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The essential mechanisms of aging: Irreparable damage accumulation of biochemical side-reactions

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Abstract

Explanations on aging mechanisms have now become unexpectedly complicated. However, it is gradually accepted that 'senescence is a collective consequence of both inheritance and environment'. Based on the achievements of biological and medical research in related fields, we pinpoint in this review that although aging is mainly considered a physiological (non-pathological) process, the biochemical structure of aged organisms is deranged, or 'sick' at the molecular level. The free radical/glycation induced carbonyl stress, the key culprit to form crosslinks, has been identified to cause stable cyclic conjugates of mainly protein-based aggregates implying entropy increase (the Second Law of Thermodynamics) during aging. When combining such key aging processes with age pigment biochemistry, a general picture of aging process can be figured out, as the main clues and results are available. In this review we also propose for the first time that by focusing on 'process' rather than on 'causes' (damages), we can then get a clear view of aging mechanisms. Through rational thinking and critical analysis, we conclude that the accumulation of irreparable damages and alternations caused by spontaneous biological side-reactions seems to be the essential and profound nature of higher animals' aging mechanisms.

Keywords: Aging mechanisms; Carbonyl stress; Entropy; Free radical oxidation; Non-enzymatic glycosylation; Age pigments

1. Introduction

Because of such extreme complexity of aging processes, such as aging processes which are caused by various factors, developed under different conditions, involved in most organs and tissues, and because of the limited knowledge of researchers in a certain field and their professional prejudice, studies on aging mechanisms, in spite of great progress and achievements (Comfort, 1979; Medvedev, 1990; Hayflick, 1998; Kirkwood, 1999; Warner, 2005; Yin and Chen, 2005), make people dazed and confused, and unable to decide which is the right explanation of the enigmatic aging mechanisms (Medvedev, 1990; de Grey et al., 2002; Olshansky et al., 2002; de Magalhaes, 2005).

In this paper, we shall first briefly review the theories on aging mechanisms, and discuss how genetic regulations may interplay with the inevitable stochastic damages on organisms during aging. Second, we suggest that in order to discuss the definitive aging process one should focus on physiological rather than pathological (accelerated) aging manifestations so that protein alterations during aging stand out evidently at the frontal. Subsequently, through elaborating the free radical/glycation biochemistry, age pigment formation biochemistry and their explanations on entropy increase, the importance of carbonyl toxification-related alteration (Yin and Brunk, 1995) during aging becomes remarkable. Finally, an essential aging mechanism stressing accumulation biochemistry due to irreparable crosslinking (mainly in proteins) is put forward to understand the aging process in physiological (health) circumstances.

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2. A brief overview of aging theories and some general remarks of aging studies

Although the deaths of a few animal species have some kind of 'switch off' characteristics (regulated directly by 'gene clock'), which is true for lower organisms such as insects, aging of higher grown up animals is understood mainly as a slow, gradual, and passive process influenced by an interplay of multiple genetic and environmental factors, which can be seen from numerous experimental data and existing theories of aging (Comfort, 1979; Medvedev, 1990; Hayflick, 1998; Yin, 2002). Some important theories of aging are listed below to provide an overview of the field and facilitate discussions.

Important theories of aging at the whole animal level are: Wear and tear theory (Sacher, 1966); Error catastrophe theory (Orgel, 1963); Stress damage theory (Selye, 1970); Autointoxication theory (Metchnikoff, 1904); The evolution theory (programmed aging theory?); Stored information theory (programmed aging theory).

Major theories of aging at the organ level are: Endocrine theory (Korencheysky, 1961); Immuno-biological theory (Waford, 1969); Brain retardation theory.

Key aging theories at the cellular level are: The cell membrane theory (Zs.-Nagy, 1978); Somatic mutation theory (Szilard, 1959); The mitochondrial theory (Miquel et al., 1980); The mitochondrial-lysosomal axis theory (Brunk and Terman, 2002); Limited cell proliferation theory (programmed aging theory).

Important aging theories at the molecular level are: Accumulation of DNA alterations (Vilenchik, 1970); Trace element theory (Eichhorn, 1979); Free radical theory (Harman, 1956, 2003); Crosslinkage theory (Bjorksten, 1968); Oxidative stress theory (Sohal and Allen, 1990; Yu and Yang, 1996); Non-enzymatic glycosylation theory (Cerami, 1985); Carbonyl toxification theory (Yin and Brunk, 1995); Garbage catastrophe theory (Terman, 2001); Gene mutation theory; Telomere shortening theory (programmed aging theory);

Some other important theories of aging are: Aging as entropy (Sacher, 1967; Bortz, 1986), Mathematic model theory and various unified aging theories (Sohal and Allen, 1990; Zs.-Nagy, 1991; Kowald and Kirkwood, 1994). It is easy to find that the majority (24 among 28) of the above-mentioned theories of aging consider aging as a consequence of diverse external damages in life process. In brief, aging is a process of passive damage accumulation.

