Review Article

Oxidative Stress Markers

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Abstract

Oxidative stress is a high-profile element among the risk factors for aging. Although several stress markers have been proposed for the evaluation of oxidative stress, there remains much room for improvement in testing and evaluation methods. In the Current Concept Session "Oxidative Stress Markers" at the of the most recent research works were presented under the titles "Present Status and New Development of Evaluation of Oxidative Stress Markers," "Oxidative Modification of Proteins and Its Quantitative Detection," and "Modification/Degeneration of Proteins and Advanced Glycation Endproducts (AGEs)".

Several markers are available for evaluation of the oxidative stress status. Methods of detecting oxidatively modified substances as new markers by mass spectrography (MS) have been developed. Oxidative modifications of highly reactive cysteine residues in several target proteins such as tyrosine phosphatase and thioredoxin-related proteins control the functions of relevant molecules, and thereby play an important role in signal transmission. Advanced glycation endproducts (AGEs) gradually accumulate with aging and are involved in the development of diabetic complications, Alzheimer's disease, and arteriosclerosis. Basic studies of the indicators of glycation are also important.

This general article outlines oxidative stress markers with a focus on oxidative modification of proteins and glycation of proteins, both of which have received attention in recent years, and introduces information regarding newly discovered markers for oxidative stress.

KEY WORDS: mass spectrometry, reactive oxygen species (ROS), reactive nitrogen species (RNS), oxidative modification of proteins, advanced glycation endproducts (AGEs)

Introduction

In Anti-Aging Medicine, which is intended to contribute to the promotion of health and improvement of QOL (Quality of Life) in daily life activities and to improve longevity, it is important to detect weak points of senility and risk factors of aging as early as possible and to undertake aggressive interventions to fight them. Oxidative stress is a high-profile element among the risk factors for aging. Although several stress markers have been proposed for the evaluation of oxidative stress, there remains much room for improvement in testing and evaluation methods ¹⁻⁴⁾.

In the Current Concept Session "Oxidative Stress Markers" at the 9th Scientific Meeting of the Japanese Society of Anti-Aging Medicine in 2009, three speakers presented their latest research under the titles "Present Status and New Development of Evaluation of Oxidative Stress Markers," by MC Lee ^{5,6)}, "Oxidative Modification of Proteins and Its Quantitative Detection," by Yoji Kato ^{7,8)}, and "Modification/Degeneration of Proteins and Advanced Glycation Endproducts (AGEs)," by Ryoji Nagai ^{9,10)}.

Oxidative stress is oxidative modification by reactive oxygen species of biological components such as proteins, nucleic acids, and lipids. This induces a variety of organ function disorders. The "free radical theory of aging" states that the accumulation of a

series of these injuries is a cause of age-related decline in biological functions (senility). However, not all free radicals are harmful substances. More recently, it has become evident that cells produce free radicals positively and utilize them to regulate cellular functions. Oxidative modifications of highly reactive cysteine residues in several target proteins such as tyrosine phosphatase and thioredoxin-related proteins control the functions of relevant molecules, and thereby play an important role in signal transmission. Methods of detecting oxidatively modified substances as new markers by mass spectrography (MS) have been developed.

Glycation (Maillard reaction) ¹¹⁾, a risk factor of aging, has primarily been investigated in the field of food chemistry and did not attract attention in the medical field until about 20 years ago. Advanced glycation endproducts (AGEs) gradually accumulate with aging and are involved in the development of diabetic complications, Alzheimer's disease, and arteriosclerosis. Basic studies of the indicators of glycation are important.

This general article outlines oxidative stress markers with a focus on oxidative modification of proteins and glycation of proteins, both of which have received attention in recent years, and introduces information regarding newly discovered markers for oxidative stress.

Antioxidative Networks in the Biological Body

The antioxidative network (**Fig.1**) acts as a defense mechanism against stress^{1,12}. The human body has antioxidative enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH), and catalase, which scavenge free radicals. These enzymes make up a preventive type of antioxidative network.

General types of antioxidative substances are able to respond directly to and eliminate free radicals and are therefore called radical-scavenging antioxidants. These are divided into water-soluble substances, such as vitamin C, and fat-soluble substances, such as vitamins A and E as well as coenzyme Q10. The water- and fat-soluble antioxidants mutually react with each other and individually form sophisticated networks that protect the body against oxidative damage.

Systems that repair and regenerate lipids, proteins, and DNAs by free radicals (repair/regeneration type of antioxidative activities) also exist. In these systems, phospholipase, protease, transferase, and DNA repair enzymes are the primary workhorses.

Antioxidative Stress Actions

Antioxidative stress actions include:

- (1) avoiding the sources of free radicals;
- (2) increasing one's own antioxidative capacity; and
- (3) intake antioxidants.

