Upregulation of Aortic Adhesion Molecules During Aging

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To investigate effects of aging on adhesion molecules (AMs), the present study assessed the expressions of aortic P-selectin and vascular adhesion molecule-1 (VCAM-1) in young (6-month-old) and old (24-month-old) Fischer 344 rats fed ad libitum (AL) or calorie-restricted diets. Results showed increased levels of aortic P-selectin and VCAM-1 in the old AL rats, causing excessive leukocyte infiltration as indicated by enhanced myeloperoxidase level. These elevations were parallel to increased oxidative stress including lipid peroxides during aging. Then involvement of redox-sensitive transcription factor nuclear factor-κB was analyzed, and greater activation of nuclear factor-κB-inducing kinase (NIK)/IκB kinase (IKK)/Inhibitor of IκB (IκB) pathway in aorta from old AL rats was found. Further, in cultured endothelial cells challenged by various oxidative stimuli, the induced redox imbalance triggered overexpression and promoter activities of P-selectin and VCAM-1. Our study documented that aortic upregulated AMs with age are closely related to activation of NIK/IKK/IκB/nuclear factor-κB pathway brought on by oxidative stress.

VASCULAR aging contributes to age-related hypertension, cardiovascular diseases, chronic heart failure, and stroke (1). However, the molecular mechanisms underlying vascular aging are still unclear. Researchers have suggested that interactions between vascular endothelial cells (ECs) and circulating leukocytes are involved in various age-associated vascular disorders (2–5). These interactions are found to be mainly regulated by activation of adhesion molecules (AMs), especially those expressed on the surface of vascular ECs (2,3).

AMs expressed on ECs consist of selectins and immunoglobulin superfamilies that orchestrate recruitment of leukocytes at different stages. The activation of selectins (including P-selectin and E-selectin) attracts leukocytes and initiates a low-affinity EC/leukocyte interaction (6). This interaction triggers production of immunoglobulin superfamily AMs, for instance, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) on the ECs, resulting in a firm adhesion of leukocytes and a subsequent transendothelial migration (7). The expression of AMs on the EC is tightly regulated to maintain an extremely low concentration during resting stage. However, significant amounts of AMs have been detected when ECs were confronted by proinflammatory mediators such as bacterial toxins and cytokine tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (6,8). Two transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), have been identified as major regulators responsible for the upregulation of E-selectin, P-selectin, VCAM-1, and ICAM-1 on ECs (6,9). An appropriate regulation of AMs is critical for host defenses; in contrast, overexpressed or improperly expressed AMs recruit excessive leukocytes, which will cause a breakdown of healthy cells and matrix components via their extravagant antimicrobial, secretory, and phagocytic activities (10).

On the basis of their potential pathogenic roles, AMs are postulated to be one of the causative factors in aging-related vascular diseases. To examine this possibility, a logical first step would be to document the status of AMs during aging. Previously, we and others reported evidence on the increased level of soluble AMs in old rats (11,12), although a full elucidation has not been presented. To date, there was still little exploration of the underpinning molecular mechanisms of altered AM expression during aging. In the present study, we looked into the molecular processes involved in aortic AM expression with age by focusing on P-selectin and VCAM-1 as representative AMs. In addition, because of the suspected redox sensitivity of these AMs, we asked whether the activation of AMs is causally linked to age-related oxidative stress. To validate our investigation, we applied calorie restriction (CR), the anti-aging experimental paradigm known for its potent anti-oxidative effects (13,14). We strengthened our current study by including an
in vitro study in which activation of AMs in cultured ECs was assessed under introduced redox imbalance. Our findings indicated a pivotal role of oxidative stress in the expression of AMs during aging.

**Methods**

**Animals and Diet**

Rat maintenance procedures for specific pathogen-free status and dietary composition of chow have been previously reported (15). Briefly, specific pathogen-free male Fischer 344 rats were fed a diet of the following composition: 21% soybean protein, 15% sucrose, 43.65% dextrin, 10% corn oil, 0.15% L-methionine, 0.2% choline chloride, 5% salt mix, 2% vitamin mix, and 3% Solka-Floc fiber. The diet was prepared by the Purina Test Diet Lab (St. Louis, MO). The ad libitum (AL) fed group had free access to both food and water. The animals designated as CR were fed 60% of the amount of food of their AL-fed littermates, beginning at 6 weeks of age. All protocols utilized for animal maintenance are approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio. This study complied with the Guide for the Care and Use of Laboratory Animals by the U.S. National Institutes of Health (Publication No. 85-23).

