HORMONES 2008, 7(2):114-122

Review

Oxidative stress and aging: the potential role of iron

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ABSTRACT

According to the free radical theory of aging proposed by Denham Harman more than 50 years ago, oxidatively modified cellular components accumulate continuously in the cells during the organism's lifespan leading to progressive decline of cellular functions. Since then, it has been shown that proteins, lipids, nucleic acids and other cell components undergo reversible and/or irreversible oxidative modifications during aging. Moreover, oxidized cell components can undergo further oxidative modifications leading to formation of products that cell degradation systems are incapable of removing. Accumulation of such non-degradable aggregates further inhibits the functionality of degradation systems, thus aggravating the effects and leading to a vicious cycle. In this presentation, we propose that the availability of intracellular iron in its redox active form (labile iron) represents the main catalyst that mediates extensive oxidative modifications of cellular components and ultimately leads to their accumulation and consequent cellular dysfunction. It is tempting to speculate that regulated restriction of labile iron may have positive effects on health in general and aging in particular.

Key words: Aging, Hydrogen peroxide (H_2O_2), Labile iron, Lipofuscin, Oxidative stress, Reactive oxygen species (ROS)

INTRODUCTION

During their lives organisms undergo progressive deleterious alterations called "aging" or "senescence". Alterations that accompany the aging phenotype are related to both genetic and epigenetic factors and ultimately lead to: a) structural disorganization, b) functional decline and c) increased probability of diseases and death. Among the numerous theories proposed to explain the molecular base of this process, the "free radical theory of aging" has attracted most interest and has accumulated substantial experimental support.[1] This theory, originally proposed more than 50 years ago by Denham Herman, maintains that "reactive free radicals" that are formed endogenously via normal oxygen-utilizing metabolic processes play an essential role in the aging process.[2] Although this theory underwent several revisions and improvements,[3,4] the basic concept that free radical-induced damage in cell components is progressively accumulated during aging remains the same until today.[5] However, the exact molecular mechanisms underlying the aging process is unclear and questions related to a) the biochemistry of intracellular ROS

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Received 21-01-08, Revised 25-02-08, Accepted 03-03-08

generation, b) the interactions of reactive oxygen radicals with the main cellular components, c) the defense and repair mechanisms that counteract the formation of irreversibly modified cell components and d) the consequences from the accumulation of oxidatively modified macromolecules on cell function remain incompletely understood and need further investigation.

In this paper we will briefly review the biochemical basis of ROS formation and the induction of oxidative stress in the cells before focusing our interest on the role of iron in the formation and accumulation of oxidatively modified macromolecules, which ultimately compromise cellular function and lead inevitably to senescence and aging.

REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS

Aerobic organisms utilize molecular oxygen as a terminal electron acceptor in order to remove the electrons that are generated during the process of energy producing oxidative catabolism. Direct reduction of molecular oxygen to water is catalyzed by the last enzyme of the respiratory chain, the "cytochrome oxidase", in a 4 electron reduction manner. However, a portion of oxygen can be reduced via a univalent pathway even under normal conditions, leading to intermediate reduction products (Figure 1). In this way, reactive intermediates such as superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) are continuously formed and removed inside the cells even under normal conditions.[6,7] This leads to an intracellular steady-state concentration of these agents, which may vary between different kinds of cells or different compartments of the same cell, as the rates of their generation and removal may differ considerably.[8] Hydrogen peroxide, superoxide anions, hydroxyl radicals and other reactive compounds derived from them are collectively called "reactive oxygen species" (ROS). Helmut Sies also introduced the term "oxidative stress" in 1985 to denote "a disturbance in the prooxidant-antioxidant balance in favour of the former", thus describing changes in the above-mentioned steady-state.[9] It has to be stressed, however, that terms like "ROS" and "oxidative stress", as used today, create a source of confusion. Molecules collectively regarded as ROS represent either relatively inactive compounds, like $O_2^{\cdot -}$ and H_2O_2, or extremely reactive ones like ·OHs, singlet oxygen, alcoxyl and peroxyl radicals, etc. Consequently, it is not clear whether "oxidative stress" refers to a change in H_2O_2 equilibrium that can trigger an adaptation response or to the generation of ·OHs which leads to severe toxicity in cells and tissues.[8,10,11] The assumption that all ROS fluctuate simultaneously is wrong and often results in erroneous conclusions. Physiological variations in the cellular "redox-equilibrium" are "sensed" by the cells, which respond by specific signalling that induces adjustments of their metabolism according to the existing conditions.[12] It is only when the level of H_2O_2 and $O_2^{\cdot -}$ surpasses a certain threshold that deleterious effects are apparent and become dangerous for cell integrity.[13-15] As will be discussed below, the availability of iron in its redox, active form represents the main mediator for the generation of reactive radicals from relative non-reactive intermediates, like H_2O_2.

