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RESEARCH ARTICLE

Differential expressions of antioxidant status in aging rats: the role of transcriptional factor Nrf2 and MAPK signaling pathway

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Abstract Antioxidant enzymes (AOEs) play an important role in the protection of cells against reactive oxygen species and facilitate the prevention of oxidative stress-induced aging. In the present study, the antioxidant indices, including the content of peroxidation product and the expression of AOEs in rat livers of varying ages (2, 12 and 18–24 months old) were evaluated. Erythrocytes haemolysis induced by free radicals showed significant age-dependent increases ($P < 0.05$). The content of oxidation products in livers showed that increasing age was associated with serious oxidative injury. The activities of AOEs decreased with increasing age. Expression of the antioxidant and age-related gene, *klotho*, decreased with increasing age. Western blot assay showed that aged rats experience higher levels of oxidative stress. Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) exhibited an age-dependent decrease. Additionally, the mitogen-activated protein kinase cascade (MAPK) played a regulatory role in signaling transduction. Overall, we suggest that age-related declines of the antioxidant defense are closely involved with the expression of Nrf2 and are regulated by the MAPK family.

Keywords Oxidative stress · Antioxidant enzyme · Aging · Signal transduction · Mitogen-activated protein kinase (MAPK) · Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2)

Abbreviations

AOEs	Antioxidant enzymes
ARE	Antioxidant response element
CAT	Catalase
ERK	Extracellular responsive kinase
GPx	Glutathione peroxidase
GR	Glutathione reductase
JNK	c-Jun NH2-terminal kinase
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
NQO1	NAD(P)H:quinone oxidoreductase 1
Nrf2	Nuclear factor-erythroid 2 p45-related factor 2
TBARS	Thiobarbituric acid reactive substances

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Introduction

Free radicals cause cumulative oxidative damage resulting in DNA, protein and lipid dysfunction. Thus, it is critical for organisms to maintain

optimal antioxidant defense against oxidative stress. Antioxidant enzymes (AOEs) play an important role in the protection of cells from reactive oxygen species and it has been shown that promoting the expression of phase II detoxifying and antioxidant enzymes, such as glutathione peroxidase, quinone reductase and NAD(P)H:quinone oxidoreductase 1 (NQO1), etc, facilitate the prevention of oxidative stress-induced disease and aging (Devasagayam et al. 2004). Several researches have shown that the decreasing expression of AOEs is associated with aging (Sanz et al. 2002).

Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) is one of the critical transcription factors involved in the regulation of drug-metabolizing enzymes (Lee and Surh 2005). This basic-region leucine zipper transcription factor mediates the transcriptional activation of genes responsive to oxidative and electrophile stress. It has been found that Nrf2 transcription factor contributes to the expression of glutathione S-transferases in mouse (Hayes et al. 2000). Recently, the study showed that the decline in Nrf2 results in the loss of glutathione synthesis and is age-dependent (Suh et al. 2004). Induction of such phase II AOE genes by Nrf2 occurs through their recruitment to the antioxidant response element (ARE) in their gene promoters (Nguyen et al. 2003). Mice, with the *nrf2* gene-knockout by targeted homologous recombination, showed lower constitutive levels of NQO1 proteins in the liver (Nioi and Hayes 2004). These researches testify that transcription factor Nrf2 indeed plays a crucial role in the regulation of antioxidant system. Furthermore, the gene named *klotho*, which is based on the name of the Greek Fate who spins the thread of life, has been found to be critically involved in the regulation of several aging phenotypes (Kuro-o et al. 2005). Mutations in the mouse *klotho* gene lead to diseases resembling aging, but few reports indicate whether there is any correlation between antioxidant status and the expression of the *klotho* gene during aging. Although the literature discusses the age-related changes in appearance and physiology, less research has been conducted on the involved molecular mechanisms.

The haemolysis assay of erythrocytes has been used as a model to evaluate oxidative damage in the blood circulation. The free radical-induced damage, including cell membrane lipid bi-layer peroxidation, protein refolding, and cell destruction, have been extensively studied (Zou et al. 2001). Some biomarkers of oxidative damage to macromolecules, including malondialdehyde (MDA), alkenals, 8-isoprostane, and protein carbonyls, have been widely used in animal aging studies (Block et al. 2003).