Rats used in this study were ages from 6 months (young) to 24 months (old). Each group consisted of six rats. Body weights of each group were as follows: 6-month-old AL/CR rats = 366.4 ± 15.9/212.3 ± 8.7 g; 12-month-old AL rats = 461.3 ± 46.9 g; 18-month-old AL rats = 491.5 ± 17.8 g; 24-month-old AL/CR rats = 463.3 ± 32.6/257.7 ± 28.8 g.

**Aorta Sample Collection**

Rats were decapitated, the chests were opened, and aortas were quickly excised, immersed in ice-cold isotonic saline, and then were rapidly frozen in liquid nitrogen and stored at −80°C until assayed. Cytosolic and nuclear proteins from aorta were extracted by using an NE-PER extraction kit (Pierce, Rockford, IL) according to the manufacturer’s instructions.

**Cell Culture and Experimental Procedure for Treatment**

Rat EC line YPEN-1 was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were grown in Dulbecco’s modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and were maintained at 37°C in a CO2 incubator. Before treatment, fresh medium containing 1% FBS was exchanged to cells.

2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) were applied as water- and lipid-soluble peroxyl radical generating systems, respectively. Administration of xanthine oxidase (XOD)/xanthine was used as a system for generation of superoxide (O2⁻) and H2O2. Buthionine sulfoximine (BSO) is a potent inhibitor of γ-glutamyl cysteine synthetase, the rate-limiting step enzyme in glutathione (GSH) synthesis. Diethyl maleate (DEM) is a substrate for glutathione S-transferase, which catalyzes the formation of GSH-DEM conjugate. The net change in redox status with BSO and DEM treatment is the decreased total cell GSH. Following incubation for 12 hours or where announced, cells were harvested and lysed in lysis buffer (10 mM Tris [pH 8.0], 1.5 mM MgCl2, 1 mM dithiothreitol, 0.1% Nonidet P-40, and protease inhibitors). After centrifugation at 13,000 g for 15 minutes, the supernatant was used for assays.

**Western Blotting Analysis for Aortic and Cellular Proteins**

Western blotting was carried out as described previously (11). Aortic or cell samples were separated by a sodium dodecylsulfate–polyacrylamide mini-gel (16). Antibodies against P-selectin, VCAM-1, myeloperoxidase (MPO), phosphorylated proteins (phosphorylated nuclear factor-xB-inducing kinase [pNIK], phosphorylated IkB-kinase α/β [pIkKα/β], phosphorylated Inhibitor of kB α [pIkBα]), p65/p50, and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-XOD antibody was a generous gift from Dr. Takeshi Nishino (Nippon Medical School, Tokyo, Japan). Antibody labeling was detected using an enhanced chemiluminescence (ECL) detection kit (Amersham Life Science, Inc. Arlington Heights, IL). Prestained blue protein marker was used for molecular weight determination. Protein amount was measured with a Sigma (St. Louis, MO) protein assay reagent kit containing bicinchoninic acid.