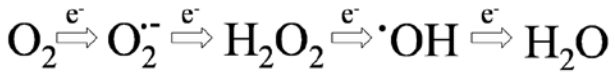

Figure 1. Schematic representation of the monovalent reduction of oxygen to water with the intermediate formation of superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals·(OH).

Mechanisms of ROS formation

Stimulated phagocytes represent the major contributor of ROS generation in the human body. These cells rapidly increase their oxygen consumption in response to appropriate stimuli, a process termed "respiratory burst". The utilized oxygen, however, is not coupled to energy production but is instead used for monovalent reduction of oxygen to $O_2^{\cdot -}$, catalyzed by the enzyme "NADPH oxidase".[16] Superoxide anion and oxidants derived from it by complex chain reactions are the main contributors in the defense against invading foreign microorganisms.[17] Although this enzymatic generation of $O_2^{\cdot -}$ was initially thought to be restricted to professional phagocytes, it is apparent today that non-phagocytosing cells are also able to produce $O_2^{\cdot -}$ in a similar way, albeit at lower rates.[18] Surprisingly, this property of non-phagocytic cells was shown to be required for normal mitogenic stimulation.[18,19] Subsequent analysis identified that

proteins similar to gp91phox, p47phox and p67phox of "NADPH oxidase" were present in different types of cells and they were accordingly named Nox1 to Nox5.[20-23]

Another important source of ROS generation is the mitochondrion where, under normal conditions, a small portion of the consumed oxygen (less than 1%) is continuously reduced by single steps of one electron reduction, thus releasing $O_2^{\cdot -}$ and other ROS.[24] Electrons from complex I and complex III of the respiratory chain can be transferred directly to oxygen to generate $O_2^{\cdot -}$ rather than following the normal redox pattern through cytochrome c and cytochrome oxidase.[25] The rate of ROS production in mitochondria is dependent upon inner membrane potential and the degree of inhibition applied to the respiratory chain.[26] It therefore appears that cells can regulate the rate of ROS production by mitochondria by adjusting membrane potential and the flow of electrons.[27] In addition to mitochondria, electron-transport chains in other organelles can also donate electrons to oxygen, generating $O_2^{\cdot -}$. Such chains have been reported to exist in the endoplasmic reticulum[28] and the nuclear envelop.[29] It has been suggested that the generation of ROS by these electron transport chains may also be regulated, as their components are targets for several signaling kinases and their phosphorylation increases the rate of ROS production.[30]

Recent experimental evidence also indicates that leakage of electrons from the respiratory chain can be mediated in a regulated manner through the action of the protein $p66^{Shc}$.[24,31] H_2O_2 produced through the action of $p66^{Shc}$ is biologically relevant, as shown by the fact that $p66^{Shc}$-null mice are refractory against induction of apoptosis[31] and mitogenic signaling through selected growth factors.[32]

In addition, ROS can be generated by the action of several other enzymes, like amino acid oxidases, cycloxygenases, lipoxygenases, xanthine oxidase, etc or during exposure to xenobiotics of varied structures and activities, like quinones, barbiturates, phorbol esters and peroxisome proliferators among others.