Recently, more attention has been paid to the importance of phase II-enzyme induction as a mechanism of chemoprevention, but there seems to be comparatively less foci on whether there is any correlation between the activity of detoxification and antioxidant enzymes and aging. In the present study, we evaluated the antioxidant indices, including free radical-mediated oxidative damage of erythrocytes, the formation of peroxidative products (MDA and carbonyl protein), and the expression of AOEs, including GPx, GR, catalase and NQO1, and the age-related gene, *klotho*, in the liver of rats with different ages (young rats: 2 months old, middle-aged rats: 1 year old, and aged rats: 1.5–2 years old). Furthermore, the content of transcription factor Nrf2 and the signaling mechanism involved were also investigated. The original intention of this study was to estimate the antioxidant status in vivo at different life phases, which mimic the entire lifespan of a human being.

Materials and methods

Chemicals and reagents

2,2′-Azo-bis (2-amidinopropane) dihydropropane (AAPH) was purchased from Wako Pure Chemical Industries (Chuo-Ku, Osaka, Japan). 1,1,3,3-tetraethoxypropane (TEP), thiobarbituric acid (TBA), and trichloroacetic acid (TCA) were purchased from Merck Chemical Co. (Darmstadt, Germany). Rabbit anti-ERK1/2, p38α, and JNK polyclonal antibodies were purchased from Chemicon International Corporation (Temecula, CA, USA) and their related phosphospecific polyclonal antibodies were purchased from Biosource International Corporation (Camarillo, CA, USA). Anti-Nrf2 antibody is the product of

Santa Cruz Biotechnology Corporation (Santa Cruz, CA, USA). All other chemicals used in this study were of the highest purity and were purchased from commercial suppliers.

Experimental animals and procedures

Male Sprague-Dawley rats were purchased from the National Laboratory Animal Center, Taipei, Taiwan. The rats were fed a laboratory rodent diet from Newco Distributors Corporation (Rancho Cucamonga, CA, USA) and given water ad libitum. The animal room was maintained at a temperature of 22 ± 5°C with a 12 h light–dark cycle (6:00–18:00) and 35 ± 5% humidity. All experimental procedures involving animals were based on the National Institutes of Health (NIH) guidelines. This experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of the National Chung Hsing University of Taichung, Taiwan. The rats [2 months old (young), 1 year old (middle-aged), and 1.5–2 years old (aged)] were sacrificed with overdosage of CO_2, blood was collected from the inferior vena cava, and livers were isolated for further examination.

Haemolysis determination

The determination of rat erythrocyte haemolysis induced by peroxyl radicals was previously described (Zhu et al. 2002). Blood was centrifuged and washed three times with 0.9% of saline. During each wash, the erythrocytes were centrifuged at 3000 rpm for 10 min to obtain a packed cell pellet. The erythrocytes were diluted with saline at a ratio of 1:200 (v/v). Erythrocyte oxidative haemolysis was performed by the presence of AAPH. Four miililitre of erythrocyte suspensions alone or with AAPH (final concentration was 15 mM) was incubated on a shaker at 37°C for 10 h. The reaction mixture was centrifuged and 100 μl of the supernatant was removed for evaluation every 2 h. The degree of haemolysis was determined from the absorbance of haemoglobin at 410 nm by FLUO star galaxy spectrophotometer (BMG LABTECH Limited Company, Offenburg, Germany).

Measurement of lipid peroxidation product: Malondialdehyde (MDA)

Determination of MDA by thiobarbituric acid (TBA) was used as an index of the extent of lipid peroxidation (Buege and Aust 1978). The supernatant of liver tissue homogenate (1 ml) was mixed with 1 ml of 7.5% (w/v) cold trichloroacetic acid (TCA) to precipitate proteins and then centrifuged at 1,500 rpm. The supernatant was mixed with 1 ml of 0.8% (w/v) TBA for 45 min in a boiling water bath. After cooling, the lipid peroxidation product (MDA) was assayed according to an improved thiobarbituric acid reactive substances (TBARS) fluorometric method after excitation at 555 nm and emission at 515 nm using 1,1,3,3-tetraethoxypropane (TEP) as the standard. The protein concentration was determined using a standard commercial kit (Bio-Rad Laboratories, Hercules, CA, USA). The results were expressed as MDA formation per milligram of protein.

