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Aging effect on myeloperoxidase in rat kidney and its modulation by calorie restriction

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Abstract

Myeloperoxidase (MPO), a heme protein existing in neutrophil and monocyte, is implicated in various stages of inflammatory conditions with the production of a variety of potent oxidants. To investigate the extent of the involvement of MPO in aging, we measured MPO activities in kidney of rats at different ages maintained with an ad libitum (AL) or a calorie restriction (CR) dietary regimen. Results showed that the MPO activities increased during aging in AL rats, but were significantly attenuated by CR. This result was consistent with altered protein level of MPO during aging. In addition, we were able to detect dityrosine that is a stable end MPO-oxidation product. The amount of dityrosine increased in old AL, but not in old CR rats. To examine the source responsible for increased MPO activity during aging for leukocyte recruitment and infiltration, the levels of vascular cell adhesion molecule (VCAM-1) protein were measured. The level of VCAM-1 showed age-dependent increase in AL rats, which was correlated with higher activity of MPO in old AL rats. Furthermore, we have found that LPS-induced inflammation increased the activity and protein levels of MPO, and VCAM-1 expression in young rat kidneys. These findings suggest that increased MPO activity with aging may related to increased recruitment of inflammatory cells, contributing to protein oxidation accumulation in the aging process. We propose that age-related alterations of MPO, dityrosine, and VCAM were modulated by CR through its anti-inflammatory action.

Keywords: *Myeloperoxidase, aging, calorie restriction, inflammation, dityrosine, vascular cell adhesion molecule*

Introduction

Aging is known to closely associate with increased pro-inflammation conditions, and "molecular inflammation hypothesis of aging" has recently been proposed to highlight the age-related shift in the cellular redox balance to pro-inflammatory status [1,2]. Sustained stimulation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive chloride species (RCS) cause chronic inflammation, which is characterized by the infiltration of various migratory inflammatory cells such as neutrophiles, macrophage, and lymphocytes [3]. Various ROS including RCS, such as HOCl and RNS are now well accepted to contribute to tissue injuries that occur during inflammatory responses [1,2].

Myeloperoxidase (MPO) is a key enzyme of neutrophil to produce potent oxidants including hypochlorous acid (HOCl), tyrosyl radical ($Tyr^{\cdot}$) and nitrogen dioxide ($NO^{\cdot}_2$), acting as a bactericidal system against invading organisms [4,5]. However, the uncontrolled formation of these oxidants leads to protein oxidation [6,7] and lipid peroxidation [8–10] that in turn cause damage in cells and tissues. It is well

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established that MPO-derived oxidants can cause cell damages *in vitro* [6–10]. Many lines of evidence have been implicated that the role of MPO in promoting oxidative stress in many inflammatory diseases including ischemia-reperfusion injury, atherosclerosis, rheumatoid arthritis, periodontal disease and proteinuric glomerulopathies [11–15].

In addition, MPO generate a family of tyrosyl radical added products, and then dityrosine is formed through interaction with two tyrosyl radical [16,17]. Protein and lipids damaged by tyrosyl radical from MPO-derived tyrosine oxidation, i.e. tyrosylation might play a role in the pathogenesis of many diseases [18]. Therefore, stable MPO-oxidation end products such as dityrosine may be used as useful biomarkers to evaluate the involvement of MPO in the aging process. Accumulated inflammatory cells are engaged in tissue damage, suggesting contribution of MPO dependent oxidants to tissue damages. Another link to associate MPO with aging is to investigate regulation of adhesion molecules (AMs) that are responsible for leukocytes infiltration. For instance, vascular cell adhesion molecule (VCAM-1) is the essential AM on the EC for infiltration of inflammatory cells, by taking charge in firm adhesion of inflammatory cells to EC. Previous studies have revealed the over-expression of AMs and the consequent accumulation of inflammatory cells in ischemic tissues [19–21]. Moreover, increases in AMs levels have been reported in aorta and serum during aging as showed recently from our laboratory [22].

CR is the only established anti-aging and anti-oxidant paradigm [23–25]. CR commonly refers to a reduction in calorie intake without a reduction in essential nutrients or malnutrition. CR has been shown to suppress a variety of oxidative cellular damages and, at the same time, maintain antioxidant defense system, including major reactive species (RS) scavengers during aging [26–28]. These powerful mechanisms to suppress cellular damage, to enhance defense system and to maintain redox balance are proposed to be responsible for CR's anti-aging action.

