Aging and the Role of Reactive Nitrogen Species

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ABSTRACT: The role of reactive oxygen species and its effects on aging has received considerable attention in the past 47 years since Dr. Denham Harman first proposed the “free radical theory of aging.” Though not completely understood due to the incalculable number of pathways involved, the number of manuscripts that facilitate the understanding of the underlying effects of reactive radical species on the oxidative stress on lipids, proteins, and DNA and its contribution to the aging process increases nearly exponentially each year. More recently, the role of reactive nitrogen species, such as nitric oxide and its by-products—nitrate (NO$_3$), nitrite (NO$_2$), peroxynitrite (ONOO$^-$), and 3-nitrotyrosine—have been shown to have a direct role in cellular signaling, vasodilation, and immune response. Nitric oxide is produced within cells by the actions of a group of enzymes called nitric oxide synthases. Presently, there are three distinct isoforms of nitric oxide synthase: neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2), and endothelial (eNOS or NOS-3), and several subtypes. While nitric oxide (NO) is a relative unreactive radical, it is able to form other reactive intermediates, which could have an effect on protein function and on the function of the entire organism. These reactive intermediates can trigger nitrosative damage on biomolecules, which in turn may lead to age-related diseases due to structural alteration of proteins, inhibition of enzymatic activity, and interferences of the regulatory function. This paper will critically review the evidence of nitration and the important role it plays with aging. Furthermore, it will summarize the physiological role of nitration as well as the mechanisms leading to proteolytic degradation of nitrated proteins within biological tissues.

KEYWORDS: nitric oxide; apoptosis; oxidants; protein nitration; denitrase

INTRODUCTION

The role of nitric oxide (NO) has received considerable attention during the past 15 years. Because of its harmful effects on the environment from automobile exhaust, short half-life, and relatively unknown therapeutic benefits, it did not receive much attention from a biological perspective until the 1980s. However in 1987, NO
was discovered to be produced in biological tissue by nitric oxide synthase (NOS). NOS acts as a catalyst to convert \( L \)-arginine to nitric oxide and \( L \)-citrulline. In 1977, Murad et al. revealed that NO has the ability to dilate blood vessels and relax smooth muscle tissue. In 1992, *Science* magazine chose NO as “molecule of the year.” Six years later, three pharmacologists were awarded the Nobel Prize in Physiology and Medicine for their discoveries pertaining to NO as a signaling molecule in the cardiovascular system. These discoveries led to the importance NO plays in cellular signaling, vasodilation, and immune response.

NO is an uncharged lipophilic molecule that contains a single unpaired electron (NO \(^*\)), which causes it to be reactive with other molecules, such as oxygen, glutathione, and superoxide radicals. Therefore, nitric oxide could function as an electron donor (oxidant) or an electron acceptor (antioxidant). After NO is produced by NOS, it diffuses across cellular membranes and into adjacent cells. It binds to soluble guanylate cyclase covalently to convert guanosine 5'-triphosphate to cyclic guanosine 3',5'-monophosphate (cGMP). The subsequent increase in cGMP level alters the activity of several main target proteins: cGMP-dependent protein kinase, cGMP-regulated phosphodiesterase, and cGMP-gated ion channels.

**AGING AND THE NITRIC OXIDE SYNTHASE ISOENZYME**

Aging is a natural and inevitable part of the life process that is characterized by a gradual and general decline in physiological functions that ultimately lead to morbidity and mortality. The process of aging is not completely understood due to the seemingly endless number of biological mechanisms and pathways. However, considerable progress has been made to understand and explain the effects of oxidants (reactive oxygen and reactive nitrogen species) on the oxidative stress on lipids, proteins, and DNA and how this contributes to the aging process.

Some of the conditions associated with aging are neurodegenerative diseases such as Alzheimer’s disease, cardiovascular disease, cancer, stroke, and a decline in the immunoresponse to pathogens, which have been shown to be directly related to the amount or incidence of nitric oxide available for biomolecular modification. Therefore, alterations in nitric oxide production could have a profound effect on normal aging and disease conditions. Examples of both aging and disease conditions will be presented. Often it is difficult to distinguish between the two (i.e., arteriosclerosis), and few studies have examined NOS activity and their by-products with normal aging.

