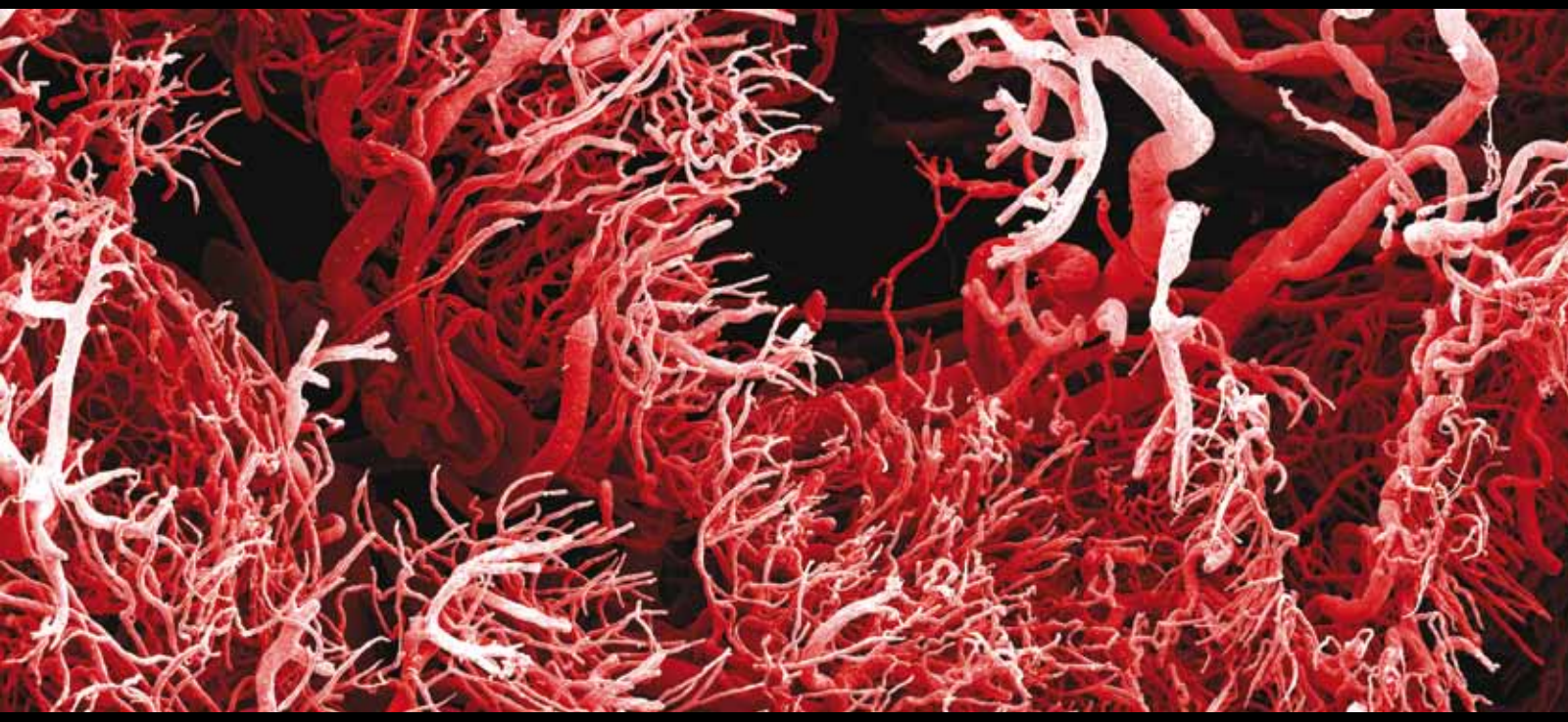
International Journal of Vascular Medicine

Inflammation and Vascular Remodeling

Guest Editors: Ken-ichi Aihara, Masaki Mogi, Rei Shibata, David Bishop-Bailey, and Muredach P. Reilly



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Editorial Board

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Editorial

Inflammation and Vascular Remodeling

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1. Introduction

Cardio- and cerebrovascular diseases are the major causes of death worldwide. Accumulating evidence has revealed that inflammation plays a pivotal role in the progression of vascular remodeling and atherosclerosis leading to cardiovascular events. In fact, several inflammatory markers such as a high-sensitivity C-reactive protein have been shown to predict cardiovascular events. In addition, metabolic disorders, including hyperglycemia, hyperinsulinemia, and dyslipidemia, injure the vascular wall and contribute to the development of vascular remodeling. Most of these metabolic stimuli initially impair homeostasis of the cardiovascular system through inflammation with recruitment of leukocytes and increased secretion of adhesion molecules, chemoattractant cytokines, and proinflammatory cytokines from endothelial cells, vascular smooth muscle cells, fibroblasts, and macrophages.

In this special issue, we invited front-line researchers and authors to submit original research and review articles that explore the interactions between inflammation and vascular remodeling. Finally, we were able to publish 6 review articles and 6 original research articles that provide pivotal evidence for understanding the pathophysiological roles of inflammation in vascular remodeling on a clinical basis and a molecular basis. The following papers will be useful for establishing treatment strategies to promote worldwide public health.

2. Review Articles

K. Ohshima et al. reviewed pathophysiological roles of peroxisome proliferator-activated receptor γ (PPAR-γ) in the process of development of atherosclerosis with vascular inflammation. They focused on novel insights into PPAR-γ activation and the immune system.

Since statins have not only cholesterol-lowering effects but also pleiotropic effects on the cardiovascular system, including anti-inflammatory and antioxidant effects and improvement of nitric oxide bioavailability, S. Yagi et al. focused on the effects of statins on cardiorenal syndrome on a clinical basis and a molecular basis.

Epoxyeicosatrienoic acids (EETs) have been shown to exert various biological effects on the vasculature including relaxation of vascular tone, cellular proliferation, and angiogenesis. S. J. Thomson et al. introduced the accumulating evidence of the anti-inflammatory effects of EETs in the cardiovascular system.

K. Yoshimura et al. previously reported the usefulness of c-Jun N-terminal kinase, a proinflammatory signaling molecule, as a nonsurgical therapeutic target for abdominal aortic aneurysm (AAA). Therefore, in their paper, they focused on recent advances in pharmacological intervention against the development of AAA.

Recent genome-wide association studies have shown that ABO blood groups are associated with various disease

phenotypes, particularly cardiovascular diseases. H. Zhang et al. reviewed the clinical importance of ABO blood groups as a locus for thrombosis, myocardial infarction, and multiple cardiovascular risk biomarkers.

MicroRNAs (miRNAs) are small noncoding RNAs of 18–22 nucleotides in length that regulate gene expression post-transcriptionally. In the past decade, miRNAs have been revealed to be novel regulators of vascular inflammation. In their paper, M. Yamakuchi overviewed the roles of miRNAs during vascular inflammation.

3. Original Research Articles

Oil thermoxidation generates oxidative free radicals that induce vascular inflammation. C. H. Ng et al. showed that prolonged consumption of repeatedly heated palm oil causes acceleration of vascular remodeling and hypertension. They concluded that the adverse phenotype is induced by increased VCAM-1 expression on endothelial cells.

A. Sakamoto et al. demonstrated that both ephrin-B1 and its cognate receptor EphB2 exhibited higher expression levels in human abdominal aortic aneurysms, and that they are expressed in macrophages, T lymphocytes and endothelial cells. They also found that membrane-bound ephrin-B1 and EphB2 inhibited chemotaxis of human peripheral blood mononuclear cells.

Y. Izumiya et al. revealed that C-type natriuretic peptide (CNP) attenuated angiotensin-II (Ang-II) induced cardiac hypertrophy, fibrosis, and contractile dysfunction through reduction in cardiac superoxide production. Their results may partly be explained by the fact that CNP reduces cardiac expression of NOX4, a subunit of NADPH oxidase.

R. L. Maurice et al. introduce an imaging-based biomarker (ImBioMark) approach for assessing *in vivo* arterial stiffness in rat models. They also presented preliminary data on the potential of ImBioMark to evaluate post-Kawasaki disease vasculitis in pediatric patients.

Since *Candida albicans* water-soluble fraction (CAWS) has a strong induction potency for murine vasculitis and shows acute lethal toxicity, N. Hirata et al. proposed that CAWS-induced arteritis is an easy and unique heart failure model without the requirement of specific experimental techniques.

The laser microdissection (LMD) method has been used for collecting target cells from the microscopic regions for malignant tumor studies. In their paper, A. Ikeda et al. showed that the LMD method enables separate collection of muscular and vascular samples and selective evaluation of gene expression in individual myocardial tissues.

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Review Article

ABO Blood Groups and Cardiovascular Diseases

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ABO blood groups have been associated with various disease phenotypes, particularly cardiovascular diseases. Cardiovascular diseases are the most common causes of death in developed countries and their prevalence rate is rapidly growing in developing countries. There have been substantial historical associations between non-O blood group status and an increase in some cardiovascular disorders. Recent GWASs have identified ABO as a locus for thrombosis, myocardial infarction, and multiple cardiovascular risk biomarkers, refocusing attention on mechanisms and potential for clinical advances. As we highlight in this paper, more recent work is beginning to probe the molecular basis of the disease associations observed in these observational studies. Advances in our understanding of the physiologic importance of various endothelial and platelet-derived circulating glycoproteins are elucidating the mechanisms through which the ABO blood group may determine overall cardiovascular disease risk. The role of blood group antigens in the pathogenesis of various cardiovascular disorders remains a fascinating subject with potential to lead to novel therapeutics and prognostics and to reduce the global burden of cardiovascular diseases.

1. Introduction

In 1901, Landsteiner identified ABO blood groups as the first recognized human blood group system. The clinical significance of ABO blood type extends beyond transfusion medicine and solid organ/hematopoietic transplantation. To date, numerous reports have suggested important associations between ABO blood groups and various diseases, for example, gastric cancer [1], periodontal diseases [2], and cardiometabolic diseases [3, 4].

According to World Health Organization (WHO) data, cardiovascular diseases (CVDs) are and will remain the leading causes of death globally: an estimated 17.3 million people died from CVD in 2008, representing 30% of all global deaths (WHO Media Centre (2011), cardiovascular diseases (fact sheet). retrieved from http://www.who.int/mediacentre/factsheets/fs317/en/index.html). Studies on the associations between CVD and ABO blood groups have a long history. In 1955, Woolf proposed an odds ratio as a measure to quantify the disease risk conferred by blood group type [5]. In 1969, Jick et al. reported a deficit of patients with blood group O among those who received anticoagulants for venous thromboembolism [6]. Prior to mutation detection in haemophilia carriership analysis, likelihood ratios of carriership of hemophilia A were based on Factor VIII levels conditional on blood group [7]. A number of later studies elucidated that ABO blood groups, particularly non-O blood groups, are associated with major cardiovascular risk factors and/or increased rate of cardiovascular events [8–13]. However, there is limited consensus regarding the magnitude and significance of the ABO effects at the population level, and whether it relates to all disorders equally or predominantly modulates thrombotic pathways and disorders [14]. This paper summarizes the basic concepts of the biochemistry of ABO blood groups and recent findings of their relations to CVD.

2. Biochemistry and Population Distribution of ABO Blood Groups

The ABO blood group is determined by the presence of A and B antigens on the surface of the red blood cells (RBCs). In addition to RBCs, these antigens are widely expressed on the membranes of a wide variety of cells, including platelets, vascular endothelium and epithelium [15] as well as in saliva and body fluids [16]. The biochemistry of the ABO blood group system has been reviewed recently [16]. Briefly, the ABH blood group antigens consist of terminal carbohydrate molecules which are synthesized by the sequential action of the ABO glycosyltransferases. The *ABO* glycotransferase (transferase A, alpha 1,3-N-acetylgalactosaminyltransferase; transferase B, alpha 1,3-galactosyltransferase) gene encodes proteins related to the ABO blood group system [17, 18]. The active ABO glycotransferases catalyze the addition of specific monosaccharides to a common core precursor antigen (H) to form distinct A and B antigens. Individuals with blood group O express only the basic H antigen [19] due to a deletion of guanine-258 in the region of the gene encoding the N-terminus of the protein which results in a frameshift and translation of a protein lacking glycosyltransferase activity [17, 18].

The frequency of the common ABO phenotypes varies among different populations. Populations with a high frequency of the A phenotype are found mainly in Northern and Central Europe [18]. The B phenotype is most frequent in Central Asia [18]. Blood group O is the most frequent phenotype globally, with parts of Africa and Australia showing highest frequencies [18]. The reasons for the observed differences among populations are not well understood, although several theories have been proposed. Evolutionary selection based on pathogen-driven blood group antigen changes may be one of the major contributors [18]. Under this theory, terminal carbohydrate modification on host proteins, lipids, and cells plays a significant role in modulating interactions with pathogens. Thus, ambient pathogens are thought to have driven the regional evolution and selection of host blood group antigens that provide survival advantage to distinct geographic pathogen exposures.

3. Genome-Wide Association Studies Confirmed ABO as a Locus for Venous Thromboembolism and Myocardial Infarction

The widespread use of genome-wide association studies (GWASs) over the last 5 years has spurred an enormous acceleration in discoveries across the entire spectrum of CVD [20]. Recent GWASs have confirmed *ABO* as a locus for venous thromboembolism (VTE), myocardial infarction (MI), and multiple cardiovascular biomarkers (Table 1).

One of the most studied aspects of the *ABO* gene is its relationship with von Willebrand factor (VWF) [17, 36, 37]. In 2003, a family-based linkage screen was carried out to determine the loci involved in VWF variation in 398 Spanish individuals. Markers at the chromosome 9q *ABO* locus region harbored the highest LOD value of 3.46 [38]. Subsequently, several GWASs have shown that carriers of single nucleotide polymorphisms (SNPs) that mark non-O blood group types have higher levels of plasma VWF when compared to O individuals. A recent GWAS of 7856 European participants in the Atherosclerosis Risk In Communities (ARIC) cohort study showed that ABO blood group O carriers had a 25% average reduction in plasma VWF levels when compared with non-O blood group carriers. SNP rs514659, which was used to tag the O blood type, contributed to 15.4% of circulating VWF variance [33]. Additionally, a genome-wide meta-analysis of 4 cohorts found that rs657152, which also tags the blood group O, was strongly associated with circulating VWF levels and that blood group O individuals had 22–30% lower plasma VWF when compared to non-blood group O individuals [28]. A relationship between Factor VIII (FVIII) plasma concentrations and ABO blood groups has also been seen. However, in a Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE) Consortium GWAS, no unique genetic variants within the ABO locus were found to affect FVIII independently of VWF [34]. As VWF binds and transports FVIII, the correlation between the ABO gene and FVIII is most likely mediated via VWF [39].

As increased plasma levels of VWF and Factor VIII are associated with greater risk of thrombosis [40, 41], many studies have examined the connection between ABO blood group and thrombotic risk. In a GWAS published in 2009, SNPs rs8176750, rs8176746 and rs8176719, which tag the A2, B, and O ABO blood groups, respectively, showed that genetically inferred blood type O had 67% lower risk of VTE than non-O blood groups. Additionally, the A2 blood group had 47% lower risk of VTE when compared to the other non-O blood group phenotypes [32]. Blood type A2 was also shown to be associated with lower VTE risk in a recently published GWAS involving 1,503 VTE patients in which rs8176704 was used to tag the A2 blood group [23]. These data suggest that the decreased risk of VTE is a result of reduced H antigen glycosylation, as the A2 allele contains a 1061delC that results in the synthesis of an enzyme that has 30–50 fold less A transferase activity than the A1 allele product [42]. Blood group genotypes may be more informative than blood group phenotypes in studying the association between blood groups and VTE since genotypes can distinguish between heterozygous and homozygous carriers of A, B, and O alleles and between A1 and A2 alleles [43, 44]. One GWAS found that the A11 allele, tagged by rs529565 and rs657152, and the B allele, tagged by rs8176749, were associated with 56% and 16% increased risk of VTE, respectively, when compared to the O11 allele. Moreover, when compared with carriers of the O1O1 diplotype, VTE risk was increased by 79% for the A11 diplotype, 82% for the B diplotype, and 170% for the AB diplotype carriers. Overall, non-O categories combined were associated with a 77% increased risk of VTE when compared to the O category [29]. The effect of ABO genotype on thrombosis risk was also investigated in a case-control study of 471 patients and 471 controls of the Leiden Thrombophilia Study (LETS) which revealed that non-OO genotypes, except

Table 1: ABO SNPs associated with cardiovascular disease and risk factors.