Nutrients taken into the body consume oxygen and enable production of the biological energy source adenosine tri-phosphate (ATP), primarily in intracellular mitochondria. In this process, reactive oxygen species, free radicals, are produced and act directly on lipids, enzyme proteins, and genes, inducing oxidative damage in tissues or cells. Free radical-related tissue damage is a cause of regressive change due to aging and is fatal to nerve cells, cardiac muscle cells, and other similar types of cells that live long with little proliferation. Factors contributing to formation of free radicals include ultraviolet rays, radiation, smoking, pollutants (e.g. NO₂ and dioxin), food additives, agrochemical residues, pathogens, stress, and excessive physical exercise. Prior to

instructing a patient on use of antioxidants, the patient needs to learn how to get away from the sources of free radicals^{1,12}.

In the body, SOD, GSH peroxidase, and catalase participate in preventive antioxidative networks. Adequate amounts of aerobic exercise produce a very small quantity of free radicals that then intensify the activity of these enzymes, and the effects of these enzymes persist for several days. Consequently, one's own antioxidative capacity is increased.

The human body contain both biosynthesized antioxidants and those obtained from the outside to fight against free radicals. Vitamins A, C, and E, coenzyme Q10, α -lipoic acid¹³, and glutathione are representative antioxidants. Some animals and plants eaten as foods contain high levels of antioxidants, such as lycopene contained in tomatoes, astaxanthin in salmon^{14,15}, catechin in green tea, polyphenols in red wine and cassis¹⁶, and anthocyanidine in blueberries. Selenium (Se) and manganese (Mn) are trace elements necessary to maintain the activity of antioxidative enzymes. GSH binds to peroxidase to exert antioxidative activity. Sulfuraphane contained in large quantities in vegetables such as broccoli, Chinese chives, and onions helps DNA repair enzymes^{17,18}. It is important to take in these ingredients in a well-balanced manner.

Evaluation of Oxidative Stress Markers

Evaluation of oxidative stress caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) is extremely important in clinical settings in the field of Anti-Aging Medicine. Attempts have been made to employ the evaluation of ROS-induced oxidative stress as a testing tool in Anti-Aging Medicine. However, at present, individual markers have not yet fully been qualified^{19,20}.

The oxidative stress profile used in medical institutions dedicated to performing a thorough physical examination from an Anti-Aging viewpoint ("Anti-Aging Dock") (**Fig.2**) measures the extent of oxidation-induced injuries and the levels of radical-scavenging type of antioxidants (<http://www.jaica.com/>), on the basis of which instructions for correction of ill-balanced nutrition, advice on how to replenish what is insufficient, and guidance for supplements are given.

Fig. 1. Antioxidative network in biological system

Fig. 2. Oxidative stress profile

A 50-year-old woman. HEL was remarkably high, possibly from eating too much cake and ice cream

8-OHdG (8-hydroxy-deoxyguanosine): A substance produced when guanine, a DNA component, is damaged by oxidative stress^{21,22}. It is excreted in urine. Its levels are increased by smoking and excessive physical exercise.

HEL (hexanoyl lysine): A product formed by oxidation of fatty acids and addition modification of lysine residue as a protein component^{7,23,24}. It is excreted in urine. Isoprostane: A product formed when phospholipids, which play an important role in the body, are subjected to oxidative stress^{25,26}. It is excreted in urine. % coenzyme Q10: Ratio of ubiquinone (coenzyme Q10's oxidized form): ubiquinol (its reduced form)²⁷. If % coenzyme Q10 is high, it will not exert its effects.

Serum LPO (lipid peroxide): A lipid peroxide formed by oxidation. The higher the serum LPO levels, the stronger the oxidative stress.

PAO (potential antioxidant): Capacity to resist oxidative stress caused by antioxidants in blood (antioxidative capacity), determined by observing a reduction reaction with copper ions. The higher the determined value, the greater the antioxidative capacity.

STAS (serum total antioxidant status): Total capacity of water-soluble antioxidants to resist oxidative stress.

Lutein + zeaxanthin: Fat-soluble antioxidants contained in green and yellow vegetables such as spinach and broccoli. Carotenoid family members are organic pigments for orange and yellow colors.

β -cryptoxanthin: A carotenoid antioxidant contained in the orange color pigment of mandarin oranges.

Lycopene: A carotenoid antioxidant contained in the red color pigment of tomatoes and watermelons.

α -carotene: A carotenoid antioxidant contained in the orange color pigment of mandarin oranges.

β -carotene: A carotenoid antioxidant contained in the pigment of carrots and spinach.

Ubiquinol: A reduced form of coenzyme Q10²⁸.