Cell adaptation in conditions of oxidative stress

Mammalian cells exhibit a broad spectrum of responses toward oxidative stress, which is dependent on the severity of the stress encountered. It has been shown that a dose-dependent temporal up- or down-regulation of the expression of several dozens of genes took place when cultured cells were exposed to increased concentrations of H_2O_2.[33] Proteins encoded by these genes usually participate in complex signaling pathways which, by acting in a concerted way, dictate concrete cell responses. Thus, exposure of cells to low concentrations of H_2O_2 influenced pathways that led to increasing cell proliferation, while slightly higher amounts induced a transient cell arrest state as expressed by inhibition of cell division and activation of repair mechanisms for oxidized cellular components.[33,34] If H_2O_2 was removed at this point and sufficient time was allowed, a "cell adaptation" state was achieved, which was characterized by an elevated defense capacity of the cells against a subsequent exposure to oxidative stress. Such mechanisms underlie the well known phenomenon in which stimulatory responses to low doses of otherwise harmful compounds can improve the resistance of the individuals to subsequent exposure to stressful conditions. However, higher levels of oxidative stress switch mitotic cells into a permanent growth-arrested, senescence-like state. Faced with even higher oxidative stress, cells "sacrifice themselves" by apoptosis, thereby protecting surrounding healthy tissue from further damage, while severe oxidative stress conditions induce necrotic death with inflammatory immune responses. This remarkable array of responses makes it possible for the organisms to counteract conditions of temporary oxidative stress with minimal adverse effects.

Participation of ROS in cell signaling

Extensive experimental research during the last decade led to exciting progress regarding redox biochemistry. Hence, it is clear today that ROS (especially H_2O_2) act as second messengers representing an integral part of the cellular signal transduction networks.[24,35] This development led to an unexpected modification in the way that oxidative stress was traditionally viewed, from the simplistic model that predicted oxidant production as inherently damaging to a more physiologically oriented view where a regulated increase of certain ROS can be essential for optimal cellular function. Thus, the expression of numerous genes is coordinately regulated by a number of transcriptional factors which are activated (or inactivated) in conditions of oxidative stress. The

notion that ROS act as second messengers in complicated signaling processes requires the existence of receptor sites for specific action of these species. Although the precise molecular mechanisms are not as yet clear, it is assumed that specificity is determined by the intensity of the challenge and the subcellular location of the produced oxidants, which further increases the complexity of redox signaling.[36,37] On the other hand, cysteine residues represent ideal initial targets due to the reversible nature of their initial oxidation products.[37,38] It is therefore now clear that cysteine oxidation in particular proteins can induce conformational changes which modulate the functions of these proteins, just as phosphorylation modulates the kinase signaling cascades. The cysteine-dependent signaling pathways are rapidly delineated and it is apparent that the range of the participating cellular processes is extensive. Unfortunately, identification of the precise intracellular location and quantification of the subcellular fluctuations of H_2O_2 is virtually impossible at present.

Oxidation of cysteine residues in particular proteins, which leads to modulation of their function in a positive or negative direction, has been reported for a number of proteins, with tyrosine phosphatases, peroxyredoxins and protein kinases representing typical paradigms.[39-41] It should be stressed that not all cysteine residues in an individual protein are equally sensitive to H_2O_2-induced oxidation. Formation of negatively charged sulfhydryl anions at neutral pH (low pKs of the sulfhydryl groups) seems to be an important determinant of cysteine vulnerability toward H_2O_2. Such oxidations of thiol residues lead either to reversible modifications, like formation of disulfide bonds, sulfenic and sulfinic acids, or to sulfonic acid, which represent a paradigm of irreversible modifications.[38] The exact chemical nature of the reaction by which H_2O_2 induces the oxidation of cysteine is not clear at present, but at least in one case "labile" iron was required.[41] Whether this is an isolated observation or it represents a generalized phenomenon dictating H_2O_2-induced oxidations of sulfhydryl groups remains to be explored.