SNP	Major allele	Minor allele	Position on Ch9 (NCBI Build 137)[a]	Location within *ABO* locus[a]	MAF (NCBI Build 137)[a]	MAF in studied population	Tagged ABO Blood Group	Associated Physiological/ Pathophysiological Trait[b]	Reference
rs558240	G	A	136157133	5′ UTR	0.260	0.39	—	sE-selectin Variance	Qi et al. [21]
						0.38	—	sE-selectin Variance	Paterson et al. [22]
rs495828	G	T	136154867	5′ UTR	0.189	0.261	O	VTE Risk	Heit et al. [23]
						0.17	—	ACE Variance	Chung et al. [24]
rs649129	C	T	136154304	5′ UTR	0.189	—	A1	sICAM-1 Variance	Barbalic et al. [25]
						0.22	—	LDL Variance	Teslovich et al. [26]
rs579459	T	C	136154168	5′ UTR	0.189	—	A1	sICAM-1 Variance	Barbalic et al. [25]
						—	A1	sICAM-1 Variance	Kiechl et al. [27]
						0.2	A1	sE-selectin Variance	Paterson et al. [22]
						—	A1	sE-selectin Variance	Kiechl et al. [27]
						—	A1	sP-selectin Variance	Barbalic et al. [25]
						—	A1	sP-selectin Variance	Kiechl et al. [27]
rs651007	C	T	136153875	5′ UTR	0.190	0.2/0.22	—	VWF Variance	Zabaneh et al. [28]
						—	A1	sICAM-1 Variance	Qi et al. [21]
						—	A1	sICAM-1 Variance	Kiechl et al. [27]
						0.22	A1	sE-selectin Variance	Qi et al. [21]
						0.21	—	Cholesterol Variance	Teslovich et al. [26]
rs630014	G	A	136149722	Intron 1	0.460	0.48/0.48	—	VWF Variance	Zabaneh et al. [28]
						0.48	—	sE-selectin Variance	Paterson et al. [22]
rs529565	T	C	136149500	Intron 1	0.361	—	A11	VTE Risk	Wiggins et al. [29]
						0.37	O/A	MI Risk	Reilly et al. [4]
rs507666	G	A	136149399	Intron 1	0.164	0.2	A1	sICAM-1 Variance	Paré et al. [30]
						0.2	A1	sICAM-1 Variance	Paré et al. [31]
						—	A1	sICAM-1 Variance	Kiechl et al. [27]
rs505922	T	C	136149229	Intron 1	0.348	0.37	O/A	MI Risk	Reilly et al. [4]
						—	—	VTE Risk	Tregouet et al. [32]
rs500498	C	T	136148647	Intron 1	0.481	0.45	—	sICAM-1 Variance	Paré et al. [31]
						0.47	—	sE-selectin Variance	Paterson et al. [22]
rs674302	T	A	136146664	Intron 1	0.376	0.37	O/A	MI Risk	Reilly et al. [4]
rs8176668	A	T	136144059	Intron 1	0.325	0.4	—	sE-selectin Variance	Qi et al. [21]
rs612169	A	G	136143442	Intron 1	0.376	0.37	O/A	MI Risk	Reilly et al. [4]
						—	O	sICAM-1 Variance	Qi et al. [21]
						0.34	O	sE-selectin Variance	Qi et al. [21]
rs545971	C	T	136143372	Intron 1	0.376	0.37	O/A	MI Risk	Reilly et al. [4]
rs643434	G	A	136142355	Intron 1	0.391	0.39	O/A	MI Risk	Reilly et al. [4]
rs644234	T	G	136142217	Intron 1	0.389	0.39	O/A	MI Risk	Reilly et al. [4]
						0.35	—	sE-selectin Variance	Paterson et al. [22]
rs514659	A	C	136142203	Intron 1	0.376	—	O	VWF Variance	Campos et al. [33]

Table 1: Continued.

SNP	Major allele	Minor allele	Position on Ch9 (NCBI Build 137)[a]	Location within *ABO* locus[a]	MAF (NCBI Build 137)[a]	MAF in studied population	Tagged ABO Blood Group	Associated Physiological/ Pathophysiological Trait[b]	Reference
						—	O	VWF Variance	Smith et al. [34]
						0.37	O/A	MI Risk	Reilly et al. [4]
rs8176672	C	T	136142185	Intron 1	0.113	—	B	sE-selectin Variance	Qi et al. [21]
rs2519093	C	T	136141870	Intron 1	0.166	0.235	—	VTE Risk	Heit et al. [23]
rs8176681	T	C	136139754	Intron 1	0.333	0.42	—	sE-selectin Variance	Qi et al. [21]
rs657152	C	A	136139265	Intron 1	0.384	0.34/0.36	O	VWF Variance	Zabaneh et al. [28]
						0.4	O/A	MI Risk	Reilly et al. [4]
						—	A11	VTE Risk	Wiggins et al. [29]
						—	A11	VTE Risk	Tregouet et al. [32]
						0.37	—	sICAM-1 Variance	Paré et al. [31]
						0.38	—	sE-selectin Variance	Qi et al. [21]
						0.35	—	sE-selectin Variance	Paterson et al. [22]
						0.373	O	Phytosterol Variance	Teupser et al. [35]
rs8176694	T	C	136137646	Intron 1	0.116	0.19/0.18	—	VWF Variance	Zabaneh et al. [28]
rs687289	G	A	136137106	Intron 2	0.367	0.37	O/A	MI Risk	Reilly et al. [4]
						0.34	O	sICAM-1Variance	Paré et al. [31]
rs687621	A	G	136137065	Intron 2	0.373	—	—	VWF Variance	Smith et al. [34]
						0.37	O/A	MI Risk	Reilly et al. [4]
						0.34	—	sICAM-1 Variance	Paré et al. [31]
rs688976	C	A	136136770	Exon 3	0.289	0.23/0.22	—	VWF Variance	Zabaneh et al. [28]
rs8176704	G	A	136135552	Intron 3	0.051	—	A2	VWF Variance	Campos et al. [33]
						—	A2	VWF Variance	Smith et al. [34]
						—	A2	VTE Risk	Heit et al. [23]
						—	A2	sICAM-1 Variance	Paré et al. [31]
						—	A2	sICAM-1 Variance	Qi et al. [21]
						—	A2	sICAM-1 Variance	Barbalic et al. [25]
						—	A2	sE-selectin Variance	Qi et al. [21]
						—	A2	sP-selectin Variance	Barbalic et al. [25]
rs549446	C	T	136135238	Exon 4	0.420	0.25/0.24	—	VWF Variance	Zabaneh et al. [28]
rs638756	A	C	136134472	Intron 4	0.300	0.25/0.24	—	VWF Variance	Zabaneh et al. [28]
rs512770	G	A	136133506	Exon 5	0.262	0.21/0.2	O2	VWF Variance	Zabaneh et al. [28]
						—	O2	VWF Variance	Smith et al. [34]
rs8176719	Del	G	136132908	Exon 6	0.348	—	O	VTE Risk	Tregouet et al. [32]

Table 1: Continued.

SNP	Major allele	Minor allele	Position on Ch9 (NCBI Build 137)[a]	Location within *ABO* locus[a]	MAF (NCBI Build 137)[a]	MAF in studied population	Tagged ABO Blood Group	Associated Physiological/ Pathophysiological Trait[b]	Reference
						0.416	O	VTE Risk	Heit et al. [23]
						0.34	O	sE-selectin Variance	Paterson et al. [22]
rs8176722	C	A	136132754	Intron 6	0.131	0.08/0.08	B	VWF Variance	Zabaneh et al. [28]
						—	B	VWF Variance	Tregouet et al. [32]
rs8176734	G	A	136132079	Intron 6	—	—	O12	VTE Risk	Wiggins et al. [29]
rs8176740	A	T	136131472	Exon 7	0.267	0.25/0.24	—	VWF Variance	Zabaneh et al. [28]
rs8176746	G	T	136131322	Exon 7	0.123	—	B	VTE Risk	Tregouet et al. [32]
						—	B	sICAM-1 Variance	Paré et al. [31]
						0.16	—	ACE Variance	Chung et al. [24]
rs8176749	C	T	136131188	Exon 7	0.123	—	B	VWF Variance	Smith et al. [34]
						—	B	VTE Risk	Wiggins et al. [29]
rs8176750	C	Del	136131059	Exon 7	—	—	A2	VTE Risk	Tregouet et al. [32]
rs7857390	G	A	136128546	3′ UTR	0.320	0.4	—	sE-selectin Variance	Qi et al. [21]

ACE: angiotensin-converting enzyme; LDL: low density lipoprotein; MAF: minor allele frequency; MI: myocardial infarction; sICAM-1: soluble intercellular adhesion molecule-1; VTE: venous thromboembolism; VWF: von Willebrand factor.

[a]The chromosomal location and MAF for each SNP table entry was obtained by querying each SNP rs number in the NCBI Single Nucleotide Polymorphism database (dbSNP), build 137 on the 13th of September, 2012 (http://www.ncbi.nlm.nih.gov/projects/SNP/).

[b]The associated physiological/pathophysiological traits were extracted from the National Human Genome Research Institute (NHGRI) GWA catalog database (http://www.genome.gov/gwastudies/) and queried publications from PubMed (http://www.ncbi.nlm.nih.gov/pubmed).

homozygous A2 and A2-O combinations, were associated with increased thrombotic risk when compared to OO genotypes. The relative thrombotic risk of AB genotypes and A1-combinations was increased by 90–110% when compared to OO genotypes and the relative thrombotic risk of the homozygous B genotype and B-O combinations was increased by 60% [45].

The ABO locus has also been associated with arterial thrombosis in studies of MI. Our group reported that all 11 SNPs that exceeded genome-wide significance for MI in patients with established coronary atherosclerosis mapped to the ABO locus. The risk alleles at rs514659 (odds ratio 1.21; $P = 7.62 \times 10^{-9}$) and rs687289 (odds ratio 1.19; $P = 7.75 \times 10^{-9}$) were perfect tags for the loss of function ABO O blood group demonstrating that functional ABO glycotransferases conferred increased risk of MI. Further analysis found that rs514659 was associated with coronary artery diseases (CADs) when complicated by MI but not with CAD without MI, suggesting that the primary relationship of ABO to clinical CAD is through modulation of coronary thrombosis or plaque rupture in patients with established coronary atherosclerosis rather than through primary promotion of atherosclerosis per se.

The increase in MI risk for non-O blood type individuals has been suggested for some time through epidemiological studies, although there has been debate as to which ABO blood group phenotypes confer the largest increase in risk [8, 11, 46–49]. Just recently, He et al. reported results of two large prospective studies of incident coronary heart disease (CHD) as well as a meta-analysis of all prospective data [50]. The Nurses' Health Study (including 62,073 women ages 30 to 55 at baseline) and the Health Professionals Follow-up Study (including 27,428 men ages 40 to 75 at baseline) were followed up to 2006 and recorded 2,055 cases of CHD in the two cohorts. Individuals with self-reported non-O blood type had an age-adjusted hazard ratio (HR) of 1.09 (95% CI 1.03 to 1.17, $P = 0.005$) for risk of developing CHD. Associations between blood type and CHD risk were not modified by age, physical activity, alcohol consumption, smoking status, or diabetes history. A meta-analysis of an additional six prior cohorts, for a combined total of 114,648 individuals and 5,741 CHD cases, also showed a significant pooled relative risk for CHD in patients with non-O blood type of 1.11 (95% CI 1.05 to 1.18, $P = 0.001$). Among participants in the cohort, those with type O blood were significantly less likely to develop CHD when compared against types B (HR 1.11, 95% CI 1.01 to 1.23) and AB (HR 1.23, 95% CI 1.10 to 1.37), with a trend toward a higher risk for patients with type A blood (HR 1.05, 95% CI 0.98 to 1.13).

It is plausible that ABO modulation of VWF-related thrombosis accounts for the ABO association with MI. However, ABO antigens are expressed also on distinct platelet proteins, including GPIIb, a subunit of the fibrinogen receptor heterodimer [51–54], and may therefore modulate specific platelet functions in arterial thrombosis and MI (see below). In addition, ABO modulation of atherosclerotic plaque rupture and atherosclerosis itself cannot be discounted without further study.

4. Associations of the ABO Locus with Markers of Endothelial Function and Serum Lipoproteins

The ABO glycotransferase may have broader impact on atherosclerotic CVD than simply through modulation of thrombosis. A series of GWAS have linked the ABO locus to circulating levels of soluble intercellular adhesion molecule-1 (sICAM-1), soluble P-selectin (sP-selectin), and soluble E-selectin (sE-selectin). Notably, mechanistic studies in rodent models have implicated these proteins in atherosclerosis [55–59] and their blood levels in humans correlate with increased risk of CVD events [60, 61]. Paré et al. showed that SNP rs507666, located in Intron 1 of the *ABO* gene and a perfect tag for ABO blood group A1, was associated with decreased levels of sICAM-1 ($P = 5.1 \times 10^{-29}$) when compared to the O allele and contributed to 1.5% of the total sICAM-1 concentration variance [31]. The same group confirmed this relationship between rs507666 and circulating sICAM-1 levels in a larger GWAS published in 2011 [30]. Blood levels of sP-selectin and sE-selectin are also associated with SNPs in the ABO region [21, 22, 25]. A recent meta-analysis showed that, compared with major allele homozygotes, heterozygote and minor allele homozygote individuals for the rs507666, rs579459, and rs651007 SNPs, which demonstrate a high degree of linkage disequilibrium with each other ($r^2 \sim 0.96$) and tag the ABO A1 subtype, had lower plasma levels of sICAM-1, sP-selectin, and sE-selectin [27]. A recent GWAS has also identified a potential relationship between ABO and circulating levels of angiotensin-converting enzyme (ACE); when compared to the ABO blood group O, mean ACE activity in carriers of blood group B was significantly increased ($P = 2.3 \times 10^{-10}$) while ACE activity in blood group A was decreased ($P = 1.5 \times 10^{-8}$) [24].

Past epidemiological studies, some dating as far back as 50 years ago, have suggested evidence for ABO association with circulating levels of cholesterol, with non-O groups appearing to have higher levels [47, 62–65]. For example, in 1976, Garrison et al. published an epidemiological analysis in the Framingham Heart Study showing consistent elevations of serum cholesterol levels in non-O blood groups when compared to the O blood group [47]. Some recent GWASs and their meta-analyses support this potential role for ABO genotypes in modulating circulating levels of total and LDL cholesterol, as well as phytosterols, established causal risk factors for atherosclerotic heart diseases [26, 35, 66]. A meta-analysis of 46 lipid based GWAS reported an association between *ABO* SNPs and serum cholesterol levels. Total cholesterol was increased by 2.3 mg/dL in heterozygote individuals for the *ABO* rs651007 SNP when compared to major allele homozygotes ($P = 8.66 \times 10^{-21}$), while LDL cholesterol was increased by 2.05 mg/dL in heterozygote individuals for the *ABO* rs649129 SNP when compared to major allele homozygotes ($P = 7.85 \times 10^{-22}$) [26]. A GWAS published in 2010 found that the ABO locus showed genome-wide-significance for association with phytosterol levels. Specifically, rs657152, which tightly tags the O1 allele, was found to be associated with decreased levels of

circulating phytosterols. In a separate analysis, the study reported that individuals with the O allele had decreased campesterol concentrations when compared to the A and B alleles. Furthermore, additional analyses revealed that rs657152 was associated with reduced CAD risk ($P = 4.0 \times 10^{-5}$) when compared to alleles that were associated with increased phytosterols [35]. These epidemiological and genetic associations of ABO blood type with levels of circulating lipoproteins and sterols underscore the need for functional studies that define the mechanistic basis of these relationships and the potential for therapeutic translation.

Overall, these pleiotropic associations with cardiovascular risk biomarkers suggest a complex role of ABO in atherosclerotic and vascular diseases with distinct ABO genotypes and ABO functions contributing to multiple causal pathways, for example, ABO genotypes related to blood group O and loss of glycotransferase function protect against VTE and MI while distinct genotypes relating to specific A and B blood subgroups and glycotransferase functions may have a more subtle and distinct impact on endothelial function, lipoproteins, and atherosclerosis (Table 1).

One paradox that has limited clinical interpretation of GWAS discoveries, including that for the *ABO* locus, relates to the highly significant *P* values reported for common disease variants despite very small effect sizes (e.g., odds ratio). Unlike rare disease causing mutations, common variants for complex diseases have either minor functional effects or are simply crude markers for rare functional variants; their strength of association with disease is often very small despite highly significant *P* values (which relate mostly to the sample size of GWAS). However, the weak effect sizes of these variants should not be mistaken for a lack of clinical importance of a particular gene (or protein/biomarker) or its therapeutic manipulation in a complex disease process. For example, rare variants in 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase have quite weak associations with LDL cholesterol (LDL-C) levels and are well down the list of top GWAS findings for LDL-C [26]. Thus, rather than attempting to infer clinical relevance based on the strength of associations, the key importance of GWAS is simply to identify novel genes and proteins for human disease that warrant further study to establish their mechanistic role and clinical importance. Over time, the totality of evidence derived from human, animal, and functional studies allows an interpretation of the clinical and therapeutic importance of the initial significance level for a locus in a GWAS, the magnitude of effect on a protein level, and the relative risk of a given protein level for a disease phenotype. For the *ABO* locus, further mechanistic and functional studies are required in order to define the clinical and therapeutic possibilities.

5. Potential Mechanisms for the Association between ABO and CVD

Following recent GWAS discoveries, there is a resurgent interest in identifying the mechanistic links underlying the association of the ABO locus and glycotransferase functions with CVD. Most attention until recently was focused on the role of ABO in regulating VWF bioactivity and related thrombotic pathways, but emerging work is defining more broadly the role of ABO glycotransferase activity in modulating multiple endothelial, platelet, and cardiometabolic pathways and exploring whether these effects are causally involved in cardiometabolic diseases.