The oxidative stress markers described above are used to evaluate ROS-induced changes in antioxidative systems and ROS-oxidized products, but do not directly determine ROS and RNS, which are the causes of oxidative stress. Electron spin resonance (ESR) methods are known to be useful in detecting free radicals (*i.e.* chemical species with electron spin [unpaired electrons]) and directly and specifically detecting ROS with properties similar to free radicals, such as superoxides ($O_2^{\bullet-}$), hydroxyl radicals ($HO^{\bullet}$), and nitrogen monoxide ($NO^{\bullet}$)^{6,19,20,29-31}. ESR methods used in evaluation of oxidative stress are characterized by a qualitative advantage, which enables identification of ROS types inducing oxidative stress, and by a quantitative advantage, which enables determination of the amount of ROS produced. In addition, when these methods are applied to biological systems (small rodent models), they enable information regarding redox reactions including oxidative stress, which is evidence of ROS production. Indeed, detection of ROS by *in vitro* ESR methods requires a special technique, as ROS such as $O_2^{\bullet-}$ and $HO^{\bullet}$, free radicals present in the body, are extremely unstable. This technique is called the "spin trap method," as a spin trap agent is used to trap unstable ROS and convert them into stable radical species, followed by determination using an ESR method^{19,20,32}. When antioxidative capacity is evaluated using an *in vitro* ESR method, based on the procedures for detecting ROS, it is possible to identify which type of ROS is reduced by the drug, food, or drink eliminates the relevant ROS type (qualitative evaluation). In other words, we are able to directly evaluate an antioxidative capacity against a given ROS type. The ESR Laboratory of Kanagawa Dental College has used an *in vitro* ESR method to evaluate

antioxidative capacity and has established a method of assessing oxidative stress in an experimental animal brain model of lifestyle-related diseases in which oxidative stress causes hypertension or stroke. The laboratory has further applied this method to evaluation of antioxidative properties of a drug in brain^{5,6,31,33}. In the future, assessment strategies using ESR methods may help further identify antioxidative properties of drugs, foods, drinks, and supplements, on the basis of which new drugs, foods, drinks, and supplements with excellent antioxidative properties would then be developed.

Oxidative Modification of Proteins and Possible Biomarkers

Proteins in the body undergo a variety of post-translational modifications. Among these modifications, oxidative modifications, are substantially involved in aging and disease. When tyrosine and lysine residues in proteins are oxidized, the modified products are relatively stable. We have investigated a possibility that chemical or immunochemical quantification of these relatively stable products may provide a biomarker of oxidative stress³⁴. Measurement of modified tyrosines can provide an inflammatory oxidative stress marker to trace halogenation or nitration (**Fig.3**). Halogenation is caused by enzymes such as myeloperoxidase (MPO) and therefore shows activation of immune cells such as neutrophils. Reactive nitrogen species (RNS) are derived from $NO^{\bullet}$ and can be a marker indicating activation of macrophages having inducible nitric oxide synthase (iNOS)⁸. We paid particular attention to the enzyme MPO and hypothesized that inhibition of the enzyme may prevent inflammation. Certain food ingredients may exert anti-inflammatory activity by *in vivo* inhibition of MPO enzyme. Indeed, polyphenols such as flavonoids have been reported to exert a strong inhibitory effect on MPO activity, as evaluated by an MPO-inhibition assay^{35,36}.

Oxidation of lipids in the body can also be estimated from covalent modification of proteins by the peroxidized lipids (**Fig.4**). Hexanoyl lysine (HEL)^{7,23,24} and propanoyl lysine (PRL)³⁷ have attracted attention because they are derived from the reaction of lysine with lipid hydroperoxides of ω -6 and ω -3 polyunsaturated fatty acids, respectively. HEL is one of the parameters measured for the oxidative stress profile in the "Anti-Aging Dock."

Modified tyrosines and peroxidized lipid-modified lysines are excreted in greater quantities in urine of patients with diabetes^{7,8}. Histological examination of experimental arteriosclerotic lesions detected HEL and dityrosine in the regions where CRP-positive immuno-reactions were noted³⁸. There is a possibility that these adducts may be used as a biomarker for food-aided health maintenance and promotion or Anti-Aging. Because a biomarker is to be determined both in patients and healthy individuals, non-invasive sampling such as urine collection is desirable.

Thus far, quantitative and sensitive detection of oxidative stress markers have been carried out using mass spectrometers, such as LC/MS/MS or by ELISA. While these conventional methods are excellent in light of their high sensitivity (LC/MS/MS) and great convenience (ELISA), they have disadvantages. For example, LC/MS/MS requires expensive measuring devices and complicated pretreatment procedures, and both methods use samples of at least 100 μ l in quantity. Recently, researchers have focused on development of protein chips that immobilize specific antibodies on glass slides via an azopolymer coating. Given that this technology requires only several microliters of urine or serum samples and enables measurement of many samples at a time, it is therefore expected to facilitate evaluation of oxidative stress.