Although the molecular aspects of pro-inflammatory proteins have been investigated based on the oxidative stress hypothesis [1,2], the detailed information and question regarding the status of MPO in pro-inflammatory aging have not been fully examined. In the present study, we provide evidences that MPO is associated with aging, and the implication of MPO in the aging process is modulated by CR.

Materials and methods

Animals

Specific pathogen-free (SPF) male Fischer 344 rats (6, 12, 18, and 24 months old) were obtained from University of Texas Health Science Center at San Antonio, U.S.A. They were fed a diet of the following composition: 21% soybean protein, 15% sucrose, 43.65% dextrin, 10% corn oil, 0.15% α-methionine, 0.2% chlorine chloride, 5% salt mix, 2% vitamin mix, and 3% Solka-floc. The ad libitum (AL) fed group had free access to both food and water. The animal designated as CR regimen was fed 60% of the food intake of AL-fed, beginning at 6 weeks of age. Histopathological analysis found no sign of nephritic lesions in these soy-protein fed rats even at the advanced age of 24 months [40]. All protocols utilized in the animal maintenance are approved by an Institutional Animal Care and use Committee at University of Texas Health Science Center at San Antonio, U.S.A. Rats at the 6, 12, 18, and 24 months old were decapitated. SPF male Fischer 344 rats at 6 and 24 months of age were used for lipopolysaccharide (LPS) experimentation. LPS was intraperitoneally administered at a dose of 5 mg/kg to 6 months old Fischer 344 rats. Five hours after the injection, rats were sacrificed. The tissues were immediately frozen in liquid nitrogen and stored at −80°C until analyses were performed.

Materials

All chemical reagents were obtained from Sigma (MO, USA). Western blotting detection reagents were obtained from Amersham (Bucks, UK). Antibodies against MPO were obtained from Santa Cruz Biotechnology (CA, USA). Anti-goat IgG-horseradish peroxide—conjugated antibody and anti-mouse IgG-horseradish peroxide conjugated antibody were obtained from Amersham. Antibodies (IC3) against dityrosine were kindly provided from Dr Yoji Kato, School of Humanity for Environment Policy and Technology, Himeji Institute of Technology, Japan.

Kidney sample preparation

One gram of kidney was homogenized with polytron homogenizer in homogenization solution of the following compositions: 50 mM phosphate buffer (pH 7.4), 0.5 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediaminetetraacetic acid disodium salt, 1 uM leupeptin, 80 mg/l trypsin inhibitor. The homogenate was centrifuged at 900*g* at 4°C for 15 min and supernatants (post-mitochondrial fraction) were used for further experiments in this study.

MPO activity assay

This assay was designed to measure the production of tetramethylbenzidine (TMB) oxidation by MPO/H_2O_2. Samples were mixed with reacting reagent (15 mM TMB, 100 mM sodium acetate, 60 mM hydrogen peroxide). The reaction was monitored at 650 nm for 30 min on a Genios TECAN Spectrophotometer (Austria). Data was

expressed mU/mg, protein. One unit of activity is defined as the amount of enzyme that will utilize 1 umol of hydrogen peroxide per minute. In this assay, one mill unit of MPO will cause absorbance change of $0.0114\,min^{-1}$ at a 650 nm.

Western blot analysis

Western blotting was carried out with different aged rat kidney (6, 12, 18 and 24 months old) post-mitochondrial fractions from AL and CR rats. The samples were boiled for 5 min with a gel-loading buffer pH 6.8; 0.125 M Tris (hydroxymethyl) amino-methane, 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol, and 0.2% bromophenol blue in a ratio of 1: 1. Total protein-equivalents for each sample were separated on an SDS—polyacrylamide mini gel using 10% acrylamide gels as described by Laemmli et al. (1970) at 100 V and transferred to a poly (vinyldene fluoride) membrane at 60 V for 1 h in a semi-dry transfer system. The membrane was immediately into a blocking solution (5% skim milk) at 25°C for 30 min. The membrane was incubated with a primary antibody (0.5% skim milk, diluted 1:200) at room temperature for 2 h. The membrane was incubated with secondary antibody polyclonal anti-goat antibody and anti-mouse antibody (0.5% skim milk, diluted 1:4000) in TBS-Tween buffer at room temperature for 1 h. Horseradish—conjugate secondary antibody labeling was detected by enhanced chemiluminesence (ECL) Western botting reagents and analysis (Amersham Bioscience, NJ) following the manufacturer's instructions. Prestained blue protein markers were used for molecular weight determination.