It should be noted that among the four theories of aging, which have been classified as 'programmed aging theory', the so-called 'cellular aging' observed and studied by proliferation senescence and telomere shortening is quite different from aging of the whole animal. The concept of 'non-dividing cells' does not mean 'aged cells', just as post-mitotic neurons and the majority of myocytes, though not proliferating after finishing differentiation at the prophase of life (the embryonic period), may remain healthy within organisms for life (Sohal, 1981; Porta, 1990). Moreover, the shortening of telomere can hardly be related to the functional decline of whole animals based on the general definition and knowledge of animal aging. The cause of telomere shortening was recently proposed due to mild chronic oxidative stress by von Zglinicki and others (von Zglinicki, 2003). Using senescent somatic cells from a bovine fetus, Lanza and coworkers have bred successfully six heads of cloned-cattle (Lanza et al., 2000), whose telomeres are longer than control cattle of the same age. Similar evidences that argue against an 'aging clock' which decides directly higher animals' aging studies are plenty and will not be discussed in detail here (Wakayama et al., 2000; Cristofalo et al., 2004).

The understanding of various effects of either genetic factors or environmental damages on aging has experienced a long and difficult period, whereas each explains aging phenotypes in its own way at the beginning. Through industrious exploration by genetic scientists for over half a century, some dozens of genes related to aging and longevity have been identified (Finch and Tanzi, 1997; Warner, 2005;), for example, *age-1, Chico, clk-1, daf-2, daf-16, daf-23, eat-2, gro-1, hsf-1, hsp-16, hsp-70, Igf1r*$^{+/-}$, *indy, inR, isp-1, KLOTHO, lag-1, lac-1, MsrA, mth, αMUPA, old-1, p66*^{sh}, *Pcmt, Pit-1, Prop-1, ras2p, spe-26, sag, sir2, SIRT1, sod1,* etc. (Hamet and Tremblay, 2003; Warner, 2005;). These longevity-related genes have been found mainly within four catagories: (1) anti-stresses (e.g. anti-heat shock and anti-oxidative stress systems); (2) energy metabolisms (e.g. insulin/IGF-1 signaling pathway, caloric intake and mitochondrial functions); (3) mutation prevention (repairing and restoration on nuclear stability); (4) protection of hormone homeostasis or mammalian sperms; yet the biological functions of some 'longevity genes' are not clear.

Furthermore, proliferation regulators of cells such as CDK1, IGF-1, MAPK, P13K, P16, etc. (Wang et al., 2001; de Magalhaes, 2005) are found relevant to cell division and cellular aging as well. Therefore, life scientists have consciously realized that genes related to aging and longevity do exist, but the aging process is found neither merely dependent on a single gene, nor dependent on a small group of genes. Longevity is regulated and/or controlled by hundreds of genes working together (Holliday, 2000; Warner, 2005). Relevant to physiology and pathology, aging is a general result of genetic networks in the regulating, controlling, defending and restoring systems, especially in keeping homeostasis and metabolisms (Warner, 2005). These systems working in harmony resist various environmental damages. In short, it is gradually accepted that aging is dependent on an interplay of both inherent (inborn) and acquired (environmental) factors.

Recognizing the above-mentioned features of higher animal aging in general, research on aging mechanisms of humans can thus be focused on damage accumulation and the efficiency/capability of defensive restoration.

3. The physiological feature of aging and the underlying molecular 'killer' of lives

To discuss the aging mechanism in its true meaning, it is necessary to discriminate aging and aging-related diseases. Generally speaking, it is widely agreed that aging is not a disease. Thus, definitive aging mechanism study should mainly deal with the physiological alterations of healthy organism developed with age. Aging as to whether the whole animal level, the organ level or the cellular level is the reflection of alterations at the molecular level, so called 'structure to settle character', although they have different features corresponding to different levels. There are many kinds of 'non-disease' aging manifestations, for example, aging-related blood vessel stiffening, slight high blood pressure, decreasing elasticity of lung fiber, falling vital capacity caused by gradual collagen crosslinking, skin slacking, eyesight degeneration, joint stiffening, lipofuscin formation, etc. All these bear substantial molecular changes in higher animals (Bailey, 2001). Clinically, such kind of change is not a typical 'disease', judging from the whole animal level or the organ level, the molecular structures of organisms, however, are already 'sick' or abnormal. Generally speaking, the crosslinking hardening of protein is a most commonly seen molecular 'disease' which incessantly kills the vital force. As a result, the protein structure of even an old man who has died of no disease at all is totally different from that of a young man.

On the other hand, genetic instability was thought to be involved in aging. However, Kirkwood (1989) summarized that the accumulation of simple somatic mutations with aging has not been experimentally supported (Clark and Rubin, 1961; Lamb, 1965). Numerous studies, nevertheless, have suggested that the DNA damage and mutation increased with aging cause mainly pathologic events (Bohr, 2002; Warner, 2005), such as, a variety of mitochondrial DNA diseases (Holliday, 2000; Wallace et al., 2003). With the concern of physiological aging as mentioned above, the genetic alterations can be interpreted as less important during aging in comparison with versatile comprehensive post-translational protein alterations (Kirkwood, 1999; Ryazanov and Nefsky, 2002; Yin and Chen, 2005).