5.1. VWF and FVIII as Candidate Mechanisms. VWF and FVIII are glycoproteins (GPs) that circulate together in normal plasma as a noncovalent complex and both play important roles in normal hemostasis. VWF is a carrier for FVIII and protects it from inactivation. VWF recruits platelets to the site of clot formation during primary hemostasis. Factor VIII is released from VWF by the action of thrombin and participates in the coagulation cascade. Thus, both VWF and FVIII are key proteins in the formation of occlusive thrombi in injured vessels [67]. As described in preceding sections, relative to non-group O, carriers of ABO blood group O have significantly lower circulating plasma VWF and FVIII levels [68]. Although this clinically important effect of ABO group on plasma VWF-FVIII levels is well established, the mechanism through which it is mediated is not completely resolved. ABO appears to have direct functional effects on circulating VWF and indirectly (via influence of VWF levels) modulates FVIII levels. The presence of abundant ABH carbohydrate molecules on the VWF oligosaccharide side chains provides the mechanistic basis for ABO regulation of VWF levels. The active ABO A and B glycotransferase enzymes, found in Golgi of endothelial cells, generate terminal carbohydrate modifications, A and B antigens, on the existing VWF "H" oligisacharides, whereas the enzymatically inactive ABO O protein cannot modify these VWF H antigens. The addition of A or B terminal carbohydrate antigens to VWF in endothelial cells might influence circulating VWF levels and function by several mechanisms: altering the rate of VWF synthesis and/or secretion, regulating VWF proteolysis induced by its major protease, ADAMTS13, modulating VWF clearance, or changing VWF biological activity: or perhaps some combination of these events [17]. A number of studies suggest that it is unlikely that ABO effects on VWF levels are mediated by alterations in the biosynthesis and secretion of VWF [17]. In contrast, it has been demonstrated that the activity of ADAMTS13 differs against VWF of different blood groups, with both the level of ADAMTS13 activity [69], and the rate of VWF proteolysis by ADAMTS13 [70], being higher in blood group O as compared to non-O individuals. Thus, the absence of VWF terminal carbohydrate modifications in individuals with ABO blood group O increases the susceptibility to and rate of proteolysis by ADAMTS13, although data available so far are not in support of the role of proteolysis by ADAMTS13 in VWF clearance from the circulation [71]. Due to their physical association in blood, lower circulating VWF results in lower levels of FVIII. Whether VWF in platelets (a relatively abundant source) undergoes any modification by ABO remains controversial; such modification could alter platelet

production and subsequent turnover of VWF, particularly locally during platelet-driven arterial thrombosis, although this remains to be established. Indeed, current knowledge suggests that ABO does not modify platelet VWF [72], but the role of ABO in regulating platelet VWF and platelet function in thrombosis requires greater study (see below).

5.2. Other Endothelial Molecules and Platelet Proteins as Potential Mediators. Soluble levels of multiple adhesion molecules, mostly derived from endothelial cells and platelets, have been associated with coronary heart disease [61], and other cardiovascular conditions [73]. In large-scale genomic studies, plasma levels of sP-selectin and sICAM-1 were associated with ABO gene variants, but no association was found between platelet-bound P-selectin levels and ABO blood group, suggesting that the ABO blood group may influence proteolysis and clearance from the circulation, rather than its production and cellular presentation [25]. However, the specific mechanisms of ABO regulation of circulating levels of these endothelial GPs are largely unknown. Further, the implications for their function in atherosclerosis and clinical CVD require elucidation.

Blood group ABO antigens are known to be carried by several platelet GPs, for example, GPIb, GPIIb, GPIIIa, and platelet endothelial cell adhesion molecule (PECAM) [53], that play important roles in platelet function. Platelet ABH expression is a stable, donor-specific characteristic with 5% of A1 donors typing as either ABH high- or low-expressers [74], with high levels of A antigen on various GPs from high-expresser platelets, especially GPIIb and PECAM (CD31) [51]. GPIIb is an integral component of the GPIIb-GPIIIa fibrinogen receptor complex, which represents the critical final common pathway for platelet-driven thrombosis in hemostasis and pathologic arterial thrombosis including acute MI. Genetic variation in GPIIb that modulates fibrinogen binding has been associated with altered risk of thrombosis and MI [75–77], so it is conceivable that ABO-driven carbohydrate modification of GPIIb might alter its functional interactions with fibrinogen and thus platelet-mediated thrombosis. However, this hypothesis has not been adequately addressed to date. Besides GPIIb and PECAM, blood group A antigen is also expressed on other uncharacterized platelet proteins (70–90 kDa) having electrophoretic mobilities closely resembling those of GPIV and GPV [52]. Thus these and other uncharacterized ABO-expressing platelet proteins may also act as potential functional modulators of the ABO associations with arterial thrombosis and cardiovascular events.

The list of GPs carrying blood type ABO antigens is growing. Some of the most abundantly expressed GPs, such as PECAM and VWF, represent major proteins recognized to carry ABO antigens. However, there is a great need to identify all ABO target proteins and glycolipids in order to understand the nature and/or properties of GPs carrying ABO antigens and to reveal novel, potentially cell-specific, mediators of CVD.

6. Perspectives

Despite the relative simplicity of the A and B antigens, especially considering the modest biochemical difference between them, the ABO blood group system appears to be one of the most interesting, both clinically and scientifically. It divides the world's population into four major groups. The common frequency of each major blood group, especially blood group O (the loss of glycotransferase function ABO type), suggests important evolutionary selection pressures distinct from heart disease which now have subtle but significant impact at the population level on complex diseases in modern society. Much larger epidemiological and genetic studies are required to determine risks of CVD with particular major and minor ABO blood group. Such human data will inform translational and mechanistic studies by pointing to specific ABO glycan modifications that appear to be the drivers of increased or decreased risk. However, such studies are inherently limited because they fail to illuminate the specific cell/tissue where glycan modifications are mediating their actions and do not define the proteins or lipids carrying such modifications that are functionally altered and mediate disease.

In future studies, it is of critical importance to establish clear mechanistic basis for the association between ABO blood groups and CVD. Advances in our understanding of the physiologic importance of the glycan structures of VWF have provided a model and elucidated one mechanism, possibly the major one, through which ABO blood group determines risk of thrombosis and acute cardiovascular events. However, there are several other known endothelial and platelet GPs, discussed above, that are potentially functionally modified by ABO and candidate causal mediators in atherosclerosis and CVD. How and where these proteins are modified and whether their carbohydrate alterations have functional impact on disease requires further study. The increased risks associated with non-O blood groups might be attributed to higher levels or functional modification of specific endothelial-derived GPs, specific platelet GPs, both sources, and/or GPs from additional cells and tissues although leukocytes are not likely primary sources as they lack the ABO enzyme [78].

The study of glycobiology in complex disease is in its relative infancy. Unbiased approaches are required to discover and identify the population of proteins and lipids that are modified by ABO (and other glycotransferases), in cells and tissues of specific relevance to atherothrombosis, so that we can define all potential mediators of specific aspects of risk and specific disease manifestations. Technological and bioinformatics advances, including mass-spectrometric approaches, are beginning to emerge which will permit such unbiased work. The tissue-specific expression and function of ABO and many known glycotransferases [79] suggest strongly that their actions and targets will be regulated at a cell- and tissue- specific levels coincident with roles in paracrine- and cell-specific events in atherothrombosis. A naïve impression might suggest that inhibition of ABO glycotransferase functions, akin to the naturally occurring ABO O blood group, might provide a therapeutic strategy

for lowering the risk of thrombosis and CVD. However, such a pharmacological approach would likely have untoward hematological and immunological consequences and/or nonspecific off target actions in cells and tissues not relevant to vascular diseases. Identification of the specific protein and lipid targets of ABO which mediate the functional effects in thrombosis, atherosclerosis and CVD is likely to provide greater opportunity for targeted therapeutic development and clinical translation. Whether targeting VWF carbohydrate modifications might provide clinically meaningful opportunities for treatment of thrombosis and MI is an open question.

There is much to be done to understand the role of ABO and glycobiology in CVD, and the next decade should see many advances in the basic biology, mechanistic actions, and diagnostic, prognostic, and therapeutic possibilities in humans. Thus, future studies to further define the association between ABO blood groups and cardiovascular events and risks, and to elucidate biochemical mechanisms responsible for these associations, are not only of basic scientific interest but also of translational clinical importance.

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Review Article

MicroRNAs in Vascular Biology

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Vascular inflammation is an important component of the pathophysiology of cardiovascular diseases, such as hypertension, atherosclerosis, and aneurysms. All vascular cells, including endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), and infiltrating cells, such as macrophages, orchestrate a series of pathological events. Despite dramatic improvements in the treatment of atherosclerosis, the molecular basis of vascular inflammation is not well understood. In the last decade, microRNAs (miRNAs) have been revealed as novel regulators of vascular inflammation. Each miRNAs suppresses a set of genes, forming complex regulatory network. This paper provides an overview of current advances that have been made in revealing the roles of miRNAs during vascular inflammation. Recent studies show that miRNAs not only exist inside cells but also circulate in blood. These circulating miRNAs are useful biomarkers for diagnosis of cardiovascular diseases. Furthermore, recent studies demonstrate that circulating miRNAs are delivered into certain recipient cells and act as messengers. These studies suggest that miRNAs provide new therapeutic opportunities.

1. Introduction

Atherosclerosis is the major cause of death in western countries; atherosclerosis leads to cardiovascular diseases such as peripheral artery disease, acute coronary syndromes, and aneurysms [1]. The pathology of atherosclerosis develops in discrete stages: normal vessel wall, fatty streaks, atherosclerotic plaques, and ruptured plaques with thrombosis. The cellular and molecular events that lead to these pathological changes are well studied and include endothelial dysfunction, monocyte adherence and entry into the vessel wall, monocyte development into foam cells, smooth muscle cell migration and proliferation, and platelet adhesion and aggregation [2, 3]. Vascular inflammation drives the entire process of atherogenesis [4, 5]. Healthy endothelial cells (ECs) control vascular tone, limit vascular smooth muscle cells (VSMCs) proliferation, inhibit leukocyte adherence, and block thrombosis [6]. ECs release a set of factors that promote vascular homeostasis, including nitric oxide and prostacyclin [7]. However, a variety of vascular injuries destroy the ability of the endothelium to protect the vessel wall. Diabetes, hypertension, hyperlipidemia, and smoking can damage ECs [8–10]. Dysfunctional ECs make less nitric oxide and less prostacyclin [11, 12]. Furthermore, injured ECs express proinflammatory soluble and membrane bound mediators, including chemokines and p-selectin and vascular cell adhesion molecule-1 (VCAM-1), which increase leukocyte trafficking, as well as von Willebrand factor (VWF) which promotes thrombosis [13]. Several inflammatory pathways in the vasculature have been well defined [14]. For example, oxidized LDL can activate the nuclear factor κB (NF-κB) pathway, inducing the expression of a set of inflammatory genes [15]. Also, angiotensin II (AngII) activates Ets-1, a key endothelial transcription factor, leading to expression of VCAM-1 by several stimuli [16].

Recent work by several investigators has revealed that microRNAs (miRNAs) can also control vascular inflammation. This paper summarizes the role of miRNAs in vascular inflammation and highlights recent evidence that circulating miRNAs are not only biomarkers for disease but also serve as cell-to-cell messengers.

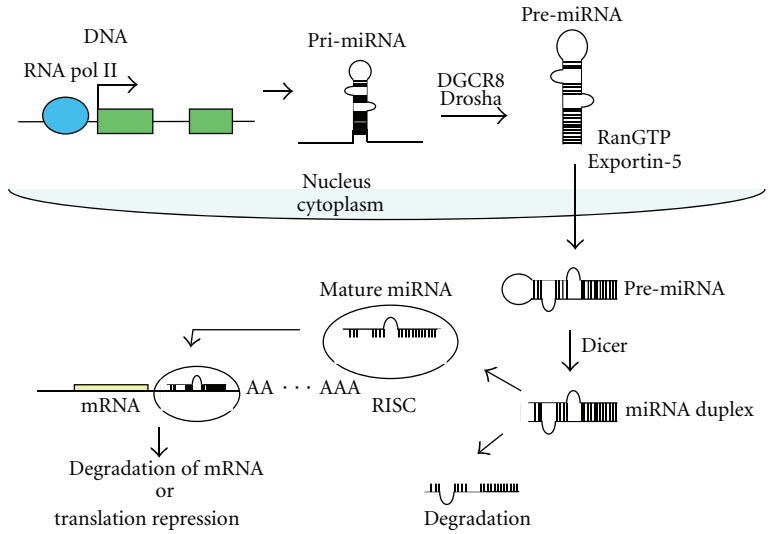

Figure 1: Schema of miRNA biogenesis. Primary miRNA (pri-miRNA) is transcribed by RNA polymerase II. Drosha-DGCR8 complex cleaves pri-miRNA into hairpin-loop structural pre-miRNA. Pre-miRNA is exported to cytoplasm by exportin-5 and RanGTP, then Dicer processes into miRNA duplex. One of the single strands (mature miRNA) is incorporated into RISC and binds to 3′UTR of target mRNA, following translational repression or mRNA degradation.

2. Biogenesis of miRNAs (See Figure 1)

MicroRNAs (miRNAs) are small noncoding RNAs of 18–22 nucleotides in length, which regulate gene expression posttranscriptionally [17–19]. miRNAs regulate diverse biological functions, including cell proliferation, apoptosis, senescence, differentiation, metabolism, tumorigenesis, and developments. Mature miRNAs are generated from primary miRNAs (pri-miRNAs) by two RNase III enzymes—Drosha and Dicer [20]. The Drosha complex processes pri-miRNAs into hairpin miRNA precursors (pre-miRNAs) in the nucleus; then Dicer cleaves these pre-miRNAs into miRNA duplexes in the cytoplasm. Recently it has shown that some miRNA precursors are generated by a Drosha-independent pathway [21]. One strand of this miRNA duplex is incorporated into the RNA-induced silencing complex (RISC) and acts to guide the RISC complex to its targets.

In mammals, mature miRNAs can bind to the 3′ untranslated region (3′-UTR) of target genes by partial complementarity. The interaction of the 5′ end of miRNAs (the seed sequence) with the target mRNA is sufficient to stop translation of target genes. miRNAs limit gene expression by (1) degradation of mRNA or (2) inhibition of translation initiation [19]. More than 1000 miRNAs are encoded in the human genome (http://www.mirbase.org/). Computer algorithms predict that most miRNAs have multiple potential target genes, based on potential interactions between the 3′UTR of mRNA and the miRNA seed sequences. In fact, it is predicted that miRNAs can manage the regulation of at least 60% of protein-coding genes in humans [18, 22].

3. Endothelial miRNAs (See Figure 2)

The physiological and pathological roles of miRNAs have been widely studied. Dysregulation of miRNAs cause a variety of diseases, including cancer [23, 24], neuropsychiatric disease [25, 26], diabetes [27], and renal failure [28]. miRNAs expressed in the vasculature play important roles in cardiovascular diseases [29]. A series of miRNAs control inflammation and oxidative stress in vascular cells including ECs, VSMCs, and inflammatory cells [20, 30, 31]. ECs control vascular homeostasis [32, 33]. miRNAs play an integral role in endothelial regulation of vessel function. Elimination of most endothelial miRNAs by knockdown of Dicer in ECs inhibits proliferation and tube formation in vitro [34]. Moreover, EC-specific Dicer knockout mice have impaired blood vessel development [35, 36]. These findings suggest that miRNAs in ECs are indispensable for the maintenance of vascular homeostasis. Which miRNAs are important in ECs and why?

3.1. miR-126: A Guardian miRNA in ECs. miRNA profiling data suggest that miR-126 is expressed mainly in ECs and platelets [34, 37, 38]. Interestingly, miR-126 is located in the intron of epithelial growth factor like domain containing protein 7 (EGFL7), an endothelial-specific protein involved in development of the vasculature [39]. During splicing of EGFL7 pre-mRNA, miR-126 is excised. miR-126 itself plays a central role in vascular development. Knockout of miR-126 in mice and zebrafish decreases vascular integrity and impairs proliferation, migration, and angiogenic activity of ECs [40, 41]. miR-126 knockout in mice is partially embryonic lethal, and surviving miR-126 knockout mice have defective cardiac neovascularization after myocardial infarction [41]. miR-126 enhances VEGF signaling by inhibiting Sprouty-related protein (SPRED1) and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2/p85-beta) to maintain vascular integrity [40–42]. Thus miR-126 acts as a proangiogenic miRNA by increasing PI3K and MAP kinase signaling.

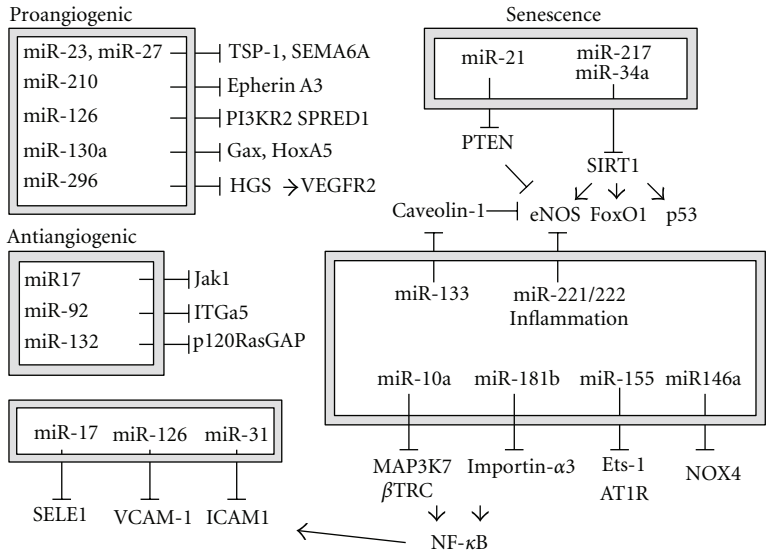

Figure 2: miRNAs regulate ECs functions. Schematic summary of endothelial miRNAs implicated in vascular inflammation. Senescence associated miRNA regulates SIRT1 or PI3K signaling. miRNAs directly involving in inflammatory response regulate angiotensin II signaling, redox signaling, and adhesion molecules. miR-146a suppresses NADPH oxidative subunit NOX4 expression [43]. Proangiogenic miRNAs (miR-23, -27, 210, 126, 130a, 296) and antiangiogenic miRNAs (miR-17, -92, -132) are also important for endothelial homeostasis [44–46].

