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Aortic Arch Calcification Detectable on Chest X-ray Films is Associated with Plasma Diacron-reactive Oxygen Metabolites in Patients with Type 2 Diabetes but without Cardiovascular Disease

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Abstract

Aims: This study aimed to evaluate the relationship between aortic arch calcification (AAC) detectable on chest X-ray films and plasma diacron-reactive oxygen metabolites (d-ROMs) in patients with type 2 diabetes but without cardiovascular disease.

Methods: Forty-nine patients with type 2 diabetes but without cardiovascular disease were evaluated with chest X-ray examinations and divided into those with AAC (n=26) and those without AAC (n=23). Biochemical variables, including plasma levels of d-ROMS, high-sensitivity C-reactive protein (hsCRP), plasminogen activator inhibitor-1 (PAI-1), and lipoprotein (a) (Lp(a)), were evaluated after an overnight fast. The relationships of AAC with both inflammation and oxidative-stress variables were evaluated.

Results: The plasma level of d-ROMs in subjects with AAC was significantly higher than that in subjects without AAC, whereas plasma levels of hsCRP, PAI-1, and Lp(a) in subjects with AAC were higher, but not significantly so, than those in subjects without AAC. Multivariate linear regression analysis with AAC grade as the dependent variable and plasma levels of d-ROMs, hsCRP, PAI-1, or Lp(a) as independent variables demonstrated a significant association of AAC grade with plasma levels of d-ROMs but not with plasma levels of hsCRP, PAI-1, or Lp(a).

Conclusions: The plasma level of d-ROMs is associated with AAC in patients with type 2 diabetes but without cardiovascular disease. Hence, the results of the present study suggest that AAC in these patients is strongly associated with oxidative stress. Furthermore, patients with type 2 diabetes and AAC may be at high risk for the development and progression of various diabetic complications induced by oxidative stress.

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Introduction

Atherosclerotic lesions, at they develop and progress, show different histological and histochemical compositions. Calcification of the artery wall appears in advanced atherosclerotic lesions, especially in type IV atherosclerotic lesions, according to Stary's classification[1]. Arterial calcification is classified as either intimal or medial. Intimal artery calcification, so called atherosclerotic calcification, occurs at sites of atherosclerotic plaques, where there is a combination of cellular necrosis, inflammation, and cholesterol deposition[2]. As a typical example of atherosclerotic calcification, coronary artery calcification is associated with an increased risk of mortality related to coronary artery disease inclusive of cardiac death and fatal myocardial infarction[3–5]. In contrast to atherosclerotic calcification, medial artery calcification, also known as Mönckeberg's sclerosis, proceeds through a process similar to matrix vesicle-mediated intramembranous bone formation with no cartilage intermediate required[2]. Medial artery calcification, which has been recognized in diabetes[6], chronic kidney disease[7], and aging[8], appears as continuous linear deposits along the internal elastic lamina, a so-called railroad track pattern (type V, VI, or VII atherosclerotic lesions in Stary's classification)[1,2]. Medial artery calcification is primarily associated with the elastic elements rather than with muscle cells and contributes to arterial stiffening (arteriosclerosis). Arterial stiffening affects systolic and diastolic pressures and small arteries and causes target-organ damage[8]. Hence, arterial stiffness is a strong predictor of future cardiovascular events and all-cause mortality[9]. Techniques available for detecting and quantifying arterial calcification include: 1) plain radiology with or without tomography, 2) fine-detail radiology (xeroradiography), 3) fluoroscopy (e.g., during coronary angiography), 4) ultrasound scanning, 5) quantitative computed tomographic (CT) scanning, 6) electron beam CT scanning (applicable to coronary artery calcification), 7) electrocardiography-gated spiral CT scanning (applicable to coronary artery calcification), and 8) histological examination of arterial specimens[7]. Of these techniques, chest X-ray examination is noninvasive and can be used to detect aortic arch calcification (AAC) and evaluate aortic calcification. Several studies have indicated that calcified lesions of the aortic arch are significantly associated with risk factors of cardiovascular disease[10,11]. Furthermore, AAC is a strong and independent predictor of cardiovascular events beyond traditional risk factors[12]. These studies have demonstrated that AAC is a suitable marker for evaluating the risk of cardiovascular disease.