**Reverse Transcription–Polymerase Chain Reaction for Aortic AMs**

Total RNA from the aorta sample was isolated by a previously described method (17). Briefly, the aorta sample was homogenized in the presence of RNAzolB (2 mL/100 mg of tissue) with a Polytron homogenizer (BioSpec Products, Inc., Bartlesville, OK). Chloroform (1/10 of volume) was added to the above sample mixture. The samples were shaken vigorously then placed on ice for 5 minutes. After centrifugation twice at 13,000 g at 4°C for 15 minutes, supernatant was removed. The RNA pellet was precipitated by isopropanol, washed with 75% ethanol, dried, and redissolved in diethylpyrocarbonate-treated water. Complementary DNA was synthesized using Im-Prom-II reverse transcriptase (Promega, Madison, WI). Polymerase chain reaction was carried out by standard protocol. Primers were designed for AMs as follows: P-selectin (sense strand [ss] 5′-CGA CGT GGA CCT ATA ACT AC-3′ and antisense strand [as] 5′-CCA CAC TCT TGG ACG TAT TC-3′); VCAM-1 (ss 5′-CTT GGA GAA CCC AGA TAG AC-3′ and as 5′-CAG AAA ATC TCA GGA GCT GG-3′). Glyceraldehyde-3-phosphate dehydrogenase was used as internal control.

**Immunohistochemical Staining for AMs in Aorta**

Frozen sections (5-μm thick) were cut on a Reichert cryostat and were placed on 3-aminopropyltriethoxysilane–coated slides. After drying, the cryosections were fixed in acetone for 20 minutes at −20°C. Immunostaining was performed using the avidin–biotin complex method. In brief,
the sections were incubated in phosphate-buffered saline solution containing 3% \( \text{H}_2\text{O}_2 \) for 20 minutes. After washing, the sections were incubated with 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 minutes, and were incubated with each of the primary antibodies at 4°C overnight. Then, the sections were incubated for 60 minutes at room temperature with biotinylated donkey anti-mouse immunoglobulin G (1:100; Jackson ImmunoResearch Laboratories) and with the avidin-biotin complex reagent (Vectastain ABC reagent Elite Kit; Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. The sections were developed with a 0.05% 3,3-diaminobenzidine and 0.003% \( \text{H}_2\text{O}_2 \) medium under microscopic control to visualize peroxidase activity. Afterward, the sections were counterstained with Mayer’s hematoxylin, and mounted in

![Figure 1. Effects of aging and calorie restriction (CR) on expressions of P-selectin and vascular adhesion molecule-1 (VCAM-1) in rat aorta. Aortic expressions of P-selectin and VCAM-1 in protein level (A) and messenger RNA (mRNA) level (B) from ad libitum-fed (AL; open bar) and CR (filled or hatched bar) rats. C, Aortic expressions of P-selectin and VCAM-1 in AL rats of different ages. β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as control. Six rats were used for each experiment, and one representative image of each gene is shown from at least three experiments that yielded similar results. Blots or bands were quantified by densitometry as a percentage of the level of a 6-month-old AL rat. Statistical significance: *p < .05, **p < .01 vs 6-month-old AL rats; #p < .05, ##p < .01 CR vs age-matched AL rats.](https://academic.oup.com/biomedgerontology/article-abstract/63/3/232/678442)
a xylene-based Entellan mounting medium (Merck, Darmstadt, Germany).

Light microscopy slides were observed and photographed using an Olympus BX50 microscope (Tokyo, Japan). All photomicrographs were taken with an Olympus c-3030 digital camera.

Assessment of Oxidative Status

Lipid peroxidation assay.—Lipid peroxidation was determined using the Bioxytech LPO-586 kit (Oxis International, Portland, OR) that measures combined amounts of malondialdehyde and 4-hydroxy-2-nonenal. Briefly, after protein normalization, aorta homogenates (cytosolic fraction) were incubated with N-methyl-2-phylindole and methanesulfonic acid at 45°C for 60 minutes, and then were centrifuged at 13,000 g for 15 minutes. Afterward, the supernatant was transferred to a 96-well microplate, and the optical density was measured at 560 nm using a microplate reader (GENios Plus; Tecan Group Ltd., Salzburg, Austria).

Quantification of \( \cdot O_2^- \) and \( H_2O_2 \).—For quantification of \( \cdot O_2^- \) and \( H_2O_2 \) in aorta homogenate, which are mainly produced through XOD activation, the 2',7'-dichlorofluorescin diacetate (DCFH-DA) method was applied as described previously (11) with the minor modification that 10 μM oxypurinol, an XOD inhibitor, was added to the reaction mixture. The change in fluorescence intensity by non-fluorescent DCFH-DA converting to fluorescent 2',7'-dichlorofluorescin (DCF) via reactive species (RS) generated in homogenates was monitored at two time points (0 and 30 minutes) using a microplate fluorescence reader (Tecan Group Ltd.) at excitation 485 nm/emission 530 nm. \( \cdot O_2^- \) and \( H_2O_2 \) status were calculated as fluorescence per minute milligram protein. The difference in RS generation with and without oxypurinol treatment was considered as the XOD-derived \( \cdot O_2^- \) and \( H_2O_2 \).