While emphasizing protein alterations, the genetic-damage-based view that 'aging is the maintenance of the accuracy of protein synthesis' presented by Orgel as the 'error catastrophe hypothesis of aging' (Orgel, 1963) has basically failed, (Gallant and Palmer, 1979; Harley et al., 1980) and scientists in the field concluded that the alterations of protein after expressing are the main and inevitable manifestation of aging. Because aging-associated protein alterations can be observed from every part of an aging body, the aging damage and modification of protein within organisms is biochemically the definitive and universal phenomena of aging to date. As a matter of fact, aging protein damage is referred to or implied in almost every aging theory. Therefore, the analysis and discussion in this review will mainly focus on the damages and alterations of proteins.

In general, the synthesis, damage and renewal of proteins run through the whole process of life. When a mammal is mature, the process (or speed) of protein synthesis and degradation will be in a dynamic balance, which will incline with age (Bailey, 2001; Terman, 2001). There are numerous reports on damages and changes of structural or functional proteins in aging organisms (Stadtman, 1992, 2003; Rattan, 1996; Ryazanov and Nefsky, 2002). Such alteration can be found either intracellular within both mitotic cells and post-mitotic cells or extracellular, such as the crosslinking hardening of skin, eye lens, blood vessel, lung tissue, glomerular basement membrane, and other tissues, which will lead to slow, gradual organ-specific functional declines, from quantitative to qualitative changes (Sell and Monnier, 1995; Rattan, 1996; Bailey, 2001; Stadtman and Levine, 2003).

The question then comes down to what are the culprits of such alterations?

Considering normal aging is a universal, gradual, cumulative, and irreversible physiological process, the causes for aging related consequences should be damaging factors with certain universality (Strehler, 1977). Biological reactions related to aging and diseases that can take place spontaneously and cause damages with universality show several main types as follows (Baynes, 2000):

Free radical oxidative stress: accumulation of physiological and pathological damage and modification of biologic molecules caused by oxidative stress. It is the most popular harmful biological reaction leading to pathological changes and aging of organisms (Harman, 1981; Yu, 1996; Sohal, 2002).

Non-enzymatic glycosylation (glycation): another important age-related biological reaction responsible for aging and various diseases initiated by diabetes (Baynes and Monnier, 1989; Cerami, 1985; Sell and Monnier, 1995). It can react spontaneously without assistance of biological enzymes.

Other important reactions related to post-translational modification of proteins during aging process are also recognized. They are isomerization, racemization, deamination, thiol oxidation, wrong folding, crosslinking reaction, DNA methylation decline, uncontrollable histone ethylation, retardation of protein degradation, etc. (Rattan, 1996; Baynes, 2000; Clarke, 2003). Unlike oxidation and glycation involved in energy producing process, spontaneous isomerization, racemization and are not metabolism-required or related, which can be efficiently eliminated enzymatically. For example, L-isoaspartyl residue and racemized D-aspartyl residue can be recognized and repaired by a methyltransferase (Clarke, 2003). Clinic and experimental gerontology has also found they are much slower and less important troublemakers during aging as they do not form irreparable polymers. The aging-related

decline of DNA methylation, deamination, protein degradation as well as disorder of histone ethylation are enzyme-sustained processes. Their decline is more likely the result of aging rather than the cause of spontaneous aging. Thiol oxidation, protein wrong folding, and crosslinking reaction are potential aging-related modifications caused by free radical oxidation and non-enzymatic glycation, which are only named differently referring to different research branches and interests.

To sum up, free radical oxidation and non-enzymatic glycation are the most important aging-related biological reactions that can go along spontaneously. Since these biological reactions do not belong to normal physiological and biological processes, they are specially termed as 'biological side-reactions' in this review. In fact, these two kinds of biological side-reaction have covered most (if not all) modification possibilities of biological 'fuel' associated damaging process (Bortz, 1986; Yin, 2003).

4. Intensive debate on the free radical theory and the importance of glycation theory of aging

Based on radiation-related free radical studies, Dr Harman formally put forward the free radical theory of aging in 1956 (Harman, 1956, 2003). Among other successes, some aging-associated achievements deduced from the theory are as follows (Halliwell and Gutteridge, 1999): (1) The free radical theory of aging explains to some extent the increase of metabolic rate and speedup of aging caused by free radicals and reactive oxygen species. (2) It clarifies preliminarily that oxygen free radical damages on cells and sub-cellular organelles (liposome, mitochondrion, etc.) can lead to damages, apoptosis, aging and even death of cells. (3) It explains the relativity of longevity differences between animal phyla and anti-oxidative capability. 4) Subsequent studies have found damages caused by oxygen free radicals which will engender age pigment-like fluorophores similar to lipofuscin (Sohal, 1981; Yin, 1996; Szweda et al., 2003). It has later been found that free radicals take part in almost every disease, acute or chronic. More importantly, almost all the recessive diseases related to aging, including cardio-vascular diseases, cancers, senile dementia, and diabetes, have close relationship with free radicals and damages they caused. Such pathological and physiological achievements seem to serve as a consolidated backbone for the free radical theory of aging.

However, experimental studies to verify the free radical theory of aging have confronted a number of bewilderments and refutations.