Enzyme-linked immunosorbent assay (ELISA) for dityrosine formation

The indirect ELISA was performed as described [29–31] with modification. Briefly, 0.01 mg/ml of proteins was dispensed into wells and kept overnight at 4°C. After the coating, the plate was washed with PBS containing 0.25% Tween 20 (TPBS) and water. The wells were blocked with 200 ul of 4% skim milk for 1 h at 37°C. After washing, the plate was incubated with the monoclonal antibody (0.1–0.2 mg/ml TPBS) for 2 h at 37°C. After washing, the binding of the antibody to the modified protein was evaluated by incubation with a peroxidase-labeled anti-mouse Ig G antibody (diluted 1/5000) for 1 h at 37°C. After washing, the color development was performed by reaction of the conjugated peroxidase with *o*-phenylenediamine and H_2O_2. Once adequately color developed, reaction was stopped by adding 50 ul 2N H_2SO_4. The plate read using ELISA reader at 490 nm. Data represent the mean of triple determinations.

Statistical Analysis

The statistical significance of the differences between an ad libitum and a calorie restriction (CR) regimen was determined by ANOVA with PLSD. Values of $P < 0.05$ were considered statistically significant. Analyses were performed using Statview® software.

Results

MPO activities were measured in kidney homogenates taken from rats of different ages (6, 12, 18, and 24 months old) maintained on either AL or CR regimen. MPO activity was significantly increased with age at the 18 and 24 months old AL, which was unchanged in CR (Figure 1A). Compared to the MPO activity of 6 months old AL, the activities were significantly elevated by 70% ($P < 0.01$) and 85% ($P < 0.01$) at the 18 and 24 months old AL, respectively.

However, elevated MPO activities in old AL rats were modulated by CR regimen. Levels of MPO protein were closely related with the altered MPO

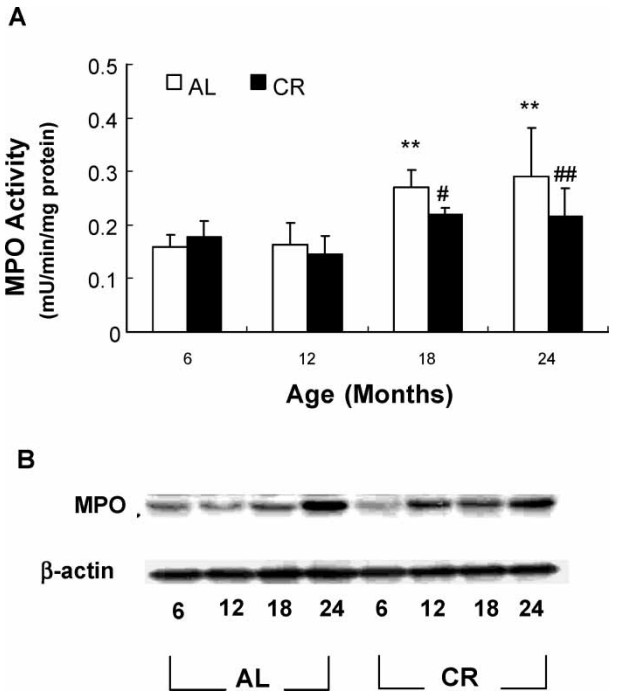

Figure 1. *Both activity and protein level of MPO were measured in rat kidneys.* (A) MPO activities were detected by specific MPO activity assay in AL group, (6, 12, 18, and 24 months old) and the same aged CR regimen. MPO activities significantly were increased in AL, but not in CR regimen during aging. Values are the mean ± S.D. ($n = 4$ or 5 rats/group). $^{**}P < 0.01$ vs. 6 months old AL rats; $\#P < 0.05$ and $\#\#P < 0.01$ vs. age-matched AL rats (ANOVA with PLSD test). (B) Western blot analysis was performed to detect protein levels of MPO. The protein levels of MPO were consistent with altered of MPO activity in both AL and CR rat kidneys. Levels of β-actin were used to normalize the levels of proteins loaded. One representative blot of each protein was shown from three experiments that yielded similar results.