NO is produced within cells by the actions of a group of enzymes called nitric oxide synthases. Presently, there are three distinct isoforms of nitric oxide synthase: neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2), and endothelial (eNOS or NOS-3), with several additional subtypes (nNOS\(\mu\) and mtNOS) of the aforementioned isoforms. All three isoforms bind calmodulin, but iNOS carries a permanently bound molecule of calmodulin that allows this isoform to function at low cytoplasmic Ca\(^{2+}\) levels. Both nNOS and eNOS are expressed constitutively and are Ca\(^{2+}\) dependent, whereas iNOS is independent of the Ca\(^{2+}\) concentration.

While each isoform varies in its tissue specificity, different NOSs occur at multiple locations within the body. It appears that several isoforms can be found in the same tissue but may have different functions. For example, nNOS is found in a va-
riety of neurons in both the central and peripheral nervous system, but eNOS is expressed in some neurons. While eNOS can be stimulated by shear stress in the vascular endothelium, iNOS may occur in normal epithelium such as the lung. In a recent study by Grange et al., nNOS and eNOS knockout mice were studied to see which isoform contributes to a decrease in smooth muscle myosin regulatory light chain (smRLC) phosphorylation in contracting fast-twitch muscle. From their studies, they suggest that NO derived from nNOS contributes to vascular smooth muscle cell relaxation during skeletal muscle contraction; however, that vascular regulation overall requires eNOS.

Neuronal NOS regulates synaptic transmission and is found in the cytoplasm of neurons in the nervous system tissues, skeletal muscle, and in lung epithelium. nNOS plays a direct role in the physiological activity of contracting skeletal muscle. While nNOS and eNOS may be activated during repetitive muscle contractions, nNOS is probably the predominant NO producer during contractile activity. Aging in the brain is characterized by a loss of activity in neuronal cells, which leads to a loss of memory and a reduction in learning capacity. Since NO is an important neurotransmitter in the central nervous system (CNS), Cheng et al. examined the changes in nNOS from isolated cerebellum of Wistar rats aged 2 to 24 months to see if there was a correspondence between nNOS levels and the lowering of activity in the CNS during aging. In rats aged 6 months, there is an increase of NOS activity, which returned to the level of 2-month-old rats and/or 12-month-old rats, and further decreased in the 24-month group. While this reduction in activity could be attributed to a decrease in the number of cells in brain tissue, it does show a correlation between nNOS expression in the cerebellum region and the aging process.

Endothelial NOS, which is the only isoform of the three to be membrane associated, produces NO that is responsible for the regulation of blood pressure. eNOS is located primarily in endothelial tissue, cardiac myocytes, and hippocampal pyramidal cells. Furthermore, eNOS is widely available in very vascular tissues, such as the kidney, spleen, and liver. eNOS is associated with skeletal muscle mitochondria and acts to limit oxidative metabolism by the production and influence of NO on electron transport. NO production from eNOS regulates platelet aggregation, vasorelaxation, and production of vascular smooth muscle cells. In addition, it also mediates sexual function in males (penile erection) by diffusing NO to smooth muscle tissue. This release stimulates the enzyme guanylyl cyclase to elevate cGMP levels that, in turn, trigger a reduction of cytoplasmic Ca\(^{2+}\) and the subsequent relaxation of the corpora cavernosa. The tautness and duration of the erection is dependant on the balance among the levels of NO synthesized in the penile nerves, the compliance of the smooth muscle, and the release of contractile factors. In the heart, in vivo studies using NO donor or eNOS knockout mice have demonstrated that NO inhibits neutrophil-mediated injury by inhibiting neutrophil adhesion to the endothelial cells and preserves endothelial function, resulting in myocardial protection. Eosinophil-dependent NO synthesis regulates arterial pressure and is defective in human essential hypertension. Hence, a deficiency in the production of NO may therefore provide a plausible mechanism linking cardiovascular disease in humans.