It has been revealed by more and more experiments that free radical reaction, as an elementary part of life chemistry, is an inevitable step of many biochemical reactions, and an intermediate step in redox-reactions of many oxidative enzymes besides its important defensive function in immunity; NO, for example, one of the oxygen free radicals and a neurotransmitter as well, has important physiological functions (Tuteja et al., 2004). The free radical hypothesis was thus questioned by Prof. Yu (1996) for revision due to its obvious defects: '(1) The source of reactive species responsible for oxidative damages is not limited to oxygen-derived free radicals; (2) Free radical generation is not stoichiometrically coupled to either oxygen consumption or metabolic rate; (3) Biological aging is not likely caused by the pathogenicity' of oxygen free radicals.

A number of experiments show that antioxidants resisting oxygen free radicals and oxidative stress are not elixir (Tochopherol Group, 1994; Olshansky et al., 2002). The redox situation within tissues is a balance of various antioxidants, which are substitutional and compensatory. Anti-oxidative enzymes may either increase, or decrease, or remain invariable with aging (Sohal and Allen, 1990).

For the above-mentioned troubles of the free radical theory of aging, Dr Sohal and others, while verifying the free radical theory of aging (Orr and Sohal, 1994), has tried also to build a new flagship explaining aging mechanisms: 'the oxidative stress hypothesis of aging' (Sohal and Allen, 1990; Yu and Yang, 1996). The reformed theory is reasonable, but it is unsatisfactory to consider oxidative stress to be the genuine aging mechanism. When even the oxidative stress theory was found failed with respect to maximum lifespan (MLS), Prof. Sohal explained this with several crucial reasons (Sohal et al., 2002): (1) 'ROS are produced at specific sites and attack nearby sensitive targets, which are not accessible to antioxidants'; (2) It may be 'too expensive for the cells to maintain (antioxidants required), either energetically and biochemically'; (3) 'A certain level of ROS production is beneficial, either in the immune response… or in the regulation of gene expression by signal transduction pathway …'.

While the understanding of aging mechanisms becomes much more complicated than ever before, we hereby suggest to illustrate the problem with a quite simple formula as given below (Formula 1):

As shown clearly in the formula, oxidative stress and associated damages are neither parallel with aging alterations nor correlated with maximum life span because there are uncertain repairing processes following up (Sohal et al., 2002; Park and Gerson, 2005). It is the remaining alterations after repairing which may mean the aging-related changes, e.g. non-degradable crosslinkages, amyloids and lipofuscin accumulated with age. Generally speaking, oxidative damages bring up acute pathologies, while irreparable alterations define the physiologic and chronic changes,

Aging alterations (may relate to MLS) = **Damages** (including by ROS) – **Repairing (?)**
(crucial aging processes) *(various aging causes)* *(largely unknown mechanisms)*

Formula 1. Aging mechanisms (and processes) in a general view.

namely aging changes. These may explain afore mentioned contradictory data to correlate ROS with MLS (Sohal et al., 2002). Detailed discussion about damage and accumulation chemistry will be given in the following sections.

In fact, oxidative stress damage is just one of the various biological side-reactions related to aging. Accelerated aging phenotypes of diabetic patients have been found solely due to high glucose content ensued hyperglycemia (Cerami, 1985; Monnier, 1990; Litchfield et al. 1999). The glycation-related aging process, basically (such as at the initiating stage) does not depend on oxidation process (Yin and Brunk, 1995; Litchfield et al. 1999; Baynes, 2001).

Researches on biochemical mechanism of aging in recent years give more attention to another important aging theory-the glycation theory of aging which is closely related to the energy metabolizing of organisms and thus represents another important universal theory of aging at the molecular level. Since Maillard primarily reported glycosylation reactions (Maillard, 1912), it is also called glycation/Maillard reaction theory of aging.

According to relevant researches, under certain physiological conditions glucose can react with many amino acids, peptides and amino-groups in proteins and result in Shiff bases, whose reconstruction with molecules can produce comparatively steady Amadori products (Maillard, 1912; Hodge, 1953; Cerami, 1985). Further degradation, like deamination and hydrolyzation, of this product can create secondary products, various unsaturated carbonyls, e.g. glyoxal, methylglyoxal, deoxyglucosones (Thornalley et al., 1999; Baynes, 2000). These secondary products are similar to those produced in lipid oxidation, and their reactivity is very similar to that of advanced products in lipid oxidation (ALEs). For example, they crosslink and polymerize with the amino-group of proteins and nucleic acids, thus producing browning biological garbage and fluorophores in various shapes (Baynes, 2000; Metz et al., 2003). At present these products are generally called advanced glycation end-products (AGEs), such as pyrimidines and 2-(2-furoyl)-4-(2-furannyl)-lH-imidazole (Pongor et al., 1984), pyrralines or pyrroles (Hayase et al., 1989), pentosidines (Grandhee and Monnier, 1991; Sell et al., 1991), naphthyridinium salts (also called crosslines) (Nakamura et al., 1992), vesperlysines (Tessier et al., 1999), *N*-(carboxymethyl)lysine (CML) (Thorpe and Baynes, 2002) et al. and their biochemical complex in vivo (Scheme 1).

The glycation theory points out that the crosslinking damage of proteins caused by glycation is the primary reason of aging. Crosslinking and denaturation of proteins caused by glycation are the key factors for early aging-related alterations of blood vessel, kidney, lung and joint (Monnier, 1990; Miyata et al., 2003). Important amino residues that react with toxic carbonyls are Lys, Asp, His, Tyr, Trp, Ser, Thr (Stadtman, 1992, 2003). This may results in the hardening of structural proteins, damages of functional proteins like aging damages of anti-oxidative enzymes and DNA restoration enzymes, and aging changes such as decreasing supply of energy, decline of metabolizing function, disruption of physiological homeostasis, etc.