Assay of carbonyl proteins

Carbonyl proteins were analysed through the determination of absorbance (Hipkiss et al. 2001). Protein samples were added to an equal volume of 0.1% 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl and incubated 1 h at room temperature. 20% TCA was added to the mixture and the precipitate was extracted three times with ethanol/ethyl acetate (v/v, 1:1). The pellets were dissolved in 8 M guanidine hydrochloride (containing 13 mM EDTA, 133 mM Tris, pH 7.4) and the absorbance was measured at 365 nm. The concentration of carbonyl groups was calculated using the absorbance of 1 nmol/ml of carbonyl at 365 = 0.021. Data are expressed as the amount of nmol carbonyl protein formed per mg of total protein.

Assays of antioxidant enzyme activity

Liver homogenates were prepared by homogenizing tissue in 0.075 M NaCl containing 3 mM EDTA, pH 7.4, to obtain a 10% solution. The samples were immediately centrifuged (12,000 rpm for 10 min) at 4°C to obtain the supernatant of liver tissue. The protein content of liver

homogenates was determined using a Bio-Rad protein assay kit.

The glutathione peroxidase (GPx) activity was determined spectrophotometrically (Mohandas et al. 1984). The following solutions were pipetted into a cuvette: 0.1 ml of homogenate and 0.8 ml of 100 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 unit/ml GSH reductase, and 1 mM GSH. This mixture was preincubated for 5 min at 37°C. After, the reaction was initiated by adding 0.1 ml of 2.5 mM H_2O_2. Enzyme activity was calculated by the change in absorbance value at 340 nm for 5 min. The non-enzymatic reaction rate was correspondingly assayed by replacing the homogenate sample with potassium phosphate buffer. GPx activity was expressed as nmole of NADPH per minute per milligram of protein.

The glutathione reductase (GR) assay monitored the oxidation of NADPH consumed in the reduction of glutathione disulfide (GSSG) by the change in absorbance at 340 nm (Reed et al. 1980). The following solutions were pipetted into a 1 cm spectrophotometric cuvette: 0.1 ml of homogenate and 0.9 ml of 0.10 M phosphate buffer, pH 7.0, containing 1 mM $MgCl_2 \cdot 6H_2O$, 50 mM GSSG, and 0.1 mM NADPH. This mixture was preincubated for 5 min at 37°C. GR activity was calculated by the change in absorbance value at 340 nm for 5 min. GR activity was expressed as nmol of NADPH per minute per milligram of protein.

Catalase (CAT) was measured as the method described by Aebi (Aebi 1984). About 100 μl of liver homogenate with 900 μl of 50 mM H_2O_2 as the substrate. The reaction was monitored over 2 min at 240 nm in a recording spectrophotometer.

The method for determining the activity of NAD(P)H:quinone oxidoreductase 1 (NQO1) was well defined (Jaiswal et al. 1988). Reduction of cytochrome *c* (50 μM) in the presence of liver homogenate, 10 μM menadione and 1 mM NADPH was monitored for 2 min. The reactions were carried out in 100 mM potassium phosphate buffer, pH 7.7, containing 0.04% triton X-100 at 25°C. Activity of dicumarol-inhibitable menadione reductase was determined at 550 nm spectrophotometrically.

Reverse transcriptase-polymerase chain reaction analysis

Total RNA from hepatic tissues was extracted by using the Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA synthesis and subsequent PCR were performed through Titanium™ One-Step system (BD Biosciences Corporation, San Jose, CA, USA). Specific primer pairs for different genes were designed as shown in Table 1.

Western blot assay

The tissue homogenates were mixed with 4× sample buffer (8% SDS; 0.04% coomassie blue R-250; 40% glycerol; 200 mM Tris, pH 6.8 and 10% 2-mercaptoethanol) and boiled for 10 min. Samples were electrophoresed in a 10% SDS-PAGE minigel and then transferred onto polyvinylidenedifluoride membranes (PVDF; Millipore Corp., Bedford, MA, USA) with transfer buffer