During vascular inflammation, miR-126 is involved in suppressing inflammation signals in ECs. Inflammatory cytokines increase a series of adhesion molecules on the surface of ECs. Harris et al. showed that VCAM-1 is a direct target of miR-126. Knockdown of miR-126 promotes leukocyte adherence to ECs by enhancing TNF-α stimulated VCAM-1 expression [37].

3.2. Senescence Associated miRNAs. Aging is an independent risk factor for cardiovascular disease [47]. Senescent ECs have increased apoptosis, induce inflammation, and have decreased nitric oxide production by endothelial nitric oxide synthase (eNOS), causing endothelial dysfunction, followed by progression of atherosclerosis [48, 49]. In cultured ECs, both replicative senescence and stress-induced premature senescence release proinflammatory mediators and decrease expression of anti-inflammatory proteins such as eNOS [50, 51]. Several miRNAs are identified as senescent associated miRNAs in many cancers and fibroblasts [52–54]. The profiling of miRNAs in senescent human primary ECs shows that a set of miRNAs, such as miR-17-5p, miR-21, miR-216, miR-217, miR-31b, and miR-181a/b, are highly expressed by aging cells [55]. In addition, some miRNAs such as miR-146a are decreased in senescent ECs. miR-146a regulates NOX4, which is one of NADPH oxidase isoforms and contributes to generation of reactive oxidative stress (ROS) [43]. Since ROS promotes ECs senescence [56], miR-146a suppresses senescence by inhibiting NOX4, suggesting that the decrease level of miR-146a in senescent ECs may promote more aging by enhancing NOX4 expression.

3.2.1. miR-217. miR-217 is minimally expressed in normal ECs, but miR-217 expression increases in senescent cells. miR-217 represses silent information regulator 1 (SIRT1) expression [55]. SIRT1 is a NAD+-dependent deacetylase that control gene expression by deacetylating target proteins. SIRT1 promotes longevity and prevents stress-induced senescence in ECs [57, 58]. SIRT1 controls a variety of transcription factors such as p53, FoxO (forkhead box O), and PGC-1a (peroxisome proliferators activated receptor gamma coactivator-1a). Overexpression of miR-217 decreases SIRT1 expression, which increases acetylation of FoxO1 in young ECs [55]. Since ectopic expression of FoxO1 inhibits ECs migration and tube formation [59], miR-217 blocks angiogenic property in ECs by inhibiting SIRT1-FoxO1 function. Menghini et al. also demonstrated that miR-217 is negatively correlated with SIRT1 expression in human atherosclerotic plaques [55]. These results suggest that miR-217 has an important role in the pathogenesis of atherosclerosis in vitro and in vivo.

3.2.2. miR-34a. miR-34a expression increases in senescent ECs. Ito et al. demonstrated that the expression of miR-34a in heart and spleen are higher in aged mice than in young mice [60]. Ectopic expression of miR-34a induced senescence and cell cycle arrest in ECs. Since SIRT1 has been shown to be a direct target of miR-34a, miR-34a promotes aging of ECs through SIRT1 inhibition. miR-34a also inhibits endothelial progenitor cells (EPC) mediated angiogenesis by induction of senescence [61]. EPCs are involved in new blood vessel formation to maintain ECs homeostasis and the number of EPCs is reduced in atherosclerotic patients [62], indicating that miR-34a may be implicated in the progression of atherosclerosis; however, the relationship between miR-34a and atherogenesis is not defined yet.

3.2.3. miR-21. Several miRNAs including miR-21 and miR-214 are downregulated in senescent human aortic endothelial cells (HAEC) compared with young HAEC [63]. miR-21 regulates cell proliferation by suppressing phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a potent negative regulator of PI3K/Akt signaling pathway. PTEN suppresses Akt signaling, which decreases eNOS activity and PTEN also inhibits VCAM-1 expression in TNF-α-stimulated ECs [64], suggesting that miR-21 promotes inflammation in ECs. Several pathological conditions lead to increased miR-21 levels. Shear stress induces miR-21 expression and high miR-21 level is observed in vessels during pulmonary hypertension [65, 66]. miR-21 also contributes to endothelial-to-mesenchymal transition (EndMT). EndMT is a phenotypic change of ECs into fibroblastic cells. Blockage of miR-21 suppresses TGF-β-induced EndMT by inhibiting phosphatase and tensin homolog (PTEN) in ECs [67]. Pressure overload of left ventricular in mice increases miR-21 expression and fibroblast markers in cardiac ECs; miR-21 antagomir blocks this effect [67]. These data indicate that miR-21 modulates vascular homeostasis through PTEN and Akt.

3.3. Angiogenesis Associated miRNAs. Angiogenesis plays an important role in the development of atherosclerosis [68, 69]. Recent studies identified several miRNAs involved in angiogenesis. These miRNAs are separated in two groups: proangiogenic miRNAs and antiangiogenic miRNAs.

3.3.1. miR-17-92 Cluster. The miR-17-92 cluster is a polycistronic miRNA gene (c13orf25 transcript), containing six tandem stem-loop hairpin structure that produce six mature miRNAs: miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a, and miR-92 [70, 71]. Moreover, two miR-17-92 cluster paralogs exist, miR-106a-363 and miR-106b-25. This miRNA polycistron is functionally categorized into four families: (1) miR-17 family, (2) miR-18 family, (3) miR-19 family, and (4) miR-92 family. The c13orf25 transcript containing miR-17-92 precursor is often elevated in many cancers [72–75]. In ECs, the expression level of miR-17-92 cluster is high [76]. Impaired angiogenic activity by knockdown of Dicer in ECs is rescued by adding individual miRNAs in the miR-17-92 cluster [36]. Bonauer et al. showed that miR-92a in ECs suppresses angiogenesis in vitro and in vivo. Overexpression of miR-92a targets integrin α5 (ITGa5) and inhibits angiogenic activity in ECs. Administration of antagomir-92a blocks neovascularization in mouse hindlimb ischemia model and limits tissue injury in myocardial infarction [77]. How do the other miRNAs of these clusters function? Overexpression of miR-17, miR-18a, miR-19a, and miR-20a inhibits endothelial sprouting in vitro. In vivo, inhibition of miR-17 and miR-20a increase the number of lectin-perfused vessels in Matrigel plugs, but knockdown of miR-18a and miR-19a does not [78]. These findings indicate that individual miRNAs in this cluster function as negative regulators of angiogenesis.

3.3.2. miR-23-27-24 Cluster. The miR-23-27-24 clusters are enriched in ECs [79]. There are two highly conserved clusters: an intergenic miR-23a-27a-24-2 cluster and an intronic miR-24b-27b-24-1 cluster [80]. miR-23a is upregulated during hypertrophy by pressure overload or isoproterenol treatment [81, 82]. miR-27 is involved in the initiation and progression of atherosclerosis [83]. miR-27b targets thrombospondin-1 (TSP-1), an endogenous angiogenesis inhibitor [34, 79]. Inhibition of miR-27b reduced in vitro sprout formation [34]. TSP-1 deficiency accelerates atherosclerotic plaque maturation in ApoE knockout mice and dysregulates VSMCs activation in the arterial wall [84, 85]. These results suggest the possibility that miR-27b may promote angiogenesis by TSP-1 inhibition. Another study identified semaphorin 6A (SEMA6A) as a target of miR-27a/b. miR-27a/b negatively regulates ECs sprout formation and knockdown of miR-27a/b blocks embryonic vessel formation in zebrafish [86]. Zhou et al. demonstrated that knockdown of miR-23 and miR-27 impairs sprouting of aorta ring cells, migration, and tube formation of ECs in vitro. miR-23 and miR-27 inhibit expression of sprouty2, semaphorin 6A, and semaphorin 6D, which inhibit angiogenesis. Inhibition of these miRNAs regulates retinal vascular development and choroidal neovascularization in mice [79]. The miR-23-27-24 clusters are therefore involved in angiogenesis and atherosclerosis.

3.4. Hypoxia and miRNAs. A constant oxygen supply is necessary to maintain cellular function. Hypoxia triggers special programs to protect cells from irreversible damage [87]. Under normoxia, cells express prolyl hydroxylase domain protein 2 (PHD2 or EGLN), which hydroxylates prolyl residues on hypoxia-inducible factor-1 alpha (HIF-1α) [88]. Prolyl hydroxylated HIF-1α is immediately degraded by binding to von Hippel-Lindau (VHL) [88]. However, hypoxia suppresses PHD2 activity, stabilizing HIF-1α, which then forms heterodimers with its partner hypoxia-inducible factor-1 beta (HIF-1β). This complex is translocated into the nucleus and promotes expression of hundreds of hypoxia regulated gene, such as vascular endothelial growth factor (VEGF) [87]. These hypoxia-regulated proteins increase ECs proliferation and migration to promote angiogenesis.

3.4.1. miR-210. Hypoxia induces miR-210 expression in ECs as well as in cancer cells [89]. In cancer, the expression level of miR-210 is correlated with poor survival in cancer patients [90, 91]. Interesting target genes of miR-210 include glycerol-3-phosphate dehydrogenase (GPD1L) [92] and mitochondrial components (NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4 (NDUFA4) and succinate dehydrogenase complex, subunit D (SDHD)) [90]. Moreover, miR-210 expression is associated with expression of VEGF signaling molecules in clinical breast cancer [93]. In ECs, miR-210 controls the receptor tyrosine kinase ephrin-A3 (EFNA3) that is involved in vascular remodeling. Overexpression of miR-210 increases ECs migration; inhibition of miR-210 decreases ECs tube formation under hypoxia [94]. These data suggest that miR-210 promotes angiogenesis. HIF-1α directly binds to hypoxia responsive element (HRE) on the promoter of miR-210, following production of miR-210 transcripts [89].

3.4.2. miR-424 and miR-503. miR-424 and miR-503 are derived from a polycistronic precursor miR-424-503. These miRNAs are induced during monocyte differentiation [95] and myogenesis [96]. miR-503 expression in ECs is upregulated by high glucose or the absence of growth factors [97]. miR-503 targets cdc25a and cyclinE1 (CCNE1) protein [97]. cdc25a is a protein phosphatase that drives cell cycle by activating cyclin-dependent protein kinases (CDKs) and CCNE1 functions as a regulator of CDKs [98]. Both promote ECs proliferation by controlling cell cycle progression [99]. Therefore miR-503 overexpression inhibits ECs proliferation by suppressing cdc25A and CCNE1. miR-503 expression is increased in ischemic adductor muscles of hindlimb ischemia model in streptozotocin-induced diabetic mice and administration of miR-503 decoy to inhibit miR-503 recovers postischemic angiogenesis [97]. miR-424 is also induced by hypoxia in several cell type including ECs [100]. An ubiquitin ligase scaffold protein cullin-2 (CLU2) destabilizes HIF-1α to assemble an E3 ubiquitin ligase complex [101]. Hypoxia-induced miR-424 decreases CLU2 protein expression, which in turn stabilizes HIF-1α and promotes hypoxia regulated gene expression, which increases proliferation and migration of ECs and angiogenesis in mice [100]. Ghosh et al. also studied the transcriptional mechanism of miR-424. C/EBPα levels increase in hypoxic ECs. C/EBPα bound with RUNX-1 activates the PU.1 promoter and increased PU.1 then induces the expression of miR-424 [100]. Another group demonstrated unique functions of miR-424 in ECs [102]. VEGF and fibroblast growth factor 2 (FGF2) increase miR-424 and miR-16, and these miRNAs target VEGF receptor 2 (VEGFR2) and FGF receptor 1 (FGFR1) [102]. miR-16 and miR-424 are located in different gene locations but have the same seed sequence, so it is not surprising that miR-16 and miR-424 share the same target genes. In this case, miR-424 overexpression reduces proliferation and migration in ECs [102]. Interestingly VEGF and FGF2 increase mature miR-424, but not pri-miR-424 in ECs, suggesting that increase of miR-424 expression by VEGF and bFGF stimulation are not because the induction of transcription, but due to a positive regulation of miRNA processing from the preexisting primary transcript [102].

3.5. Inflammation and miRNAs. Vascular inflammation is an early step in atherogenesis, and many miRNAs are induced in inflamed ECs.

3.5.1. miRNAs Regulating NF-κB-Dependent Pathway. Proinflammatory cytokines (TNF-α, IL-1β) and LPS increase a set of adhesion molecules in ECs, which recruit inflammatory cells to the site of inflammation [103]. This induction of adhesion molecules is mainly mediated by the NF-κB pathway [104]. Among many different homodimers and heterodimers in the NF-κB/Rel family, the p50/p65 heterodimer is predominant in ECs [105]. In resting ECs, NF-κB binds to IκB protein, an inhibitor protein of NF-κB, and localized in the cytoplasm [106]. Once ECs are activated, IκB kinase (IKK) complex is phosphorylated, which rapidly degrades IκBα by the 26S proteasome. This leads to translocate NF-κB heterodimers into nucleus immediately, following the induction of the number of inflammatory response genes [107].

Sun et al. showed that TNF-α treatment decreases miR-181b expression in ECs [108]. Overexpression of miR-181b blocks the induction of adhesion molecules, such as VCAM-1, in vitro and in vivo. Systematic administration of miR-181b mimics reduces leukocyte accumulation and ECs activation in LPS-induced lung injury. Sun et al. demonstrated that miR-181b targets importin-α3, which is required for nuclear translocation of NF-κB, suggesting that the inhibitory effects of miR-181b on TNF-α induced expression of adhesion molecules are mediated by repression of NF-κB nuclear translocation.

Fang et al. demonstrated that miR-10a/b expression is lower at athero-susceptible arterial sites compared with athero-protected sites in dorsal thoracic aorta from swine [109]. Fang et al. then showed that miR-10a directly inhibits two key molecules of IκBα degradation, mitogen-activated kinase kinase kinase 7 (MAP3K7 or TAK1) and beta-transducin repeat-containing gene (betaTRC). Knockdown of miR-10a decreases the expression of MAP3K7 and beta-TRC, which upregulates phosphorylation of IκBα, causing more nuclear transport of NF-κB p65 and upregulation of the inflammatory cytokines such as MCP-1, IL-6, IL-8, VCAM1, and E-selectin (SELE) [109]. This suggests that miR-10a contributes to the regulation of inflammatory response through NF-κB pathway in ECs.

3.5.2. miR-31 and miR-17. miR-31 and miR-17 are induced by TNF-α in human umbilical cord endothelial cells (HUVEC) and miR-31 regulates SELE and mIR-17-3p targets intercellular adhesion molecule 1 (ICAM1) in ECs [110]. Both miRNAs control neutrophil adhesion to ECs in vitro, suggesting that miR-31 and miR-17-3p limit vascular inflammation by regulating the expression of adhesion molecule [110].

3.5.3. miR-155. TNF-α treatment of ECs induces other miRNAs such as miR-155, miR-221, and miR-222 [111]. These miRNAs are enriched in ECs and target Ets-1, a key endothelial transcription factor [111]. Stimulation with angiotensin II increases downstream genes of Ets-1, including VCAM1, monocyte chemotactic protein 1 (MCP1), and fms-related tyrosine kinase 1 (FLT1); overexpression of miR-155 partially restores this effect, suggesting that miR-155 regulates adhesion of T cells to activated ECs. Angiotensin II type 1 receptor (AT1R) is another target of miR-155 [112]. Interestingly, the human AT1R contains a +1166 A/C polymorphism, which enhances AT1R activity [113]. Since this +1166 A/C mutation destroys miR-155 binding element (the seed sequence), this mutation often maintains high AT1R activity [112].

3.5.4. miR-221 miR-222. TNF-α increases miR-221/222 expression in ECs. Dicer knockdown enhances eNOS expression in HUVEC, and miR-221 and miR-222 overexpression

rescues the enhanced eNOS suppression [20, 35]. Of note, the 3′UTR of eNOS has no target sequence for miR-221/222, suggesting that this regulation is indirect. miR-221/222 inhibits proliferation and migration of ECs [20, 35]. In contrast, miR-221/222 increases VSMCs proliferation and migration [114]. Liu et al. have shown that miR-221/222 targets p27 (Kip1) to suppress endothelial proliferation and growth of VSMCs is promoted by inhibiting c-kit [114]. These opposing cellular effects of miR-221/222 are observed in vivo. miR-221/222 increases neointimal growth but decrease reendothelialization in balloon-injury rat carotid artery model [114].

3.6. Kruppel-Like Factors and miRNAs. Krüppel-like family of transcription factor, the zinc finger family of DNA-binding transcription factor, is regulated by several stimuli such as laminar flow and statins in ECs [115]. Kruppel-like factor 2 (KLF2) and KLF4 are implicated in protection of atherogenesis through anti-inflammatory and anticoagulant pathways [116, 117]. Especially KLF2 plays a pivotal role in endothelial biology [117]. KLF2 inhibits cytokine-mediated induction of VCAM-1 and SELE expression, resulting in decreasing inflammation in ECs [118]. KLF2 induces thrombomodulin (TM), a cell surface factor involved in antithrombotic function on the surface of ECs [119]. KLF2 also induces eNOS expression and activity to maintain vasoreactivity and vascular tone [115].

miR-92a negatively regulates KLF4 and KLF2 expression in arterial endothelium [120, 121]. miR-92a, a member of the miR-17-92 cluster, has been identified as an endogenous repressor of angiogenesis (see Section 3.3.1). Overexpression of miR-92a inhibits the expression of eNOS and TM, downstream molecules of KLF2, and administration of miR-92a into mice decreases the expression of KLF2 and eNOS in the arteries [120]. Fang and Davies also demonstrated that atheroprone flow increases the interaction between miR-92a and KLF2 mRNA with Ago proteins, one of the major RNA induced silencing complex, indicating direct evidence that miR-92a regulates KLF2 expression. miR-92a regulates KLF4 expression as well as KLF2 [121]. TNF-α increases the expression of Monocyte chemotactic protein 1 (MCP-1), VCAM-1, and SELE in human aortic endothelial cells (HAEC). Fang and Davies demonstrated that knockdown of miR-92a partially suppresses these TNF-α-induced endothelial inflammatory mediators through KLF4 and miR-92 knockdown inhibits TNF-α-induced leukocyte adhesion to ECs in vitro [121]. These findings suggest that miR-92a in ECs acts as an atheroprotective miRNA by regulating KLF2 and KLF4.