Glycation has an important bearing on oxidative aging because glycation reaction is very slow, many experiments of it are under ambient oxygen, and oxidation process related to oxygen free radicals surely speed up and promote glycation reaction. (Monnier, 1990; Baynes, 2000).

In 1992, Doctors Kristal and Yu (1992) suggested to combine free radical oxidation and non-enzymatic glycation into a new theory of aging-the free radical/glycation reaction theory of aging so that the two theories can complement each other to explain many problems concerning aging mechanisms that remain unsolved at present.

5. Free radical oxidative stresses and the crucial role of unsaturated carbonyls during aging

According to the free radical theory of aging, senescence and a variety of degenerative diseases associated with it are attributed primarily to the deleterious attack of oxygen free radicals on cellular constituents, including chromosomes, mitochondrial DNA and connective tissues (Halliwell and Gutteridge, 1999; Esterbauer et al., 1991). Univalent reduction of oxygen gives rise to damaging oxidative species:

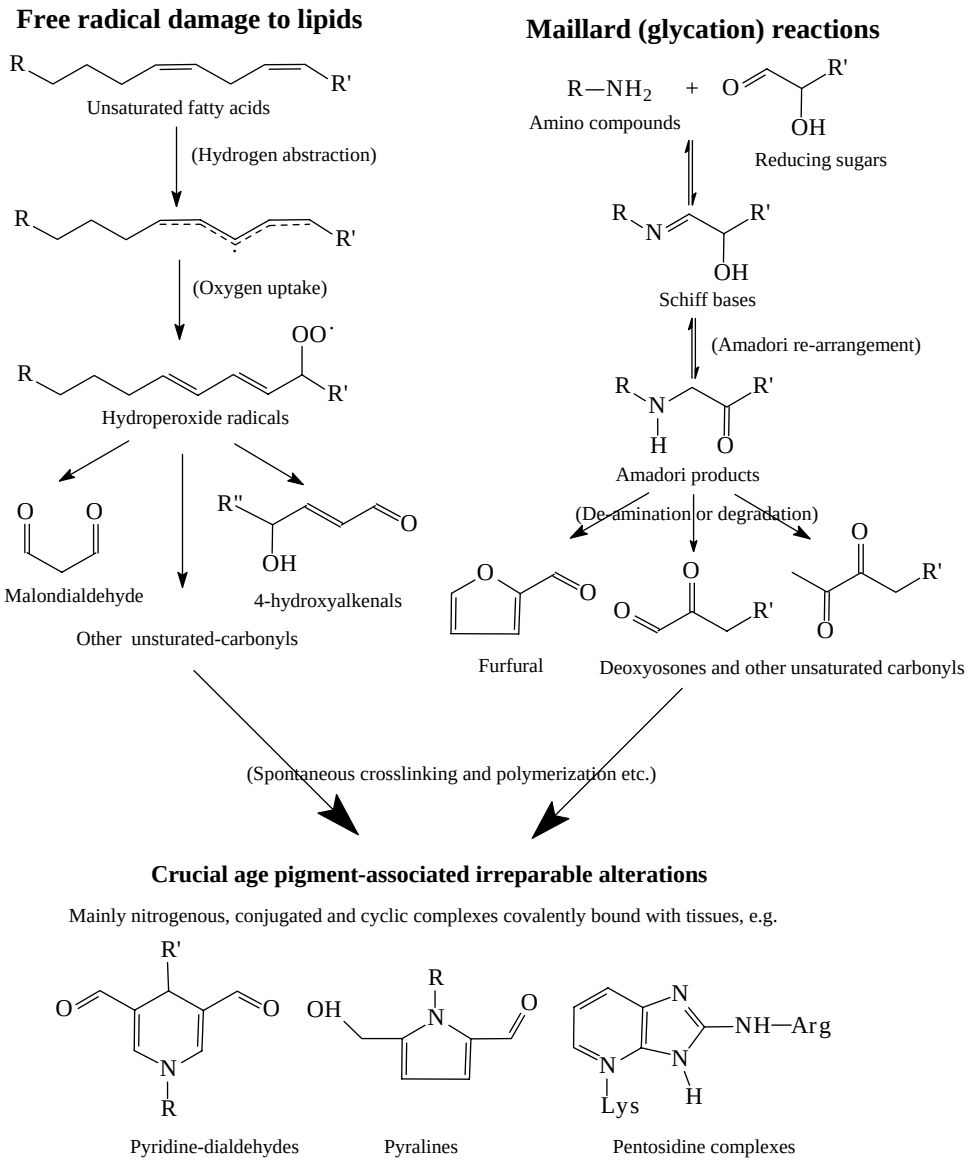
$$O_2 \xrightarrow{e} O_2^{-\cdot} \xrightarrow{e} H_2O_2 \xrightarrow{e} HO^{\cdot} \xrightarrow{e} H_2O$$

However, the stepwise oxidation of various essential biological components, shown in Scheme 2, may need to receive more considerations.

