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GLUTATHIONE, OXIDATIVE STRESS AND AGING

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ABSTRACT

The free radical theory of aging proposes that the impairment in physiological performance associated with aging is caused by the detrimental effects of oxygen free radicals. This is interesting because it provides us with a theoretical framework to understand aging and because it suggests a rationale for intervention, i.e., antioxidant administration. Thus, the study of antioxidant systems of the cell may be very important in gerontological studies. Glutathione is one of the main nonprotein antioxidants in the cell which, together with its related enzymes, constitute the "glutathione system." The involvement of glutathione in aging has been known since the early seventies. Several studies have reported that reduced glutathione is decreased in cells from old animals, whereas oxidized glutathione tends to be increased. Recent experiments from our laboratory have underscored the importance of cellular compartmentation of glutathione. Mitochondrial glutathione plays a key role in the protection against free radical damage associated with aging. Oxidative damage to mitochondrial DNA is directly related to an oxidation of mitochondrial glutathione. In fact, aging is associated with oxidative damage to proteins, nucleic acids, and lipids. These molecular lesions may be responsible for the low physiological performance of aged cells. Thus, antioxidant supplementation may be a rational way to partially protect against age-associated impairment in performance. Apoptosis, a programmed cell death, is an area of research which has seen an explosive growth. Glutathione is involved in apoptosis: apoptotic cells have lower levels of reduced glutathione, and administration of glutathione precursors prevent, or at least delay, apoptosis. Age-associated diseases constitute a major concern for researchers involved in aging. Free radicals are involved in many such diseases; for instance, cancer, diabetes or atherosclerosis. The key role of glutathione and other antioxidants in the pathophysiology of aging and age-associated diseases is discussed in this review.

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INTRODUCTION

Glutathione is the most abundant nonprotein thiol in the cell. Its role in cell metabolism and physiological functions has been emphasized (Viña, 1990). The importance of glutathione in aging was initially studied by Pinto and Bartley (1969) and by Lang and his colleagues (Hazelton and Lang, 1980).

The free radical theory of aging proposed by Harman (1956) suggests that antioxidants may be administered to diminish age-associated impairments in physiological performance. Maintenance of an adequate glutathione status may be important in understanding aging.

Cell compartmentation of antioxidants, especially glutathione, is important because many of the free radical species generated in the cell are highly reactive and will attack preferentially those cell components which are close to the organelle in which the radicals are generated. This is especially important for mitochondria. About 2% of all oxygen used by the cell is not converted to water but to reactive oxygen species. Most of these are generated in mitochondria. Miquel and co-workers have emphasized the role of damage to mitochondria, and especially mitochondrial DNA, in aging (Miquel et al., 1980). We recently found that mitochondrial glutathione and, to a greater extent, extramitochondrial glutathione, is oxydized with aging and correlates with mitochondrial DNA damage (García de la Asunción et al., 1996).

These facts suggest an approach to minimize the damaging effect of aging on cell function; i.e., the administration of antioxidants, which may counteract in part the damaging effect of free radicals in cell physiology. Indeed, the protective effect of antioxidants, especially antioxidant vitamins, has been proven. We found that those antioxidants which protect against glutathione oxidation are effective in partially protecting against the loss of physiological performance which is observed in aging *in vivo* (Viña et al., 1990).

Free Radical Theory of Aging

Aging is characterized by a decline in physiological performance in many organs. The aging process is multifactorial, and many theories have been put forward to explain it (for review see Medvedev, 1990). One of the most prominent is the free radical theory of aging, which was first proposed by Harman in 1956. According to this theory, oxygen-derived free radicals cause damage to cells, which leads to age-associated impairments in functions at cellular and organic levels (Harman, 1956).

Knowledge of free radical reactions and their roles in biological systems has increased rapidly during the last three decades (Pryor, 1986, Ames et al., 1993). Reactive oxygen species, a term used for oxygen free radicals, peroxides and singlet oxygen, are generated continuously in aerobic cells through different mechanisms. These include the mitochondrial respiratory chain, the microsomal cytochrome P-450 system, phagocytosis, peroxisomes, prostaglandin synthesis, ionizing radiations, signal-transduction pathways and the activity of oxidases such as xanthine oxidase.

In order to detoxify reactive oxygen species, cells are provided with enzymatic and nonenzymatic antioxidant systems. Enzyme activities involved in cellular defense are mainly superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase; nonenzymatic antioxidants include reduced glutathione (GSH), ascorbate, tocopherol, carotenoids, etc.

