- $\kappa$ B activation that was dependent on tyrosine nitration of I $\kappa$ B $\alpha$  but independent of its serine phosphorylation and degradation. This activation did not decrease in the presence of I $\kappa$ B $\alpha$  super-repressor. Moreover, prolonged exposure to peroxynitrite resulted in nontransient NF- $\kappa$ B activation and high iNOS expression. When applied, proteasome inhibitor N-[(Phenylmethoxy)carbonyl]-L-leucyl-N-[(1*S*)-1-formyl-3-methylbutyl]-L-leucinamide (MG-132) did not diminish SIN-1–induced NF- $\kappa$ B activation, whereas tyrosine nitration inhibitor epigallocatechin gallate (EGCG) reestablished transient NF- $\kappa$ B activation through I $\kappa$ B $\alpha$  degradation after SIN-1 treatment. EGCG but not MG-132 decreased SIN-1–dependent iNOS expression. Accordingly, we have shown that peroxynitrite activates NF- $\kappa$ B in skeletal myocytes through an alternative mechanism, in which I $\kappa$ B $\alpha$  is nitrated on tyrosine and



dissociated from NF- $\kappa$ B, thus enabling its nontransient activation. This resulted in prolonged iNOS expression. Hence, we propose that peroxynitrite may exacerbate inflammatory responses mediated by NF- $\kappa$ B. Recently, Yakovlev and co-workers have demonstrated that peroxynitrite caused nitration of tyrosine 181 of I $\kappa$ B $\alpha$  and its dissociation from NF- $\kappa$ B [180]. In another study, peroxynitrite abrogated the classic pathway of NF- $\kappa$ B activation triggered by TNF $\alpha$  or lipopolysaccharide while concurrently inducing the processing of IKK $\alpha$  by NF- $\kappa$ B inducing kinase (NIK), the nonclassic NF- $\kappa$ B pathway [58]. Shuttling of these upstream kinases between the cytosol and nucleus is important because activated IKK $\alpha$  regulates many chromatin events in the nuclear phase of the NF- $\kappa$ B program such as phosphorylation of histone H3 and removal of corepressors from NF- $\kappa$ B-dependent promoters [181, 182]. Thus, the possibility that peroxynitrite may also activate IKK $\alpha$  signaling in muscle atrophy in old age cannot be excluded.

According to the classic model of NF- $\kappa$ B regulation, activation of NF- $\kappa$ B requires degradation of the inhibitory I $\kappa$ Bs, and their re-synthesis is considered a master terminator of the NF- $\kappa$ B response. However, NF- $\kappa$ B activity was found terminated in I $\kappa$ B $\alpha$ <sup>-/-</sup> 3T3 cells by proteosomal degradation of p65 in the nucleus, suggesting that I $\kappa$ Bs re-synthesis is not a sole terminator of the NF- $\kappa$ B response [183]. Actually, recent reports have found E3 ubiquitin ligase complexes COMMD1 and PDLIM2 to be responsible for the polyubiquitination targeting of NF- $\kappa$ B p65 and its subsequent degradation by the 26S proteasome in the nucleus [184, 185]. Currently, the effects of nitrosative stress on the process of NF- $\kappa$ B p65 nuclear ubiquitination and degradation in aging skeletal muscle under atrophy conditions are not known, although all three forms of NOS are present in skeletal muscles of mammals [186], and they may be transcriptionally regulated by the aging process [187]. Also, one has to take into account that during aging, the retention of NF- $\kappa$ B proteins in the nuclei is increased [142]. Thus, nitration by peroxynitrite may be a causative agent in modulation of the NF- $\kappa$ B network in skeletal muscle atrophy in old age by changing the feedback axis between I $\kappa$ Bs re-synthesis and the level of proteosomal degradation of p65 in the nucleus.

### ***3.7 Nitration by Peroxynitrite and NF- $\kappa$ B Activation Is Supported by Proinflammatory Conditions – Link to Inflamm-aging***

One of the features of NF- $\kappa$ B signaling in pathologic conditions, including cancer, is NF- $\kappa$ B nontransient (continuous) activation. In addition to cytokines such as TNF $\alpha$ , the generation and the release of the ROS and RNS such as superoxide and nitric oxide, as well as the generation of peroxynitrite in reaction between these two radicals, also contribute to the activation of the inflammation-related pathway of IKK $\beta$ -mediated NF- $\kappa$ B activation. However, the possibility that nitrosative stress may cause prolonged NF- $\kappa$ B signaling to account for disease development has not yet been fully investigated, although simultaneous production of superoxide radical and NO can be increased by 1,000-fold under proinflammatory conditions, resulting in the increased formation of peroxynitrite by as much as 1,000,000-fold [59].

Although it has a short half-life at physiologic pH ( $\sim 10$  ms), peroxynitrite has an excellent ability to cross cell membranes [188, 189], which implies that it could influence target cells within one to two cell diameters ( $\sim 5\text{--}20\text{ }\mu\text{m}$ ) [190].