Fig. 3. Modified tyrosines and sources of ROS

Fig. 4. Modification of proteins by lipid peroxidation (amido-type lysine adduct)

Glycation and Antiglycation

Non-enzymatic reaction between sugars and proteins is called the Maillard reaction (hereafter referred to as glycation), and advanced glycation endproducts (AGEs) are formed as late-stage products of this reaction¹¹. Formation of AGEs in the body is a risk factor of accelerated aging and is a cause of diabetic complications³⁹⁻⁴¹. Under hyperglycemic conditions, amino residues of proteins react with aldehydes of reducing sugars such as glucose in a non-enzymatic manner to form a reversible Schiff base (Fig. 5). Amadori rearrangement makes the Schiff base become a stable Amadori compound, which subsequently undergoes many complicated reactions, including among others dehydration, condensation, oxidation, and rearrangement to produce highly reactive dicarbonyl compounds such as 3-deoxyglucosone (3DG), glyoxal, and methylglyoxal. Additional pathways include methylglyoxal production through the glycolysis pathway⁴², 3-deoxyglucosone production by reaction with fructosamine-3-kinase⁴³, glucosone and glyoxal generation by oxidation of glucose⁴⁴, and glycolaldehyde formation from hypochlorous acid via myeloperoxidase produced by activated neutrophils. These dicarbonyl and carbonyl compounds promptly react with proteins *in vivo* to produce AGEs⁴⁵. This chemical reaction is an irreversible post-translational modification/degeneration mechanism in biological systems.

The term AGEs does not denote a single compound, but is a general term for the endproducts of glycation. Many compounds have so far been identified as AGEs, for example *N*^ε-(carboxymethyl)lysine (CML)⁴⁶, pentosidine (Pent)⁴⁷, pyrraline⁴⁸, crossline⁴⁹, GA-pyridine⁴⁵, *N*^ε-(carboxyethyl)lysine (CEL)⁵⁰, *N*^ω-(carboxymethyl)arginine (CMA)⁵¹, and 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole⁵². Various antibodies have been developed for the purpose of identifying AGEs^{10,53}, and with these antibodies the presence of AGEs have been confirmed in sclerotic tissues of the renal glomerulus⁵⁴ and renal arteries⁵⁵ of patients with diabetic nephropathy.

Reported means by which AGEs induce progress of diabetic complications include functional disorders as a result of formation of AGEs from intracellular proteins, organ disorders from intracellular accumulation of AGEs, and involvement of receptors for AGEs (RAGE)⁵⁶. In addition, glycation in skin collagen and the corneal layer of the epidermis accelerates formation of protein cross-linkages, which reduce elasticity, and are a factor of advancing senility⁵⁷. The quantity of AGEs in skin collagen increases with aging⁵⁸ and the amounts of glycated protein in skin, nails, and hair are greater in diabetic patients than in non-diabetic patients⁵⁹.

In the ophthalmologic field also, proteins rich in AGEs and D-type amino acids are clustered in retinopathy and keratopathy as diabetic complications as well as in cataract, pinguecula, spheroid

Fig. 5. Maillard reaction and advanced glycation endproducts (AGEs)

degeneration, and macular degeneration as age-related ocular changes⁶⁰). In addition, proteins rich in AGEs and D-type amino acids cannot display the functions that they should normally exert, because their conformations have changed. These findings indicate that ophthalmologic complications of lifestyle-related diseases such as diabetes and age-related ocular lesions are caused by changes in amino acids making up the eye or those at the level of atoms. In other words, ophthalmologic complications of lifestyle-related diseases and age-related ocular lesions can be regarded as disease conditions where proteins that have undergone post-translational modification, AGEs, or D-type amino acids are accumulated⁶⁰.

Antiglycation Compounds

The effects of aminoguanidine⁶¹ and benfotiamine⁴² in inhibiting hyperglycemia-induced excessive formation and accumulation of AGEs have been investigated. The design of aminoguanidine enables it to block a carbonyl group within the 3DG molecule that is an intermediate product of many glycation reaction pathways, stopping the subsequent reactions. Studies have demonstrated that aminoguanidine is effective in treating diabetic retinopathy⁶², diabetic nephropathy⁶³, and diabetic arteriosclerosis⁶⁴. In addition, aminoguanidine inhibits cross-linking of collagen-containing AGEs, which is caused by a volume-overloaded heart or heart failure⁶⁵.

As these AGE formation-inhibiting agents delay the onset of diabetic nephropathy or retinopathy, we can conclude that AGEs are not merely waste matter but are highly likely to actually cause these diseases. AGEs therefore attract attention as targets of new drugs to be developed. Anemia, appearance of autoantibodies, and hepatic function impairment have all been reported as adverse reactions of aminoguanidine treatment⁶⁶, and therefore development of safer antiglycation substances (AGE formation-inhibiting agents) that are effective at lower concentrations is desired.