Quantification of GSH.—Aorta GSH levels were determined according to the method of Tauskela and colleagues (18). Briefly, 25% meta-phosphoric acid was added to aorta cytosolic fractions, followed by centrifugation at 13,000 g for 15 minutes to yield supernatant. To assay GSH, 1 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline was added to the supernatant followed by addition of o-phthalaldehyde. After incubation at room temperature for 20 minutes, the fluorescence was measured at excitation/emission wavelength of 340 nm/505 nm.

Transient Transfection and Luciferase Reporter Assay

VCAM-1 and P-selectin promoter activity induced by oxidative stresses was examined using luciferase plasmid pGL3-VCAM-1-LUC (generously provided by Dr. William Aird, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA), which contains a human VCAM-1 (hVCAM-1) promoter region spanning –1716 to +119 bp and mp1379LUC containing wild-type murine P-selectin (mP-selectin) promoter (generously provided by
Transient transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. After transfection, cells were treated with reagents per experimental design. Briefly, YPEN-1 EC were seeded into a 48-well plate (1 × 10^5 cells/mL, and 250 μL/well) and cultured overnight in DMEM containing 10% FBS. For transfection, the cells should be >90% confluent. Following transfection, cells were cultured for 24 hours, and then exposed for an additional 8 hours to DMEM containing 1% FBS with or without designated reagents. Luciferase activity was measured by the Steady-Glo Luciferase Assay System (Promega) and detected by a luminometer (Tecan Group Ltd.). The obtained raw luciferase activities were normalized by protein concentration per each well.

**Statistical Analysis**

For western blotting, one representative blot was shown from three independent experiments. Analysis of variance was conducted to analyze significant differences among all possible ages and diet pairs. Differences among the means of individual groups were assessed by the Fischer’s protected least significant differences post hoc test. Values of p < .05 were considered statistically significant.

Figure 2. Alterations in aortic P-selectin and vascular adhesion molecule-1 (VCAM-1) by aging and calorie restriction (CR) detected by immunohistochemical staining. Vascular expressions of P-selectin (A) and VCAM-1 (B) were localized by immunohistochemical stain. Arrow indicates endothelial cell layer; * = smooth muscle cells. Representative data from three slides are shown. 6AL: Young (6 months old) and ad libitum-fed (AL); 6CR: Young (6 months old) and CR; 24AL: Old (24 months old) and AL; 24CR: Old (24 months old) and CR. Scale = 100 μm.
Results

Effects of Aging and CR on Aortic P-Selectin and VCAM-1 Expression

Expressions of P-selectin and VCAM-1 in rat aorta were measured by western blotting. As shown in Figure 1A, compared to that in 6-month-old AL rats (6AL), the expression of P-selectin was elevated by 80% ($p < .01$) in the 24-month-old AL rats (24AL). The age-related increase in VCAM-1 expression was about 45% for the 24AL rats ($p < .01$). However, increases of P-selectin and VCAM-1 were significantly attenuated by CR (Figure 1A). To obtain the clear evidence of aging effects on the expression of aortic AMs, an additional experiment was carried out to measure the expression of P-selectin and VCAM-1 in AL rats aged 6, 12, 18, and 24 months. Expressions of both AMs were found to gradually increase with age (Figure 1C).

To examine whether aging affects AMs through transcriptional regulation, we assessed messenger RNA (mRNA) levels of P-selectin and VCAM-1. Increased mRNA levels of P-selectin and VCAM-1 with aging were apparent. Figure 1B shows a 15% increase ($p < .05$) in the upregulation of P-selectin in 24AL compared to 6AL and an increase in VCAM-1 of about 4-fold ($p < .01$) in the 24AL group compared to the 6AL group. A significant finding was that the upregulation of AMs was blunted by CR. When comparing protein levels, we found that more elevated VCAM-1 mRNA levels were revealed in 24AL animals (Figure 1B), suggesting a posttranscriptional modification of VCAM-1 with age.