Among other molecular groups shown in Scheme 2, carbonyl compounds (biomolecules that contain a carbon–oxygen double bond, or carbonyl group, mainly aldehydes and ketones) are active intermediates, particularly when they are conjugated with a secondary functional group. A large body of knowledge (see Scheme 1) about their biological occurrence, mechanism of formation, reactivity and bio-toxicity has been obtained mainly through studies of lipid peroxidation (Comporti, 1985; Halliwell and Gutteridge, 1999).

Lipid peroxidation is mainly initiated by hydrogen abstraction from unsaturated fatty acids by oxygen-centered radicals followed by the formation of hydroperoxides. Degradation of hydroperoxides results in a variety of derivatives including various carbonyl products (Schauenstein and Esterbauer, 1979; Esterbauer et al., 1991). Such unsaturated carbonyls include enals, dienals, trienals, hydroxylenals, 2-ketoaldehyes, deoxyosones and various reductons that are all very reactive and toxic to almost all cellular and extracellular biomolecules (Esterbauer et al., 1991; Yin and Brunk, 1995; Baynes, 2000).

Many carbonyls react readily, even at neutral pH and room temperature, with important biochemical groups, such as amino, thiol or hydroxyl. A secondary functional

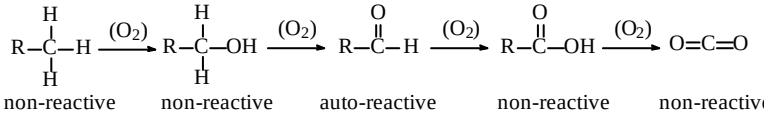


Scheme 1. Biological side reaction induced irreparable complex during aging.

group of the carbonyls increases the reactivity potential and may induce irreversible reaction products, or result in crosslinking reactions (Bjorksten, 1968). Napetschnig (1981) reported that 4-hydroxylalkenals could react with nearly all amino acids under appropriate conditions. Reactions of 4-hydroxylalkenals and malondialdehyde (MDA) with amino groups in proteins and nucleic acids have also been extensively studied (Wolman, 1980; Winter et al., 1986).

Due to their reactivity, the carbonylic products, particularly α,β-unsaturated carbonyls of lipid peroxidation, are implicated in various types of cell damage, including depletion of glutathione, protein modification, disturbance of calcium homeostasis, retardation of respiration and glycolysis, cell membrane destruction, tissue injuries, enzyme inhibition and decreased DNA, RNA and protein synthesis (Esterbauer et al., 1991; Schaur, 2003; Eckl, 2003; Zarkovic, 2003).

Compared with vanishing oxygen free radicals, toxic carbonyls can pass a long distance through organ tissues and cellular membrane and reach almost any place of our body (Esterbauer et al., 1991).



Scheme 2. Principle of stepwise oxidation of biomolecules.

While oxidative damage is an external cause, carbonyl toxification causes internal changes. External causes are causal factors of aging; internal alterations carry the subject, our body, to senesce. When free radicals disappeared after quick attack, or clearance, or restoration, carbonyl related crosslinkage remain at any possible biological corner. If garbage scavengers (e.g. repairing enzymes) do not appear, aging-related changes appear for sure.

6. Studies of age pigments evolving into a carbonyl stress hypothesis of aging

Up to date, a large number of biomedical studies indicates that both lipid peroxidation and non-enzymatic glycation will lead to formation of lipofuscin-like substances (age pigments) via unsaturated carbonyls (Wolman, 1980; Yin, 1996; Szweda et al., 2003). Taking thiobarbituric acid reactive substances (TBARS) for example (Uchiyama and Mihara, 1978), a most popular index of unsaturated carbonyls, diseases such as inflammation, fever, stroke, and operation can result in higher content of TBARS in body fluid (Yagi, 1982; Kosugi et al., 1994). Even normal tension or stress will speed up this aging-related process in which unsaturated carbonyls are enhanced. For example, TBARS in the blood serum of old people is twice as much as that of young people (Yagi, 1982), while in that of diabetic patients is one and a half times as much as that of normal people (Sato et al., 1979).

Not only related with aging and diseases, unsaturated carbonyls are found increased during daily activities like exercise and sleep. Urine TBARS of a human subject after intensive exercise is 5–6 times more than that of normal people, while in that of a sleep-deprived subject is 8–10 times more than that of control (Kosugi et al., 1994). It remains almost certain that whenever the content of TBARS becomes higher, carbonyl stress leading to aging-related alterations will be speeded up (Comporti, 1985; Esterbauer et al., 1991; Yin, 1995). An apparent feature of such a reaction is a higher content of ceroid (misnamed as melanin in some reports), precursor of age pigments in the human body (Porta, 1990; Yin, 1996; Hegedus, 2000).

In 1992, Yin pointed out that carbonyl toxification, concurrent occurring in the two important biological side-reactions, the free radical oxidation and non-enzymatic glycosylation, is the key process of age pigments' formation (Yin, 1992). Yin also has further clarified that carbonyl stress may be the key process of the slow inevitable aging process, because the crosslinkages from carbonyl stress are mostly irreparable (Yin, 1995; Nilsson and Yin, 1997).