4. The Communication of miRNAs between Cells

Human studies have revealed a set of miRNA in blood, joint fluid, and other extracellular locations [122, 123]. Extracellular miRNAs have been used as biomarkers to classify diseases and progression of diseases [124, 125]. Recent studies have revealed that miRNAs also serve as messengers between cells [126–128].

4.1. miRNA Secretion (Figure 3). How do miRNAs exit cells? One mode is by passive leakage from necrotic or apoptotic cells [129]. The other mode is by active secretion from living cells within microvesicles (MVs) or in RNA-lipid/protein complexes [130]. Cytokines or shear stress induce ECs-derived MVs release [131]. In response to these stresses, ECs release three types of MVs: exosomes, microparticles, and apoptotic bodies [132]. Exosomes, lipid bound particles about 30–100 nm in size, are generated through the endosomal pathway from multivesicular bodies (MVB), and then secreted by the fusion of endosome and plasma membrane [133]. Microparticles are released by budding from the outer layers of plasma membranes, and their size is larger than exosomes (100 nm–1 μm) [134]. The much larger size of apoptotic bodies, about 1–3 μm in size, contains miRNAs, DNA, and histones [135]. Apoptotic bodies are released by ECs in atherosclerotic lesions and can fuse to other vascular cells, delivering their contents [128]. Some miRNAs are incorporated into RNA-binding proteins such as Argonaute 2 or nucleophosmin 1 (NPM1) and high-density lipoprotein (HDL) and exist as MVs-free conditions [32, 33, 136]. However, the function of these extracellular miRNAs complexes is still unclear. How are miRNAs packaged into MVs? Kosaka et al. raised one possible answer. Neutral sphingomyelinase 2 (nSMase2) controls ceramide biosynthesis and inhibition of nSMase2 by GW4869 or a silencing RNA decreases secretion of miRNA [137], suggesting that ceramide pathway is involved in MVs secretion.

MVs protect miRNAs from degradation [20, 138]. Naked extracellular miRNAs are immediately degraded by ribonuclease (RNase) [122]. MVs are released into microenvironments near their origin and can be detected in plasma, urine, bile, ascites, cerebrospinal fluid, and breast milk [132]. Circulating miRNAs are also detected in body fluids, such as serum, plasma, urine, and saliva [124, 139–141]. Previous studies demonstrated that MVs play important roles in diverse vascular events. MVs derived from ECs and platelet are elevated in hypertensive patients, suggesting that pressure induced activation of ECs and platelets increase MVs productions [142]. Human atherosclerotic plaques contain a lot of microparticles, which comes from other origins such as platelets, ECs, and monocytes [143, 144]. Platelet MVs and macrophage MVs accumulate in the lipid core of atherosclerotic plaques [145]. Moreover, MVs affect the progression and development of human atherosclerotic lesions by transferring adhesion molecules and cytokines [146]. Interestingly, chronic treatment with antioxidants decreases ECs-derived MVs in patients with heart failure [147]. These reports suggest that molecules including miRNAs inside MVs can regulate functions of recipient cells.

4.2. Biomarkers. A variety cells secrete miRNAs, including T cells, monocytes, endothelial cells, adipocytes, and cancer

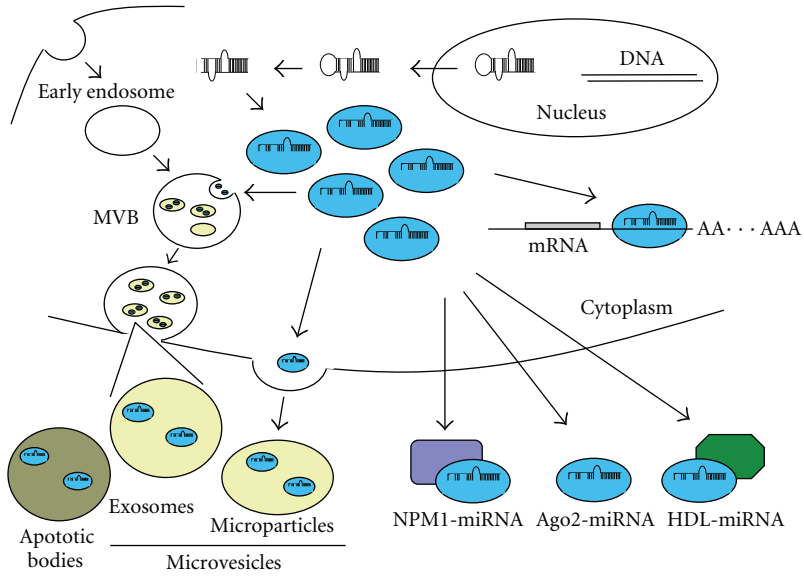

Figure 3: Pathways involved in miRNAs secretion. Extracellular miRNAs (circulating miRNAs) are secreted by incorporation into RNA binding protein, such as Ago2, HDL, and NPM1, or packaged into microvesicles (MVs), including exosomes, microparticles, and apoptotic bodies.

cells [126–128, 148–150]. Since many cell types express a set of miRNAs, circulating miRNAs released from the cells represent their original source. Therefore circulating miRNAs have diagnostic and prognostic potential. For example, high levels of miR-208b, miR-499, and miR-133a are detected in plasma of myocardial infarction patients [151, 152]. Especially, high levels of miR-133a and miR-208b are significantly associated with the risk of death in acute coronary syndrome [153]. Since these mIRNAs are expressed well in the heart, most of these miRNAs might be released from cardiomyocytes or fibroblasts during myocardial injury or infarction. In plasma of type 2 diabetes patients, many miRNAs including miR-126 are detected in low levels [154]. Vascular miRNAs, such as miR-126, miR17, miR-92a, and miR-155 are significantly lower levels in serum of patients with coronary artery disease compared with healthy subjects [155]. Possible reasons for this reduction of miRNA levels are (1) lack of miRNAs storage and production in vascular cells after dramatic release and activation of vasculature, (2) increased uptake of miRNAs into blood cells or into atherosclerotic lesions, or (3) decrease of nSMase activity in blood vessels.

4.3. Functional Messengers (Figure 4). Several recent studies show that circulating miRNAs can affect target cells [126–128]. miRNAs released by ECs can regulate the biology of vascular cells, including VSMCs, leukocytes and other ECs.

4.3.1. ECs to Distal ECs: miR-126. Zernecke et al. demonstrated that apoptotic bodies from ECs trigger CXCL12 production in other cells in the vascular wall in a paracrine manner [128]. miR-126 is packaged in ECs derived apoptotic bodies, and directly suppressed a set of genes, including regulator of G protein signaling 16 (RGS16), which is known to negatively regulate CXCR4, the CXCL12 receptor. This upregulation of CXCR4 by miR-126 uptake promotes CXCL12 production through an autoregulatory feedback loop. Transfer of miR-126 enriched apoptotic bodies or even miR-126 itself into the ApoE knockout mice reduces the size of lesions, suggesting that the antiatherosclerotic effect of ECs derived apoptotic bodies is at least partially performed by mIR-126.

4.3.2. Monocyte to ECs: miR-150. Various stimuli, including LPS, oxidative stress, and advanced glycosylated end-product (AGE), trigger miR-150 release from monocytes in vitro [127]. miR-150 is packaged into 20–400 nm sized MVs and these MVs deliver miR-150 into human cultural ECs and inhibit c-Myb expression [127]. In vivo, miR-150 enriched MVs decreased ECs proliferation after injection into mice. miRNAs represent a novel mechanism for communication between monocyte and ECs. Communication via miRNAs between vascular cells might play a role in inflammatory events leading to atherosclerosis.

4.3.3. ECs to VSMCs: miR-143/145. Kruppel-like factor 2 (KLF2) is a key molecule induced by atheroprotective shear stress, and it regulates a set of genes expressed in ECs described above (see Section 3.6). In vitro, KLF2 expression is upregulated by laminar flow or statin [117]. Hergenreider et al. discovered that physiological shear stress and statin treatment activate expression of the miR-143/145 cluster through KLF2 in ECs [126]. MiR-143 and miR-145 are intergenic miRNAs, which control the VSMCs phenotypic switch, tumorigenesis, and adipocyte differentiation [156–158]. Interestingly miR-143/145 synthesized in ECs are secreted into extracellular vesicles and transported into VSMCs [126]. MiR-143 and miR-145 are highly expressed

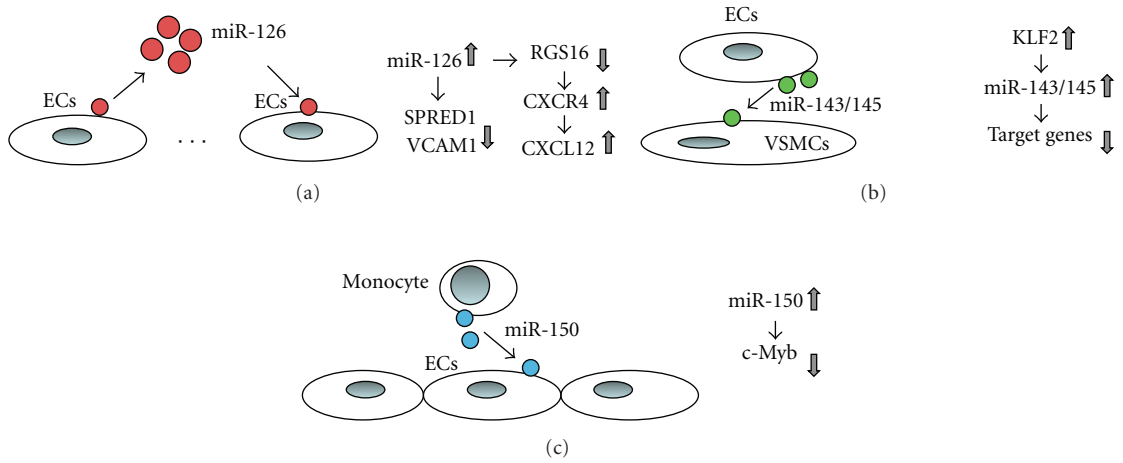

Figure 4: Cell-to-cell communication. ECs are involved in cell-to-cell communication. Schematic representation of three reported communications is shown. (a) ECs-derived apoptotic bodies are transferred to recipient ECs. These apoptotic bodies contain miR-126 that suppress RGS16 expression, enabling CXCR4 to induce CXCL12 production. miR-126 can suppress SPRED1 and VCAM-1 expressions. (b) Shear stress stimulated ECs released miR-143/145 enriched microvesicles, which are transferred into VSMCs. (c) miR-150 is selectively loaded into monocyte derived microvesicles. These microvesicles are transferred to ECs and inhibit c-Myb expression in ECs.

in VSMCs and heart, not usually in resting ECs [159]. ApoE knockdout mice showed low levels of vascular miR-143/145 [160]. Overexpression of miR-143/145 inhibits neointimal formation in acute vascular injury in rats [159]. In contrast, incomplete differentiation of VSMCs is observed in aortas from miR-143/145 knockout mice [136]. Hergenreider et al. demonstrated that injection of ECs derived MVs containing miR-143/145 reduces the formation of atherosclerotic lesion in ApoE knockout mice [126]. These studies demonstrated that atheroprotective flow increases miR-143/145 expression and secretion, and miR-143/145 can be transferred into VSMCs, preventing dedifferentiation. This elegant work suggests that miRNA mediate communication between ECs and VSMCs. Although the in vitro evidence is compelling, definitive in vivo evidence for intercellular communication through miRNA is still lacking.

5. Summary

miRNAs function as fine tuners of various biological processes to maintain homeostasis and play a key role in atherogenesis. miRNAs within ECs and VSMCs and monocytes regulate their proliferation, migration, and inflammatory profile. miRNAs can be released by cells and taken up by vascular cells, modulating their cellular biology. miRNA profiles in the blood of humans provide diagnostic and prognostic information during acute vascular events. The rapid development of RNA chemistry has led to the invention of novel modifications of RNA bases and the synthesis of artificial antisense miRNA or antagomir, which may be used as novel therapeutic tools in the future to manipulate miRNA and control vascular inflammatory diseases.

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Review Article

Recent Advances in Pharmacotherapy Development for Abdominal Aortic Aneurysm

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Abdominal aortic aneurysm (AAA) is a common disease causing segmental expansion and rupture of the aorta with a high mortality rate. The lack of nonsurgical treatment represents a large and unmet need in terms of pharmacotherapy. Advances in AAA research revealed that activation of inflammatory signaling pathways through proinflammatory mediators shifts the balance of extracellular matrix (ECM) metabolism toward tissue degradation. This idea is supported by experimental evidence in animal models that pharmacologic intervention at each pathological step can prevent AAA development. Previously, we identified c-Jun N-terminal kinase (JNK), a pro-inflammatory signaling molecule, as a therapeutic target for AAA. Abnormal activation of JNK in AAA tissue regulates multiple pathological processes in a coordinated manner. Pharmacologic inhibition of JNK tips the ECM balance back towards repair rather than degradation. Interventions targeting signaling molecules such as JNK in order to manipulate multiple pathological processes may be an ideal therapeutic strategy for AAA. Furthermore, the development of biomarkers as well as appropriate drug delivery systems is essential to produce clinically practical pharmacotherapy for AAA.

1. Introduction

Abdominal aortic aneurysm (AAA) is a common and fatal disease that is among the top 15 causes of death in elderly men. The incidence of AAA has greatly increased over recent decades, although a recent report suggests that the incidence of AAA may now be declining [1, 2]. Because most AAA patients have no symptoms until the catastrophic event of aneurysmal rupture, the main purpose of treatment is to prevent this rupture, thereby improving prognosis. Patients with large aneurysms are at high risk for rupture and, therefore, are treated by open or endovascular repair. When these surgical treatments are not applicable, an AAA inevitably progresses by gradually increasing its diameter and, concomitantly, the risk of rupture. In addition, close observation is recommended for patients with small aneurysms because of the lack of effective non-surgical treatment options [3]. Therefore, medical treatment, especially pharmacotherapy, for AAA has long been desired.

2. Pathological Features of Human AAA

It is generally accepted that an AAA is characterized by chronic inflammation and degradation of the extracellular matrix (ECM) by proteolytic enzymes, such as matrix metalloproteinases (MMPs), leading to segmental dilatation of the aortic wall and eventual rupture with a high mortality rate [4–7]. Importantly, these pathological changes are not distributed homogeneously throughout the aneurysmal wall. We, as well as Curci et al., have pointed out that the histopathology of human AAA exhibits three distinct regions: inflammatory, active, and amorphous [7–10]. These distinct regions are characterized by the cellular components and ECM architecture. The wall of a normal-sized aorta without aneurysmal change shows some intimal thickening and a media composed of well-preserved elastic lamellae with orderly layers of vascular smooth muscle cells (VSMCs), but no inflammatory cell infiltration. The inflammatory region of the vascular wall of an AAA is characterized by

a large number of inflammatory cells frequently localized on the adventitial side of the media. The inflammatory infiltrates, including T and B cells, macrophages, mast cells, and neutrophils, secrete proinflammatory mediators and accelerate chronic inflammation. However, elastic lamellae and VSMCs are still preserved in this region. As elastin-degrading enzymes, such as MMP-9, increase and the number of VSMCs, which produce elastin fibers, decreases, there is increased destruction of elastic lamellae. Thus, the area where elastin degradation is actively ongoing is defined as the active region. Finally, most of the maximally dilated area in the walls of a large AAA is characterized by amorphous tissue with abundant fibrocollagenous ECM. The absence of elastic lamellae and VSMCs is particularly striking (Figure 1). Therefore, each distinct region of an AAA wall includes distinct cells and a distinct extracellular environment. An increasing number of studies continue to be performed in basic and translational AAA research, but only a limited number of studies have focused on the regional heterogeneity of AAA. A strategy for developing clinically effective pharmacotherapies based on a better understanding of such heterogeneous molecular processes in human AAA is needed.

3. Therapeutic Targets for Treatment of AAA

3.1. Overview. Activation of proinflammatory signaling pathways through proinflammatory mediators shifts the balance of ECM metabolism towards tissue degradation. Various inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1), are involved in the pathogenesis of AAA by causing and maintaining the inflammatory response including inflammatory cell infiltration. Intracellular signaling molecules, such as c-Jun N-terminal kinase (JNK) and nuclear factor-κB (NF-κB), are activated by most proinflammatory mediators, while activated signaling pathways enhance expression of proinflammatory mediators, thus propagating a vicious cycle of chronic inflammation. Proinflammatory signaling pathways, in turn, activate ECM degradation enzymes, such as MMP-9 and MMP-2, and simultaneously reduce the expression of ECM synthetic enzymes, such as lysyl oxidase (LOX), thereby causing an overall loss of elastic fibers and AAA progression. This view is supported by accumulating evidence that the development of AAA in animal models can be suppressed by pharmacologic intervention at each step in its molecular pathogenesis. Many reported drugs are effective in suppressing proinflammatory mediators, modulating the intracellular signaling pathways, and inhibiting ECM degradation (Figure 2). Here, we summarize recent advances in pharmacotherapeutic strategies for AAA, which have been examined primarily in animal models (Table 1).