On the other hand, of the many risk factors for arterial calcification, inflammation and oxidative stress are considered additive risk factors, whereas hypertension, dyslipidemia, diabetes, age, genetics, and smoking are considered traditional risk factors[13]. In fact, the relationships between cardiovascular disease and both inflammation and oxidative stress have previously been reviewed[14,15].

The aim of the present cross-sectional study was to evaluate the relationships between AAC and both oxidative stress and inflammation in patients with type 2 diabetes but without cardiovascular disease.

Subjects and Methods

Study Subjects

The subjects were 47 patients (24 men and 23 women; average age, 65.1±10.7 years) without cardiovascular disease being treated for type 2 diabetes in our division. All subjects were ambulatory and were free of anorexia or stress

conditions possibly affecting glycemic conditions, inflammation, or oxidative stress. Furthermore, patients were excluded if they used steroids or nonsteroidal anti-inflammatory drugs, had had an acute illness in the 3 months before the study, or had an estimated glomerular filtration rate of less than 50 mL/min/1.73 m^2, according to the Cockcroft-Gault formula, because of a possible effect on oxidative stress production or inflammation. All subjects had received a diagnosis of diabetes because they met at least 1 of the following criteria: 1) a fasting plasma glucose level of ≥126 mg/dL, 2) a 2-hour value of ≥200 mg/dL on a 75-g oral glucose tolerance test, or 3) a casual plasma glucose level of ≥200 mg/dL[16].

Informed Consent and Ethics Regulations

The study was designed in accordance with the principles of the Declaration of Helsinki. Before the start of the study, written informed consent was obtained from all subjects after they had received a clear explanation of the study protocol.

Assessment of Aortic Arch Calcification

Posteroanterior chest X-ray films of subjects were reviewed in a manner previously reported[11,12], and subjects were divided into those with AAC (n=26) and those without AAC (n=23). Furthermore, study subjects were divided into 4 groups based on the extent of AAC in each chest X-ray film as follows: grade 0, no visible calcification (n=23); grade 1, small spots or a single thin area of calcification (n=8); grade 2, 1 or more areas of thick calcification (n=8); and grade 3, circumferential calcification (n=10). Also, the frequency of each grade of AAC was evaluated in all subjects, male subjects, female subjects, and subjects <65 years or ≥65 years.

Assessment of Oxidative Stress and Inflammation Variables

The activation of oxidative stress was estimated by measuring the plasma level of diacron-reactive oxygen metabolites (d-ROMs) as an index of products of reactive oxygen species (ROS), as previously reported[17]. Plasma levels of d-ROMs show significant associations with cardiovascular risk factors, such as lipid metabolism and inflammatory markers[18,19]. Furthermore, the plasma level of d-ROMs is a predictor of cardiovascular disease[20]. Hence, the plasma d-ROMs level is a suitable marker for evaluating the risk of cardiovascular disease. Plasma levels of d-ROMs were measured by a single observer using the Free Radical Analytical System (FRAS4, Wismer LL Co., Tokyo, Japan). The measurement unit is the Carratelli unit (U.CARR), which corresponds to 0.08 mg/dL H_2O_2. The correlation coefficient for repeated measurements of plasma levels of d-ROMs was 0.966 ($P<0.001$), and the coefficient of variation was 3.2%. Inflammatory status was evaluated by measuring the plasma level of high-sensitivity C-reactive protein (hsCRP) with the latex-enhanced immunonephelometric method.

For additive variables of oxidative stress and inflammation variables, plasminogen activator inhibitor-1 (PAI-1) and plasma lipoprotein (a) (Lp(a)) were evaluated. The PAI-1 promoter and gene expression are activated by inflammatory cytokines[21] and oxidative stress[22]. The plasma PAI-1 level was measured in citrated plasma with a latex agglutination method that detects total PAI-1, including free PAI-1 and tissue plasminogen activator-bound PAI-1. Lp(a) leads to stenosis, occlusion, and cardiovascular events by increasing thrombosis and impairing fibrinolysis at sites of plaque rupture[23]. Also, Lp(a) is a predictor of ischemic heart disease, as previously reported[24]. The plasma Lp(a) level was measured with the latex agglutination method.