OXIDATIVE STRESS AND AGING

Experiments with genetically modified animals

Over the last two decades, genetically modified animals have been used extensively in studies attempting to clarify the molecular mechanisms of aging. Transgenic experimental animal models targeting either ROS-generating or ROS-scavenging systems have been used in these studies.

The over-expression of Superoxide Dismutase (SOD) enzymes did not lead to conclusive results, most probably because the action of SOD, while removing $O_2^{\cdot -}$, simultaneously generates H_2O_2. Thus, on certain occasions mammalian cells that over-express CuZnSOD are more sensitive to ensuing oxidative stress, a factor that was prevented by increasing catalase or glutathione peroxidase activities.[42] Mice carrying inactive mitochondrial MnSOD ($Sod2^{-/-}$) died within the first week of life, while $Sod2^{+/-}$ mice showed decreased $O_2^{\cdot -}$ scavenging capacity but normal lifespan.[43] In addition, synthetic SOD analogues that removed $O_2^{\cdot -}$ did not increase lifespan when delivered to *Drosophila melanogaster* or *Caenorhabditis elegans* experimental models.[44] These results indicate that scavenging $O_2^{\cdot -}$ cannot be used as an anti-aging strategy. In contrast, simultaneous over-expression of SOD and H_2O_2 removing systems (catalase or glutathione peroxidase) resulted in some cases in increased resistance to oxidative stress and extended lifespan.[45,46] Surprisingly, targeted deletion of $p66^{Shc}$, a mitochondrial protein that reduces O_2 to $O_2^{\cdot -}$ by diverting electrons from the respiratory chain, was shown to prolong lifespan and reduce age-associated degeneration diseases in mice.[47-50] In addition, mice over-expressing catalase which was specifically targeted to mitochondria showed prolonged lifespan.[51] Hence, the generation of H_2O_2 in mitochondria was proposed as being involved in the aging process through endocrine signaling pathways that modulate the expression of aging-related genes.[24,52,53]

H_2O_2-induced and iron-mediating oxidation of cell components

The initial ROS produced in almost all cases related to oxidative stress is $O_2^{\cdot -}$, which is rapidly converted to H_2O_2 by the action of SODs. Neither $O_2^{\cdot -}$ nor H_2O_2 are strong oxidizing agents and inefficient of interacting directly with any intracellular target other than iron or iron-contained molecules. However, when "labile" iron ions (able to participate in redox-active reactions) are available, the so-called

"Fenton-type" reaction takes place, leading to formation of extremely reactive intermediates and ·OH (reaction 1), as indicated below:

$$Fe_2^{+} + H_2O_2 \rightarrow \text{reactive intermediates} \rightarrow Fe^{3+} + {\cdot}OH + OH^{-}$$

Although other metals, like copper, are able to catalyze reaction 1 even more effectively than iron, the latter, due to its wide availability in biological systems, represents the main catalyst in living cells.[54,14] The reactivity of ·OHs is diffusion controlled, meaning that they interact with target positions in the vicinity of their generation. Consequently, the location of available redox-active iron also determines the specificity of H_2O_2-mediated oxidation in a site-specific manner.[55] It is therefore not surprising that nature handles iron with the utmost care and iron homeostasis is carefully regulated by sophisticated mechanisms in order to avoid such injurious interactions.[56-59] Our knowledge about the mechanisms that are responsible for iron transport and homeostasis has increased dramatically during the last decade. Moreover, development of new methodologies for estimation of intracellular levels of labile iron in human cells will certainly help to clarify the role of iron in a variety of pathophysiological conditions, including the aging process.[60,61] By using a flow cytometric methodology, we recently observed a positive association of labile iron levels in human blood cells with the age of the blood donors.[60] This observation may offer a mechanistic explanation for the increased oxidative modifications of major cellular components during aging, as previously reported.[62,63] The possible establishment of labile iron as the main catalyst for the oxidation of cell components under conditions of oxidative stress may contribute to far-reaching considerations regarding potential interventions aiming to improve health in general and to modulate the aging process in particular.