Researches on 4-hydoxylnonenal (HNE) toxicities, highlighted recently by activities of an international HNE Club, have demonstrated a variety of evidences about toxification of HNE in different systems (Esterbauer et al., 1991; Schaur, 2003; Eckl, 2003) from physiology to pathology (Dianzani, 2003; Zarkovic, 2003). Studies of AGE receptors and immunological detection of AGEs and ALEs have also shown genotoxic and cytotoxic evidences from autopsy of corpse and from samples of patients with inflammation, diabetes mellitus, atherosclerosis, nephrosis and neuronal degenerative diseases (Yan et al., 2003; Yamagishi et al., 2003; Chavakis et al., 2004).

Stadtman and co-workers in the National Institutes of Health presented another important type of aging-related bio-molecular alterations (Stadtman, 1992, 2003). They have found that carbonyls content in protein of aged animals are much higher than those of young ones. Accordingly that, 40–50% of protein in aged animals has been oxidized and changed into protein carbonyls (Stadtman, 1992, 2003). These data (although variable, the tendency is clear) and the results of lipid peroxidation studies, which confirm each other, account for the fact that carbonyls increase with age. Undoubtedly, the increase of protein carbonyls will lead to the spontaneous carbonyl-amino crosslinking and accumulation of irreversible changes associated with aging.

The toxic carbonyls inducing protein aggregation was actually the key process in the crosslinkage theory of aging as described by Bjorksten in 1968 (Bjorksten, 1968). Compared with the steady-state physiological concentration of unsaturated carbonyls (a few micromolar), the other crosslinking factors are much trivial and conditional, and other important crosslinking factors have not been recognized and reported since then.

According to the above-mentioned data and studies on the formation biochemistry of age pigments, Yin and Brunk have put forward a carbonyl toxification (stress) hypothesis of aging in 1995 (Yin, 1995; Yin and Brunk, 1995). Recent developments about the carbonyl toxification theory will be presented in a separated review.

A critical confusion in understanding aging process is to tell causes from consequences (de Magalhaes, 2005). The carbonyl stress as profoundly studied recently by Miyata and coworkers (2000, 2003), although appears to be a secondary cause after different stresses, is a virtually indisputable causal factor inducing aging-related changes. The carbonyl stress is more essential than oxygen free radicals during aging simply because it contributes almost directly to the 'crucial aging process' part of the afore mentioned formula (Formula 1).

Another confusion often encountered in the field is mixing up acute and chronic changes of organisms. A basic notion of the carbonyl toxification hypothesis of aging is to emphasize the slow daily biochemical modifications resulted from a few common biological side-reactions, hence stressing also the importance of producing and elimination of daily biological garbages (Yin and Brunk, 1995; Terman, 2001). A de-carbonylation process was also proposed to be a daily anti-aging chemistry during sleep (Yin, 2000).

Although a clear picture of biological aging mechanisms cannot be obtained directly through a retro-deduction of the accumulation chemistry of biological garbage, a general

picture of aging process starts to emerge. Because no matter how complex the aging process is, the main clues and the final results are available, especially the net result of impairments and restoration, which are the accumulation of age pigments, the hallmark of aging (Sohal, 1981; Porta, 1990; Yin, 1996).

7. Discriminating causes (damages) from irreparable alterations during aging

It is necessary to point out that the 'causal factors' and the 'process' of aging can be considered two different concepts, just as a causal factor of a disease may not be the process of the disease per se (see also Formula 1). Free radical oxidative stress should undoubtedly be regarded as a causal and also an accelerative factor of aging (such kind of damage is mostly non-crosslinking); however, the crosslinking reaction of unsaturated carbonyls with amino compounds seems to be a more important reaction inducing aging-related alterations. This is simply due to the fact that unsaturated carbonyl has a molecular structure of 'second bite' (namely, it contains two conjugated carbonyls) so that a further reaction is possible if a detoxification process fails to follow. As a result, steady crosslinking (of low energy potential due to cyclization and/or conjugation!) and irreversible by-products can be produced. Organisms have no enzyme to break down such side-reaction induced by-products (e.g. AGEs and ALEs) with their limited (gene programmed) types of enzymes, so they have to put up with them, then pile them up (Yin, 1995; Terman, 2001).

In most cases, both free radical oxidation and non-enzymatic glycation will lead to damages of biologic molecules in all kinds of forms. Oxygen free radical will cause substantive acute damages, especially when a patient suffers from diseases. However, organisms never cease to repairing and renewing, including via apoptosis at the cellular level, lysosomal endophagocytosis at the sub-cellular level, reductive degenerating (GSH reduce and sulfur bridge disassociation, etc.) at the molecular level, degradation and renewal of protein, repair of DNA (DNA repairing speed is some dozens of times higher than that of damaging), and so on (Park and Gerson, 2005). As a result, though vital tissues get injured intensely and violently every day, thanks to repair and restoration in time, they are still not on the verge of death or destruction. It is mainly the crosslinking (second bite)-related alterations which cannot be completely repaired or renewed so that they will accumulate. Belonging to time-dependent modification, it is such an accumulation being the irreparable impairment of aging.

