TISSUE PROTEIN CARBONYLATION IN AGING: A STRATEGIC ANALYSIS OF AGE-RELATED PROTEIN MODIFICATION

ABSTRACT

Free radicals generated in a variety of biological systems have been implicated in mechanisms of aging and age-related pathologies. This study strategically revealed the varying levels of carbonylated proteins in 3 different tissues of 40 Wistar rats of varying ages. Their ages include 25-30, 45-50 and 65-70 days. The brain, heart and kidney tissue homogenates were prepared and biochemically analyzed for products of protein oxidation using 2,4-dinitrophenylhydrazones and autoantibodies against carbonylated proteins. This study revealed a direct proportional relationship between age and protein carbonylation in brain, heart and kidney tissue homogenates. The level of carbonylated proteins were significantly (*P*≤0.05) increased in the assayed tissues as all test groups advanced in age. Oxidative modification of proteins in brain and kidney tissues showed similar trend. These age-related biochemical manifestations may be as a result of increased generation of free radicals at mitochondrial level or decreased anti-oxidant defenses as living organisms advance in age.

Key words; *Aging*, *Carbonylated proteins*, *Free radicals*, *2,4-dinitrophenylhydrazone*

INTRODUCTION

Reactive oxygen species (ROS) have been implicated in changes that occur in a wide variety of biological systems [1]. These active oxygen species have, for centuries, generated interest most especially due to the fact that they are suspected to be the causative factor for aging and age-related pathologies like neurodegenerative diseases [2], cardiovascular, renal and diabetic complications. Reactive oxygen species are capable of modifying cell proteins [1] and therefore, over a given period of time, induce damage to cells that make up tissues of vital organs such as nervous and pulmonary tissues [3]. Some of the changes induced by reactive oxygen species occur extensively and are directly proportional to the level of metabolic activity in the organism[4].Some scientists have postulated that we age according to the quantity of free radicals we generate especially at mitochondrial level[5][6]. Nucleic acids, proteins and membrane lipids are major targets of reactive oxygen species [6].In hyperglycemic conditions, the accumulation of free radicals increases the tendency for hemoglobin to be glycated. Hydroxyl radical ($OH^{\bullet}$) is the most potent free radical[7]. Highly reactive hydroxyl radicals are thought to be generated in vivo by catalytic action of transition metals such as iron and copper that bind to appropriate sites of proteins and can modify nearby amino acid residues[6][7]. Amino acids which are easily modified by free radicals include proline, arginine, threonine and lysine. These residues undergo oxidative modifications to carbonyl derivatives. ROS leading to protein oxidation and modification include free radical species such as superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$),peroxyl ($RO^{\bullet}_2$), alkoxyl (RO), hydroperoxyl ($HO^{\bullet}_2$),and nonradical species such as hydrogen peroxide(H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$)[8].Protein carbonyl content is actually the most general indicator and by far the most commonly used marker of protein oxidation[1][2][9], and accumulation of protein carbonyls has been observed in several human diseases including Alzheimer's disease (AD), diabetes mellitus, inflammatory bowel disease (IBD), and arthritis[9]. Scientific investigations have revealed that enzymes with altered activity are increased in tissues of senescent animals, some of which have been mimicked in vitro by metal-catalyzed oxidation, suggesting the involvement of active oxygen species in protein modifications in aging process [10]. There is, however, controversy regarding this claim with respect to the level of modified proteins in tissues of aging animals. This study was aimed at investigating any change in level of protein carbonyl in the brain, heart and kidney tissue homogenates in Wistar rats of varying ages.

MATERIALS AND METHODS

Animal procurement

This study included 40 male Wistar rats procured from the matrices of Experimental Animals Unit, Madonna University. All animals were confirmed to be healthy by a veterinarian in the institution. The animals were randomly sampled into 4 groups with 10 rats per group. They were exposed to normal day/night cycle and pelleted feed was provided *ad-libitum*. Proper handling of the animals was ensured to avoid stress due to restraint and infection due to coprophagy and improper sanitary techniques.

Study design

The animals were grouped according to age in days as follows;

Table 1; animal grouping

Groups	Age (in days)
a	25-30
b	45-50
c	65-70
d	85-90

N=10

The study period lasted for 90 days. All animals were collected after birth. Samples were collected exactly 12:000am on days 25-30, 45-50, 65-70 and 85-90.

Tissue preparation

All animals were anaesthetized with pentobarbital sodium salt (0.5 ml i.p.) and transcardially perfused with saline (0.9% NaCl) followed by 4% paraformaldehyde (PFA) in phosphate buffer (PB; 0.1 M; pH 7.4). The brain, heart and kidney tissues were removed and after overnight post-fixation at 4^{o}C, they were stored in the refrigerator (4^{o}C) in phosphate buffer with 0.01% sodium azide until use for biochemical analysis.

Biochemical analysis

Protein carbonyl assay

Cayman's Protein Carbonyl Assay Kit manufactured by IBL International with catalogue number CM10005020, was used for this assay. This assay utilized dinitrophenylhydrazine (DNPH) reaction to measure the protein carbonyl content in tissue homogenates in 96-well format Enzyme-linked immunoabsorbent assay (ELISA), with autoantibodies against carbonylated proteins. The amount of protein-hydrazone produced was quantified spectrophotometrically at an absorbance between 360-385nm. The carbonyl content was then standardized to protein concentration.