Further, through immunohistochemical staining we observed aging-induced expression of AMs in aortic tissue. The clearly higher level of P-selectin was seen in the vascular EC region of the 24AL rats (Figure 2A). In addition, aging-increased generation of VCAM-1 was...
detected in both EC and smooth muscle cells (SMC) in the AL group (Figure 2B). Also, as shown in Figure 2, little change in AMs was detected in CR rats with age.

Leukocyte Recruitment by Upregulation of AMs
To evaluate whether alterations in expression of AMs affect leukocyte infiltration during aging, we examined MPO levels in the aorta homogenates. The level of MPO has been widely used as a reliable marker for leukocyte infiltration/accumulation (19,20). As shown in Figure 3, there was a significant increment in MPO amount (about 40% increase, p < .01) in the 24AL group whereas MPO was maintained at a low level in both young and old CR groups.

Increased Oxidative Stress in Aorta During Aging
To delineate mechanisms involved in aging-induced AM upregulation, we investigated oxidative stress in aorta. First, we measured lipid peroxide levels, the major markers of oxidative stress (21), among the young and old AL and CR
compared with 6AL rats (p \(<\) .05). In contrast, the CR regimen suppressed this age-related upregulation (p \(<\) .05). To quantitate the contribution of XOD activation to oxidative stress, RS derived from XOD, which are mainly \(\cdot\)O\(_2\) and H\(_2\)O\(_2\), were measured by the fluorescent dye DCFH-DA with and without the specific XOD inhibitor, oxypurinol. The level of RS measured in the 24AL group was 1.5-fold that of the 6AL group (p \(<\) .05) (Figure 5B). And, as expected, RS generation was suppressed in the aged CR rats (p \(<\) .05).

**Activation of NF-\(\kappa\)B in Aorta During Aging**

To confirm oxidative stress as a responsible factor in aging-induced upregulation of AMs, the status of redox-sensitive transcription factor NF-\(\kappa\)B was examined in aorta homogenates. NF-\(\kappa\)B is a key enhancer of the expression of P-selectin and VCAM-1. The NF-\(\kappa\)B pathway can be activated by the mitogen-activated extracellular-signal regulated kinase kinase kinase (MEKK)/nuclear factor-\(\kappa\)B-inducing kinase (NIK) family of proteins that phosphorylate and activate a cytosolic enzymatic complex IkB kinase (IKK). Consequently, IkB proteins that sequester dimeric NF-\(\kappa\)B complexes in cytosol are phosphorylated, rapidly ubiquitinated, and degraded. As a result, the associated NF-\(\kappa\)B is free to move into the nucleus and activate transcription by binding to a specific DNA sequence in the enhancer region of target genes. Our data demonstrated that there is higher phosphorylation of NIK and IkB\(\alpha/\beta\) in old AL rats (p \(<\) .05), indicating activation of NF-\(\kappa\)B upstream with age. This activation resulted in increased level of phosphorylated IkB\(\alpha\) (p \(<\) .05), as shown in Figure 6. Subsequently, excessive translocation of p65/p50 complex into the nucleus was observed (p \(<\) .05) (Figure 6).

**Upregulation of AMs in ECs by Oxidative Stress**

Further, we attempted to verify whether oxidative stress that we detected in aged tissue can induce AM upregulation in cultured ECs. We challenged rat EC line YPN-1 with various oxidants. These cells express AMs in response to proinflammatory cytokines, such as TNF-\(\alpha\) and IL-1\(\beta\) (Figure 7A). Figure 7A also shows TNF-\(\alpha\) induced dose-dependent (at 10 ng/mL, 50-fold increase; p \(<\) .01) and time-dependent increase of VCAM-1 (p \(<\) .01) in YPN-1.