Progressive impairment of cellular degradation capacity and accumulation of oxidized proteins

The strategies used by the cells to repair their damaged components vary depending on the particular component. Sophisticated surveillance systems exist in order to detect oxidative modifications in DNA, which subsequently are removed and replaced by new nucleotides through such processes as "base excision repair" or "nucleotide excision repair". Irreversibly oxidized proteins, however, are totally degraded and their amino acid residues are used for synthesis of new proteins. There are several such protein-degradation systems which are located in different cell compartments: the proteasomes and calpains in the cytosol, the Lon proteases in the mitochondrial matrix, the triple A proteases in the mitochondrial membrane and the lysosomes, which probably represent the most important cellular degradation system.[64-66] Apart from oxidatively modified cytosolic proteins, lysosomes can degrade cell organelles, like mitochondria or part of the cytoplasm.[67] Oxidatively modified cellular organelles are taken up into lysosomes by specific pathways, such as macroautophagy, microautophagy and chaperon-mediated autophagy.[68,69]

Cellular capacity for protein degradation, however, is not unlimited. In conditions of increased oxidative stress in combination with elevated levels of redox-active iron, the rate of protein oxidation is elevated and the overall time needed for removal of the modified proteins increased. During this period, additional more profound oxidative modifications can take place, including the formation of covalent bond adducts or covalent intra- or inter-protein bonds. The advanced chemical complexity of these compounds exceeds the capacity of cellular proteolytic systems, which are unable to cope with the degradation of these over-oxidized products. An unavoidable consequence of this situation is a progressive accumulation of strongly modified materials (usually called lipofuscin) mainly in lysosomes.[69,70] Accumulation of non-degradable compounds over time, mainly in post-differentiated cells such as heart and brain cells, have an inhibitory effect on the proteolytic capacity of the cells, which further increases the turnover time for proteins. As a consequence, the chance of over-oxidation of already modified proteins is increased, thus facilitating a vicious cycle of oxidation, over-oxidation and progressive impairment of cellular degradation capacity. The final outcome of these procedures is the decline of cellular functions, as observed in aging and senescence.

CONCLUDING REMARKS

Aging is a multifactorial process that is controlled by complex molecular networks. There are no indications that any single factor predominates dur-

ing aging but it seems likely that combinations of different factors contribute toward the appearance of variable aging phenotypes, even within the same species. These considerations raise the question about a common determinant among the various aging mechanisms.

Primary and secondary modifications of cell components during their turnover period represent a common phenomenon in all variants of the free radical theory of aging. Cell degradation machineries are unable to hydrolyze over-oxidized materials, such as proteins, lipids, carbohydrates and ribonucleotides, thus leading to intracellular accumulation of non-functional aggregates. Moreover, these components can exert inhibitory effects on major cellular degradation systems, like proteasomes and lysosomes,[64] thereby further contributing to elevated accumulation of over-oxidized non-degradable materials.

Oxidative stress in mitochondria seems to be intimately related with the aging process. Oxidatively damaged mitochondria as well as other organelles are taken up by lysosomes as units by a specific process called macrophagocytosis.[67] Inside lysosomes, the various mitochondrial components are degraded and their building blocks are transported out in order to be reused. If non-degradable materials are present in the ingested mitochondria, this process is hindered, thus exacerbating the accumulation of non-functional materials inside the lysosomes.