iNOS is located in macrophages and liver cells and is induced by endotoxin and by inflammatory cytokines, such as interleukin-1 or tumor necrosis factor. Because it is independent of Ca\(^{2+}\) concentrations, it is produced in higher amounts
and for longer periods of time. High amounts of NO are released by iNOS in response to inflammatory stimuli, where it is involved in host-defense against pathogens. Excess NO production, often involving iNOS may occur in certain diseases and has been hypothesized as a major contributor in the disease pathway and also may play a significant role with aging. Hence, many groups are currently studying physiological or synthetic agents that inhibit cytokine-induced iNOS expression at the transcriptional level. For example, nitrones are used in the aging brain to attenuate iNOS activity and may explain the increase in the life span of animals receiving radical spin-trap compounds. One of the characteristics seen in stroke victims is the increased activity of myocardial iNOS. High levels of nitric oxide and peroxynitrite (formed from nitric oxide) produced by iNOS is particularly neurotoxic. Alpha-phenyl-N-tert-butyl nitron (PBN) inhibits iNOS production by inhibiting the induction of cytokines. Therefore, it appears that nitrones show promise in inhibiting cytokine-induced iNOS expression at the transcriptional level by mediating proinflammatory conditions in the brain.

NOS has been primarily investigated in endothelial cells, but recent experiments have been performed that indicate that NOS in the mitochondria (mtNOS) produce NO. Recently, NO was detected using electron paramagnetic resonance with spin-trapping techniques. Giulivi et al. isolated nitric oxide synthase from Percoll-purified rat liver mitochondria. Several different mitochondrial preparations, such as toluene-permeabilized mitochondria, mitochondrial homogenates, and a crude preparation of NOS, were incubated with the spin trap N-methyl-D-glucamine-dithiocarbamate-Fe II, which produced a signal ascribed to the NO spin adduct. It has been suggested that mitochondrial NOS and NO production may not only have an important role as a cellular transmitter, messenger, or regulator, but that it is also an active player in oxidative metabolism. In addition, mtNOS stimulation has been shown to induce mitochondrial cytochrome c release and increase lipid peroxidation (LPO), which, in turn, may mediate Ca2+-induced apoptosis.

In summary, the production of NO can result from an immunoresponse against macrophage activation of tumoral cells, from induction of iNOS in tumoral cells themselves, or from the vasodilation (eNOS) of blood vessel cells (endothelial and smooth muscle cells) by proinflammatory cytokines. Cytokines could also effect the production of nitric oxide by neuronal NOS, which may be inhibited by antioxidants and/or antiinflammatory compounds. Alterations of the levels of mtNOS and nitric oxide production could also have an influence on mitochondrial metabolism and oxidant production. Therefore, with aging, a variety of events, such as sickness, disease or injury, exposure to toxins, or physical activity levels could influence the levels of NOS and the production of nitric oxide.

BRIEF CHEMISTRY OF PROTEIN NITRATION

Nitric oxide itself is a relatively unreactive radical and cannot nitrate proteins irreversibly. However, it is able to form other reactive intermediates that could have an effect on protein function and on the function of the entire organism. For example, the reaction between NO+ and O2− produces a very reactive oxidant, peroxynitrite (ONOO−). Peroxynitrate reacts with tyrosine in proteins to form 3-nitrotyrosine (FIG. 1). However, besides nitration by peroxynitrate, other chemical reactions can
generate specific reactive nitrogen species, which could form 3-nitrotyrosine. Nitryl-chloride (NO$_2$Cl) a gas formed from the reaction of hypochlorous acid and nitrite (NO$_2^−$)—a major decomposition product of NO—can generate the nitrogen dioxide radical (NO$_2^+$). This very reactive oxidant is also able to oxidize tyrosine to 3-nitrotyrosine. Moreover, NO$_2^−$ at physiological or pathological levels is a substrate for the mammalian peroxidases myeloperoxidase and lactoperoxidase and forms NO$_2$ via peroxidase-catalyzed oxidation of NO$_2^−$. This provides an additional pathway contributing to cytotoxicity or host defense associated with increased NO production and an alternative pathway for the formation of 3-nitrotyrosine.