Characteristics of Study Subjects

Age, sex, body-mass index (BMI), smoking habit, hypertension, statin use, systolic and diastolic blood pressures, and biochemical variables, including inflammation and oxidative stress markers, were evaluated in all subjects. Biochemical variables were evaluated after an overnight fast. Serum levels of total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, uric acid, and creatinine were measured with an automatic analyzer. The HbA_{1c} (Japan Diabetes Society: JDS) was measured with high-performance liquid chromatography (JDS Lot 3). Also, the HbA_{1c}

(JDS) was transformed into A1C (NGSP) as follows: A1C (NGSP)=HbA_{1c} (JDS) + 0.4[25]. Patients with hypertension were defined as patients treated for hypertension, or patients with blood pressure of ≥140/90 mm Hg measured in a hospital, on the basis of the Japanese Society of Hypertension Committee for Guidelines for the Management of Hypertension[26]. Blood pressure was measured with the patient in a sitting position in the morning after an overnight fast. Patients with dyslipidemia were defined as those having a low-density lipoprotein cholesterol level of ≥140 mg/dL, or a high-density lipoprotein cholesterol level of <40 mg/dL, or a triglyceride level ≥150 mg/dL after an overnight fast, on the basis of the Executive Summary of Japan Atherosclerosis Society Guideline for Diagnosis and Prevention of Atherosclerotic Cardiovascular Disease of Japanese[27].

Statistical Analysis

The Mann-Whitney U-test and the chi-squire test were used to compare clinical characteristics, including inflammation and oxidative stress variables, between subjects with AAC and subjects without AAC. The Kruskal-Wallis test and the Mann-Whitney U-test were used to compare oxidative stress and inflammation variables between different AAC grades. Multivariate linear regression analysis was used to identify associations of AAC grades with both inflammation and oxidative stress variables. In this multivariate linear regression analysis, we assumed that AAC was a dependent variable and that clinical characteristics, including inflammation and oxidative stress variables, were independent variables. Data are presented as means±SD, number (%), or β coefficients (95% confidence interval [CI]). Statistical significance was defined as $P<0.05$. All analyses were performed with IBM SPSS Statistics for Windows Version 12.0J (IBM Corp., Armonk, NY, USA).

Results

Relationship between AAC and Clinical Characteristics

In subjects with AAC, age (68.3±9.8 years) was significantly higher, and both diastolic blood pressure (66.5±9.6 mm Hg) and frequency of statin use (42.3%) were significantly lower than in subjects without AAC (61.6±10.7 years, $P<0.05$; 73.1±7.8 mm Hg, $P<0.05$; and 73.9%, $P<0.05$, respectively, **Table 1**). However, no other characteristics differed significantly between subjects with AAC and subjects without AAC. In particular, the duration of diabetes in subjects with AAC was higher, but not significantly so, than that in subjects without AAC ($P=0.073$).

Relationship between Plasma d-ROMs and Both Age and Duration of Diabetes

No significant correlation was observed between plasma d-ROMs and either age or duration of diabetes ($r=0.216$, $P=0.135$, and $r=0.061$, $P=0.675$, respectively).

Frequency of AAC Grade

The overall frequencies of AAC grades 0, 1, 2, and 3 in subjects were 47%, 16%, 16%, and 20%, respectively (**Fig. 1A**). However, no difference in the frequencies of AAC grades was observed between male subjects (57%, 13%, 17%, and 13%) and female subjects (36%, 20%, 16%, and 28%, **Fig. 1B**) or between subjects younger than 65 years and subjects 65 years or older. However, the frequency of AAC grade 3 in subjects 65 years or older (28%) was higher, but not significantly so, than that in subjects younger than 65 years (10%, **Fig. 1C**).