A crucial concept that remains poorly understood is the nature of over-oxidized materials as well as the molecular mechanisms that underlie their formation. There is strong experimental evidence indicating that hydrogen peroxide (especially that generated in mitochondria) represents the unifying aging mediator (for a review, see ref 24). However, H_2O_2 by itself is a

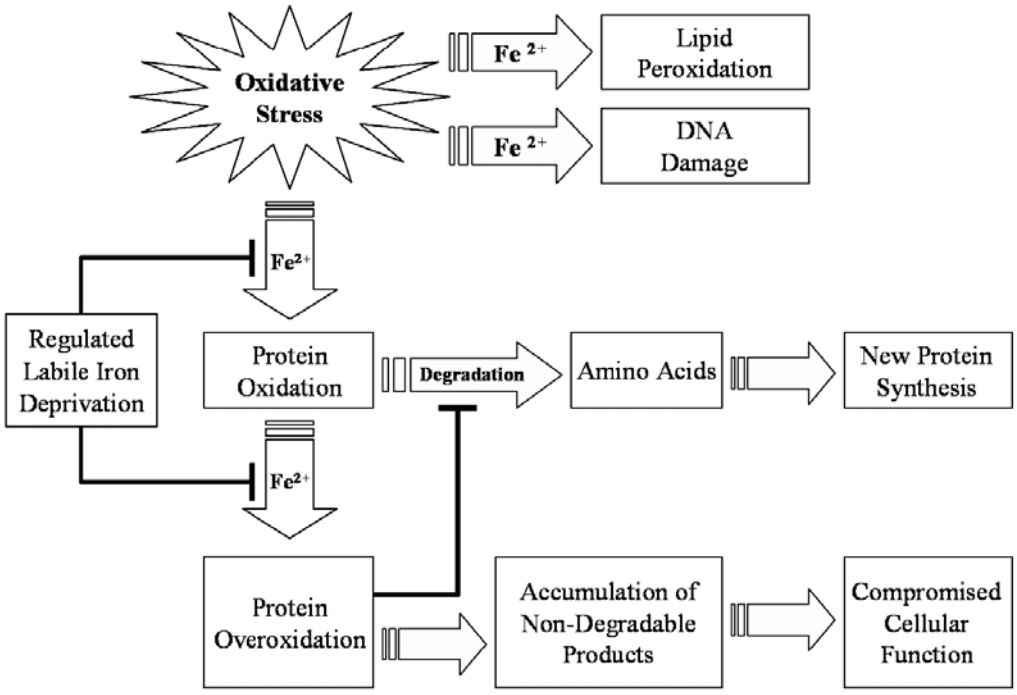

Figure 2. Proposed schematic representation of the steps that take place during aging. Oxidative stress conditions lead to formation of $O_2^{\cdot-}$ and H_2O_2, which can oxidize cellular macromolecules mainly through the mediation of iron. Normally, the oxidized proteins are degraded to the constituting amino acids, which are subsequently used for synthesis of new proteins. However, under conditions of elevated levels of H_2O_2 and/or iron or decreased cellular degradation capacity, secondary oxidation of already oxidized proteins takes place and leads to formation of over-oxidized products that are not capable of further processing. The consequences of the formation of over-oxidation products are dual. First, they inhibit the cellular degradation systems, and secondly they progressively accumulate inside the cells, thus compromising the cells' structures and functions. These effects are especially apparent in terminally differentiated non-divided cells. It is proposed that regulated suppression of labile iron levels may decrease the rate of accumulation of over-oxidized materials and in this way favourably influence the aging process.

rather weak oxidizing agent which is unable to interact directly with any cell component except with redox-iron-containing materials. The main part of synthesis of heme and iron-sulfur complexes takes place in mitochondria and for this reason they need increased amounts of available iron for immediate use. These particular conditions, i.e. increased generation of ROS in combination with elevated redox-iron, create an ideal environment for secondary oxidizing reaction on already oxidized mitochondrial components.

In conclusion, it is argued that any metabolic process that may contribute to accumulation of deleterious modifications in cell components over time may eventually limit lifespan. However, reactive free radicals generated through normal oxygen metabolism in combination with the presence of increased levels of labile iron represent the main contributors in the formation of non-degradable materials inside the cells. This accumulation ultimately leads to structural disorganization and functional decline of cell function characteristic of the aging process.[71,72] Based on the above description, it is tempting to speculate that regulated suppression of labile iron levels, as illustrated in Figure 2, may decrease the rate of accumulation of over-oxidized materials inside the cells and in this way favourably influence the aging process per se.

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