Three stimuli were applied to promote oxidative stress, and the range of doses used in our experiments was dependent on the cell viability assay. At the highest concentration, the cell survival ratio was 90%–95% (data not shown) under our experimental conditions (i.e., 12 hours in 1% FBS-containing DMEM).

Oxidative challenge to cells was carried out with AAPH and AMVN, which generate carbon-centered radicals through thermal decomposition and yield peroxyl radicals under aerobic conditions. Our data showed that both AAPH and AMVN induced upregulation of AMs, but that hydrophilic AAPH was more effective than AMVN. And, as expected, RS generation was suppressed in the aged CR rats (p \(<\) .05).

![Figure 6. Nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) activation in aorta was induced by aging. Aging effects on NF-\(\kappa\)B signaling were analyzed by western blotting in aortic tissue from ad libitum-fed (AL) and calorie-restricted (CR) rats. Phosphorylated signaling proteins: phosphorylated nuclear factor-\(\kappa\)B-inducing kinase (pNIK), phosphorylated IkB-kinase \(\alpha/\beta\) (pIkB\(\alpha/\beta\)), phosphorylated inhibitor of \(\kappa\)B \(\alpha\) (pIkB\(\alpha\)), and phosphorylated IkB\(\alpha\) were detected in cytosol fraction (densitometry data expressed as a percentage of 6AL [100 \(\pm\) 3\%] for pNIK; 24AL, 524 \(\pm\) 28\%; 6CR, 161 \(\pm\) 23\%; 24CR, 93 \(\pm\) 19\%; pNIK, 6AL, 100 \(\pm\) 3\%; 24AL, 160 \(\pm\) 17\%; 6CR, 99 \(\pm\) 4\%; 24CR, 76 \(\pm\) 13\%; for pIkB\(\alpha\): 6AL, 100 \(\pm\) 1\%; 24AL, 233 \(\pm\) 12\%; 6CR, 115 \(\pm\) 6\%; 24CR, 147 \(\pm\) 13\%), whereas p65 and p50 were detected in nuclear extracts (densitometry data expressed as a percentage of 6AL for p65 [100 \(\pm\) 7\%]; 24AL, 350 \(\pm\) 19\%; 6CR, 120 \(\pm\) 14\%; 24CR, 201 \(\pm\) 21\%). For p50: 6AL, 100 \(\pm\) 2\%; 24AL, 187 \(\pm\) 6\%; 6CR, 47 \(\pm\) 12\%; 24CR, 44 \(\pm\) 6\%). One representative blot is shown from three independent experiments that yielded similar results.](https://academic.oup.com/biomedgerontology/article-abstract/63/3/476/678442)
XOD/xanthine was used to generate \( \cdot O_2^- \) and \( H_2O_2 \). Expressions of both P-selectin and VCAM-1 were induced dose dependently by XOD/xanthine (\( p < .05 \)) (Figure 7B), indicating the role of reactive oxygen species in triggering AM expression.

The additions of BSO and DEM were to induce intracellular redox imbalance through the depletion of GSH. As detected in the present study, BSO (0.1, 0.5 mM) and DEM (4, 20 \( \mu \)M) induced the upregulation of P-selectin in ECs but at different rates (\( p < .05 \)) (Figure 7B). In contrast, VCAM-1 was upregulated by BSO but not by DEM (Figure 7B).

**Elevated Promoter Activities of AMs by Oxidative Stress**

To further elucidate the effects of oxidative stress on transcriptional activation of AMs, we performed transient transfection. The plasmids mp1379 and pGL3-VCAM-1-LUC contain mP-selectin and hVCAM-1 promoter region, and they are linked to luciferase reporter construct to illustrate transactivation of mP-selectin and hVCAM-1. IL-1\( \beta \) was used as a positive control. As shown in Figure 8, XOD and xanthine system (10 \( \mu \)M XOD and 25 \( \mu \)M xanthine) and GSH depletion system (treatment with BSO 0.5 mM or DEM 20 \( \mu \)M) stimulated transactivation of mP-selectin (increased by 7-fold) as strongly as did IL-1\( \beta \). Lipid peroxidation (treatment with AAPH 40 \( \mu \)M) also induced robust mP-selectin transactivation (by 5-fold), although it was weaker than that induced by IL-1\( \beta \). In our experiments, the strongest hVCAM-1 transcription activity was induced by IL-1\( \beta \) treatment, whereas GSH depletion caused by BSO also stimulated significant promoter activation of hVCAM-1 (by 7-fold). The other two oxidative stress systems showed the capacity to induce hVCAM-1 transactivation to different extents (Figure 8).