**PHYSIOLOGICAL RELEVANCE OF PROTEIN NITRATION**

Modification of tyrosine residues in receptor molecules has been shown to impair signaling pathways. For example, a specific modification, such as nitration of a tyrosine residue would compromise one of the most important mechanisms of cellular regulation, the cyclic interconversion between the phosphorylated and unphosphorylated form of tyrosine. This possibility is underscored by the demonstration that nitration of tyrosine residues in model substrates prevents the phosphorylation of these residues by protein tyrosine kinases. It is postulated that the nitration of tyrosine residues is an irreversible process and may lock the enzyme into a relatively inactive form. However, it appears that specific systems may be in place for the removal of potential unwanted nitrogen dioxide groups (see later section and Fig. 1).

The physiological importance of nitration has become more apparent in its effects on neurotransmission, vasodilation, and immunology. Growing attention is being given to endothelial function in hypertension and to the possibility that an inadequate NO-mediated vasodilation may have a direct impact on sexual function. Blood
pressure is controlled by the release of NO by the vascular endothelium—inhbiting the production of NO can cause elevated levels.\textsuperscript{78} The erection of the penis during sexual excitation is controlled by the release of NO. Relaxation of these vessels causes blood to pool in the blood sinuses producing an erection. The popular prescription drug sildenafil citrate (Viagra\textsuperscript{79}) inhibits the breakdown of NO by acting on the nitric oxide-cyclic GMP pathway that mediates penile erection and thus enhances its effect.\textsuperscript{79} Recent evidence shows that there is significant nitration (3-nitrotyrosine) and induction of iNOS in the normal aging rat penis.\textsuperscript{43} These findings could lead to targeted interventions to reduce iNOS activity and may point to possible tissue-specific nitration of a tyrosine residue on signaling proteins. Therefore, nitration intermediates can induce a number of covalent modifications in various signaling proteins that lead to functional or structural changes.

A specific protein that could become nitrated in a given tissue (i.e., brain or penis) could be the platelet-derived growth factor (PDGF) receptor. This receptor has five known tyrosine autophosphorylation sites. Mutations or modifications in specific tyrosine residues in the receptor—for example, tyrosines 1009 and 1021—prevent the binding and activation of phospholipase C-gamma (PLC-gamma), an important signaling protein. If PLC-gamma does not bind to the tyrosine residues, then the inositol phospholipid signaling pathway is not activated.\textsuperscript{80} Other important receptors, such as the insulin receptor also contains key tyrosine residue for phosphorylation and may become inactivated due to nitration. Thus, a site-specific modification of a single amino acid by an oxidant could result in the decline of a protein’s activity and a specific tissue this protein is abundant in. Hence, the role of reactive nitrogen species on biological aging is of great importance, and more research needs to be carried out in order to better understand if reactive nitrogen species play a role with age and what interventions can inhibit or reverse the aging process.

There are many other physiological effects of reactive nitrogen species. For example, the free radical gas nitric oxide has been shown to have a wide variety of biological effects, including the ability to act as an inhibitor of mitochondrial electron transport.\textsuperscript{81} Nitric oxide binds reversibly to cytochrome oxidase and can completely inhibit mitochondrial oxygen consumption.\textsuperscript{81} Another example concerns mitochondrial aconitase, a key enzyme in the citric acid cycle, which is a major target of superoxide and peroxynitrite mediated disruption of the [4Fe-4S] prosthetic group. This results in significant losses of aconitase activity.\textsuperscript{82} Interestingly, aconitase activity declines as a function of age both in skeletal and heart muscle.\textsuperscript{83–85} Therefore, this may be a mechanism by which enzyme function declines with age. However, even postmitotic cells are continuously repaired, and cellular components are entirely replaced, making it unclear what the physiological effects are during the aging process.