Relationships of AAC with Both Oxidative Stress and Inflammation Variables

The plasma level of d-ROMs was significantly higher in subjects with AAC (382.9±98.1 U.CARR) than in subjects without AAC (307.6±68.2 U.CARR, $P<0.01$, **Fig. 2**) and increased significantly with the AAC grade (307.6±68.1, 348.4±75.5, 396.1±95.2, and 399.8±117.2 U.CARR, respectively, $P<0.05$ by the Kruskal-Wallis test, **Table 2**). Furthermore, levels of d-ROMs in subjects with either grade 2 or 3 AAC were significantly higher than those in subjects with grade 0 AAC ($P<0.05$ by the Mann-Whitney U-test, **Table 2**). However, no significant difference in plasma PAI-1, hsCRP, or Lp(a) was observed

Table 1 Clinical characteristics of study subjects

Clinical characteristics	Without AAC (n=23)	With AAC (n=26)
Male sex (%)	14 (60.9)	10 (38.5)
Age (years)*	61.6 ± 10.7	68.3 ± 9.8
Duration of diabetes (years)	9.4 ± 6.6	13.1 ± 10.0
Body-mass index (kg/m^2)	25.14 ± 3.70	23.75 ± 5.54
Smoking habits		
None	14 (60.9)	16 (61.5)
Recent	3 (13.0)	3 (11.5)
Current	6 (26.1)	7 (26.9)
Hypertension	13 (56.5)	18 (69.2)
Statin use*	17 (73.9)	11 (42.3)
Blood pressure (mm Hg)		
Systolic	124.4 ± 11.4	124.5 ± 15.7
Diastolic*	73.1 ± 7.8	66.5 ± 9.6
Low-density lipoprotein cholesterol (mg/dL)	100.3 ± 30.0	112.7 ± 44.7
High-density lipoprotein cholesterol (mg/dL)	49.2 ± 14.9	52.4 ± 17.7
Triglyceride (mg/dL)	165.8 ± 130.1	149.7 ± 117.9
Uric acid (mg/dL)	5.69 ± 1.66	5.30 ± 1.46
Serum creatinine (mg/dL)	0.75 ± 0.19	0.72 ± 0.20
Fasting plasma glucose (mg/dL)	147.9 ± 52.6	155.6 ± 47.4
Glycosylated hemoglobin (%)	8.77 ± 1.75	9.01 ± 1.79

Mean ± SD, n (%)
AAC: aortic arch calcification, *P<0.05.