activities during aging (Figure 1B). In particular, there were significantly elevated levels of MPO protein at the 24 months old AL. However, CR regimen attenuated the age-dependent increase of MPO protein. To determine whether the age related alterations of MPO activity and protein level are involved in the MPO derived protein oxidation, we measured levels of dityrosine that is one of major MPO-oxidation products, using ELISA (Figure 2A) and immunoblot analysis (Figure 2B). Levels of protein-dityrosine were elevated at the 24 months old AL, compared to 6 months old AL approximately 25% ($P < 0.01$). However, CR regimen decreased the levels of dityrosine approximately 20% ($P < 0.01$) and 15% ($P < 0.01$) at the 18 and 24 months old, respectively, compared to the age-matched AL group. Western blot result was similar to the ELISA results (Figure 2B). To assess the alteration of MPO involvement in the recruitment or infiltration of inflammatory cells during aging, we measured levels of VCAM-1 protein at the different aged AL and CR rat kidney. Levels of VCAM-1 were significantly increased with age in AL (Figure 3). However, levels of VCAM-1 protein in rat kidneys taken from CR were unaltered during aging. We measured the activity and protein levels of MPO, and VCAM-1 expression in LPS treated rat kidneys to verify whether LPS-induced inflammation has a similar effect as the aging process. The result show that MPO activities were increased in LPS treated 6 months old rat kidneys (Figure 4A). The increase of protein levels of MPO and VCAM-1 was accompanied by LPS treatment in 6 months old rat kidneys (Figure 4B and C). These findings indicated that MPO and VCAM-1 are co-expressed in inflammatory regions and LPS-induced inflammation could mimic the aging effects on MPO and VCAM-1 in rat kidneys.

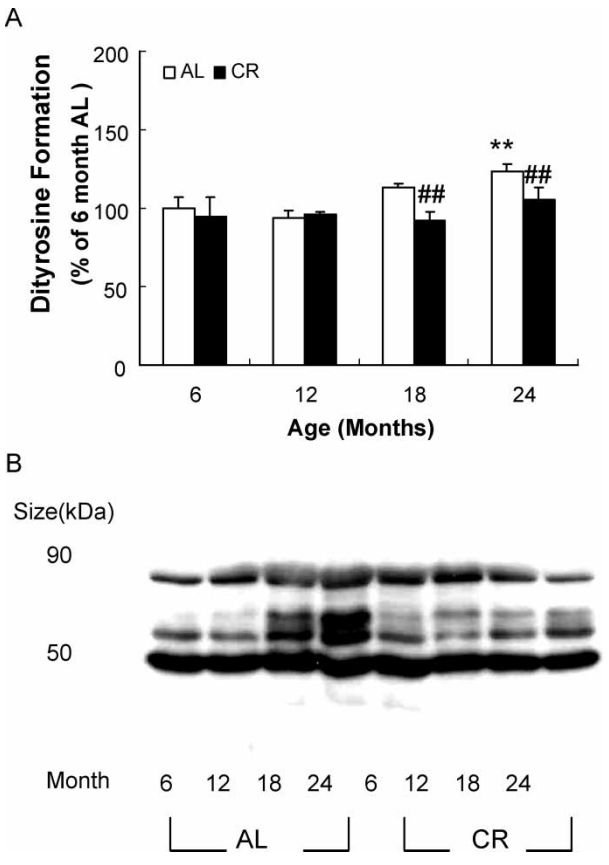

Figure 2. *Effects of aging and CR on dityrosine formation were analyzed in rat kidneys*. (A) Dityrosine Formations were detected by ELISA in AL group, (6, 12, 18, and 24 months old) and the same aged CR regimen. AL significantly increased dityrosine formation during aging. No significant changes of dityrosine formation in CR regimen. Values are the mean ± S.D. ($n = 4$ or 5 rats/group). ** $P < 0.01$ vs. 6 months AL rats; ##$P < 0.01$ vs. age-matched AL rats (ANOVA with PLSD test). (B) Dityrosine formations in homogenates of rat kidneys from AL and CR group (6, 12, 18, and 24 months old) were subjected to immunoblot analysis using antibody that recognized protein-dityrosine. Whole proteins were screened and size between 50 and 90 kDa of protein were severely in creased dityrosine formation in AL, but modulated by CR regimen. One representative blot of each protein is shown from three experiments that yielded similar results.