Table 1 Primer sequences for the targeted genes

Gene (Accession No)	Primer sequences
Actin (V00481)	Forward: 5′-ACCTTCAACACCCCAGCCATGTACG-3′ Reverse: 5′-CTGATCCACATCTGCTGGAAGGTGG-3′
CAT (M11670)	Forward: 5′-GCGAATGGAGAGGCAGTGTAC-3′ Reverse: 5′-GAGTGACGTTGTCTCATTAGCACTG-3′
GPx (L24896)	Forward: 5′-CTCTCCGCGGTGGCACAGT-3′ Reverse: 5′-CCACCACCGGGTCGGACATAC-3′
GR (U73174)	Forward: 5′-GGGCAAAGAAGATTCCAGGTT-3′ Reverse: 5′-GGACGGCTTCATCTTCAGTGA-3′
Klotho (AB017820)	Forward: 5′-GGCCGACCATTTCAGGGATTAC-3′ Reverse: 5′-ATCGGGCAGCAGGGATGAGA-3′
NQO1 (BC083542)	Forward: 5′-ATAGCAAGAATGAGGCGGAGAC-3′ Reverse: 5′-TCAAACAATGGCTGAGGGACTA-3′

(48 mM Tris; 39 mM glycine; 0.0037% SDS and 20% methanol) at 350 mA for 60 min. The membranes were blocked with 5% nonfat milk in PBS solution containing 0.1% Tween-20 (PBST) for 1 h. The membrane was immunoblotted with primary antibodies of rabbit anti-rat Nrf2 and MAPK family in PBST solution containing 5% bovine serum albumin overnight at 4°C. After 30 min of consecutive PBST washes, the membrane was incubated with horseradish peroxidase-labeled secondary antibody for 60 min at room temperature and washed with PBST for 30 min. Final detection was performed with enhanced chemiluminescence (ECL^{TM} kit) western blotting reagents (Amersham Pharmacia Biotech, New Jersey, USA).

Statistical analysis

All data are presented as means ± SD. Statistical significance relative to the untreated group was calculated by a one- or two-way analysis of variance (ANOVA) and individual group means were then compared with the Student's *t* test.

Results

The levels of haemolysis and oxidative products

To evaluate the antioxidant ability against free radical induced damage, erythrocytes from rats of different ages were treated with or without peroxyl radical for up to 10 h at 37°C. Erythrocytes exposed to 15 mM AAPH underwent progressive haemolysis and the release of haemoglobin was determined. As shown in Fig. 1, the middle-aged and aged rats exhibited lower resistance to AAPH-mediated oxidative damage in red blood cells. The haemolysis percentages of middle-aged and aged rats were significantly ($P < 0.05$) elevated after 8 h incubation as compared to the young rats.

The liver performs critical functions, which include anti-oxidation and detoxification. Lipid peroxidation levels, which have been recognized as biomarkers for tissue injury, were measured to ascertain whether any changes in liver integrity occur throughout the aging process. In Fig. 2a, aged rats had higher contents of MDA as compared to the young rats ($P < 0.05$). Furthermore, oxidation of protein always results in the formation of carbonyl proteins, which has been used as a marker of protein dysfunction. As shown in Fig. 2b, the content of carbonyl proteins was associated with increasing age and the aged rats showed 2.2 times greater oxidative levels relative to the young rats.

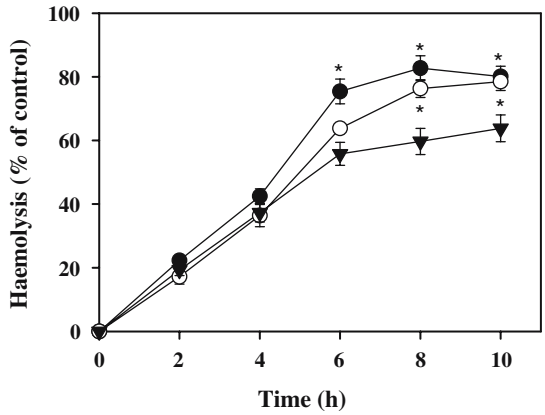

Fig. 1 Time course of haemolysis in rat erythrocytes induced by AAPH. Erythrocyte suspensions were incubated with or without 15 mM AAPH at 37°C. Significant differences in the young group (2 months old) compared with middle-aged group (1 year old) and aged group (1.5–2 years old) of rats (*$P < 0.05$). ▼: 2 months old; ○: 1 year old; ●: 1.5–2 years old

The expression of antioxidant enzymes

The different expression of various phase II antioxidant enzymes, including GPx, GR, CAT and NQO1, was elevated. As shown in Fig. 3a and b, the aged group of rats displayed lower glutathione-related antioxidant enzymes activities. The activity of GPx and GR in aged rats significantly decreased to 23% and 49%, respectively, as compared with young rats. The aged group of rats also exhibited lower activities of CAT and NQO1 (Figs. 3c, d) than other groups, which significantly decreased 26% and 86% relative to the young group, respectively. These trends were similar to the changes in GPx and GR.