3.2. Proinflammatory Mediators

3.2.1. α-Tocopherol. Human AAA is associated with a local increase in the production of reactive oxygen species, which may act as proinflammatory mediators. α-Tocopherol (vitamin E), a lipid-soluble antioxidant, was found to significantly attenuate the formation of AAA in two animal models [11, 13]. In a study using the angiotensin II- (Ang II-) induced AAA model, treatment with vitamin E decreased the 8-isoprostane content (a marker of oxidative stress) and reduced macrophage infiltration [11]. These findings indicate that oxidative stress may play a significant role in these AAA models. However, a large randomized study reported that long-term supplementation with vitamin E did not reduce hospital admissions for elective AAA repair or reduce the rate of AAA rupture [12].

3.2.2. 17β-Estradiol. Females are much less susceptible to AAA than males [9]. This gender difference is lost after menopause, suggesting that reproductive hormones, including estrogens, may play protective roles against the development of AAA. Ailawadi et al. reported that rats treated with 17β-estradiol exhibited less macrophage infiltrate and lower MMP-9 levels and developed smaller aneurysms after elastase infusion compared to controls [14]. Treatment with 17β-estradiol also reduced expression of MCP-1, activity of NF-κB, and aneurysm size in an Ang II-induced AAA model [15]. These data provide evidence of gender-related differences in AAA development, which may reflect an estrogen-mediated reduction in proinflammatory mediators and MMP-9.

3.2.3. Inhibitors of Renin-Angiotensin System (RAS). Angiotensin-converting enzyme (ACE) inhibitors are widely used in the treatment of hypertension, congestive heart failure, and other cardiovascular disorders. Liao et al. reported the effects of ACE inhibitors on the development of AAA created by elastase infusion in rats. They examined three drugs—captopril, lisinopril, and enalapril. All three prevented the development of AAA and attenuated the degradation of medial elastin without diminishing the inflammatory response [16]. Interestingly, the aneurysm-suppressing effects of ACE inhibitors were dissociated from their effects on systemic hemodynamics. Fujiwara et al. reported the effects of an angiotensin II type 1 (AT1) receptor antagonist, valsartan, on development of AAA created by elastase infusion in rats. Treatment with valsartan prevented AAA development and infiltration of macrophages, while suppressing NF-κB activation and MMP-9 expression [19]. Moreover, a case-control study by Hackam et al. demonstrated that administration of ACE inhibitors was associated with a reduced risk of AAA rupture, though AT1 receptor blockers did not exhibit a significant benefit in terms of preventing AAA rupture [17]. In contrast, Sweeting et al. reported an association of ACE inhibitors with increased AAA expansion [18]. Thus, evidence is inconsistent for the RAS as a therapeutic target in human AAA.

3.2.4. Statins. A class of cholesterol-lowering drugs, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, have gained a great deal of attention because of their pleiotropic effects, which

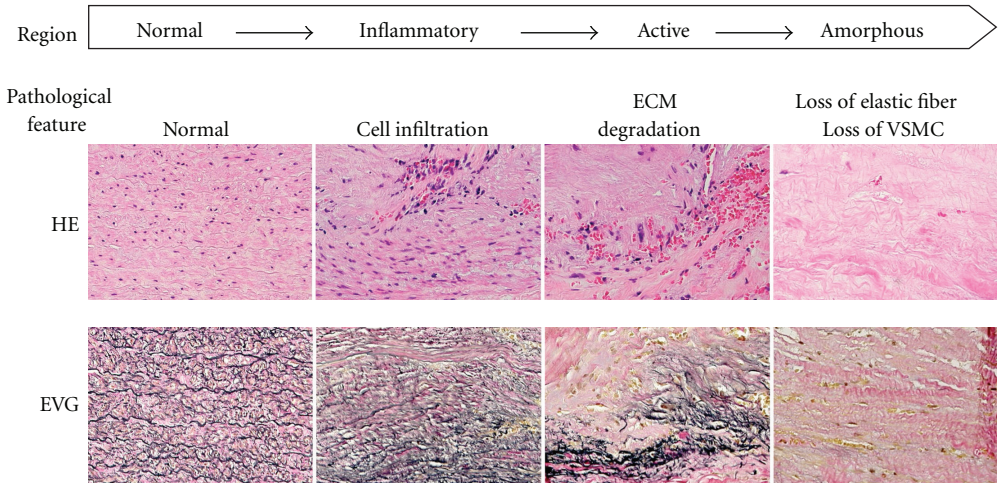

Figure 1: Heterogeneity of the histopathology of human abdominal aortic aneurysm (AAA). Regional heterogeneity within three distinct regions—inflammatory, active, and amorphous—is demonstrated. The order of these distinct regions may correspond to AAA progression from early to advanced phases. ECM: extracellular matrix; VSMC: vascular smooth muscle cell; HE: hematoxylin and eosin; EVG: elastica Van Gieson.

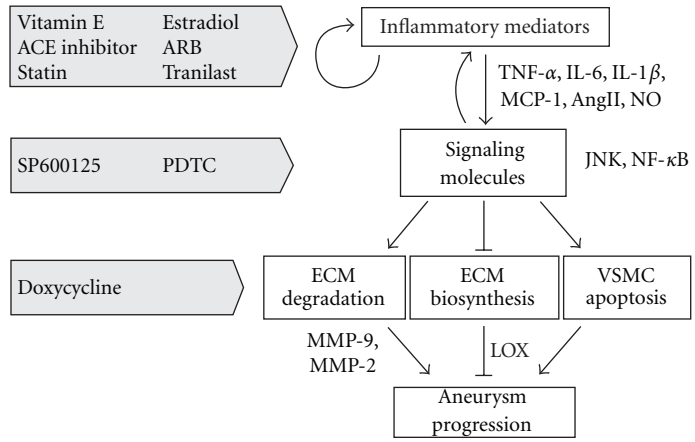

Figure 2: Pathogenesis of abdominal aortic aneurysm (AAA) with potential therapeutic drugs. Intracellular signaling molecules are activated by several types of proinflammatory mediators, while activated signaling pathways also enhance inflammatory mediators. Moreover, the activated signaling molecules shift the balance of extracellular matrix metabolism toward degradation, leading to AAA progression. Pharmacologic agents targeting each aspect of the pathological processes are demonstrated. ACE: angiotensin converting enzyme; ARB: angiotensin II receptor blocker; PDTC: pyrrolidine dithiocarbamate; TNF: tumor necrosis factor; IL: interleukin; MCP: monocyte chemoattractant protein; Ang: angiotensin; NO: nitric oxide; JNK: c-jun N-terminal kinase; NF: nuclear factor; MMP: matrix metalloproteinase; LOX: lysyl oxidase; ECM: extracellular matrix; VSMC: vascular smooth muscle cell.

may be beneficial in various vascular diseases [50–52]. Steinmetz et al. reported that simvastatin suppressed aneurysm formation in the elastase infusion model in both C57BL/6 wildtype and hypercholesterolemic $ApoE^{-/-}$ mice [28]. Importantly, treatment with simvastatin had no effect on serum cholesterol levels in either normal or hypercholesterolemic mice, suggesting that the benefit of simvastatin to aneurysm development is independent of its cholesterol-lowering effect. Kalyanasundaram et al. also demonstrated that simvastatin suppressed aneurysm formation in an elastase-induced AAA model in rats and reduced protein levels of MMP-9 and NF-κB [29]. Transcriptome analysis provided evidence that simvastatin treatment downregulated proinflammatory mediators in the aortic wall, including IL-1, TNF-α, inducible nitric oxide synthase (iNOS), and several chemokines. Shiraya et al. reported the effects of atorvastatin on development of AAA created by elastase infusion in rats. Treatment with atorvastatin prevented AAA development and macrophage infiltration into the aortic wall, while suppressing MMP-9 and MCP-1 secretion [30]. In addition, we and others reported that statins inhibit the secretion of MMP-9 and MCP-1 in the walls of human AAA in vitro and in vivo [20–23]. Several clinical studies demonstrated an association between statin administration and decreased AAA growth [24–27]. However, the beneficial effect of statins has not been confirmed in large clinical trials [18, 31, 32].

On the other hand, there seems to be consistent evidence that statin therapy improves perioperative and postoperative outcome after AAA repair [53, 54].

3.2.5. Mast Cell Stabilizer. Mast cells are one of the inflammatory cell types found in the walls of human AAA and are likely to play a role in its pathogenesis because activation of mast cells leads to the release of proinflammatory cytokines, such as IL-6 and IFN-γ. Sun et al. demonstrated that mast cell activation induced by compound 48/80, a mast cell degranulation agent, increased AAA expansion induced by elastase infusion in mice, whereas disodium cromoglycate (DSCG), a mast cell stabilizer, significantly reduced AAA expansion [33]. In addition, the inhibition of AAA development by DSCG corresponded to preservation of the elastic lamina, decreased infiltration of mast cells and macrophages, and reduced IFN-γ, IL-6, and MMP activity. Tsuruda et al. also reported that pharmacological intervention with tranilast, another inhibitor of mast cell degranulation, attenuated AAA development in two rodent models [34]. Although these data raise considerable interest in the use of mast cell stabilizing drugs for the treatment of patients with AAA, no clinical trial results have been reported revealing their efficacy in reducing AAA expansion.

3.3. Intracellular Signaling Pathways. Recently, intracellular signaling pathways have attracted attention as therapeutic targets of AAA. We and others demonstrated that the pharmacologic inhibition of certain signaling pathways is effective in treating experimental AAA, including the Rho/Rho-kinase, NF-κB, and JNK pathways [35, 36, 55].

Wang et al. reported that treatment with fasudil, a Rho-kinase inhibitor, resulted in reduction of both the incidence and severity of Ang II-induced AAA [55]. They also demonstrated that fasudil treatment inhibited both VSMC apoptosis and proteolysis by MMP-9 and MMP-2. Parodi et al. studied the effects of NF-κB inhibition by pyrrolidine dithiocarbamate (PDTC) on the development of elastase-induced AAA [35]. Treatment with PDTC suppressed NF-κB activity, expressions of MMP-9 and proinflammatory cytokines, and formation of AAA in mice. These results were further supported by other studies demonstrating the suppression of experimental AAA by NF-κB/Ets decoy oligonucleotide [56, 57].

We identified JNK as a therapeutic target for AAA treatment by screening the activation status of signaling molecules in human AAA tissue [5, 36, 37, 58]. JNK is a key regulator of AP-1, which is a critical transcriptional regulator of MMP-9 and various proinflammatory cytokines. SP600125, a specific JNK inhibitor, completely prevented the development of calcium-induced AAA in mice and significantly reduced MMP-9, macrophage infiltration, and the preservation of elastic lamellae. Moreover, treatment with SP600125 after the establishment of AAA formation resulted in a striking reduction of the aneurysm diameter and restored the once-disrupted elastic lamellae, indicating that JNK inhibition enhances the repair of tissue architecture (Figure 3). In addition, treatment with SP600125 resulted in reduced aneurysm diameters of Ang II-induced AAA in $ApoE^{-/-}$ mice. These data demonstrated for the first time that pharmacological treatment causes regression of an established AAA in animal models [36]. SP600125 also suppressed the secretion of MMP-9 and MMP-2 in the walls of human AAA in ex vivo culture. Thus, pharmacological inhibition of JNK is thought to be a potentially effective therapeutic option for the treatment of AAA [59, 60].

3.4. Enzymes for ECM Metabolism. The development, progression, and rupture of AAA are closely associated with connective tissue destruction. In particular, the biophysical properties of the aneurysmal aorta are largely due to a loss of medial and adventitial elastin. It is widely accepted that elastolytic MMPs, particularly MMP-9 and MMP-2, cause degradation of ECM, thereby leading to the development of AAA [5, 9, 44, 61]. Petrinec et al. first reported that treatment with doxycycline, a tetracycline derivative, caused a significant reduction in the incidence of AAA induced by elastase infusion in rats [38]. They also observed that doxycycline reduced MMP-9 production and prevented the destruction of elastic lamellae without decreasing inflammatory cell infiltration. Their results were further supported in various experimental conditions [39–41, 44–49]. Because of the promising results in these animal studies, the effect of doxycycline on human AAA has been investigated. Curci et al. demonstrated that preoperative treatment with doxycycline decreased MMP-9 expression in aneurysm tissue [42]. Mosorin et al. first published a randomized trial of doxycycline in patients with small AAA [62]. They showed that the expansion rate of AAA treated with doxycycline (1.5 mm/year) was lower than those treated with placebo (3.0 mm/year), but the difference did not reach statistical significance. Baxter et al. conducted a clinical trial evaluating the effect of doxycycline in patients with small AAA [43]. They demonstrated that doxycycline was safe and well tolerated and was associated with a gradual reduction of plasma MMP-9 levels. In addition, Lindeman et al. reported that doxycycline reduced inflammation in the walls of human AAA by inhibiting infiltration of neutrophils and cytotoxic T cells [63]. Thus, MMP inhibitors, especially doxycycline, are the most extensively tested pharmacotherapy for AAA treatment in both animal models and clinical trials. However, further clinical studies are still needed to confirm the beneficial effects of MMP inhibitors on the growth of human AAA.

ECM biosynthetic enzymes, such as LOX, are essential for stabilization of collagen and elastin fibers. A critical role of ECM biosynthesis in aortic wall integrity was demonstrated by the observation that disruption of the LOX gene leads to aneurysm formation and aortic rupture [64]. Reduced LOX expression in experimental AAA has also been reported [36, 40]. Indeed, we demonstrated that adenoviral expression of LOX ameliorated experimental AAA progression, indicating that impaired ECM biosynthesis plays a critical role in the development of AAA [36]. More interestingly, we found that enhancement of LOX activity not only stabilized the ECM

Table 1: Pharmacotherapy for abdominal aortic aneurysm in animal models.

Target	Drug	Model	Mechanism of AAA inhibition	Effects in human AAA
Oxidative stress	α-Tocopherol (vitamin E)	ATII/*ApoE* $^{-/-}$ mice [11] Elastase/rat [13]	ROS ↓, macrophage infiltration ↓	No effect on growth [12]
Estrogen receptor	17β-estradiol	Elastase/rat [14] ATII/*ApoE*$^{-/-}$ mice [15]	Macrophage infiltration ↓, MCP-1 ↓, NF-κB activity ↓, MMP-9 ↓, preserved elastin	No evidence
RAS	ACE inhibitor (captopril, lisinopril, and enalapril)	Elastase/rat [16]	Preserved elastin	Rupture risk ↓ [17] growth ↑ [18]
	ARB (valsartan)	Elastase/rat [19]	Macrophage infiltration ↓, NF-κB activity ↓, MMP-9 ↓	No effect on rupture risk [17]
Mevalonate pathway	Statin (simvastatin and atorvastatin)	Elastase/mice Elastase/*ApoE* $^{-/-}$ mice [28]	Macrophage infiltration ↓, IL-1 ↓, MCP-1 ↓, NF-κB activity ↓, MMP-9 ↓,	MCP-1 ↓, MMP-9 ↓ [20–23] growth ↓ [24–27]
		Elastase/rat [29, 30]	Preserved elastin	No effect on growth [18, 31, 32]
Mast cell	DSCG [33] Tranilast [34]	Elastase/mice $CaCl_2$/rat ATII/*ApoE* $^{-/-}$ mice	Mast cell and macrophage infiltration ↓, IFN-γ ↓, IL-6 ↓, MMP activity ↓, preserved elastin	No evidence
NF-κB	PDTC [35]	Elastase/mice	Cellular infiltration ↓, IL-1β ↓, IL-6 ↓, NF-κB activity ↓, MMP-9 ↓, preserved elastin	No evidence
JNK	SP600125 [36]	$CaCl_2$/ mice ATII /*ApoE* $^{-/-}$ mice	Macrophage infiltration ↓, MMP-9 ↓, preserved elastin, regression of established AAA	MMP-9 ↓, TIMP-3 ↑ [36, 37]
MMP	Doxycycline	Elastase/rats [38–41] Elastase/mice [44, 45] $CaCl_2$/mice [46] ATII/*ApoE* $^{-/-}$ mice [47, 48] Thioglycolate plus plasmin/rats [49]	MMP-9 ↓, preserved elastin	MMP-9 ↓ [42, 43]

AAA: abdominal aortic aneurysm; ATII: angiotensin II; ROS: reactive oxygen species; MMP: matrix metalloproteinase; NF: nuclear factor; MCP: monocyte chemoattractant protein; RAS: renin-angiotensin system; ACE: angiotensin converting enzyme; ARB: angiotensin receptor blocker; IL: interleukin; DSCG: disodium cromoglycate; IFN: interferon; PDTC: pyrrolidine dithiocarbamate; JNK: c-Jun N-terminal kinase; TIMP: tissue inhibitor of metalloproteinase.