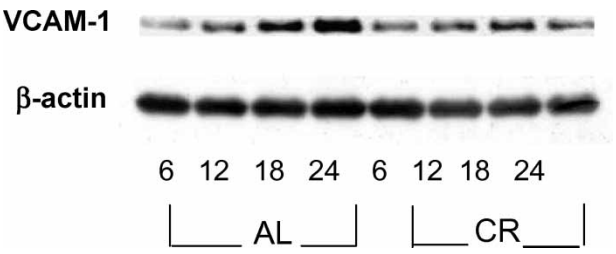

Figure 3. *Western blot analysis of VCAM-1 was performed in rat kidneys*. Representative western blot showed relative levels of VCAM-1 protein in AL group (6, 12, 18, and 24 months old) and the same aged CR regimen. Note that levels of VCAM-1 protein were age dependently increased in AL, but not changed in CR regimen during aging. This representative result of each protein was shown from three experiments that yielded similar results.

Discussion

Many studies demonstrated the presence of MPO-containing cells as well as MPO protein and activity in many renal diseases [36,37]. In addition, MPO has been implicated in pathogenesis of a variety of vascular abnormalities [38,39]. However, to date, there are few reports on the status of MPO during aging. Therefore, we investigated aging effect on MPO in kidney and its modulation by the anti-oxidative action of CR regimen.

We were able to document in the present study that both activity and protein level of MPO, dityrosine formation, and level of VCAM-1 protein were increased in AL rats during aging, but remained unchanged in CR rats. Total MPO activity and protein level clearly increased in rat kidney during aging. These findings (Figure 1A and B) indicate that the increased activity and protein level of MPO in the AL

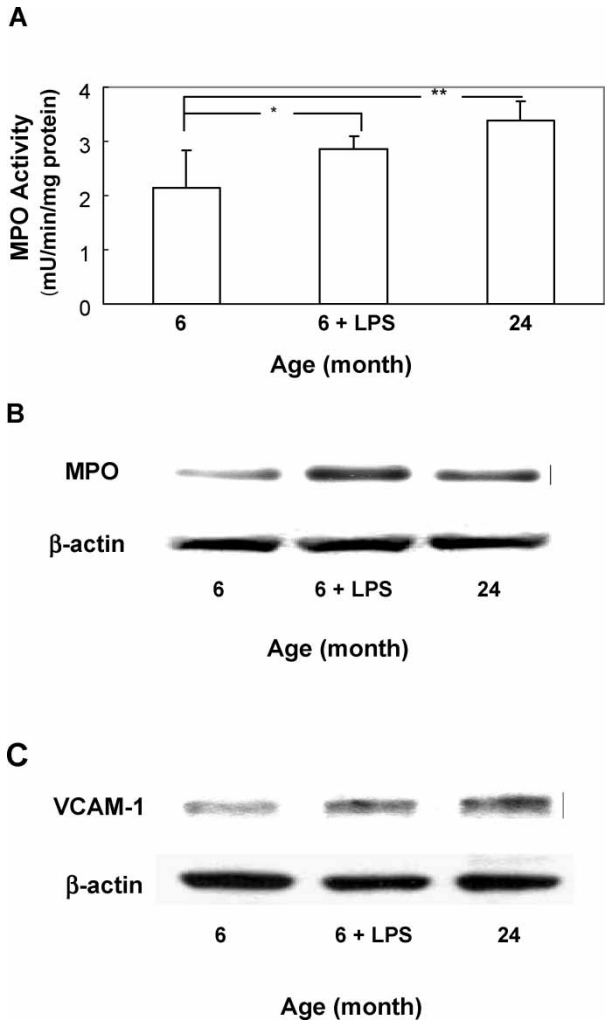

Figure 4. *Effects of LPS-induced inflammation on MPO and VCAM-1 in rat kidneys*. (A) MPO activities were detected by specific MPO activity assay in LPS treated young group (6 months old) and in old group (24 months old); LPS, group treated with LPS 5 mg/kg of LPS; control, treated with an equal volume of vehicle as saline. MPO activities were increased in LPS treated 6 months old rat kidneys, compared with control 6 month old rat kidneys. Values are the mean ± S.D. ($n = 4$ or 5 rats/group). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control 6 months old rat kidney; (ANOVA with PLSD test). (B) Protein levels of MPO were detected by western blot analysis. Protein levels of MPO in LPS treated 6 months old rat kidneys was significantly increased compared to that of control 6 months old rat kidneys. β-actin were used as a protein loading control. One representative blot of each protein was shown from three experiments that yielded similar results. (C) Expression of VCAM-1 in LPS treated rat kidneys was measured by western blot analysis. The protein levels of VCAM-1 were increased in LPS treated 6 months old rat kidneys compared to that of control 6 months old rat kidneys. β-actin were used as a protein loading control. This representative result of each protein was shown from three experiments that yielded similar results.

rat kidneys likely resulted from age-related elevation of activated inflammatory cells. The elevated MPO activity during aging might be caused by increased recruitment of activated inflammatory cells at inflammatory sites or by increased activity of MPO in the recruited cells. This was verified by measuring that MPO activity and protein expression levels in LPS treated young rat kidneys (Figure 4A and B). LPS-induced inflammation has accompanied with increased MPO activity and protein levels in young rats. All of the data we obtained in the current study are consistent and are supportive of the molecular inflammation hypothesis of aging that was proposed from this laboratory [1].