Figure 4a indicates the mRNA expressions of antioxidant enzymes, including GPx, GR, CAT,

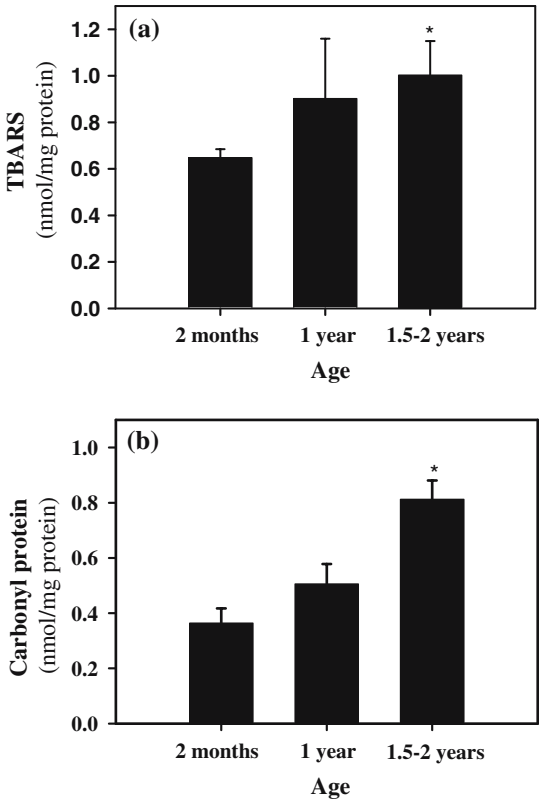

Fig. 2 Age-related levels of (**a**) TBARS and (**b**) carbonyl-protein formation during aging. Young group: 2 months old; middle-aged group: 1 year old; and aged group: 1.5–2 years old. The values presented as nmol TBARS formation/mg protein, and $*P < 0.05$ versus young group of rats

NQO1, and the age-related klotho. The data displayed different expression patterns in the three age groups. The results showed that the activities of all genes in aged rats were dramatically decreased (declined about 53%, 60%, 62%, 69%, and 72% in GPx, GR, NQO1, CAT and klotho, respectively) as compared to the young group of rats.