Trounce \textit{et al.}\textsuperscript{86} isolated intact skeletal muscle mitochondria from 29 subjects aged 16–92 years. State-3 (activated) mitochondrial respiration rates using several substrates were assayed and showed a negative correlation between respiration rate and age with all substrates tested. In addition, respiratory enzyme activities assayed in muscle homogenate also declined. They suggested that a substantial fall in mitochondrial oxidative capacity in aging muscle might contribute to reduced exercise capacity in elderly people. However, no direct evidence of a decrease in bioenergetics (i.e., ATP production) was presented, and the role of nitric oxide has not been investigated. Others have found very similar findings in the decline of respiratory
function in skeletal muscle and liver of aged animals. Furthermore, nitric oxide, some of its intermediates, and/or by-products may promote the induction of apoptosis with time. Apoptosis may therefore be involved in aging where it could serve to eliminate nonfunctional, harmful, abnormal, or misplaced cells, especially in advanced age. The death of cells in the heart and skeletal muscle fibers could therefore explain a decline in function.

**REVERSIBILITY OF NITRATION**

As mentioned earlier, the mechanisms for oxidative cellular damage during aging are poorly understood. However, oxidative damage on biomolecules leads to age-related diseases due to structural alteration of proteins, inhibition of enzymatic activity, and interferences of the regulatory function. Specifically, one of the mechanisms that plays a direct role in cellular signaling and cytotoxic host defense mechanisms may also directly contribute to a mechanism for aging, due to the nitration of intracellular proteins. This could contribute to a variety of disease states, such as cardiovascular disease, and to neurodegenerative diseases, in which 3-nitrotyrosine has been detected. However, few studies have investigated whether 3-nitrotyrosine nitration is reversible and if specific proteolytic mechanisms are in place for the removal of covalently bound reactive intermediates.

In general, the level of oxidized or nitrated proteins in a tissue reflects the balance between the relative rates of protein oxidation and clearance. For example, lens or collagen proteins turn over extremely slowly and thus should accumulate products of oxidative and nitrosative damage over time. Protein turnover and repair in skeletal muscle is also relatively slow compared to more mitotically active tissues. Oxidized and nitrated proteins may accumulate in muscle tissues due to the slower repair capacity. The production of reactive oxygen and reactive nitrogen species in old compared to young animals may be of key importance because it is likely to increase the rate at which proteins are damaged.

Starke-Reed, Stadtman, and coworkers have performed several studies addressing how proteins are oxidized and subsequently, proteolytically degraded and how these systems may change with age. They found that with age there is less efficient removal of oxidized proteins through proteolytic cleavage, which may cause the accumulation of protein carbonyls with aging. Several proteolytic enzymes responsible for degrading oxidized proteins decline with age in tissues. These proteases rapidly degrade oxidized enzymes but do not affect native nonoxidized enzymes. Several multicatalytic proteases provide major intracellular pathways for protein degradation.