The free radical theory of aging assumes that cellular antioxidant systems are not able to cope with the reactive oxygen species generated continuously throughout cell life. Thus, cellular aging would be associated with a "chronic" oxidative stress, which was defined by Sies (1986) as a disturbance in the balance between prooxidants and antioxidants, in favor of the former.

At present, a great deal of experimental evidence supports the free radical theory of aging (Harman, 1991; Ames et al., 1993). This includes:

a) the inverse relationship between the average life spans of mammalian species and their basal metabolic rates;
b) the increase in the mean life span of transgenic flies expressing high levels of enzymatic antioxidants;
c) evidence of free radical damage to cell components upon aging;
d) the increase in the mean life spans of several species following dietary supplementation with antioxidants;
e) evidence of involvement of oxygen free radicals in the pathogenesis of degenerative diseases associated with aging.

Points (a) and (b) will be discussed here, while points (c), (d) and (e) will be commented on in detail later.

In 1908, Max Rubner was the first to point out the inverse relationship between longevity and the amount of energy necessary for increases of weight by body growth. This finding prompted Rubner to propose the rate of living theory of aging. According to this theory, there is an inverse relationship between the rate of oxygen consumption as an index of basal metabolic rate and the maximum life span potential (MLSP). This theory explains the differences in MLSP among species in most, but not all, cases. Exceptions to this theory are birds and primates (Ku et al., 1993; Barja et al., 1996). These groups exhibit high oxygen consumption and high longevity simultaneously. Nevertheless, it has been shown that mitochondrial production of free radicals is lower in pigeons than in rats (Ku *et al.,* 1993; Barja et al., 1996). Thus, the exceptions in the rate of living theory are not contradictory to the free radical theory of aging, as mitochondria from birds may use oxygen more efficiently and exhibit less free radical leakage through the respiratory chain.

Clear-cut evidence in favor of the free radical theory of aging is that simultaneous overexpression of copper-zinc superoxide dismutase and catalase genes in transgenic *Drosophila* extends their mean and maximum life span (Orr and Sohal, 1994). Furthermore, the overexpression of these enzymes slows the aging process in flies, since transgenic flies exhibited a delayed loss of physical performance and a lower amount of oxidative protein damage (Orr and Sohal, 1994). However, overexpression of copper-zinc superoxide dismutase alone or catalase alone in transgenic flies did not affect the life span (Seto et al., 1990; Orr and Sohal, 1992). These results point to the key role of the joint action of different antioxidants in the aging process.

Barja and co-workers have also obtained support in favor of the role of antioxidant defense in aging. They have found that a simultaneous induction of superoxide dismutase, glutathione reductase, GSH and ascorbate synthesis, following complete inhibition of catalase activity, increases the mean life span in frogs (López-Torres et al., 1993). Again, an extension of life span is obtained by increasing different components of antioxidant defense.

The Glutathione System: Role in Aging

The involvement of glutathione metabolism in aging has been known since the work of Pinto and Barley (1969) in the late sixties. If free radicals are related to aging, it seems obvious that glutathione, a major antioxidant in mammals, would be oxidized in the cells of old animals. Glutathione oxidation produces an increase in its oxidized form (GSSG) and probably a decrease in GSH, the reduced form. Different authors found an age-related glutathione oxidation in several animal models (Hamilton and Lang, 1980; Allen and Sohal, 1986; Viña et al., 1992) or even in humans (Goldschmidt, 1970). Glutathione oxidation in aging can be due to an increase in the production of oxidative species, a decreased reductive capacity, or both factors.

Changes in glutathione redox status can be induced by variations in the activity of glutathione related enzymes, as seen in Figure 1. The aging effect on these enzymes has been studied in several laboratories using various cell types and approaches (Pinto and Barley, 1969; Santa María and Machado, 1987; Al-Turk et al., 1987; Yen et al., 1989; Benzi et al., 1989; Viña et al., 1992).

In general terms, aging is associated with a decrease in the activity of enzymes which catalyze reactions tending to reduce GSSG, such as glucose-6 phosphate dehydrogenase or glutathione reductase, rather than with an increase in the activity of those enzymes which favor oxidation of glutathione, such as glutathione peroxidase or transferase (Viña et al., 1992).