Antiglycation compounds have been reported in natural products, such as tea leaf⁶⁷, herbs^{68,69}, and oriental medicines⁷⁰. A mixture of herbal extracts from *Anthemis nobilis* (Chamomile: flower, AN), *Crataegus oxyacantha* (Hawthorn: berry, CO), *Houttuynia cordata* (Doku-dami: whole plants, HC), and *Vitis vinifera* (Grape: leaf, VV) has been found to be as effective as aminoguanidine in inhibiting formation of AGEs^{41,71}. Further, astragalosides isolated from *Astragalus radix* have been shown to remarkably inhibit CML and pentosidine, which have oxidation-dependent AGE structures, and they are expected to be effective in the human body as well⁷².

AGE Receptors and Glycolipid Metabolism

It had been considered that AGEs in the body are primarily formed by glucose over a long period of time. More recently, however, it has been clarified that aldehydes, which are highly capable of modifying proteins, are involved in formation of AGEs. Aldehydes are generated by glycolysis, inflammatory reactions, and oxidation reactions. They promptly produce AGEs and have various effects on glucose and lipid metabolism⁷³. Fat cells under hyperglycemic conditions have been shown to develop a notable fumarate-induced post-translational modification pathway⁹.

New knowledge has come to light for AGE receptors as well. Where macrophages are present, class A macrophage scavenger receptors (SR-A) function also as AGE receptors⁷⁴. In liver sinusoid endothelial cells, the oxidized LDL receptor CD36⁷⁵ and HDL receptor class B type I scavenger receptor (SR-BI)⁷⁶ function as AGE receptors in addition to the SR-A. SR-BI is

involved in selective uptake of HDL-cholesterol ester into hepatocytes and contributes to removal of cholesterol from peripheral cells. These functions of SR-BI are inhibited when AGEs bind to SR-BI⁷⁶. It is therefore anticipated that increased AGEs in blood under diabetic conditions inhibit the cholesterol transport system via SR-BI, accelerating the progress of arteriosclerosis.

Conclusion

From an Anti-Aging viewpoint, great expectations have been placed on antioxidative therapy. Despite the ubiquitous information available on functional food or supplements, no sufficient medical evidence of their effectiveness is available in the relevant literature.

Before relying on supplements, lifestyle-related habits should be improved for the purpose of improving one's own antioxidative capacity. It is important to establish oxidative stress markers to promote reasonable spread of antioxidative interventions. For clinical studies that are currently ongoing independently, data need to be accumulated in preparation for a large-scale meta-analysis to be performed in the future.