Ischiropoulos et al. established that a nitrated protein (tyrosine hydroxylase) is selectively degraded in vivo by chymotrypsin. In addition, it was found that protein nitration enhances susceptibility to proteolytic degradation by the proteasome. In their experiments, peroxynitrite was used in the nitration of tyrosine hydroxylase and detected by immunoprecipitation with a monoclonal antityrosine hydroxylase antibody. After the first two hours, there was no detectable change in the amount of nitrated protein; however, there was a significant decrease (~50%) in the antinitrotyrosine immunoreactivity after four hours. Hence, it is apparent that the chymotrypsin activity may be critical for the induction of accelerated proteolytic
degradation of nitrated proteins \textit{in vivo} and provides a model for studying the structural basis for the removal of oxidatively modified proteins.$^{106}$ It is therefore feasible that specific systems for the removal of nitrating species exist to reduce the toxicity of reactive nitrogen species. One indication of this possibility is provided by the studies performed by Murad \textit{et al}.$^{107}$ As mentioned earlier, it is postulated that the nitration of tyrosine residues is an irreversible process. However, in a study by Murad \textit{et al}.$^{107}$ they identified a possible “nitrotyrosine denitrase” that reversed protein nitration (Fig. 1). In their study,\textit{107} they examined tissues (spleen, lung, liver, and kidney) from rats treated with lipopolysacharide—a cytokine, which increases nitric oxide and superoxide production—to see what effect it had on the modification of nitrated bovine serum albumin (BSA). It was concluded that homogenates (containing the “denitrase enzymes”) from rat spleen and lung could modify 3-nitrotyrosine-containing BSA; however, no activity was observed in homogenates from rat liver and kidney, suggesting that there may also be some tissue specificity for the apparent denitrase activity.$^{107}$ We show (see next section) that 3-nitrotyrosine in liver tissues tends to increase with age, which may be partly explained by the lack of activity of denitrase. Hence, the presence of a denitrase within human tissues could have an acute and significant chronic effect on cell signaling pathways.

\section*{3-NITROTYROSINE IN PROTEINS AND TISSUES OF AGING ANIMALS}

Caloric restriction has widely been investigated and in almost every species studied, it has been found to impede the aging process. One of the postulated mechanisms is that a reduction in reactive oxygen species (and hence peroxynitrate production) can lower the chronic constant oxidative stress with age and therefore attenuates protein oxidation, DNA damage, and lipid peroxidation.$^{11-14}$ Thus, during the aging process, protein oxidation is increased in a wide variety of human and animal tissues. However, the exact pathways for oxidative cellular damage are poorly understood because the reactive metabolites are very short-lived and difficult to detect directly \textit{in vivo}.

We have determined 3-nitrotyrosine levels in liver homogenate of young, mid-aged, and old mice and young and old rats (Fig. 2). We found that 3-nitrotyrosine tended to increase with age in the liver of both old mice and rats. In addition, caloric restriction in the mice lowered the levels of 3-nitrotyrosine. However, the results from an isotope-dilution gas chromatography mass spectrometry study\textsuperscript{10} suggest that proteins oxidized by reactive nitrogen species do not accumulate significantly with normal aging in the skeletal muscle, heart, and liver of rats, though there was an apparent increase of 3-nitrotyrosine in the liver of old rats. Though the results were somewhat surprising, it did suggest that reactive nitrogen species damage proteins during biological aging; however, the accumulation of these proteins may have been prevented by removal mechanisms. Thus, proteolytic degradation of intracellular proteins may account for the relatively constant level of protein oxidation products seen in these tissues. The study on mice was performed on a small number of animals. Consequently, additional investigations are needed to better quantify the effects of reactive nitrogen species on the oxidation of proteins; however, it does appear that caloric restriction plays an integral role in the reduction of nitrosative stress. In addition, these findings are suggestive that nitrosative stress has a greater
impact on some tissues over others, possibly due to the availability of a “denitrase” enzyme.

A study by Weindruch et al. showed that caloric restriction lowers oxidative and nitrosative damage in aging primates. They were able to quantify the age-dependant accumulation of oxidative damage in mammalian skeletal muscle as well as characterize its attenuation by caloric restriction. Using immunogold light microscopy (LM), an age-dependant decrease of 3-nitrotyrosine in skeletal muscle was observed, whereas no change was observed using immunogold electron microscopy (EM). However, the authors mention that these differences could be explained by the increased sensitivity due to the enhanced preservation of the tissue with immunogold EM as well as inconsistencies in software analysis using immunogold LM. Additionally, it appeared that caloric restriction reduced the level of nitration by 20% using both techniques. The authors go on to suggest that if an age-dependant in-
crease in oxidative damage is pivotal for the sarcopenia in mammalian skeletal muscle, the target of oxidative stress is more crucial than the subcellular site of free radical production. These findings are in agreement with an earlier report from Leeuwenburgh et al. who also did not find increases in 3-nitrotyrosine in rat skeletal muscle homogenate with age.