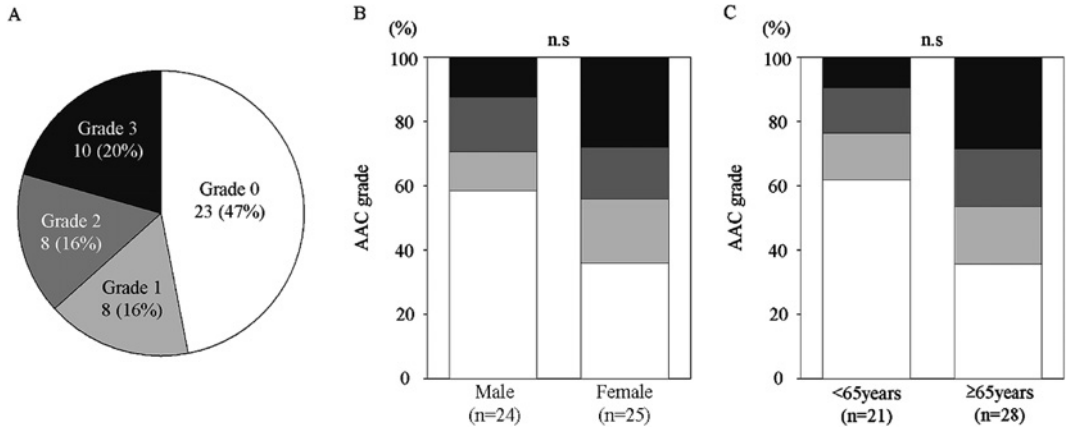


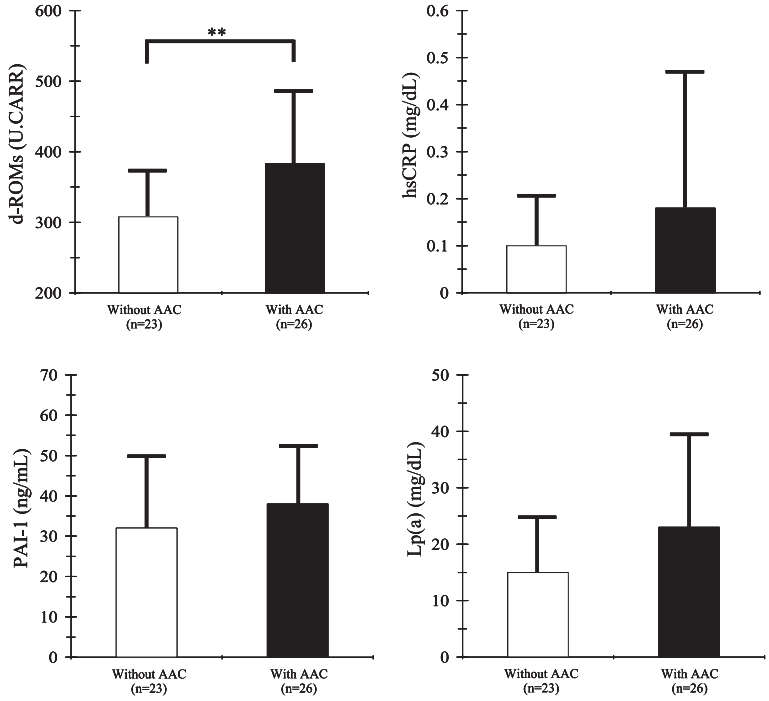
Fig. 1 Frequency of each grade of AAC in study subjects. **A**: all subjects; **B**: male or female; **C**: subjects <65 years or ≥65 years.
AAC: aortic arch calcification.

between subjects with AAC and subjects without AAC or in relation to the AAC grade.

Linear regression analysis adjusted for the subjects' characteristics showed significant correlations of AAC grade with the plasma level of d-ROMs (β=0.006, t=3.073; P<0.01, **Table 3**) but not with the level of PAI-1, hsCRP, or Lp(a).

Discussion

The results of the present study have demonstrated a relationship between plasma levels of d-ROMs and AAC in patients with type 2 diabetes but without cardiovascular disease. Hence, the results of the present study suggest that AAC in these patients is strongly associated with



**P<0.01

Fig. 2 Comparisons of plasma levels of d-ROMs, hsCRP, PAI-1, and Lp (a) between subjects with AAC and those without AAC. d-ROMs: diacron-reactive oxygen metabolities; hsCRP: high-sensitivity C-reactive protein; PAI-1: plasminogen activator inhibitor-1; Lp(a): lipoprotein(a).

Table 2 Plasma levels of d-ROMs, hsCRP, PAI-1, or Lp(a) according to AAC grade

	AAC grade			
	grade 0 (n=23)	grade 1 (n=8)	grade 2 (n=8)	grade 3 (n=10)
(1) d-ROMs (U.CARR)	307.6 ± 68.1	348.4 ± 75.5	396.1 ± 95.2*	399.8 ± 117.2*
(2) hsCRP (mg/dL)	0.103 ± 0.108	0.236 ± 0.433	0.208 ± 0.270	0.120 ± 0.153
(3) PAI-1 (ng/mL)	31.6 ± 20.9	40.8 ± 14.2	44.9 ± 24.8	27.8 ± 9.1
(4) Lp(a) (mg/dL)	15.0 ± 9.1	26.6 ± 13.9	22.4 ± 13.2	20.9 ± 22.2

d-ROMs: diacron-reactive oxygen metabolities; hsCRP: high-sensitivity C-reactive protein; PAI-1: plasminogen activator inhibitor-1; Lp(a): lipoprotein(a). *P<0.05 vs. grade 0.

oxidative stress. To our knowledge, the present study is the first to evaluate the relationship between oxidative stress and AAC clinically. In addition, oxidative stress is related not only to the development and progression of cardiovascular disease but also to other complications of diabetes. Oxidative stress develops from hyperglycemia and induces β-cell dysfunction[28] and insulin resistance[29]. Furthermore, oxidative stress is related to the development of many diseases, such as heart failure[30] and cancer[31]. The results of these studies and the present study suggest that patients with type 2 diabetes and AAC are at high risk for the development and progression of various diabetic complications induced by oxidative stress.