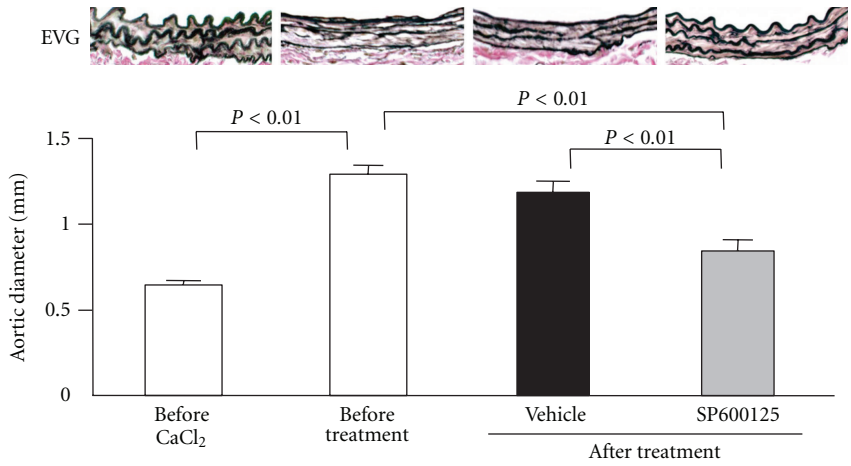

Figure 3: Regression of abdominal aortic aneurysm (AAA) with c-Jun N-terminal kinase (JNK) inhibitor in a mouse model. Six weeks after stimulation of mouse aorta with $CaCl_2$, the AAA model was established in association with elastic lamellae disruption and increased aortic diameter. After AAA establishment, pharmacologic treatment with SP600125, a JNK inhibitor, was initiated. After six weeks of SP600125 treatment, there was a significant reduction in aneurysmal size compared with vehicle treatment as well as before treatment. The regression of AAA was accompanied by a repair of tissue architecture. EVG: elastica Van Gieson (modified from Yoshimura et al. [36]).

but also reduced inflammatory responses, including MCP-1 secretion, macrophage infiltration, and JNK activation, thereby preventing AAA progression in mice [65]. Therefore, enhancement of LOX activity may represent a new therapeutic target for the treatment of AAA.

4. Future Directions

Since AAA is predominately localized to a limited site on the aorta, local delivery of pharmacologic agents, which would increase therapeutic efficacy and reduce systemic side effects, is a reasonable approach. The efficacy of this therapeutic option was previously reported using doxycycline and rodent models of AAA [41, 45]. The combination of local drug delivery with other interventions, such as endovascular stent grafting, is one possibility. Because ongoing aortic wall degeneration and subsequent failure of aneurysm exclusion are the major concern, after endovascular repair, adjuvant pharmacotherapy would provide an ideal solution for these concerns. Indeed, patent applications claiming a device composed of stent graft and drug delivery system have been filed by us and others [66, 67]. Further progress with drug delivery systems could advance the development of less invasive therapeutic strategies for the treatment of AAA.

Today, several pharmacologic agents targeting various pathological components, including ECM degrading enzymes, proinflammatory mediators, and intracellular signaling pathways, are documented to be effective at least in AAA animal models. Therefore, it seems very possible for us to manipulate some aspects of the biological process, such as the enhancement of proinflammatory mediators, the activation of intracellular signaling pathways, and the shift of ECM metabolism toward degradation. The challenge is to diagnose the predominant aspect in a given patient, or even in a given area of the AAA tissue. Another challenge is monitoring tissue response during pharmacotherapy to optimize the therapeutic regimen to fit individual patients' needs. To this end, it is necessary to identify biomarkers that accurately reflect the biological activity in AAA, as proposed by Golledge and Powell [68]. Several circulating biomarkers for AAA have emerged as candidates [69, 70]. However, they have yet to be tested in a large standardized study to reveal their potential utility in the prediction of AAA progression as well as in the diagnosis of disease activity.

5. Conclusions

In past decades, several studies have identified classes of drugs that can ameliorate various pathological activities in AAA. Human AAA will be treatable by pharmacological therapy if we can optimize the therapeutic regimen for the individual patient with the aid of biomarkers. Despite current improvements in therapeutic options, including open surgical and endovascular aneurysm repairs, nonsurgical prevention of AAA growth and rupture has yet to be achieved. Once established, pharmacotherapy could play a crucial role in the management of patients with AAA.

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Review Article

Role of Peroxisome Proliferator-Activated Receptor-γ in Vascular Inflammation

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Vascular inflammation plays a crucial role in atherosclerosis, and its regulation is important to prevent cerebrovascular and coronary artery disease. The inflammatory process in atherogenesis involves a variety of immune cells including monocytes/macrophages, lymphocytes, dendritic cells, and neutrophils, which all express peroxisome proliferator-activated receptor-γ (PPAR-γ). PPAR-γ is a nuclear receptor and transcription factor in the steroid superfamily and is known to be a key regulator of adipocyte differentiation. Increasing evidence from mainly experimental studies has demonstrated that PPAR-γ activation by endogenous and synthetic ligands is involved in lipid metabolism and anti-inflammatory activity. In addition, recent clinical studies have shown a beneficial effect of thiazolidinediones, synthetic PPAR-γ ligands, on cardiovascular disease beyond glycemic control. These results suggest that PPAR-γ activation is an important regulator in vascular inflammation and is expected to be a therapeutic target in the treatment of atherosclerotic complications. This paper reviews the recent findings of PPAR-γ involvement in vascular inflammation and the therapeutic potential of regulating the immune system in atherosclerosis.

1. Introduction

Atherosclerosis is the primary cause of cerebrovascular and coronary artery disease through slowly progressive lesion formation and luminal narrowing of arteries. This vascular remodeling leads to thrombotic complications including acute coronary syndrome, myocardial infarction, and stroke. Atherosclerosis is well known to be an inflammatory disease, and the underlying pathology is characterized by a persistent inflammatory process of the arterial wall [1]. With increasing prevalence of risk factors such as hypertension, diabetes, and obesity [2], it is critical to control vascular inflammation in order to decrease mortality and improve public health. To solve this problem, peroxisome proliferator-activated receptor (PPAR)-γ has emerged as an important player.

PPAR-γ belongs to the nuclear receptor family of ligand-activated transcription factors, which also include the steroid and thyroid hormone receptors [3]. PPAR-γ forms heterodimers with the retinoid X receptor (RXR) and activates transcription by binding to a specific DNA element known as the PPAR response element (PPRE) [4]. In the absence of ligand, PPAR-RXR heterodimers bind a number of corepressors, including nuclear receptor corepressor and the silencing mediator of the retinoid and thyroid hormone receptors, to suppress the target genes. In the presence of selective ligands, PPAR-γ undergoes a conformational change facilitating the dissociation of corepressors and the recruitment of co-activators, leading to transcriptional activation of the target genes [5, 6]. To date, a variety of endogenous and synthetic ligands in addition to its co-activators have been detected (Table 1). PPAR-γ is known to have four splice isoforms: PPAR-γ1, γ2, γ3, and γ4. PPAR-γ1 and γ2 have been identified in mouse, whereas in humans and other species, at least two other isoforms, PPAR-γ3 and γ4, have also been detected [7]. PPAR-γ3 and γ4 encode the same protein as PPAR-γ1, which is expressed in endothelial cells (ECs), vascular smooth muscle cells (VSMCs), macrophages, and cardiomyocytes. On the other hand, PPAR-γ2 is mainly expressed in adipocytes [8].

PPAR-γ plays an important role in regulation of adipocyte differentiation and insulin resistance [9]. The thiazolidinedione (TZD) class of synthetic PPAR-γ ligands reduces

Table 1: Endogenous and synthetic ligands for PPAR-γ and genes for PPAR-γ related coactivator.

Ligands for PPAR-γ		Genes for PPAR-γ related co-activator
Endogenous ligands	Synthetic ligands	
Unsaturated fatty acids	Rosiglitazone	CBP/p300
15-deoxy-$\Delta^{12,14}$-prostaglandin J_2	Pioglitazone	SRC-1
15-HETE	Troglitazone	SRC-2
9-HODE	Ciglitazone	SRC-3
13-HODE	Tyrosine derivatives	PGC-1α
Oxidized LDL	Farglitazar	PGC-1β
	GW7845	PBP
		PRIP
		PRIC285
		BAF60c

BAF60c: BRG1/Brm-associated factor of 60 kDA subunit of c; CBP: cyclic-AMP responsive element binding protein (CREB)-binding protein; HETE: hydroxyeicosatetraenoic acid; HODE: hydroxyoctadecadienoic acid; LDL: low-density lipoprotein; PBP: PPAR-binding protein; PGC: PPAR-γ coactivator; PPAR: peroxisome proliferator-activated receptor; PRIC: PPAR-α-interacting cofactor; PRIP: PPAR interacting protein; SRC: steroid receptor coactivator.

peripheral insulin resistance and has been widely used to treat type 2 diabetes mellitus. For instance, several reports using high-fat diet-induced obese mice demonstrated that PPAR-γ agonists had beneficial effects on improving insulin resistance and inflammation [10–13]. In addition, recent large clinical studies have demonstrated that a PPAR-γ agonist had beneficial effects not only on glycemic control but also in preventing atherosclerotic disease [14–17]. The lines of evidence derived from study of EC specific PPAR-γ null mice [18–20] and from virus-mediated constitutive expression of PPAR-γ in human ECs [21] have also shown important roles of PPAR-γ on atherogenesis. Increasing evidence has demonstrated that PPAR-γ plays important roles in the immune system, since PPAR-γ is expressed in inflammatory cells such as macrophages, T cells, B cells, and dendritic cells [22]. These results suggest that PPAR-γ activation is an important regulator in vascular inflammation and is expected to be a therapeutic target in the treatment of atherosclerotic complications (Figure 1). The present paper focuses on the role of PPAR-γ in vascular inflammation beyond its beneficial effects on glycemic control and discusses the potential therapeutic roles of regulating PPAR-γ activation.

2. PPAR-γ and Monocytes/Macrophages

Monocytes/macrophages are key players in vascular inflammation and atherosclerosis [23]. PPAR-γ has been detected in rodent macrophages [24], and human macrophages in atherosclerotic lesions [25]. Differentiated macrophages show two acquired phenotypic characteristics, the classically activated (M1) phenotype and the alternatively activated (M2) phenotype [26]. M1 activation is triggered by stimulation such as by bacterial lipopolysaccharide (LPS) and is associated with the production of proinflammatory cytokines including interferon-γ (IFN-γ) and interleukin-12 (IL-12), which are linked to T helper 1 (Th1) immune responses. In contrast to M1, M2 activation is triggered by IL-4 and IL-13, which are linked to Th2 responses [27]. M1 macrophages produce a number of proinflammatory cytokines and express a high level of reactive oxygen species (ROS), having antimicrobial activity. On the other hand, M2 macrophages generate anti-inflammatory products and are involved in pathogen sequestration, wound healing, and phagocytosis of apoptotic cells [28, 29]. The balance between these two subsets is thought to be important in regulating vascular inflammation.

In vitro studies have demonstrated that PPAR-γ agonists attenuated the gene expression and secretion of proinflammatory cytokines associated with M1 macrophages in human monocytes, such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 [30], and reduced the activity of macrophages including the transrepression of nuclear factor kappa B (NF-κB) [24]. In addition, troglitazone and rosiglitazone, PPAR-γ agonists, inhibited monocyte chemotactic protein 1 (MCP-1)-directed monocyte migration through modulation of matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) production [31]. These results suggest that PPAR-γ activation may be involved in vascular inflammation through regulating macrophage activation.

PPAR-γ has been also reported to be an invaluable transcriptional regulator of monocyte phenotypic differentiation. Crosstalk between PPAR-γ and IL-4 signaling is thought to be important for M2 macrophage polarization [32, 33]. In macrophages, IL-4-mediated signaling activates the transcription factor, signal transducers, and activators of transcription 6 (STAT6), resulting in upregulation of the expression of PPAR-γ, PPAR-γ coactivator-1β (PCG-1β), and ARG1. Increased PCG-1β enhances STAT6 action on these genes and other genes relating to mitochondrial biogenesis, oxidative metabolism, and M2 differentiation. Additionally, other recent studies have demonstrated that PPAR-γ-deficient macrophages were resistant to M2 polarization and promoted insulin resistance [29, 34].

Foam cell formation of macrophages is also important in the progression of atherosclerosis. Another function of PPAR-γ in macrophages is regulation of lipoprotein uptake and cholesterol efflux. Tontonoz et al. have demonstrated that PPAR-γ ligands induced the differentiation of human

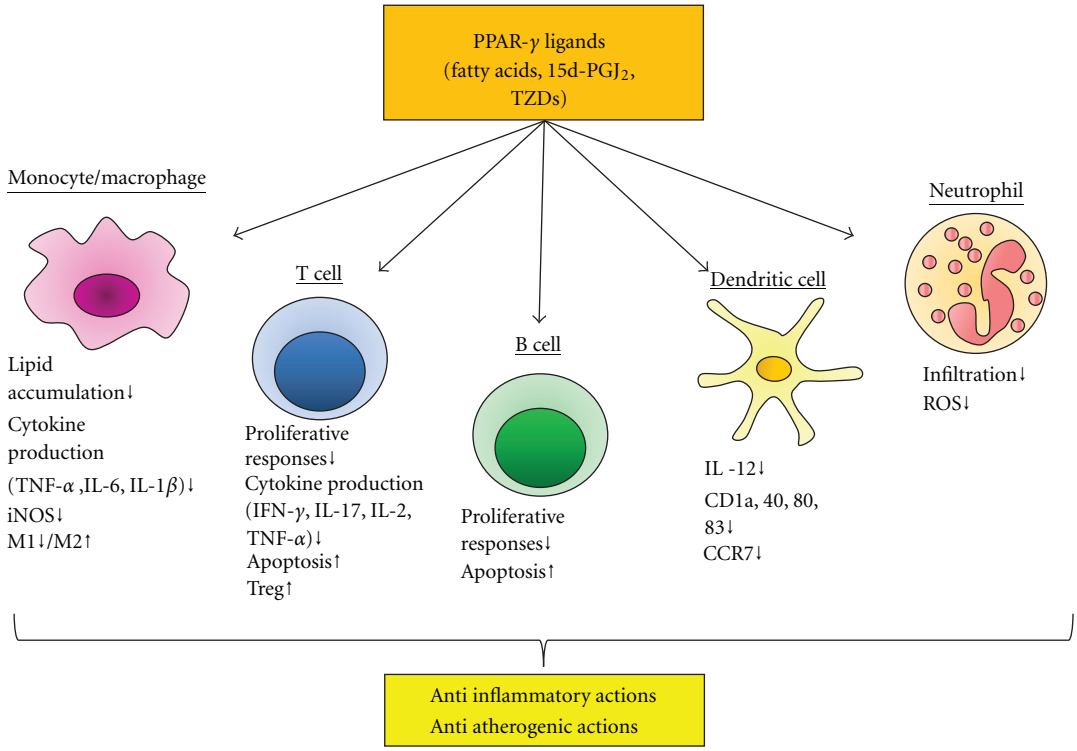

Figure 1: Effects of PPAR-γ activation on various immune cells in vascular inflammation. PPAR-γ is expressed in various immune cells such as monocyte/macrophage, lymphocyte, dendritic cell, and neutrophil. PPAR-γ activation by endogenous and synthetic ligands could regulate inflammatory responses induced by these cells, leading to anti-inflammation and antiatherogenicity. CCR, chemokine (C-C motif) receptor; 15d-PGJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; IFN, interferon; IL, interleukin; ROS, reactive oxygen species; TNF, tumor necrosis factor; Treg, regulatory T cell; TZD, thiazolidinedione.

monocytes into macrophages and enhanced the transcription of a scavenger receptor for oxidized low-density lipoprotein (oxLDL), CD36 [35]. In addition, oxidized lipids inside the oxLDL particle, including 9-hydroxyoctadecadienoic acid (9-HODE) and 13-HODE, enhance PPAR-γ activation [36]. Thus, PPAR-γ activation in the presence of oxidized lipids could lead to a positive feedback loop to promote foam cell formation [37, 38]. On the other hand, *in vivo* studies revealed that TZD treatment could increase macrophage CD36 expression, but did not enhance foam cell formation, suggesting that PPAR-γ could activate other pathways that enhance cholesterol efflux and reduce intracellular cholesterol level. The enhancement of cholesterol efflux was mediated by the cholesterol-phospholipid transporter ABCA1, which is an indirect target gene of PPAR-γ via liver X receptor α [39, 40]. These results suggest that PPAR-γ activation couples oxLDL uptake to cholesterol efflux, thus enhancing the removal of oxLDL from the vessel wall.

3. PPAR-γ and T Cells

PPAR-γ is expressed in T cells, and its expression is increased in activated T cells [41]. It is reported that PPAR-γ activation modulates the expression of proinflammatory Th1 cytokines in CD4-positive lymphocytes. For instance, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ$_2$), an endogenous PPAR-γ ligand, and TZDs reduced IL-2 secretion from murine T cell clones [42] and inhibited IL-2 production and phytohemagglutinin-inducible proliferation in human T cells in a dose-dependent manner [43]. In addition, PPAR-γ activators inhibited the expression of proinflammatory cytokines such as interferon-γ (IFN-γ), TNF-α, and IL-2, leading to attenuation of human monocyte CD64 expression and human endothelial cell major histocompatibility complex class II induction [44]. In a well-established mouse colitis model, it is reported that TZDs attenuated intestinal inflammation, at least in part, due to immune deviation away from Th1 and towards Th2 cytokine production [45].

Th17 cells and a proinflammatory cytokine, IL-17, secreted by them have been reported to be involved in the pathogenesis of atherosclerotic disease. Recently, Klotz et al. have indicated that PPAR-γ activation can regulate the differentiation and function of Th17 cells, a newly identified T cell subset [46]. PPAR-γ activators could suppress the differentiation of Th17 cells by inhibiting the upregulation of retinoic acid receptor-related orphan receptor γt (RoRγt), the key transcriptional factor of Th17 differentiation, in response to Th17 cell-promoting cytokines, such as TGF-β and IL-6. Therefore, PPAR-γ activation selectively suppressed Th17 cell differentiation, but not the differentiation of Th1, Th2, or regulatory T cells (Treg). Pharmacologic activation of PPAR-γ prevented removal of the silencing mediator for retinoid and thyroid hormone receptors corepressor from the RoRγt promoter in T cells, thus interfering with RoRγt transcription. Both T cell-specific PPAR-γ knockout and

endogenous ligand activation revealed the physiological role of PPAR-γ in continuous T cell intrinsic control of Th17 differentiation.