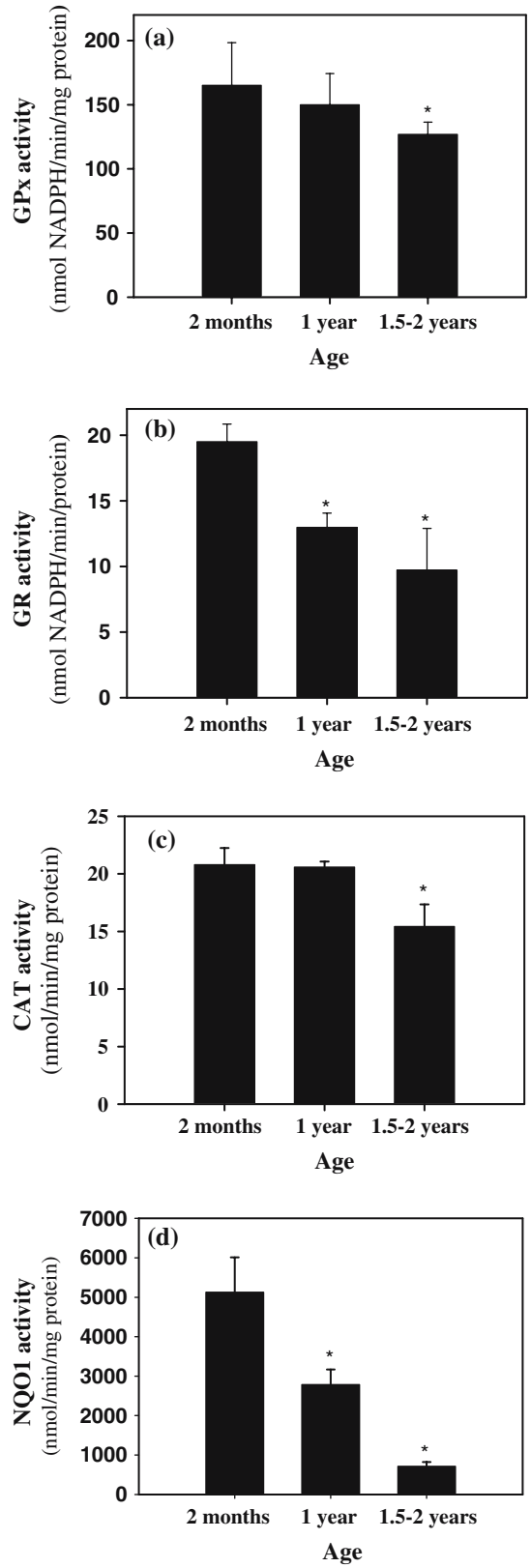

Fig. 3 Effect of aging on the activities of antioxidant enzymes. (**a**) Glutathione peroxidase (GPx). (**b**) Glutathione reductase (GR). (**c**) Catalase (CAT) and (**d**) NAD(P)H:quinone oxidoreductase 1 (NQO1). The results are expressed as nmol/min/mg protein in the case of GP, GR, CAT and NQO1. $*P < 0.05$ and $**P < 0.01$ as compared with young group of rats

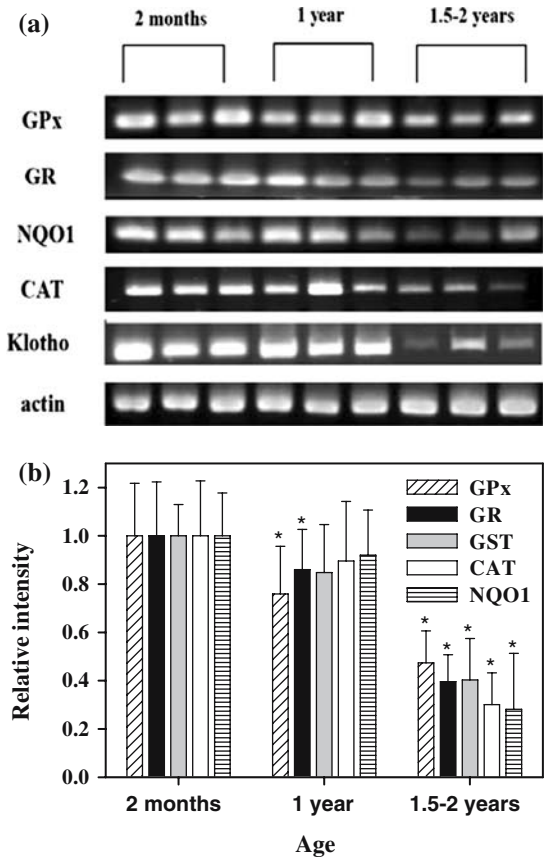

Fig. 4 Phase 2 antioxidant enzymes and age-related gene expression during aging. (**a**) mRNA expression of specific genes at various ages. Young group: 2 months old; middle-aged group: 1 year old; and aged group: 1.5–2 years old. (**b**) *$P < 0.05$ compared to young group of rats ($n = 6$–8)

Age-related decline in transcription factor Nrf2

The transcription factor, Nrf2, plays a crucial role in the regulation and activation of the transcription of antioxidant enzymes. Thus, we examined whether Nrf2 levels are negatively affected during aging. The age-dependent expression of transcription factor Nrf2 in rats with different ages was detected (Fig 5). Rats in each group were used to determine the total cytosolic Nrf2 activity in liver tissue. Results of the western blot assay showed that aged groups of rats exhibited significantly lower Nrf2 content than young group of rats (46.4% decreased, $P < 0.05$).