Concerning the frequency of AAC grades in the present study, the frequency of AAC grade 3 in subjects 65 years or older was higher, but not significantly higher, than that in subjects younger than 65 years. This result suggests that the effect of aging on AAC in patients with diabetes is weaker than that in the general population. A previous study has also found that that the AAC grade in subjects with diabetes is significantly higher than

Table 3 Multivariate linear regression analysis assuming AAC grade as the dependent variable and plasma d-ROMs, hsCRP, PAI-1, or Lp(a) as independent variables

PI	β Coefficient (95% CI)	t-value	P-value
(1) d-ROMs (U.CARR)			
Nonadjusted	0.006 (0.002–0.009)	3.328	0.002
Adjusted*	0.006 (0.002–0.009)	3.073	0.004
(2) hsCRP (mg/dL)			
Nonadjusted	0.391 (−1.219–2.002)	0.489	0.627
Adjusted*	1.569 (−0.346–3.484)	1.665	0.105
(3) PAI-1 (ng/mL)			
Nonadjusted	0.001 (−0.018–0.019)	0.043	0.966
Adjusted*	0.014 (−0.011–0.039)	1.153	0.257
(4) Lp(a) (mg/dL)			
Nonadjusted	0.016 (−0.009–0.041)	1.289	0.204
Adjusted*	0.019 (−0.012–0.050)	1.254	0.218

d-ROMs: diacron-reactive oxygen metabolities, hsCRP: high-sensitivity C-reactive protein, PAI-1: plasminogan activator inhibitor-1, Lp(a): lipoprotein(a), CI: confidence interval.
*adjusted for age, sex (female), body-mass index, smoking habit (current), duration of diabetes, hypertension, statin use, low-density lipoprotein cholesterol, triglyceride, uric acid, serum creatinine, and glycosylated hemoglobin.

that in subjects without diabetes[11]. Also, the accumulation of atherosclerotic risk factors, including diabetes, is positively correlated with AAC grade[11]. Furthermore, the presence of severe AAC is strongly associated with hypertension and dyslipidemia, rather than with aging[10]. In fact, the prevalence of hypertension in our subjects was high. Therefore, in the present study, the clustering of risk factors, such as diabetes with hypertension, may attenuate the relationship between aging and AAC. Also, the effect of AAC grade 3 in female subjects was greater than that in male subjects in the present study. The prevalence of AAC in females is significantly higher than that in males in the general population, and the effect of accumulation of atherosclerotic risk factors, including diabetes, on AAC affects males, rather than females[10]. The trend of a sex difference concerning the incidence of AAC in the present study was similar to findings of other studies.

The many regulatory factors in aortic calcification are divided into 3 types: metabolic, inflammatory, and developmental[32]. Excess lipids, phosphate, and glucose in the setting of chronic disease have direct and indirect effects on vascular calcification. Oxidative stress is a central downstream mediator of these changes and their link with inflammatory and developmental processes of metabolic abnormalities[32]. Several studies have demonstrated a relationship between aortic calcification and oxidative stress[33–35].