The importance of tyrosine nitration as a posttranslational modification in cell signaling came to light after the pioneering discovery of Kong et al. that peroxynitrite-mediated nitration of a single tyrosine residue in purified cdc2, a cell cycle kinase, prevented its phosphorylation on tyrosine [191]. Gow and co-workers have demonstrated that exposing bovine pulmonary artery endothelial cells to authentic peroxynitrite would result in a decrease in the levels of tyrosine-phosphorylated proteins with concomitant increase in nitrotyrosine-containing protein levels, a finding implying that tyrosine nitration interferes with the process of phosphorylation [89]. On the other hand, it has been shown also that nitration of tyrosine residues may simulate phosphorylation and, as a consequence, may result in the activation of T lymphocytes [192], pancreatic carcinoma cell tyrosine nitration of c-Src kinase resulting in increased tyrosine phosphorylation [167, 193], and tyrosine phosphorylation of the major intrinsic membrane protein, band 3, in erythrocytes [194].

Importantly, tyrosine phosphorylation and nitration are not mutually exclusive events of the cell signaling pathways. For example, Mourad and co-workers [195] have reported the existence of dynamic interplay between nitration and phosphorylation of tubulin cofactor B (TCoB) in the control of microtubule dynamics; p21-activated kinase 1 (Pak1) phosphorylates TCoB on Ser-65 and Ser-128 and plays an essential role in microtubule regrowth. However, TCoB is efficiently nitrated, mainly on Tyr-64 and Tyr-98, and nitrated TCoB attenuates the synthesis of new microtubules. Additionally, optimal nitration of TCoB requires the presence of functional Pak1 phosphorylation sites, thus providing a feedback mechanism to regulate phosphorylation-dependent microtubule regrowth [195]. Thus, the influence of tyrosine nitration on phosphorylation cascades may be more subtle and modulatory as it was initially postulated.

It should be noted that peroxynitrite not only nitrates but also potently oxidizes proteins such as oxidizing and inactivating regulating phosphotyrosine phosphatase (PTPase), which in turn would lead to enhanced tyrosine phosphorylation [196]. Recently, Levrand et al. [58] have shown that authentic peroxynitrite has inhibited NF- $\kappa$ B activation triggered by inflammatory stimuli (TNF $\alpha$  or lipopolysaccharide) in cardiac and endothelial cell lines. The inhibition of NF- $\kappa$ B–DNA binding was completely prevented with the SOD mimetics and antioxidant agent Mn(III)-tetrakis-(4-benzoic acid) porphyrin (MnTBAP), which leads to the conclusion that peroxynitrite-induced NF- $\kappa$ B inhibition may be dependent on peroxynitrite oxidative chemistry [58]. On the other hand, Levrand and co-workers have demonstrated that peroxynitrite, while inhibiting a classic NF- $\kappa$ B pathway, strongly activates phosphorylation of NIK and IKK $\alpha$ , the components of the alternative, noncanonical NF- $\kappa$ B activation pathway, even in the absence of stimulatory signal by LPS or TNF $\alpha$ , suggesting that peroxynitrite molecule alone is involved in the nonclassic pathway of NF- $\kappa$ B stimulation [58]. As mentioned before, we have also proved that total IKK activity has increased after exposure to peroxynitrite *in vitro*, although the

separate measurements of IKK $\alpha$  were not performed [96]. From our experiments, we have concluded that when tyrosine nitration is not blocked, the noncanonical activation of NF- $\kappa$ B would take place. On the other hand, when nitration is blocked with an agent such as EGCG, the canonical NF- $\kappa$ B pathway ensues. However, blocking nitration would not exclude the engagement of the oxidatively induced alternative pathway of NF- $\kappa$ B activation by peroxynitrite via NIK and IKK $\alpha$  phosphorylations. Thus, these two peroxynitrite chemistries, oxidative and nitrosative, should not be considered mutually exclusive events. In fact, it may be assumed that the alternative pathways of NF- $\kappa$ B activation and posttranslational modifications may be triggered by peroxynitrite through nitration and/or oxidation, which may be a matter of redox balance. It will be challenging to assess the relative contributions of either type of reaction of peroxynitrite, tyrosine nitration, or oxidation to the observed signaling effects.