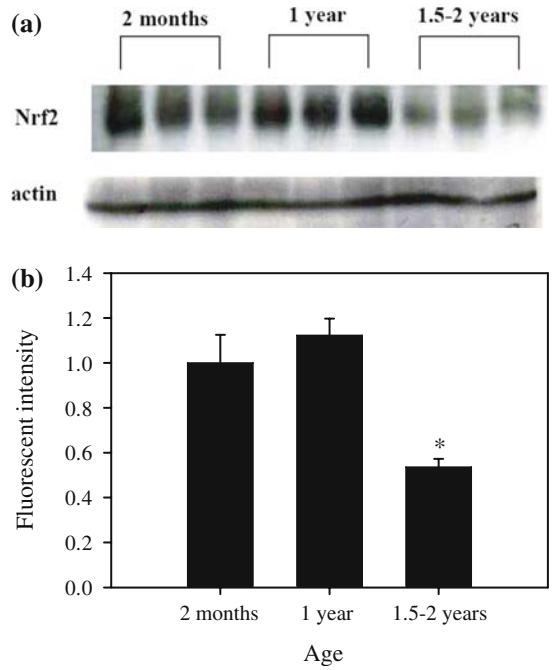

Fig. 5 Nrf2 expression in rats of varying ages. (**a**) Western blot analysis of rat liver total Nrf2 expression from young group: 2 months old; middle-aged group: 1 year old; and aged group: 1.5–2 years old. (**b**) Significant difference between the aged and young groups of rats ($P < 0.05$)

MAPK family participate in the signaling transduction

To evaluate the mechanism involved in the regulation of signals that stimulate the expression of the transcription factor, Nrf2, the members of MAPK cascades were examined. The MAPK pathway is thought to participate in the upstream mediation of Nrf2. Figure 6 illustrates the expression of p47phox. The increase in oxidative stress was accompanied by increasing age and age-related deficits in Nrf2 were regulated by the different activities of phosphorylated ERK1/2 and p38. The expression of phospho-ERK1/2 was decreased and the phospho-p38 level was increased with age, but the expression of phospho-JNK did not change significantly (data not shown). The data suggests that the activities of antioxidant enzymes were affected by the expression of transcription Nrf2, which was regulated by signaling mediators, MAP kinases.

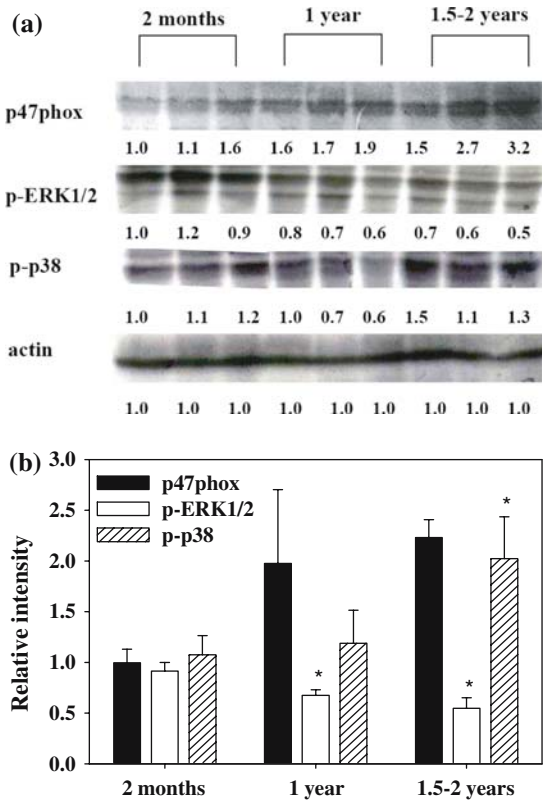

Fig. 6 Changes in the expression of p47-phox and MAPK family in aging rats. Western blot analysis of rat liver MAPK family expression from young group: 2 months old; middle-aged group: 1 year old; and aged group: 1.5–2 years old

Discussion

The chronic process of aging leads to a functional imbalance, increased pathology and death. It has been hypothesized that senescence is the result of accumulated oxidative injury. Methods of promoting cellular antioxidant capacity and reducing oxidative stress are areas of intense investigation.