Others have looked at subcellular modifications of reactive nitrogen species in aged skeletal muscle. A study by Viner et al. stated that the level of nitration of the SERCA2a isoform of calcium-ATPase in sarcoplasmic reticulum vesicles isolated from rat skeletal muscle increases with age; there are approximately one and four nitrotyrosine residues per young and old Ca-ATPase, respectively. In addition, nitration was undetectable in a closely related form of the protein, which strongly suggests that certain calcium-ATPases are selectively modified by reactive nitrogen species. In vitro studies suggest that this level of protein oxidation may alter the function of SERCA2a in vivo. These observations raise the possibility that specific proteins accumulate oxidative damage during aging. Therefore, it is plausible that increases in nitric oxide and/or superoxide production with age in, for example, the mitochondria discussed previously could become deleterious to mitochondrial respiratory enzymes and critical cellular components.

OTHER INDICATORS OF THE LEVELS OF REACTIVE NITROGEN SPECIES AND AGING

Many of the studies on the effect of NO on aging have shown that there is a decrease in the level of basal secretion with increased age. Because of the short half-life and low levels of NO in tissues and biological fluids, estimations of NO are based mostly on measurements of nitrite and nitrate—end products of NO metabolism. Nitrite and nitrate are either partly formed by auto-oxidation of nitric oxide with oxygen and/or derived from the diet (see next section). In a recent study on age-associated changes in NO metabolites in humans, Toprakci et al. found that NO release declines with age in healthy people, with the most pronounced decrease between 46 and 60 years of age. Consequently, this reduction in NO synthesis with age could help in explaining the onset of vascular disease with increasing age due to the decline in endothelium-dependant vasodilation.

Nitrite (NO$_2^-$) can be formed from nitrate (NO$_3^-$) by a chemical process called reduction. Nitrate is relatively harmless unless it is reduced to nitrite. The vast majority (80–90%) of the nitrate most people consume comes from vegetables; however, because very little of the nitrate in vegetables is converted to nitrite, any health problem is unlikely. While meat products account for very little of nitrate in the diet (<10%), it does account for 60 to 90% of the nitrite consumed. Nitrites are unstable and can combine readily with other compounds in the digestive tract to form carcinogenic nitrosamines. In an earlier study by Witter et al., the distribution of $^{13}$N-labeled nitrate in humans and rats was observed. The radiolabeled nitrate rapidly distributed in the bloodstream throughout the body. The radioactivity accumulated almost linearly with time in a small region of the abdomen, which was probably due to the swallowing of salivary nitrate. Nitrate salts are the predominant preservatives used in cured or processed meats. Based on early results of a major new study, eating lots of preserved meats, such as salami, bacon, cured ham, and hot dogs could in-
crease the risk of bowel cancer by 50%.\textsuperscript{113} Hence it appears that there is a strong correlation between dietary nitrite consumption and the mechanisms of such diseases as vascular disease and cancer. Finally, besides dietary consumption of nitrates and nitrites, there are other sources for intake of harmful nitrites into biological tissues and fluids. Municipal drinking water, though not as severe a problem since the passage in 1972 of the Clean Water Act, used to be a huge source of nitrite exposure in humans. For this reason, the U.S. Environmental Protection Agency has set a maximum contaminant level, requiring that the maximum nitrate concentration content not exceed 1 part per million in public drinking water supplies. However, private sources of water, such as wells in rural areas, are still susceptible to high levels of nitrate and nitrite. Smoking is another source of nitration for biological tissues. Tobacco contains specific carcinogenic nitrosamines, which are derived from nicotine. These compounds may be among the causative agents for the various cancers (lung, oral cavity, esophagus, bladder, and pancreas) that are associated with tobacco usage.\textsuperscript{114} Therefore, exogenous levels of nitrate and nitrite could significantly affect mean and maximum life span potential. In addition, when investigating the levels of nitrite and nitrate with age in blood or urine, dietary and environmental influences could play a major role in the cumulative levels.

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