In this relationship, vascular smooth muscle cells (VSMCs) play an important role in the pathogenesis of aortic calcification. Apoptotic bodies derived from VSMCs act as nucleating structures for calcium crystal formation and induce atherosclerotic calcification[36]. Also, the mineralization-competent matrix vesicles released from VSMCs play an important role in calcium crystal formation and the development of aortic calcification[37,38]. Furthermore, induction of bone formation by osteoblastic cells, which are VSMCs that have undergone a phenotypic change in the aortic wall, also advances aortic calcification. VSMCs are induced toward the osteoblastic phenotype by bone morphogenetic proteins that are produced by several stimuli, such as hypoxia, ROS, turbulent blood flow, high pressure, inflammation, and oxidized low-density lipoproteins. These osteoblastic phenotype cells are characterized by increased expression of the core binding factor-

α1 (Cbfa1) and osterix and increased expression of alkaline phosphatase[2]. The apoptosis of VSMCs in atherosclerotic lesions is induced by inflammatory cytokines, such as tumor necrosis factor α, interferon β, and interleukin 1β, and by oxidative stress[39]. Furthermore, the elevated oxidative stress associated with Cbfa1 and alkaline phosphatase expression induces VSMCs toward osteoblastic differentiation[40]. These studies demonstrate that oxidative stress plays an important role in the arterial calcification that is induced by apoptosis and the degeneration of VSMCs.

Furthermore, the relationship between advanced glycation end products (AGEs) and arterial calcification may provide important information. AGEs induce calcification of VSMCs through osteoblast-like differentiation by means of the receptor for the AGE/p38 mitogen-activated protein kinase signaling pathway[41]. Also, AGEs has been shown to enhance medial artery calcification via AGE-modified elastin[42]. On the other hand, AGEs contribute to vascular oxidative stress by increasing the expression and activity of nicotinamide adenine dinucleotide phosphate oxidase in patients with diabetes[43]. Also, AGEs stimulate mineralization and calcium deposition in VSMCs and increase production of ROS, such as H_2O_2[35]. These studies demonstrate that AGEs play important roles in both arterial calcification and oxidative stress in patients with diabetes.

In the present study, it is remains unclear why AAC showed a significant relationship with the plasma level of d-ROMS but not with the level of hsCRP, PAI-1, or Lp(a). However, the characteristics of aortic calcification in patients with diabetes may provide additional information. As previously described, patients with diabetes often show medial artery calcification. Medial artery calcification proceeds via bone formation by osteoblastic cells, which are VSMCs that have undergone a phenotype change in the absence of atheroma, whereas atherosclerotic calcification occurs at atherosclerotic plaques, which show a combination of cellular necrosis, inflammation, and cholesterol deposition[2,44]. In fact, in the present study the frequency of grade 3 AAC, which indicates medial artery calcification, was higher than that in the general population, which includes patients with diabetes [11]. Furthermore, several studies have demonstrated that hsCRP[45,46], PAI-1[47], and Lp(a)[23,48] are strongly associated with atherosclerotic plaques, rather than with medial artery calcification. Hence, hsCRP, PAI-1, and Lp(a) might have been weakly associated with AAC in the present study because the frequency of medial artery calcification was higher than that of atherosclerotic calcification.

The present study had several limitations. First, our assessment using AAC grades was relatively crude because the simple chest X-ray films could not show every calcification. Therefore, it is possible that the true extent of calcium deposition in the aortic wall was underestimated. If we had used chest CT scans, the result of the classification of AAC grade might have differed from that in the present study. Second, the number of subjects in each AAC grade group was small. The differences in plasma d-ROMs between each AAC grade should be evaluated in a large population. Finally, the use of statins may have affected the results of our study. In fact, plasma d-ROMs in subjects receiving statins (336.1±89.4 U.CARR) was lower, but not significantly so, than that in subjects not receiving statins (362.8± 96.9 U.CARR; P=0.390). The effects of statins on AAC should be evaluated in a large population.

In conclusion, the present study has demonstrated that AAC detectable on chest X-ray films is associated with plasma d-ROMs in patients with type 2 diabetes but without cardiovascular disease. This result suggests that patients with type 2 diabetes and AAC show high plasma levels of oxidative stress. Furthermore, the results of the present study also suggest that patients with type 2 diabetes and AAC are at high risk for the development and progression of various diabetic complications induced by oxidative stress. The results of our study should be clarified in a larger population. Further prospective study is needed to confirm these results.

Conflict of Interest: The authors declare that they have no conflict of interest for this study.

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