References

- 1) Yoshikawa T, Naito Y, Kondo M: Free radical involvement in the aging process. *Neuroscience* 16; 603-612: 1990
- 2) Aoi W, Naito Y, Takanami Y, et al: Oxidative stress and delayed-onset muscle damage after exercise. *Free Radic Biol Med* 37; 480-487: 2004
- 3) Naito Y, Takano H, Yoshikawa T: Oxidative stress-related molecules as a therapeutic target for inflammatory and allergic diseases. *Curr Drug Targets Inflamm Allergy* 4; 511-515: 2005
- 4) Naito Y, Yoshikawa T: Oxidative stress involvement and gene expression in indomethacin-induced gastropathy. *Redox Rep* 11; 243-53: 2006
- 5) Lee MC, Shoji H, Miyazaki H, et al: Assessment of oxidative stress in the spontaneously hypertensive rat brain using electron spin resonance (ESR) imaging and *in vivo* L-Band ESR. *Hypertens Res* 27; 485-492: 2004
- 6) Kobayashi K, Yoshino F, Takahashi SS, et al: Direct assessments of the antioxidant effects of propofol medium chain triglyceride/long chain triglyceride on the brain of stroke-prone spontaneously hypertensive rats using electron spin resonance spectroscopy. *Anesthesiology* 109; 426-435: 2008
- 7) Kato Y, Yoshida A, Naito M, et al: Identification and quantification of N(epsilon)-(hexanoyl)lysine in human urine by liquid chromatography/tandem mass spectrometry. *Free Radic Biol Med* 37; 1864-1874: 2004
- 8) Kato Y, Dozaki N, Nakamura T, et al: Quantification of modified tyrosines in healthy and diabetic human urine using liquid chromatography/tandem mass spectrometry. *J Clin Biochem Nutri* 44; 67-78: 2009
- 9) Nagai R, Brock JW, Blatnik M, et al: Succination of protein thiols during adipocyte maturation: a biomarker of mitochondrial stress. *J Biol Chem* 282; 34219-34228: 2007
- 10) Nagai R, Fujiwara Y, Mera K, et al: Usefulness of antibodies for evaluating the biological significance of AGEs. *Ann NY Acad Sci* 1126; 38-41: 2008
- 11) Maillard LC: Action des acides amines sur les sucres; formation des melanoidines par voie methodique. *Compt Rend Acad Sci (Paris)* 154; 66-68: 1912
- 12) Naito Y: How to choose the anti-oxidative supplement. *Modern Physician* 26; 547-550: 2006 (in Japanese)
- 13) Hibino S, Yonei Y, Takahashi Y, et al: Effects and safety of the short term treatment of α -lipoic acid on female volunteers. *Therapeutic Research* 29; 1947-1961: 2008 (in Japanese)
- 14) Naito Y, Uchiyama K, Aoi W, et al: Prevention of diabetic nephropathy by treatment with astaxanthin in diabetic db/db mice. *Bio Factors* 20; 49-59: 2004
- 15) Iwabayashi M, Fujioka N, Nomoto K, et al: Efficacy and safety of eight-week treatment with astaxanthin in individuals screened for increased oxidative stress burden. *Anti-Aging Medicine* 6; 15-21: 2009
- 16) Yonei Y, Iwabayashi M, Fujioka N, et al: Evaluation of effects of cassis (*Ribes nigrum* L.) juice on human vascular function and gene expression using a microarray system. *Anti-Aging Medicine* 6; 22-31: 2009
- 17) Barcelo S, Gardiner JM, Gescher A, et al: CYP2E1-mediated mechanism of anti-genotoxicity of the broccoli constituent sulforaphane. *Carcinogenesis* 17; 277-282: 1996
- 18) Yanaka A, Zhang S, Tauchi M, et al: Role of the nrf-2 gene in protection and repair of gastric mucosa against oxidative stress. *Inflammopharmacology* 13; 83-90: 2005
- 19) Halliwell B, Whiteman M: Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean? *Br J Pharmacol* 142; 231-255: 2004
- 20) Halliwell B: The wanderings of a free radical. *Free Radic Biol Med* 46; 531-542: 2009
- 21) Floyd RA, Watson JJ, Wong PK, et al: Hydroxyl free radical adduct of deoxyguanosine: sensitive detection and mechanisms of formation. *Free Radic Res Commun* 1; 163-172: 1986
- 22) Toyokuni S, Tanaka T, Hattori Y, et al: Quantitative immunohistochemical determination of 8-hydroxy-2'-deoxyguanosine by a monoclonal antibody N45.1: its application to ferric nitrilotriacetate-induced renal carcinogenesis model. *Lab Invest* 76; 365-374: 1997