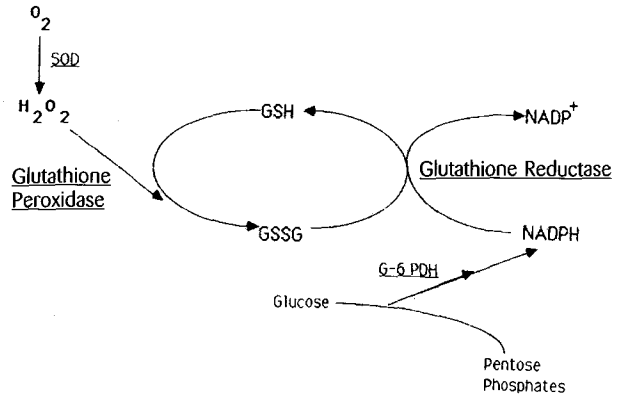

Figure 1. Glutathione redox cycle

When GSH is oxidized, GSSG tends to be released from cells (Sies et al., 1978). Thus, the oxidation of glutathione in aging may increase the rate of glutathione release from cells. We, as well as other investigators, have demonstrated that GSSG increases in blood from old rats (García de la Asunción et al., 1996). So far, glutathione compartmentation during aging has not received sufficient attention. We recently found that glutathione oxidation increases in mitochondria from liver, kidney and brain of old rats. This increase was much higher in mitochondria from brain tissue. No significant variations were found in the GSH concentration. The increase in GSSG (and in the GSSG/GSH ratio) correlates with mitochondrial DNA (mtDNA) oxidation. In a similar pattern, blood GSSG concentration was found to be higher in aged rats. These results support the idea that mitochondria are a major source of free radicals in aging as suggested by Sohal and Dubey (1994), and emphasize the relevance of mitochondria as primary targets of damage associated with aging (Miquel et al., 1980).

Changes in glutathione synthesis occur in some tissues, such as in the eye lens. Eye lenses from young rats or mice synthesize GSH from methionine or N-acetylcysteine. However, we found that lenses from old animals do not synthesize GSH from methionine due to the absence of gamma-cystathionase activity in old lenses (Ferrer et al., 1990). Thus, the impairment of the trans-sulfuration pathway may be responsible, at least in part, for the glutathione depletion found in lenses of old animals. Furthermore, Ritchie et al. (1994) showed that correlation of a glutathione deficiency in the aging mosquito increases its longevity.

Role of mitochondria in cell aging

Mitochondria generate high levels of oxygen radicals, since 2% of all oxygen used by mammalian mitochondria does not form water but oxygen-activated species (Chance et al., 1979). Furthermore, formation rates of $O_2^{-\cdot}$ and H_2O_2 by mitochondria increase with age (Nohl and Hegner, 1978). It is well known that fixed post-mitotic cells accumulate various age-related pigments, especially lipofuscin. A considerable amount of this pigment may derive from injured mitochondria (Miquel et al., 1978). All these findings prompted Miquel and co-workers to propose the mitochondrial theory of cell aging (Miquel et al., 1980). This theory suggests that senescence is a by-product of oxy-radical attack to the mitochondrial genome (mtDNA) of fixed post-mitotic cells (Miquel et al., 1980). According to Miquel and Fleming, it is essential that cells contain "differentiated" mitochondria for cellular aging to occur (Miquel and Fleming, 1986). Such mitochondria use high levels of O_2 due to the high energy requirements of somatic cells, thereby releasing O_2 radicals which exceed cellular homeostatic protection (Miquel and Fleming, 1986).

Many studies have demonstrated oxidative damage to mtDNA, protein and lipids, as well as changes in mitochondrial function and morphology upon aging (for a review see Shigenaga, 1994). The role of old mitochondria in cell aging has also been outlined by the degeneration induced in cells microinjected with mitochondria isolated from fibroblasts of old rats (Corbisier et al., 1990).

Several features of mtDNA make it especially susceptible to oxidative damage and mutation. Mitochondrial DNA mutates at least 10 times more frequently than nuclear DNA (Richter et al., 1988). Furthermore, it lacks protective histones or effective repair systems and has no introns, so that mutations are likely to affect coding DNA sequences (Johns, 1995).

Oxidative lesions in mtDNA accumulate with age in human and rodent tissues (Ames et al., 1993; García de la Asunción et al., 1996). As suggested before, this damage to mtDNA may affect transcription of mitochondrial genes. Indeed, an age-related decrease in the levels of mitochondrial transcripts in some rat tissues and in *Drosophila* has been reported (Gadaleta et al., 1990; Calleja et al., 1993).

The age-associated increase in the level of common deletions produced spontaneously in the absence of inherited cases seems to be very low (<0.1%) and may not be significant (Shigenaga et al., 1994). Nevertheless, these deletions may represent only a small portion of the numerous deletions and point mutations which might accumulate with age. Indeed, several studies have found increased deletions, point mutations and aberrant forms in mtDNA of postmitotic tissues upon aging (Linnane et al., 1989; see review of Wallace et al., 1994). On this basis, it was suggested that mtDNA mutations may be important contributors to aging and neurodegenerative diseases. (Richter et al., 1988; Linnane et al., 1989).