In the present study, we examined the antioxidant status of aging rats. Two-month-old rats (young group), weighing 200–250 g, 1 year old rats (middle-aged) weighing 400–600 g, and 1.5–2 year old rats (aged group) weighing 650–750 g, showed no difference in histological analysis and serum biochemical values, except cholesterol (aged group was 2 times over the young group) and albumin content (aged group decreased 22% as compared to the young group, data not shown). In addition, we evaluated the free radical-induced oxidative damage in the blood circulation using the haemolysis assay; the data showed that erythrocytes exposed to AAPH underwent progressive haemolysis. Furthermore, lipid damage, expressed in TBARS levels, and protein damage, measured by the liver carbonyl content in the aged rats, were significantly higher in the older rats relative to the younger rats ($P < 0.05$, Fig. 2). Similar results have shown that aged rats exhibited higher TBARS content than others (Sanz et al. 2002).

In the assays of the antioxidant enzymes, including GPx, GR, CAT, and NQO1, both the activity and mRNA were analysed. The data showed that the aged group of rats exhibited lower enzymatic activities and genetic expression than the other groups of rats. The literature indicated that many antioxidant enzymes, such as superoxide dismutase and xanthine oxidase, display lower activities in elder rabbit cornea (Cejkova et al. 2004). Furthermore, levels of the age-related gene, *klotho*, decreased with age. The expression of *klotho* is closely related to the lifespan of the organism (Takahashi et al. 2000; Kuroso et al. 2005). Recently, it has been shown that klotho increases the resistance to oxidative stress induced cell death (Yamamoto et al. 2005; Ikushima et al. 2006). This evidence may explain why aging rats experience higher levels of oxidative injury.

It has been reported that there is an age-dependent decrease in the activity of antioxidant defense systems (Semsei et al. 1989; Rao et al. 1990); however, few researches focus on the involved mechanism. We determined the content of the transcription factor, Nrf2, which mediates activation of the antioxidant responsive element (ARE)-related gene expression, such as NQO1, HO-1, and GST, etc, and thus plays a critical role in activation of phase II detoxifying and antioxidant enzymes. Our data showed that the aged group of rats exhibited lower total Nrf2 content relative to other groups (Fig. 5). Similar results showed that the decline in transcriptional activity of Nrf2 causes the age-associated loss of glutathione synthesis, which is directly involved in the expression of glutamylcysteine synthetase (Suh et al. 2004). The

mitogen-activated protein kinase (MAPK) family is well known that involves in the regulation of survival, proliferation and cell death (Johnson and Lapadat 2002; Dent et al. 2003). MAP kinase members, such as ERK, JNK, and p38, are considered to be involved in regulation of the ARE (Chen et al. 2004). Additionally, the subunit of NAD(P)H oxidase, p47phox, which has been widely defined as a sensor to the ROS, was also analysed to evaluate is tissue after oxidative stress challenge (Touyz and Schiffrin 2004). According to the data of the expression of p47phox, we observed that the increase in oxidative stress was accompanied by increasing age. Age-related deficits in Nrf2 were regulated by the different activities of specifically phosphorylated ERK1/2 and p38, which might affect the expression of antioxidant enzymes consistently. Based on the present data, we have found that declines in the expression of antioxidant enzymes, such as NQO1, GPx, GRd, and CAT, are critically involved in the transcriptional activity of Nrf2 and regulation of MAP kinase pathway, especially in the upregulation of phospho-p38 kinase and the down-modulation of phospho-ERK1/2 kinase. These results are similar to a recent study that shows ERK and JNK signaling pathways induced the recruitment of a co-activator to the transcription initiation complex and up-regulated Nrf2 transcriptional activity (Shen et al. 2004). As researchers of functional food and health enhancement, our purpose is to understand how and what we should eat to improve health throughout the aging process. Recently many studies have been focused on the significant relationship between phytochemicals and Nrf2, such as the induction of detoxifying enzymes by garlic organosulfur compounds through transcription factor Nrf2 (Chen et al. 2004). Nrf2 contributes to the expression of glutathione S-transferase in mice liver and could be induced by synthetic antioxidants (Suh et al. 2004).

In conclusion, we evaluated the correlation between aging and antioxidant status and many important antioxidant enzymes. Furthermore, a potential mechanism was also described. Our work suggests that age-related decreases in resistance to oxidative stress, might result from the lower expression of antioxidant enzymes, which are regulated by the transcriptional activity of Nrf2 and its mediators, MAP kinases. As a result, we question whether the supplement of healthy food, herbal medicine, or nutraceuticals, which are all naturally occurring compounds, will promote antioxidant activity by scavenging free radicals directly or enhancing the expression of antioxidant enzymes through biological mechanisms.

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