**DISCUSSION**

In the present study, we report two important findings: (i) expressions of aortic P-selectin and VCAM-1 were upregulated during aging; and (ii) increased activation of NF-\( \kappa \)B signaling brought on by oxidative stress during aging contributed to this upregulation. Our evidence is confirmed by results obtained from experiments using both CR paradigm and an in vitro EC culture system. To date, several researchers reported the effects of aging on expression of AMs (22–25). However, because their focus was mainly on diseases, the exploration of molecular AM changes in the context of normal aging was not carried out. Our study is among the first investigations to focus on the molecular link between aging and aortic AMs, and highlights the important association between oxidative stress and age-associated vascular alterations. This finding may also shed light on a better understanding of increased incidence of vascular diseases in elderly persons.

In the current study, we observed increased expression of aortic P-selectin and VCAM-1 in both protein and mRNA levels during aging. Using immunohistochemical staining, we revealed the expression and localization of P-selectin and VCAM-1 in aortic tissue. It is interesting to note that upregulation of P-selectin with age on the surface of vascular ECs was more pronounced than what was detected in protein and mRNA levels (Figures 1 and 2). This result suggested that the age-affected P-selectin process may undergo multiple phases: de novo synthesis, prestorage into Weibel–Palade bodies, and translocation to the cell surface. Another interesting observation from immunohistochemical staining is that both ECs and SMCs contributed to the age-related upregulation in VCAM-1. SMCs are reported to produce VCAM-1 in response to cytokines (26), facilitating T-cell transmigration (27); however, the role of increased VCAM-1 in SMCs during the aging process is still unknown. Moreover, we demonstrated that increased expression of aortic AMs with age resulted in excessive exposure of tissue to leukocytes (Figure 3), which would trigger prolonged or exaggerated inflammatory responses in older animals. In addition, leukocyte recruitment through AMs has been suspected to cause endothelial dysfunction (28). All of these conditions are exacerbated during aging, which may underlie the initiation of age-related vascular diseases.

One major question is why and how AMs are upregulated by aging. In the current study, we looked into the role of oxidative stress and age-related vascular alterations. This finding highlights the important association between oxidative stress and normal aging.
oxidative stress in the process, because available data show strong implications for redox imbalance on age-related vascular diseases (29). Oxidative stress has also been proposed to be responsible for altered cellular functions and characteristics of aged organisms (13). Here, we documented high oxidative status during aging in aorta with increased lipid peroxides (Figure 4A), which is an early indication of age-related oxidative stress detected even in 3-month-old AL rats, as shown previously (30). The high level of oxidative stress, which was partly attributed to age-induced XOD activation, was also evidenced by increased RS and depleted GSH in old AL animals. We also found that this redox imbalance was closely correlated with the upregulation of AMs (Figures 1 and 4). The conclusions drawn from these data are further supported by the results from CR experiments. Currently, CR is the only experimentally accepted anti-aging paradigm shown to consistently increase both median and maximum life spans in laboratory animals, likely due to its antioxidative ability to suppress age-related oxidative stress and to uphold the antioxidant defense system (11,13–15,31–34). In the current study, we found that CR reduced elevations of both aortic

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![Figure 7. (Continued).](https://academic.oup.com/biomedgerontology/article-abstract/63/3/232/678442)
AMs and oxidative stress. These results led to the conclusion that aging-related oxidative stress contributed to the upregulations of aortic P-selectin and VCAM-1.