Regarding peroxidation of mitochondrial lipids upon aging, it should be mentioned that this appears due in part to changes in membrane lipid composition which enhance its susceptibility to oxidative damage (Shigenaga et al., 1994). A progressive decline in the amount of linoleic acid, together with an increase in the amount of long-chain polyunsaturated fatty acids (which are more sensitive to oxidation), has been reported (Shigenaga et al., 1994).

Another important change in mitochondrial lipid composition is the age-related decrease found in cardiolipin content. It decreases with age in heart, liver and non-synaptic brain mitochondria. Cardiolipin is required for optimal catalytic activity of many inner mitochondrial enzymes. Thus, modifications in cardiolipin composition may be involved in the age-related changes of certain activities, such as those of the respiratory chain (Shigenaga et al., 1994).

Several studies have reported a decline in activities of complex I (NADH:Co Q reductase), complex II (succinate: ubiquinone oxidoreductase) and complex IV (cytochrome c oxidase). Moreover, the respiratory activity of isolated mitochondria decreases with age in liver, skeletal muscle and brain (Yen et al., 1989; Trounce et al., 1989; Beal et al., 1993). Age-related changes in mitochondrial membrane potential and transport systems have also been reported (Hansford, 1978; Paradies and Ruggiero, 1978; Tummino and Gafni, 1991; Sastre et al., 1996).

A correlation between age-associated changes in mitochondrial function and morphology has been reported (Shigenaga et al., 1994; Sastre et al., 1996; De la Cruz et al., 1990). Enlargement, matrix vacuolization, shortened cristae and damage to mitochondrial crests have been found in mitochondria from old animals (De la Cruz et al., 1990; Sastre et al., 1996). Changes in mitochondrial ultrastructure may modulate mitochondrial function (Scalettar et al., 1991). Thus, volume-dependent regulation of matrix protein packing modulates metabolite diffusion and, in turn, mitochondrial metabolism (Scalettar et al., 1991). On the other hand, the damage to mitochondrial crests which occurs in old mitochondria may be responsible, at least in part, for the age-related impairment in mitochondrial membrane potential and in mitochondrial membrane activities (Sastre et al., 1996).

An increased generation of oxygen free radicals may be responsible for the decline in the activity of mitochondrial membrane proteins, such as metabolite carriers and respiratory chain complexes. In fact, it is known that exposure of mitochondria to free radicals causes impairment of the mitochondrial inner-membrane proteins (Takeyama et al., 1993). In addition, studies in isolated mitochondria have shown that an acute oxidative stress causes an inhibition of mitochondrial respiration (Corbisier et al., 1990). Thus, the damage to mitochondria which occurs upon aging may be due to an age-associated oxidative stress. Furthermore, oxygen free radicals may be responsible for the change in mitochondrial size which is found upon aging. It is well known that acute oxidative stress causes mitochondrial swelling (Takeyama et al., 1993). Thus, we suggest that age-associated chronic oxidative stress may be the cause, at least in part, of both mitochondrial swelling and a decline in mitochondrial function.

Several studies have found an increase in peroxide generation by mitochondria from old animals (Sohal et al., 1990; Sohal, 1991; Sastre et al., 1996). Furthermore, the rate of H_2O_2 generation by mitochondria correlates inversely with intra- and inter-species variations in longevity (Sohal, 1991). These results support Sohal's hypothesis that the rate of pro-oxidant generation may be a key factor in the rate of aging (Sohal, 1991).

All of these findings support the hypothesis that mitochondrial damage plays a key role in the aging process. However, we should emphasize that most mitochondrial changes were found in experiments using isolated mitochondria. Thus, some of these effects could be due to an increased susceptibility of old mitochondria to stress caused by the isolation procedure. Moreover, when intact cells were not used, the mitochondrial-cytosolic interactions were also ignored. This might lead to errors. Therefore, whole cells, such as isolated hepatocytes, should be considered the best model for studies on mitochondrial aging (Sastre et al., 1996).

We have measured the rate of biochemical pathways which critically depend on mitochondrial function in isolated hepatocytes. Gluconeogenesis from lactate plus pyruvate, but not from glycerol or fructose, fell with aging in isolated hepatocytes. Gluconeogenesis from lactate, but not from glycerol or fructose, involves mitochondria. The lower rate of gluconeogenesis from lactate plus pyruvate is due to an impaired mitochondrial handling of malate. Indeed, we have observed that malate accumulates in liver mitochondria of old animals, and this is due to a diminished malate export from liver mitochondria of old rats. Furthermore, post-transcriptional modifications appear to be involved in the age-related impairment of the malate carrier.