Statistical analysis

Data was expressed as Mean ± SEM. IBM®SPSS Version 20.0 was used for statistical analysis. The statistical tool used was One-Way ANOVA. All values were statistically significant at a confidence interval of 95%.

RESULTS

Table 2; age-related protein oxidation

Groups	Age (in days)	Protein carbonyl (µg/ml)		
		Brain	**Heart**	**Kidney**
a	25-30	80.4±1.3	42.2±1.0	57.2±1.1
b	45-50	93.6±0.1[a]	43.4±0.3	64.4±0.4[a]
c	65-70	143.2±2.1[ab]	63.2±1.4[ab]	72.1±1.0[ab]
d	85-90	154.3±2.0[abc]	63.4±0.3[ab]	84.4±1.2[abc]

Key; [a,b,c] value is statistically significant compared to ages 25-30, 45-50 and 65-70 respectively.

Table 3; percentage change in protein carbonyl level at different ages (in days)

Tissue	Percentage change			
	a-b	***b-c***	***c-d***	***a-d***
Brain	16.4	52.9	7.75	91.9
Heart	2.84	45.6	0.31	50.2
Kidney	12.5	11.9	17.0	47.5

Key; *a-b*, *b-c*, *c-d*, *a-d* percentage change from a to b, b to c, c to d and a to d respectively.

Age-related protein carbonylation in brain tissue

Compared to age 25 to 30 days, there was a significant progressive increase at $P<0.05$ in protein oxidation as age advanced from 25 days to 90 days with the highest increase experienced from age 45 to 70 days.

Age-related protein carbonylation in heart tissue

The pattern of protein oxidation was not similar to brain tissue. Compared to age 25-30, there was a significant increase in protein carbonyl content of heart tissue in ages 65 to 90 days.

Age-related protein carbonylation in kidney tissue

There was a significant ($P<0.05$) progressive increase in protein carbonyl formation in kidney tissue. This increase was similar to what was observed in brain tissue, with the highest significant increase observed in ages 65 to 90 days.

Percentage change in protein carbonyl level at different ages (in days)

The highest percentage positive change in carbonylated proteins in brain tissue was seen from days 45-50 to 65-70 with an overall increase of 91.9. The highest percentage positive change in carbonylated proteins in heart tissue was seen from days 45-50 to 65-70 with an overall increase of 50.2. The highest percentage positive change in carbonylated proteins in kidney tissue was seen from days 65-70 to 85-90 with an overall increase of 47.5.

DISCUSSION

Oxidative stress, an imbalance towards the pro-oxidant side of pro-oxidant/antioxidant homeostasis, has been implicated in aging and age-related pathologic conditions [1]. Harman realized in 1954 that toxic free radicals might be formed in the body and might cause aging [1]; What relationships might exist among high level of protein carbonyl groups, oxidative stress, and diseases remains uncertain. A postulated mechanism suggests that reactive oxygen species (ROS) increase the tendency of proteins in living systems to be carbonylated [11]. The usage of protein carbonyl groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins[10][11]. Several study findings have added support to the claim that oxidative damage to proteins can explain the physiological decline of human bodily functions with age [12]. There exists, however, controversy regarding this claim. Another theory suggests that the rate of protein carbonylation may be determined by the amount of heavy metals like total iron in living systems which was suspected to increase with age[13], suggesting a possibility that iron is responsible for the generation of oxidatively damaged proteins in tissues. It is also conceivable that proteins in tissues are carbonylated by other mechanisms such as glycation or the reaction with aldehydes generated from lipid peroxides rather than by direct oxidation of amino acid residues[14][15].This study supports the scientific explanation that oxidative modification of organic biomolecules like proteins increases in living systems as we advance in age. This protein oxidation may be responsible for age-related physical manifestations like wrinkling of the skin of face especially close to the ocular region, shrinking of the extremities and axial body framework. It can be deduced from this study that free radical generation increases with age, and there is a positive correlation between both. If the concentration of heavy metals was assayed for, then the mechanism behind these age-related differences would have been clearly established as it may be dependent on the concentration of this factor. The higher the level of free radicals the greater the tendency of oxidative modification of proteins like enzymes, cell membrane proteins, connective tissue proteins and formation of cross-linked proteins like lipofuscin also called the 'Age-pigment'[15][16], which limits the life-span of cells and inhibits proteasome and lysosomal enzymatic degradation of oxidized proteins[16][17]. The probability that frequent exposure to any agent that has the ability to prevent, terminate or mop up free radical generation can slow down the aging process as well as prevent or ameliorate age-related pathologic conditions cannot be completely ruled out. A preferred target site for such agent may be the mitochondria, the intracellular site for oxidative phosphorylation[18].

CONCLUSION

From the outcome of this study, brain, heart and kidney tissue protein carbonylation may be positively correlated with aging. As we advance in age, the rate of free radical generation and oxidative modification of organic biomolecules in living systems increases. These changes may be responsible for the age-related physical and mental deterioration and pathologic complications that occur as we advance in age. There is a clear chance that the process of aging can be influenced by chemical agents targeted at protein oxidation reactions.

Ethical approval

This experiment was conducted in accordance with Madonna University ethical guidelines for investigations using laboratory animals.

Consent: NA

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