To further explore molecular clues about oxidative stress-induced AMs, we introduced oxidative stress to cultured YPEN-1 cells. Significantly increased expressions of AMs in ECs by peroxyl radicals from AAPH/AMVN and by XOD/xanthine treatments were detected (Figure 7B). These results implied that extracellular peroxyl radicals, $\cdot$O$_2^-$, and H$_2$O$_2$, are able to induce the generation of AMs. In a previous study, Zahler and colleagues (35) tested human umbilical vein ECs (HUVEC) with H$_2$O$_2$ challenge, after which an increase of 46% in P-selectin expression was observed. To carry out further study of the intracellular redox status, we induced a depletion of GSH using BSO/DEM and observed active expressions of AMs (Figure 7). Our results showed that oxidative activation of AMs agreed with a previous study by Kokura and colleagues (36), who suggested that the upregulation of ICAM-1, P-selectin, and E-selectin was a result of changes in the GSSG/GSH ratio in HUVEC. It was also suggested that an increased GSSG/GSH ratio was responsible for H$_2$O$_2$-induced P-selectin upregulation (35).

To obtain additional evidence for the effects of redox imbalance on triggering the activation of AMs, we transfected mP-selectin and hVCAM-1 promoter reporters into YPEN-1 cells. In view of consistent activations of mP-selectin and hVCAM-1 promoters that are triggered by lipid peroxidation, RS generation, and GSH depletion (Figure 8), we proposed that without proinflammatory stimuli, oxidative stress per se is sufficient to upregulate the expression of AMs.

To delineate the signaling responsible for oxidative stress-induced upregulation of AMs during aging, we assessed the status of NF-kB that is known to be exquisitely sensitive to oxidative stress and binds to defined DNA motifs that enhance the transcription activity of various targeting genes. The predominant regulatory role of NF-kB in upregulation of VCAM-1 and P-selectin in ECs has been established (6,37), because p50/p65 components of NF-kB are detected to bind VCAM-1 and mP-selectin promoters (9,37). However, the entire signaling process of the expression of AMs in response to oxidative stress was not completely understood. In our study, we found that aging promoted the activation of NIK/IKK/IkB pathway in the aorta, which resulted in increased nuclear translocation of the p65/p50 complex in the old AL rats. In agreement with our findings, those of Kokura and colleagues (36) also

![Figure 8. Oxidative stress elevated promoter activities of adhesion molecules (AMs). Effects of oxidative stress on P-selectin and vascular adhesion molecule-1 (VCAM-1) promoter activities in transfected YPEN-1 endothelial cells were detected by luciferase assay. Cells were transfected with reporter mp1379 (open bar) or pGL3-VCAM-1-LUC (hatched bar) plasmids, which contain murine P-selectin and human VCAM-1 promoters, respectively. Cells were treated for 8 hours with various reagents and lysed for determination of luciferase. Control, transfected, and untreated cells: 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), transfected cells challenged by 200 $\mu$M AAPH; 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN), transfected cells challenged by 80 $\mu$M AMVN; xanthine oxidase (XOD)/xanthine, transfected cells challenged by 10/25 $\mu$M XOD/xanthine; buthionine sulfoximine (BSO), transfected cells challenged by 0.5 mM BSO; diethyl maleate (DEM), transfected cells challenged by 20 $\mu$M DEM. Interleukin-1$\beta$ (IL-1$\beta$; 5 ng/mL) treatment was used as a positive control. Each value was expressed as the mean ± standard error from three independent experiments. RLU = relative light units.](https://academic.oup.com/biomedgerontology/article-abstract/63/3/232/678442/242-ZOU-ET-AL)
demonstrated that enhanced expression of P-selectin elicited by redox imbalance appears to be mediated specifically by NF-κB. However, this activated signal transduction was suppressed by CR (Figure 6). Our data documented that the activation of redox-sensitive transcription factors by oxidative stress contributed to the upregulation of aortic AMs during aging.

Conclusion

The current study documented that upregulated expressions of aortic P-selectin and VCAM-1 during aging were elicited by increased oxidative stress via the activation of NF-κB signaling. Our assertion is further supported by CR experiments in which CR was shown to suppress elevated aortic AMs by decreasing age-related oxidative stress. The molecular insights revealed in the current study can help us to gain a better understanding of vascular aging and/or vascular diseases based on increased expressions of AMs with oxidative stress that occurs during aging (29).

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