Mitochondrial glutathione plays a key role in the protection against the free radical damage associated with aging. Oxidative damage to mitochondrial DNA which occurs upon aging is directly related to an oxidation of mitochondrial glutathione (García de la Asunción et al., 1996). Similarly, glutathione oxidation may also be correlated within the oxidative damage to mitochondrial lipids and proteins related to aging. A change in the glutathione redox status would indicate that mitochondrial antioxidant systems cannot cope with the oxidant species generated throughout the cell life. Therefore, glutathione oxidation may occur prior to oxidative damage to other mitochondrial components, and it might be an initial event in oxidative stress associated with mitochondrial aging.

Evidence of Free Radical Damage to Cell Components Upon Aging

At present it is well established that oxidative damage to lipids, proteins and DNA accumulates with age (for review see Ames et al., 1993). Mitochondria is probably the main target of oxidative damage upon aging. Nevertheless, since the oxidative damage to mitochondria has been commented in detail previously, we will now focus on the oxidative lesions found in cell components apart from mitochondria.

Lipid peroxidation is considered a key factor in aging of aerobic cells (Lippman, 1985). Moreover, it is well known that lipid peroxidation plays an important part in the etiology and pathogenesis of age-associated diseases (Vladimirov and Archakov, 1972; Vladimirov, 1986). Peroxidation of unsaturated fatty acids develops in the membrane lipid bilayer and produces hydroperoxides and cyclic peroxides (Gutteridge et al., 1986). Formation of oxygen free radicals in the membranes is needed for the initiation of a lipid oxidation chain (Vladimirov, 1986). However, the rate of lipid peroxidation in biological membranes is determined by the reaction chain branching and, hence, by the levels of lipid hydroperoxides which act as intermediates (Vladimirov, 1986). In living cells, the rate of this reaction chain is limited by certain components of the antioxidant defense, such as GSH, glutathione peroxidase activity and vitamin E, which restrict or completely depress the process of lipid peroxidation (Vladimirov, 1986).

Lipid peroxidation leads to the accumulation of "age pigments" during aging. These are complex, insoluble polymeric deposits of oxidized lipids, transition metals and protein and they display a characteristic fluorescence (Wolman, 1975; Vladimirov, 1986). The finding of high concentrations of iron and copper within these pigments suggests an essential role for metal ions in their formation (Gutteridge, 1984).

Lipid peroxidation produces changes in membrane properties and in the activities of membrane proteins, which may be involved in the impairment of cell function which occurs during aging (Vladimirov, 1986; Lippman, 1989). Thus, the oxidative damage to membrane lipid components is likely to be involved in the decreased fluidity of cellular membranes during aging (Shigenaga et al., 1994). The toxicity of lipid peroxidation products is well recognized (Gutteridge et al., 1986). Lipid peroxidation gives rise to mutagenic lipid epoxides, lipid hydroperoxides, lipid alkoxyl and peroxyl radicals and enols (Halliwell and Gutteridge, 1989). The breakdown of peroxides to carbonyls is of special importance. Many of these products display potent biological activities, such as carcinogenic, enzyme-inactivating and protein-cross-linking effects (Vladimirov, 1986).

In vivo measurement of lipid peroxides using the thiobarbituric acid reactive substances assay (TBARS) leads to conflicting results in rodents, showing increases (Mizuno and Ohta, 1986; Sawada and Carlson, 1987) or decreases (Devasagayam and Tarachand, 1987; Cand and Verdetti, 1989) with age in rodents. Results obtained with the TBARS test have been criticized because as much as 80-90% of the TBARS values are not malondialdehyde but other compounds related, or not related, to lipid peroxidation (Sevanian and Hochstein, 1985). This interference can be minimized if the sample is subjected to a strong peroxidative stress before the TBARS assay, which multiplies the TBARS value by an order of magnitude (Devasagayam, 1986). Using this modification, Barja et al. (1996) have found no age-related change in lipid peroxidation in rat brain, whereas it decreases in rat liver. This decrease might be explained by the increases in lipid soluble antioxidants such as a-tocopherol found in the liver of old animals (Rikans and Moore, 1988).