- 23) Kato Y, Mori Y, Makino Y, et al: Formation of N^ε-(hexanoyl)lysine in protein exposed to lipid hydroperoxide-A plausible marker for lipid hydroperoxide-derived protein modification. *J Biol Chem* 274; 20406-20414: 1999
- 24) Arai H, Kato Y, Fukunaga K, et al: Formation of N^ε-(hexanoyl)lysine in oxidized human very-low-density lipoprotein. *J Electrophoresis* 48; 37-40: 2004
- 25) Morrow JD, Awad JA, Kato T, et al: Formation of novel non-cyclooxygenase-derived prostanoids (F2-isoprostanes) in carbon tetrachloride hepatotoxicity. An animal model of lipid peroxidation. *J Clin Invest* 90; 2502-2507: 1992
- 26) Milne GL, Morrow JD: Isoprostanes and related compounds: update 2006. *Antioxid Redox Signal* 8; 1379-1384: 2006
- 27) Yamamoto Y, Yamashita S: Ubiquinol/ubiquinone ratio as a marker of oxidative stress. *Methods Mol Biol* 186; 241-246: 2002
- 28) Hosoe K, Kitano M, Kishida H, et al: Study on safety and bioavailability of ubiquinol (Kaneka QH) after single and 4-week multiple oral administration to healthy volunteers. *Regul Toxicol Pharmacol* 47; 19-28: 2007
- 29) Lee C, Miura K, Liu X, et al: Biphasic regulation of leukocyte superoxide generation by nitric oxide and peroxynitrite. *J Biol Chem* 275; 38965-38972: 2000
- 30) Lee CL, Liu X, Zweier JL: Regulation of xanthine oxidase by nitric oxide and peroxynitrite. *J Biol Chem* 275; 9369-9376: 2000
- 31) Lee MC: Assessment of antioxidant property of drugs by electron spin resonance (ESR) spectroscopy. *Nippon Yakurigaku Zasshi* 128; 293-297: 2006 (in Japanese)
- 32) Janzen EG: Spin trapping. *Methods Enzymol* 105; 188-198: 1984
- 33) Lee MC, Shoji H, Miyazaki H, et al: Measurement of oxidative stress in the rodent brain using computerized electron spin resonance tomography. *Magn Reson Med* 5; 79-84: 2003
- 34) Kato Y, Kawai Y, Morinaga H, et al: Immunogenicity of a brominated protein and successive establishment of a monoclonal antibody to dihalogenated tyrosine. *Free Radic Biol Med* 38; 24-31: 2005
- 35) Kato Y, Nagao A, Terao J, et al: Inhibition of myeloperoxidase-catalyzed tyrosylation by phenolic antioxidants *in vitro*. *Biosci Biotechnol Biochem* 67; 1136-1139: 2003
- 36) Shiba Y, Kinoshita T, Chuman H, et al: Flavonoids as substrates and inhibitors of myeloperoxidase: Molecular actions of aglycone and metabolites. *Chem Res Toxicol* 21; 1600-1609: 2008
- 37) Hisaka S, Kato Y, Kitamoto N, et al: Chemical and immunochemical identification of propanoyllysine derived from oxidized n-3 polyunsaturated fatty acid. *Free Radic Biol Med* 46; 1463-1471: 2009
- 38) Fukuchi Y, Miura Y, Nabeno Y, et al: Immunohistochemical detection of oxidative stress biomarkers, dityrosine and N(epsilon)-(hexanoyl)lysine, and C-reactive protein in rabbit atherosclerotic lesions. *J Atheroscler Thromb* 15; 185-192: 2008
- 39) Cerami A, Vlassara H, Brownlee M: Glucose and aging. *Sci Am* 256; 90-96: 1987
- 40) Nagai R, Fujiwara Y, Mera K, et al: Usefulness of antibodies for evaluating the biological significance of AGEs. *Ann NY Acad Sci* 1126; 38-41: 2008
- 41) Yonei Y, Yagi M, Hibino S, et al: Herbal extracts inhibit Maillard reaction, and reduce chronic diabetic complications risk in streptozotocin-induced diabetic rats. *Anti-Aging Medicine* 5; 93-98: 2008
- 42) Babaei-Jadidi R, Karachalias N, Ahmed N, et al: Prevention of incipient diabetic nephropathy by high-dose thiamine and benfotiamine. *Diabetes* 52; 2110-2120: 2003
- 43) Szewgold BS, Howell S, Beisswenger PJ: Human fructosamine-3-kinase: purification, sequencing, substrate specificity, and evidence of activity *in vivo*. *Diabetes* 50; 2139-2147: 2001
- 44) Nagai R, Unno Y, Hayashi MC, et al: Peroxynitrite induces formation of N(epsilon)-(carboxymethyl)lysine by the cleavage of amadori product and generation of glucosone and glyoxal from glucose: Novel pathways for protein modification by peroxynitrite. *Diabetes* 51; 2833-2839: 2002
- 45) Nagai R, Hayashi CM, Xia L, et al: Identification in human atherosclerotic lesions of GA-pyridine, a novel structure derived from glycolaldehyde-modified proteins. *J Biol Chem* 277; 48905-48912: 2002