### ***3.8 Thus, Is NF- $\kappa$ B a Signaling Mediator of Aging?***

Genetic studies in model organisms such as yeast, worms, flies, and mice leading to life-span extensions suggest that longevity is subject to regulation. In addition, various system-wide interventions in old animals can reverse features of aging. To better understand these processes, much effort has been put into the study of aging on a molecular level. In particular, genome-wide microarray analysis of differently aged individual organisms or tissues has been used to track the global expression changes that occur during normal aging. To circumvent this problem, Adler et al. [197, 198] have recently developed a novel computational approach to discover transcription factors that may be responsible for driving global expression changes with age. They develop a systematic approach to identify combinatorial *cis*-regulatory motifs that drive age-dependent gene expression across different tissues and organisms. Integrated analysis of 365 microarrays spanning nine tissue types predicted 14 motifs as major regulators of age-dependent gene expression in human and mouse. The motif most strongly associated with aging was that of the transcription factor NF- $\kappa$ B! Accordingly, the transcription factor NF- $\kappa$ B may be a candidate activator of age-related transcriptional changes in multiple human and mouse tissues. Inducible genetic blockade of NF- $\kappa$ B for 2 weeks in the epidermis of chronologically aged mice by adenovirus-mediated expression of dominantly active I $\kappa$ B $\alpha$  (Ad-I $\kappa$ B $\alpha$ ) reverted the tissue characteristics and global gene expression programs to those of young mice [197]. Also, age-specific NF- $\kappa$ B blockade and orthogonal cell cycle interventions revealed that NF- $\kappa$ B controls cell cycle exit and gene expression signature of aging in parallel but not sequential pathways. These results identify a conserved network of regulatory pathways underlying mammalian aging and show that NF- $\kappa$ B is continually required to enforce many features of aging in a tissue-specific manner. In that way, genetic blockade of NF- $\kappa$ B in the skin of chronologically aged mice ( $\Delta$ SP-p50-ER transgenic mice) reversed the global gene expression program and tissue characteristics to those of young mice, demonstrating for the first time that disruption of a single gene is sufficient to reverse features of

aging, at least for the short-term [197]. Age-associated genes whose expression was inhibited by NF- $\kappa$ B blockade included those related to chromatin/transcriptional regulation (*RAD50*, *SMC2L1*, *SMC6L1*, and *ATRX*), protein modification/signal transduction (*STK25*, *RAMP2*, and *HIP2*), cell cycle/growth control (*DNAJC2* and *IGFBP5*), and mitochondria (*ALAS2*, *GSTK1*, and *PTEI*).

It should also be noted that experiments have shown that activation of NF- $\kappa$ B can induce muscle atrophy [157], insulin resistance [199], and neurotoxicity in Alzheimer's disease [200], three prevalent age-associated morbidities. Our work in vitro has shown that NF- $\kappa$ B activity increases in cultured muscle cells exposed to proinflammatory RNS, which are increasingly generated in aging, and that RNS peroxynitrite may induce alternative activation of the NF- $\kappa$ B classic pathway of activation [96]. In addition, in our in vivo work we have observed a biphasic pattern of NF- $\kappa$ B activation in atrophic muscles of old rats [161, 175], which was somewhat different from the pattern of NF- $\kappa$ B activation described in atrophic muscles of young rodents by Kandarian and co-workers [157, 159].

From the evolutionary point of view, the NF- $\kappa$ B system can be viewed as a pleiotropic signaling mediator and that its output is most probably age dependent. The disposable soma theory, which was proposed by Kirkwood and Holliday [201], predicts that aging occurs due to the accumulation of damage during life and that failure of defensive or repair mechanisms contribute to aging. It postulated a special class of gene mutations with antagonistic pleiotropic effects in which hypothetical mutations save energy for reproduction (positive effect) by partially disabling molecular proofreading and other accuracy promoting devices in somatic cells (negative effect). In other words, aging evolves due to the pleiotropic effect of genes that are beneficial early in life and then harmful at later ages. Consequently, inflammation and the activation of the NF- $\kappa$ B system protect tissues and organisms against pathogen attacks and traumatic tissue damage. This is especially important in young organisms to protect reproduction. However, later in life, the pleiotropic functions of the NF- $\kappa$ B system may carry out the disposable soma program through its ROS/RNS oxidative stress-induced unfavorable activation, which may be described as inflamm-aging. Therefore, NF- $\kappa$ B may be considered a signaling mediator of aging from the evolutionary point of view.

## 4 Summary and Conclusions

The accumulated data indicate that major, chronic age-related diseases are inflammation-related and that the activation of redox-sensitive transcription factors under age-related nitrosative stress are likely to be an important cause. The extent to which observed changes in NF- $\kappa$ B activity drive aging and influence life span and health in humans and other mammals is not yet clear. However, concerning deregulation of NF- $\kappa$ B activity found in human disease, NF- $\kappa$ B activation by peroxynitrite includes the induction of alternative NF- $\kappa$ B pathways and the state of its continuous activation, which is observed in age-related pathologies, including cancer. Thus, understanding mechanisms of alternative pathways of NF- $\kappa$ B activation

would raise the possibility to induce therapeutic manipulations to achieve a desirable level of activation of different NF- $\kappa$ B subunits that change in aging in distinct manners. Moreover, systemic approaches are already able to identify major regulators of age-dependent gene expression in humans. Accordingly, these results show that NF- $\kappa$ B is incessantly required to enforce many features of aging, which suggests that NF- $\kappa$ B may be a major signaling mediator of aging.

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