The importance of free radical damage to proteins in the aging process did not receive much attention until recently, as pointed out by Stadtman (Stadtman, 1992). The role of protein damage in cell aging became apparent when it was found that catalytically less active or inactive forms of some enzymes accumulate during aging (Machado, 1983; Oliver et al., 1987). Post-translational modifications seem to be responsible for this accumulation of inactive proteins (Gordillo et al., 1988). Most of these modifications may be due to oxygen radicals (Levine et al., 1983; Stadtman, 1992). In this process some amino acid residues such as proline, arginine and lysine, are oxidized to carbonyl derivatives (Stadtman, 1992). Thus, the carbonyl content of proteins may be used as a measurement of protein oxidative damage (Stadtman, 1992). On the other hand, protein oxidation may cause modification of histidine residues (Levine, 1983; Fucci et al., 1983). Indeed, modification of histidine residues appears to be involved in the loss of enzymatic activity of rat liver malic enzyme during aging (Gordillo et al., 1988).

A small but significant increase in the carbonyl content has been reported upon aging in human erythrocytes and fibroblasts (Oliver et al., 1987), in human eye lenses (Garland et al., 1988), in the occipital pole of the human brain (Smith et al., 1991) and in rat hepatocytes (Starke-Reed and Oliver, 1989). Furthermore, the carbonyl content of fibroblasts from patients with premature aging diseases such as progeria or Werner's syndrome is very much higher than that in fibroblasts of age-matched control subjects (Oliver et al., 1987).

Although most studies report only a two- to three-fold age-associated increase in the carbonyl content, this would affect 20-30% of the total cellular protein (Stadtman, 1992). Moreover, this percentage is probably underestimated, as carbonyl groups are not formed in the oxidation of some amino acids such as histidine, cysteine or methionine (Stadtman, 1992).

The accumulation of damaged protein could be due to an age-related increase in the rate of protein oxidation and also to a decrease in the ability to degrade oxidized proteins (Stadtman, 1992). A decrease in the activity of neutral alkaline protease, which degrades oxidized proteins, appears to be involved, since accumulation of oxidized proteins varies inversely with the amount of neutral alkaline activity (Carney et al., 1991). The accumulation of oxidatively damaged proteins seems to be involved in the physiological deterioration associated with aging. This is supported by the reversal of the age-related loss in spatial and temporal memory in old gerbils by chronic treatment with a spin trap agent (tert-butyl-a-phenylnitrone), which decreases the accumulation of oxidized proteins in brain (Carney et al., 1991). This finding also points out the relevance of antioxidant administration to prevent the aging process.

Ames and co-workers calculated that oxygen free radicals are responsible for approximately ten thousand DNA base modifications per cell per day (Ames et al., 1993). DNA-repair enzymes are able to remove most of these oxidative lesions, but not all of them. Thus, unrepaired oxidative lesions in DNA such as 8-oxo-7,8-dihydro-2'-deoxyguanosine accumulate with age. As mentioned above, most of this damage occurs in mitochondrial DNA, not in nuclear DNA (Richter, 1988). We have recently found that mitochondrial DNA oxidation is associated with mitochondrial glutathione oxidation (García de la Asunción, 1996). This points out the importance of maintaining a reduced glutathione status to protect cells against oxidative damage of important molecules such as DNA. Oxidative DNA damage would represent another index of oxidative stress and serves to confirm the involvement of free radical damage in aging.

Oxidative damage to proteins and DNA should not be considered separately, because they can potentiate each other. Thus, accumulation of inactive forms of DNA repair enzymes might enhance the accumulation of DNA oxidative damage. Moreover, a loss of repair enzymes leads to an increased spontaneous mutation rate when oxidative lesions to guanine residues are present. Therefore, oxidative lesions in DNA exhibit mutagenic potential. On this basis, oxidative damage to DNA appears to be involved not only in cell aging, but also in the pathogenesis of age-associated diseases such as cancer.

The glutathione system protects against oxidative damage to lipids, proteins and DNA. The presence of oxidative injury to these cell components proves that the antioxidant action of GSH and related enzymes in aged cells is not completely effective. Thus, the joint action of GSH, glutathione peroxidase and glutathione reductase cannot cope with the oxygen activated species generated in old cells. To find ways of improving the function of the glutathione system by increasing GSH levels and/or the activities of glutathione peroxidase and reductase is of great importance.

Use of Antioxidants in Aging Studies: Benefits and Risks

The free radical theory of aging is especially attractive because it provides a rationale for intervention, i.e., administration of antioxidants such as vitamins C or E which might retard the impairment in performance that accompanies aging.

The free radical theory of aging suggests that administration of antioxidants may protect against the age-associated impairment of performance. Administration of antioxidants has been used successfully in some cases either to protect against the physiological deterioration related to aging or to increase the mean life span of some species (Furukawa et al., 1987; Cutler, 1991).