- 46) Nagai R, Ikeda K, Higashi T, et al: Hydroxyl radical mediates N(epsilon)-(carboxymethyl)lysine formation from Amadori product. *Biochem Biophys Res Commun* 234; 167-172: 1997
- 47) Sell DR, Monnier VM: Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process. *J Biol Chem* 264; 21597-21602: 1989
- 48) Hayase F, Nagaraj RH, Miyata S, et al: Aging of proteins: immunological detection of a glucose-derived pyrrole formed during maillard reaction *in vivo*. *J Biol Chem* 264; 3758-3764: 1989
- 49) Ienaga K, Nakamura K, Hochi T, et al: Crosslines, fluorophores in the AGE-related cross-linked proteins. *Contrib Nephrol* 112; 42-51: 1995
- 50) Nagai R, Fujiwara Y, Mera K, et al: Immunochemical detection of N(epsilon)-(carboxyethyl)lysine using a specific antibody. *J Immunol Methods* 332; 112-120: 2008
- 51) Iijima K, Murata M, Takahara H, et al: Identification of N(omega)-carboxymethylarginine as a novel acid-labile advanced glycation end product in collagen. *Biochem J* 347; 23-27: 2000
- 52) Ulrich P, Cerami A: Protein glycation, diabetes, and aging. *Recent Progr Horm Res* 56; 1-21: 2001
- 53) Mera K, Nagai M, Brock JW, et al: Glutaraldehyde is an effective cross-linker for production of antibodies against advanced glycation end products. *J Immunol Methods* 334; 82-90: 2008
- 54) Miyata S, Monnier V: Immunohistochemical detection of advanced glycosylation end products in diabetic tissues using monoclonal antibody to pyrraline. *J Clin Invest* 89; 1102-1111: 1992
- 55) Niwa T, Katsuragi T, Miyazaki S, et al: Immunohistochemical detection of imidazolone, a novel advanced glycation end product, in kidneys and aortas of diabetic patients. *J Clin Invest* 99; 1272-1280: 1997
- 56) Yonekura H, Yamamoto Y, Sakurai S, et al: Roles of the receptor for advanced glycation endproducts in diabetes-induced vascular injury. *J Pharmacol Sci* 97; 305-311: 2005
- 57) Reiser KM: Nonenzymatic glycation of collagen in aging and diabetes. *Proc Soc Exp Biol Med* 28; 23-37: 1998
- 58) Monnier VM, Bautista O, Kenny D, et al: Skin collagen glycation, glycoxidation, and crosslinking are lower in subjects with long-term intensive versus conventional therapy of type 1 diabetes. Relevance of glycated collagen products versus HbA1c as markers of diabetic complications. *Diabetes* 48; 870-880: 1999
- 59) Sueki H, Nozaki S, Fujikawa R, et al: Glycosylated proteins of skin, nail and hair: Application as an index for long-term control of diabetes mellitus. *J Dermatol* 16; 103-110: 1989
- 60) Kaji Y: Ocular diseases caused by accumulation of proteins with post-translational modifications. *Nippon Ganka Gakkai Zasshi* 113; 424-442: 2009 (in Japanese)
- 61) Nilsson B-O: Biological effects of aminoguanidine: An update. *Inflamm Res* 48; 509-515: 1999
- 62) Raskin P, Cattran D, Williams M, et al: Pimagidine (PG) reduces progression of retinopathy and lowers lipid levels in patients with type 1 diabetes mellitus (DM). *J Am Soc Nephrol* 10; 179A: 1999 (abstract)
- 63) Appel G, Bolton K, Freeman B, et al: Pimagidine (PG) lowers total urinary protein (TUP) and slows progression of overt diabetic nephropathy in patients with type 1 diabetes mellitus (DM). *J Am Soc Nephrol* 10; 153A: 1999 (abstract)
- 64) Brownlee M, Vlassara H, Kooney A, et al: Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science* 232; 1629-1632: 1986
- 65) Herrmann KL, McCulloch AD, Omens JH: Glycated collagen cross-linking alters cardiac mechanics in volume-overloaded hypertrophy. *Am J Physiol Heart Circ Physiol* 264; H1277-H1284: 2003
- 66) Whitter F, Spinowitz B, Wuerth JP, et al: Pimagidine (PG) safety profile in patients with type 1 diabetes mellitus (DM). *J Am Soc Nephrol* 10; 184A: 1999 (abstract)
- 67) Kinae N, Yamashita M: Inhibitory effects of tea extracts on the formation of advanced glycation Products. *The Maillard reaction in the food processing human nutrition and physiology* (Point PA ed.), 221-226, Birkhauser, Basel, Boston, Berlin, 1990
- 68) Matsuura N, Aradate T, Sasaki C, et al: Screening system for the Maillard reaction inhibitor from natural products extracts. *J Health Sci* 48; 520-526: 2002
- 69) Matsuura N, Kurosaka C, Ubukata M, et al: Development of pentosidine formation inhibitor. *Bull Toyama Prefect Univ* 13; 65-69: 2003 (in Japanese)
- 70) Yokozawa T, Nakagawa T, Terasawa K: Effects of oriental medicines on the production of advanced glycation endproducts. *J Tradit Med* 18; 107-112: 2001
- 71) Yonei Y, Miyazaki R, Takahashi Y, et al: Anti-glycation effect produced by mixed herb extracts and corresponding evaluation: Placebo-controlled double-blind trial for pre-diabetes. *Anti-Aging Medicine* 7; 26-35: 2010
- 72) Motomura K, Fujiwara Y, Kiyota N, et al: Astragalosides isolated from the root of *Astragalus radix* inhibits the formation of advanced glycation end-products. *J Agric Food Chem* 57; 7666-7672: 2009
- 73) Ohgami N, Miyazaki A, Sakai M, et al: Advanced glycation end products (AGE) inhibit scavenger receptor class B type I-mediated reverse cholesterol transport: a new crossroad of AGE to cholesterol metabolism. *J Atheroscler Thromb* 10; 1-6: 2003
- 74) Nagai R, Matsumoto K, Ling X, et al: Glycolaldehyde, a reactive intermediate for advanced glycation end products, plays an important role in the generation of an active ligand for the macrophage scavenger receptor. *Diabetes* 49; 1714-1723: 2000
- 75) Ohgami N, Nagai R, Ikemoto M, et al: CD36, serves as a receptor for advanced glycation endproducts (AGE). *J Diabetes Complications* 16; 56-59: 2002
- 76) Ohgami N, Nagai R, Miyazaki A, et al: Scavenger receptor class B type I-mediated reverse cholesterol transport is inhibited by advanced glycation end products. *J Biol Chem* 276; 13348-13355: 2001