To probe the migration of inflammatory cells, we monitored VCAM-1 protein levels in several aged groups (Figure 3). Our data showed the elevated VCAM-1 protein levels in kidney during aging, indicating more recruitment of activated inflammatory cells into the aged tissue. Our laboratory reported that other adhesion molecules (AMs), E-selectin, P-selectin, and ICAM increased in aorta and serum [22]. In addition, several other reports showed that increased levels of VCAM-1 were closely associated with age-related diseases such as atherosclerosis, coronary heart disease and peripheral arterial occlusive disease [35]. These pro-inflammatory phenomena may well be related to the age-related oxidative stress, which can exacerbate the tissue damage followed by enhanced inflammatory cell infiltration during aging. The other possible mechanism underpinning increased MPO activity during aging may be that the activated inflammatory cells have more MPO activity in the aging process. Therefore, elevated MPO activity during aging partially contributes to increased incidence of various inflammatory diseases. In fact, many studies reported that protein oxidation via MPO/H_2O_2 system and hypochlorite is major oxidants generated by neutrophils and macrophages activated at inflammatory sites, such as in atherosclerotic lesions [13–15,32].

In the current study, we obtained evidence showing the increased dityrosine formation in kidney during aging. Dityrosine, one of the specific biomarkers for MPO activity, is formed via tyrosyl radical intermediates which can be generated by MPO [17]. We confirmed that dityrosine formation was increased in MPO/tyrosine/H_2O_2 system *in vitro* (Data not shown). Heinecke et al. reported that *o*,*o*′-dityrosine generated by activated phagocyte cause cross-links in proteins and lipoproteins *in vitro*. *o*,*o*′-dityrosine level in tissue are elevated in arthrosclerosis, inflammatory lung disease, neurodegenerative disorders, and aging [12,34].

Kato et al. developed novel monoclonal antibody for detecting protein dityrosine and the antibody reacted with peptidyl dityrosine, derived from Thr-Thr-Ser, rather than free dityrosine [29–31]. In the present study, we used this specific antibody for anti-dityrosine as a marker for protein oxidation during aging. Our current study demonstrated that the tyrosylation process during aging process has been elevated with association of the increased MPO

activity and its protein level (Figure 2A and B). However, we were able to get evidence that CR effectively down-regulated the elevated tyrosylation in the kidney, as the attenuated dityrosine contents in the cardiac and skeletal muscle of mice by CR [33]. It is noteworthy pointing out that some of bands appeared in western blot analysis (Figure 2B) showed more significantly increased in 18 and 24 months of AL rat kidneys as compare to ELISA results. The probable reason is that dityrosine bound proteins might be masked by the unchanged other dityrosine bound proteins in ELISA because ELISA results represented for whole dityrosine bound proteins. The densitometric analysis of whole bands in western blot was consistent with ELISA results (data not shown).

In the present study, we documented that the age-related change in MPO both activity and protein level, dityrosine formation, and level of VCAM-1 protein were significantly attenuated in CR. Although the cellular basis of CR's beneficial effects on aging has not been well elucidated, recent work on CR provided abundant evidence that CR likely exerts diverse benefits by its ability to uphold the antioxidant defense system [23,27].

Putting together, MPO activities and their protein levels, VCAM-1 protein levels are significantly increased with aging. Dityrosine formations are also elevated in aged rat kidney. Moreover, alterations of MPO activity and its protein level, VCAM-1 protein level and dityrosine formation were counteracted by the anti-aging, anti-inflammatory CR. These finding suggest that increased MPO activity may be due to increased recruitment of inflammatory cells, contributing to protein oxidation accumulation during aging. Collectively, current study suggests that the anti-aging capacity of CR attributes to its anti-inflammatory function as well as anti-aging function, which is consistent with the molecular inflammation hypothesis of aging.

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