In earlier studies, we and other investigators have observed that some antioxidants partially protect against glutathione oxidation in tissues of rodents and in *Drosophila* (Viña et al., 1992; Cutler, 1991; Ames et al., 1993). In addition, we found that antioxidants which protected against glutathione oxidation were effective in preventing impairments of physical exercise performance in the animals (Viña et al., 1992). Ritchie and his colleagues reported that methionine restriction increases longevity in rats, which is probably due to an increase in blood glutathione (Ritchie et al., 1994). We recently reported that antioxidants such as thiazolidine carboxylate derivatives or vitamins C and E protect against glutathione oxidation and mtDNA oxidative damage associated with aging (García de la Asunción et al., 1996). Meydani and coworkers have shown the protective effect of vitamin E on exercise-induced oxidative damage in young and older adults (Meydani et al., 1993).

Despite the beneficial effects we have just reported, antioxidant administration is not without risk. Indeed, the pro-oxidant effect of antioxidants has been documented. We previously found that cysteine may have a pro-oxidant effect (Viña et al., 1978), since, upon oxidation, it generates free radicals (Sáez et al., 1982).

It was found that administration of b-carotene to smokers increases the incidence of cancer in these patients (Omenn et al., 1996). Similarly, vitamin E administration decreases the acute response of neutrophils during exercise in aging (Cannon et al., 1990). Thus, administration of large doses of antioxidants to patients must be done with care.

Cell Death: Role of Free Radicals in Apoptosis

The maintenance of tissue homeostasis involves the removal of superfluous and damaged cells. Failure to accomplish these goals will induce malformations during development or cancer. This process is often referred to as "programmed cell death" (PCD), because it is believed that cells activate an intrinsic death program contributing to their own demise. It has also been termed "apoptosis", which was originally related to the morphological description of this universal mechanism. Now both terms are used as synonyms.

Apoptosis contrasts with necrosis (for a review see Buja et al., 1993; Schwartzman and Cidlowski, 1993; Vaux, 1993; Kroemer et al., 1995) . In necrosis, cell death is induced by osmotic, physical or chemical damage. These agents produce an early disruption of external and internal membranes liberating denatured proteins into the cellular space, thus inducing an inflammatory response in the vicinity of the dying cell. By contrast, in apoptosis, cells undergo nuclear condensation and shrinkage, as major morphological features. A ladder-type fragmentation affecting nuclear DNA, but not mtDNA, is typical in apoptosis. Recent studies, however, invalidate the concept that alterations of the nucleus are obligatory events in PCD (Kroemer et al., 1995). Recent experiments have shown that a change in mitochondrial activity is another common feature in apoptosis (Deckwerth and Johnson, 1993; Kroemer et al., 1995).

New evidence suggests that at least two independent cytoplasmic pathways may induce nuclear disintegration: one which requires the presence of mitochondria and the other which directly involves the action of specific proteases. This model fits with the contradictory data available in apoptosis research.

Recent reports show that reactive oxygen species play a major role in apoptosis (Hockenbery et al., 1993; Ratan et al., 1994). Apoptosis can be induced by exogenous sources of reactive oxygen species such as t-butyl hydroperoxide in several cell lines (Hockenbery et al., 1993; Buttke and Sandstrom, 1994). Glutathione depletion increases the percentage of apoptotic cells in a given population. We further examined the role of glutathione in apoptosis by increasing the levels of glutathione using glutathione monoethyl ester. This compound, unlike glutathione, is able to penetrate the cell and greatly increase the intracellular glutathione levels because it is hydrolyzed inside the cell. We found administration of glutathione monoethyl ester decreases the percentage of apoptosis in fibroblasts (Pallardó et al., 1996).

Similarly, inhibition of antioxidant pathways such as glutathione synthesis by dl-buthionine sulfoximine provokes apoptosis in different cell lines. Proto-oncogen Bcl-2 exerts its action because it acts as an antioxidant, mainly on the mitochondria. Mitochondria are an important source of reactive oxygen species. Leakage of high-energy electrons along the mitochondrial electron transport chain causes the formation of superoxide anion radicals. A recent report (Kroemer et al., 1995) shows that mitochondria specifically increase the permeability transition (PT) of these organelles during apoptosis. Permeability transition involves the opening of the so-called PT pores, which are identical to mitochondrial megachannels and are located at the inner-outer membrane contact sites. In a very interesting experiment, Kroemer and co-workers (Zamzami et al., 1996a) have shown that isolated mitochondria can induce nuclear DNA digestion in a cell-free system when PT is activated. In addition, inhibition of PT blocks apoptosis. Thus, mitochondrial PT appears to be a critical step in apoptosis (Zamzami et al., 1996b).