Although animals have substantial recovery system resisting carbonyl stress, their biological molecules are still surrounded by constantly produced unsaturated carbonyls (Esterbauer et al., 1991; Yin, 1995; Baynes, 2001). As a matter of fact, the gradual physiological variations of organism induced by various poisonous carbonyl compounds are universal and inevitable. If the instant damage of the free radical/Maillard reaction belongs to pathological cause, then the carbonyl stress induces mainly the irreversible accumulative physiological aging.

Nonetheless, damages can be caused differently and can be quick or slow, in healthy or unhealthy organisms; however, irreparable accumulation is the essence of the mild chronic alteration process, and thus is the aging mechanism.

In brief, damages are causal factors rather than the process of aging. Accumulation, a surplus of damage minus repair, is the process (Formula 1). The accumulation speed may thus understandably parallel to the speed of aging. Lipofuscin, age pigments, blood vessel stiffening and organ fibrosis etc., all of which are leftover alterations, are important manifestations of aging.

8. How life is conquered by entropy increase during aging

In order to know more profoundly the biological mechanisms of aging, it is necessary to survey this 'super-problem' of life sciences in a broader field into physics and chemistry.

It is well known that energy of material obeys the basic laws of thermodynamics. According to the second law of thermodynamics, entropy in an isolated system is sure to increase with time until to reach a lowest energy state, which is the so-called law of entropy increase (Bortz, 1986; Roth, 1993).

As early as in 1947, Schrodinger pointed out farsightedly that entropy increase must be embodied in life system (Schrodinger, 1947). Man's body is a reaction center of chemical activities and metabolizing processes. To some extent, the stability of life lies in the ability of resisting entropy increase of itself. In life chemistry there are interdependent spontaneous and non-spontaneous processes; however, because of the inevitable entropy increase, organisms experience a continuous process from ordering to disordering, and to aging and death irreversibly. In recent years, scientists in different fields have mentioned entropy increase theory of aging occasionally (Sacher, 1967; Bortz, 1986; Roth, 1993), but it has been kept in limbo for a variety of reasons.

The primary problem for the entropy theory of aging being ignored may be that the precondition of the second law of thermodynamics applies to a closed energy system whereas everyone's body and even individual cells are all open systems of energy (Kirkwood, 1999). Such open systems can easily obtain enough energy to overcome entropy. Actually, what entropy theory of aging is requested to answer, namely the crucial problem, is 'what is the exact biochemistry behind the entropy increase during aging'. Scientists have been missing an adequate resolution until recently (Yin, 2003; Yin and Chen, 2005).

Biological side-reactions, which have been discussed comprehensively in the foregoing chapters, occur

spontaneously in life activities, mainly including free radical oxidation, non-enzymatic glycation, carbonyl stress and protein crosslinking, etc. provide the best answer. The irreparable impairments mentioned above are the molecular explanation of entropy increase. For example, protein cyclic conjugation is an exoenergetic process (see Scheme 1) that makes life system to increase entropy, and stochastic by-products of crosslinking accumulate gradually because they cannot be degraded by normal proteinases, such as the crosslinked connective tissues and other protein complex like lipofuscin, amyloids, tangles or senile plaques in vivo (Yin, 1996; Bailey, 2001; Binder et al., 2005).

9. Perspectives: proposing the aging mechanisms in general

The exploration of aging mechanisms has involved almost a full scale of life science; however, at the same time, most scholars are becoming more and more puzzled about the real nature of the aging of humans. The problem is, probably, that we have ignored (or viewed only superficially) several essential biological deteriorative processes, for example, the spontaneous and universal biological side-reactions inside organisms, the producing and cleaning biochemistry of daily biological garbage. Open-mindedly, the tiredness and sleep mechanism that are ambiguous up to date are also responsible for the confusion (Yin, 2000; Prinz, 2004).

Moreover, the accumulation of minute impairments in the immune system and neuronal endocrine systems of organisms may result in a 'snowball' effect so that the aging process of animals and human beings will speed up pathologically in their late years (Bortz, 1986; Kirkwood, 1999; Yin, 2002).

In a narrow sense, crosslinking reaction such as carbonyl stress is one of the main approaches of biological aging process; however, irreparable accumulation of biological side-reactions seems to be the mechanism of aging in a broad sense. Based on the accumulation of impairments in biological side-reactions, this review proposed a general aging mechanism of higher animals. Seizing the aging essence out of the complex phenomena at the molecular level, it puts forward a novel unified biochemical theory of aging. Some other principles of maintaining health and preventing biological damages are connoted in the hypothesis. No matter what other characteristics the whole aging process may conceal, anti-aging strategies of human beings can certainly get useful inspiration from eliminating biological side-reactions as elaborated above.

In summary, direct DNA damage and mutation in comparison with protein impairments are either less important or disease-related, which may not be the crucial issue of physiological aging of higher animals. Whereas biological systems of anti-stresses, protein turnover, metabolisms and homeostasis regulated by genetic network are the key elements of aging mechanisms, various irreparable accumulations of protein alterations induced by spontaneous biological side-reactions turn out to be the center of aging biochemistry. The carbonyl stress highlighted by the free radical/glycation reactions and age pigment studies seem to represent the universal and fundamental molecular processes beyond others following the 'time's arrow'. Such crosslinking/conjugation substantiated entropy increase seems, to the best of our knowledge, to have played a critical role during physiological aging processes.

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