There are very few reports on the effect of aging on apoptosis but it has been proposed that the efficiency of apoptosis may correlate with the rate of aging. Experimental studies on rats have suggested that apoptotic cell death provides protective mechanisms by removing senescent DNA-damaged or diseased cells which might interfere with the normal functioning or might lead to neoplastic transformation (Monti et al., 1992).

Livers from old rats show a higher *in situ* rate of apoptosis. Using the dietary restriction model, which is known to retard aging, Muskhelishvili and co-workers (1995) have shown that tumor incidence may be related to the intrinsic rate of apoptosis. Diet restricted animals exhibit a higher rate of apoptosis. These authors concluded that increased apoptotic activity in livers from old animals is a cellular mechanism of defense against neoplasic degeneration.

Age-Associated Diseases: Role of Oxidative Stress and Protection by Glutathione and Other Antioxidants

Age-associated diseases include cancer, cardiovascular disease, immune-system impairment, brain atrophy and cataracts. Degeneration of post-mitotic cells appears to be the main cause of these diseases. It is well known that cancer incidence rises with age. Thus, species that live longer will have a higher frequency of cancer.

Reactive oxygen species have been involved in the pathophysiology of Parkinson's disease. Gotz et al. (1990) proposed that oxidative stress has a role in the pathogenesis of Parkinson's disease. Ikebe et al. (1990) reported that reactive oxygen species increase the number of deletions in mtDNA in the striatum in Parkinson's disease. These authors found similar damage in senescence.

Embryonic rat cortical neurons exposed to elevated concentrations of extracellular glutamate or homocysteate degenerate by apoptosis at approximately 24 h of development. Apoptosis occurs due to glutathione depletion (Murphy et al., 1990). Decreased cystine uptake leads to a decreased level of glutathione through depletion of the limiting amino acid cysteine for GSH synthesis.

Many findings support an increased oxidative stress in Parkinson's disease (for a review see Jankovic, 1994). The findings in studies of Parkinson's disease indicate an increased level of hydrogen peroxide and reactive iron, together with decreased levels of reduced glutathione and ferritin. Lipid peroxidation is also increased.

The decline in the immune system with age can be reversed by dietary glutathione supplementation in mice (Furukawa et al., 1987). In addition, the role of the immune system in the pathogenesis of different neurodegenerative diseases, including Parkinson's disease, has been gaining support from some experimental studies (Kalra et al., 1992).

As pointed out by Coyle et al. (1993), the involvement of oxidative stress in the pathophysiology of neurodegenerative diseases is indeed an important area in which basic research will no doubt provide ideas which may have clinical relevance.

The rate of cancer is very low in nondividing cells. Oxidants form a very important group of agents that stimulate cell division. Therefore, antioxidants can decrease mutagenesis, and thus carcinogenesis, by decreasing oxidative DNA damage and/or by decreasing cell division. On the other hand, selective GSH depletion by extracellular ATP decreases the rate of cell proliferation significantly in Ehrlich ascites tumor (Estrela et al., 1995).

It is well established that cataracts have an oxidative etiology. Those individuals taking tocopherol have less

risk of developing cataracts (Leske et al., 1991; Knekt et al., 1992). Pregnant mice depleted of glutathione produce offspring with cataracts (Martensson et al., 1989). It appears that antioxidant dietary treatment is the most promising treatment against cataract formation.

CONCLUSION

Many experiments support the theory that free radicals are involved in the impairment of physiological functions associated with aging. Thus, antioxidants play a key role in protecting cells against free radicals. Glutathione is a major endogenous antioxidant in cells. Aging is associated with a decrease in reduced glutathione levels and an increase in oxidized glutathione. Thus, the glutathione redox pair tends to be oxidized with aging. Compartmentation of glutathione within the cell is of major importance. Indeed, mitochondrial glutathione is critical and is oxidized to a greater extent with aging than extramitochondrial glutathione. Furthermore, oxidation of glutathione is correlated with oxidative damage to mitochondrial DNA.

Low levels of glutathione are associated with apoptosis. Furthermore, artificially increasing glutathione levels results in a decrease, or at least a delay, in the occurrence of apoptosis in cells.

Administration of antioxidants (which partially prevents oxidation of glutathione) or of glutathione precursors (which increases the rate of glutathione synthesis), is therefore of great importance to partially prevent the impairment in cell function which is associated with aging and, indeed, with cell death.

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