CD4+CD25+ Tregs also play an important role in the pathogenesis of atherosclerosis and are expected to be a novel therapeutic target to attenuate atherosclerosis and stabilize vulnerable plaques [47]. A relationship between PPAR-γ activation and regulation of Tregs has been reported. The PPAR-γ ligand, ciglitazone, enhanced the conversion of effector T cells to Tregs *in vitro* and had an enhancing effect on both natural and inducible Tregs [48]. Moreover, Lei et al. have demonstrated that PPAR-γ activation with endogenous and synthetic ligands together with transforming growth factor-β (TGF-β) elicited Foxp3 deoxyribonucleic acid (DNA) methylation through potent downregulation of DNA methyltransferases (DNMTs) such as DNMT1, 3a, and 3b, and induced potent and stable Foxp3, resulting in the generation of functional inducible Tregs [49].

4. PPAR-γ and B Cells

B cells play an important role in atherosclerosis and are thought to have atherogenic and antiatherogenic effects according to their subtype [50]. Mature B cells are categorized into three subtypes according to their surface antigens: conventional B2 B cells, B1 B cells, and marginal zone B cells [51]. The conventional B2 B cell plays an important role in adaptive immunity by producing specific antibodies to cognate antigens. The B1 B cell, which is found primarily in serosal cavities such as the peritoneal and pleural cavities, is important in innate immunity and responsible for production of natural IgM antibodies. The marginal zone B cell in splenic tissue plays a role in first-line defense against circulating blood-borne antigens. B1 B cells are thought to have a protective effect against atherogenicity [52, 53]. On the other hand, it seems that B2 B cells are involved in atherosclerosis, since native conventional B2 B cells can differentiate into two effector B cells, so-called Be1 and Be2 B cells. Be1 B cells produce Th1 cytokines including INF-γ, IL-2 and IL-12, whereas Be2 B cells secrete IL-4, IL-6 and IL-10, which are Th2 cytokines. It is reported that these cytokines secreted by Be cells enhance immunomodulation during chronic inflammation [54]. However, the detailed role of Be1 and Be2 B cells in atherosclerosis remains to be elucidated. Recently, regulatory B cells that produce IL-10 have been recognized as an important component of the immune system [55–59]. Regulatory B cells secrete IL-10, and this may lead to suppression of both Th1 and Th2 polarization and inhibition of proinflammatory cytokine production from macrophages and DC. The role of regulatory B cells in atherosclerosis also remains to be elucidated, but they may attenuate the progression of atherosclerosis.

PPAR-γ is expressed in human and mouse B cells [60, 61]. Most studies of the effect of PPAR-γ activation on B cells focus on the apoptotic effect of endogenous and synthetic ligands on normal or B lymphoma cells. Recent reports demonstrated that detailed roles of PPAR-γ and RXRα agonists in PPAR-γ agonist-induced apoptosis of B cells were activation of mitogen-activated protein kinases (MAPKs), inhibition of nuclear factor-kappa B (NF-κB), and CD40 activation [62–66]. On the other hand, a recent paper by Garcia-Bates et al. reported the role of the PPAR-γ/RXRα pathway in human B cell differentiation [67]. They demonstrated that activated B cells have upregulated expression of PPAR-γ. In addition, nanomolar levels of PPAR-γ ligands, such as 15d-PGJ_2 and rosiglitazone, enhanced B cell proliferation and significantly stimulated plasma cell differentiation and antibody production. The simultaneous addition of nanomolar concentrations of the RXRα ligand, 9-*cis*-retinoic acid, and PPAR-γ ligands to CpG-activated B cells resulted in additive effects on B cell proliferation, plasma cell differentiation, and antibody production. This result suggests that PPAR-γ activation may also regulate the function and differentiation of B cells. However, the link between PPAR-γ activation and B cell function in atherosclerosis is still unclear.

5. PPAR-γ and Dendritic Cells

DC contributes to chronic vascular inflammation, leading to atherosclerosis and its complications [68–70]. In fact, a number of DC has been observed in atherosclerotic lesions of mouse models [71–73] and in human advanced plaques [74–76]. In normal conditions, DC is professional antigen-presenting cell that presents many kinds of endogenous and exogenous antigens to T cells, providing an important link between innate and adaptive immune responses [77]. Additionally, many lines of evidence have demonstrated that DC contributes to the pathogenesis and progression of atherosclerosis [68–70]. DC accumulates in the intima of atherosclerotic lesions through vascular cell adhesion molecule-1 (VCAM-1) and CX3C chemokine receptor 1 (CX3CR1) during low-grade chronic inflammation [72, 78]. DC may differentiate from $Ly6^{low}$ monocytes that CX3CR1-dependently patrol arterial vessels, but can also differentiate from $Ly6^{high}$ monocytes, which seem to be preferentially recruited. In intimal proliferation of DC, granulocyte macrophage colony-stimulating factor (GM-CSF) is thought to be important [79, 80]. Excess lipoproteins deposited in the arterial wall accumulate within lipid-loaded $CD11c^{+}$ DC, contributing to early-stage plaque formation. DC can control lipid homeostasis possibly through lipoprotein uptake and clearance from the circulation. DC also regulates T cell activation in the vessel wall and influence helper T cell responses, with lipoprotein being able to contribute to DC maturation and activation [69]. In addition, various DC subsets can release proinflammatory cytokines [68]. For instance, conventional DC (cDC) can participate in interaction with T and natural killer T cells, which results in increased production of IFN-γ, IL-17, and TNF-α from T cells [81]. Activation of CD36 and Toll-like receptors (TLRs) in CD11b+CD11c+ DC and cDC by lipids results in increased secretion of various DC-derived cytokines, such as IL-6, IL-12, and TNF-α [82]. Plasmacytoid DC (pDC) has been shown to produce amounts of type I IFNs (IFN-α and β), which play a proatherogenic role.

PPAR-γ is expressed in murine and human DC, and PPAR-γ activation has been shown to be involved in DC

function [83–90]. PPAR-γ ligands inhibited the production of IL-12 and several cytokines such as chemokine (C-X-C motif) ligand 1 (CXCL1) and chemokine (C-C motif) ligand 5 (CCL5) [85, 86]. Moreover, PPAR-γ inhibited the maturation of DC and attenuated the expression of CD1a, CD40, CD80, CD83, and chemokine (C-C motif) receptor 7 (CCR7) [85, 88, 90]. These results indicate that PPAR-γ activation by synthetic ligands reduced the ability of DC to stimulate lymphocyte proliferation and to prime antigen-specific T cell responses.

6. PPAR-γ and Neutrophils

Neutrophils, as well as macrophages, lymphocytes, and DC, also play crucial roles in atherogenesis [91, 92]. Neutrophils and their mediators have been detected in mouse and human atherosclerotic lesions [93–95]. An increased number of circulating neutrophils are also observed in pathological conditions such as hyperlipidemia. Neutrophils are recruited into atherosclerotic lesions via specific chemokine receptors, including CCR1, 2, 5, and CXCR2 [96]. OxLDL may induce the transmigration of neutrophils and release of ROS and granule proteins from neutrophils, which trigger monocyte recruitment and extravasation directly or indirectly through upregulation of adhesion molecules on endothelial cells. In addition, apoptotic neutrophils sustain monocyte recruitment via various find-me and eat-me signals [97]. Thus, neutrophils could provide a chronic inflammation trigger sustaining atherogenesis.

Several lines of evidence have demonstrated the presence of PPAR-γ in neutrophils, and have shown a suppressive effect of PPAR-γ activation by endogenous and synthetic ligands on neutrophil infiltration in various animal models of inflammation [98–103]. A recent study by Napimoga et al. [104] reported that administration of 15d-PGJ_2, an endogenous PPAR-γ ligand, decreased leukocyte rolling and adhesion to inflamed mesenteric tissue by a mechanism dependent on NO. Specifically, pharmacological inhibitors of NO synthase (NOS) abrogated the 15d-PGJ_2-mediated suppression of neutrophil migration to the inflammatory site. Moreover, inducible $NOS^{-/-}$ mice were not susceptible to 15d-PGJ_2-mediated suppression of neutrophil migration to inflammatory sites compared with their wild type. In addition, 15d-PGJ_2-mediated suppression of neutrophil migration appeared to be independent of the production of cytokines and chemokines, since their production was not significantly affected in the carrageenan-injected peritoneal cavity. These findings demonstrated that 15d-PGJ_2 suppresses inflammation-initiated neutrophil migration in a mechanism dependent on NO production in mesenteric tissue. However, the detailed role of neutrophil regulation by PPAR-γ ligands in atherosclerosis remains to be elucidated and further studies are needed.

7. Recent Concern for Cardiovascular Risks of PPAR-γ Agonists

As described above, PPAR-γ activation is expected as a therapeutic target for improving cardiovascular risk factors. However, its safety is controversial in clinical use, since several reports pointed out an increase in risk of ischemic cardiovascular events with PPAR-γ agonists. Meta-analysis of randomized controlled trials has suggested that rosiglitazone, one of TZD, increased risk of ischemic cardiovascular events [105, 106]. In contrast, meta-analysis of clinical trials of another TZD, pioglitazone has also reported the possibility of an ischemic cardiovascular benefit by pioglitazone [107]; however, both TZDs are reported to increase the risk of congestive heart failure [108]. Recently, meta-analysis of observational studies that directly compared the risk of cardiovascular outcomes for rosiglitazone and pioglitazone among patients with T2DM has demonstrated that the use of rosiglitazone was associated with significantly higher odds of congestive heart failure, myocardial infarction, and death compared with that of pioglitazone [109]. However, whether any meaningful difference exists in the magnitude of risk between two TZDs is still unclear. The European Medicines Agency has recommended the suspension of marketing authorization for rosiglitazone, whereas the US Food and Drug Administration has allowed the continued marketing of rosiglitazone with additional restrictions [110]. Further studies are required to understand these contradictory effects of PPAR-γ agonists in the future.

8. Conclusion

Vascular inflammation-induced atherosclerosis is one of the most worrying common problems throughout the world. As described above, PPAR-γ has a wide range of roles in the pathogenesis and progression of atherosclerosis via regulation of inflammatory cells, including monocytes/macrophages, lymphocytes, dendritic cells, and neutrophils. Although regulation of PPAR-γ activity may not alter the underlying cause of the disease, it may regulate pathological conditions, resulting in clinical benefit. Several recent experimental and clinical findings have supported the potential utility of regulating PPAR-γ activity as a therapeutic approach for atherosclerosis. The roles of PPAR-γ regulation still represent huge unmet challenges in therapeutic interventions. Further accumulation of experimental and clinical evidence on the relationship between PPAR-γ and vascular inflammation may contribute to solving this problem.

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Research Article

Chronic C-Type Natriuretic Peptide Infusion Attenuates Angiotensin II-Induced Myocardial Superoxide Production and Cardiac Remodeling

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Myocardial oxidative stress and inflammation are key mechanisms in cardiovascular remodeling. C-type natriuretic peptide (CNP) is an endothelium-derived cardioprotective factor, although its effect on cardiac superoxide generation has not been investigated in vivo. This study tested the hypothesis that suppression of superoxide production contributes to the cardioprotective action of CNP. Angiotensin II (Ang II) or saline was continuously infused subcutaneously into mice using an osmotic minipump. Simultaneously with the initiation of Ang II treatment, mice were infused with CNP (0.05 μg/kg/min) or vehicle for 2 weeks. The heart weight to tibial length ratio was significantly increased by Ang II in vehicle-treated mice. Treatment with CNP decreased Ang II-induced cardiac hypertrophy without affecting systolic blood pressure. Echocardiography showed that CNP attenuated Ang II-induced increase in wall thickness, left ventricular dilatation, and decrease in fractional shortening. CNP reduced Ang II-induced increases in cardiomyocyte size and interstitial fibrosis and suppressed hypertrophic- and fibrosis-related gene expression. Finally, CNP decreased Ang II-induced cardiac superoxide production. These changes were accompanied by suppression of NOX4 gene expression. Our data indicate that treatment with CNP attenuated Ang II-induced cardiac hypertrophy, fibrosis, and contractile dysfunction which were accompanied by reduced cardiac superoxide production.

1. Introduction

C-type natriuretic peptide (CNP) belongs to the natriuretic peptides (NPs) family, which was originally isolated from porcine brain tissues and later established as a positive regulator of endochondral ossification [1]. In the cardiovascular system, CNP is secreted from endothelial cells and cardiac fibroblasts, and acts as an autocrine/paracrine regulator [2, 3]. Myocardial production and circulating CNP levels are increased in patients with chronic heart failure [4, 5], suggesting that CNP has an important role in the pathogenesis of heart failure. ANP and BNP bind to natriuretic peptide receptor (NPR)-A, inducing natriuresis and diuresis, whereas CNP preferentially binds to its specific receptor, NPR-B. Because of the relative abundance of NPR-B over NPR-A in cardiac fibroblasts and in cardiomyocytes, CNP reportedly has more potent antihypertrophic and antifibrotic properties, despite less hypotensive and less natriuretic effects compared with ANP and BNP [6, 7]. The recently designed novel compound CD-NP, which is a chimera of CNP and DNP, is a promising candidate for new-generation therapeutics using NPs [8]. It is therefore of interest to elucidate the precise role of CNP in cardiovascular pathophysiology from both a basic and clinical medicine perspective.

Previous experimental studies showed that long-term administration of CNP attenuates cardiac remodeling and maintains systolic function in murine models of myocardial infarction and myocarditis, independent of its hypotensive effect [9, 10]. Cardiomyocyte-specific overexpression of CNP attenuated cardiac hypertrophy in a murine model of myocardial infarction [11]. Conversely, transgenic rats with

Table 1: Primer sequences used for quantitative real-time PCR.

Gene		Primer sequence
ANP	Forward	5′-GAGAGACGGCAGTGCTTCTAGGC-3′
	Reverse	5′-CGTGACACACCACAAGGGCTTAGG-3′
BNP	Forward	5′-GGAAGTCCTAGCCAGTCTCC-3′
	Reverse	5′-TTGGTCCTTCAAGAGCTGTC-3′
α-MHC	Forward	5′-GACGAGGCAGAGCAGATCGC-3′
	Reverse	5′-GGGCTTCACAGGCATCCTTAGGG-3′
Collagen I	Forward	5′-GTCCCAACCCCCAAAGAC-3′
	Reverse	5′-CATCTTCTGAGTTTGGTGATACGT-3′
Collagen III	Forward	5′-TGGTTTCTTCTCACCCTTCTTC-3′
	Reverse	5′-TGCATCCCAATTCATCTACGT-3′
NOX-4	Forward	5′-TGGGCCTAGGATTGTGTTTA-3′
	Reverse	5′-CTGCTAGGGACCTTCTGTGA-3′
GAPDH	Forward	5′-TCACCACCATGGAGAAGGC-3′
	Reverse	5′-GCTAAGCAGTTGGTGGTGCA-3′

overexpression of a dominant-negative mutant of NPR-B showed exacerbated cardiac hypertrophy [12]. In vitro, it has been shown that CNP prevents agonist-induced cardiomyocyte hypertrophy via a cyclic GMP-dependent protein kinase-mediated mechanism [7, 13]. CNP also inhibits fibroblast proliferation and extracellular matrix production of cardiac fibroblasts more potently than ANP and BNP [6]. These data indicate that CNP acts directly on myocardial cells to protect against pathological stimuli.

Experimental and clinical evidence implicates a central role for angiotensin II (Ang II) in all stages of the cardiovascular continuum [14, 15], and the detrimental effects of Ang II are mediated, at least in part, by reactive oxygen species (ROS) [16–18]. To this end, the antioxidant activity contributes to the reported cardioprotective effects of ANP [19]. However, little is known about the effects of CNP on Ang II-induced cardiac ROS generation. Here, we investigated whether chronic CNP infusion could attenuates myocardial superoxide production in a mouse model of cardiac hypertrophy and remodeling induced by Ang II.

2. Materials and Methods

2.1. Animals. Ten-week-old male C57BL/6 mice were used in the present study. Ang II (Peptide Institute) or saline alone was subcutaneously infused via an osmotic minipump (Alzet model 1002) at the rate of 3.2 mg/kg/day for 2 weeks [20]. Simultaneously with the initiation of Ang II treatment, mice were infused with CNP (Peptide Institute, 0.05 μg/kg/min) or vehicle by continuous subcutaneous infusion for 2 weeks. Systolic blood pressure (SBP) and heart rate (HR) of the conscious mice were monitored by the tail-cuff method (MK-2000ST; Muromachi). Transthoracic echocardiography was performed 2 weeks after the Ang II infusions. Mice were then euthanized and the hearts were weighed and harvested for further analysis. The serum level of CNP was measured using the CNP-22 EIA Kit (Phoenix Pharmaceuticals, Karlsruhe, Germany) according to the manufacturer's protocol [21]. All procedures were performed in accordance with the Kumamoto University animal care guidelines, which conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Echocardiography. Transthoracic ultrasound cardiogram (UCG) was performed using a Xario system (Toshiba, Tokyo, Japan) equipped with a 12-MHz linear array transducer. M-mode images were recorded from the short-axis view at the high papillary muscle level. Left ventricular end-diastolic dimension (LVDd), end-systolic dimension (LVDs), intraventricular septum (IVS), and posterior wall thickness (PW) were measured. Fractional shortening (%FS) was calculated using the following equation: %FS = (LVDd − LVDs)/LVDd $\times$ 100. All recordings were performed in conscious mice. All echocardiography was performed by investigators who were blinded to the identity of the treatment group.

2.3. Real-Time PCR. Total RNA was prepared using a Qiagen RNeasy fibrous minikit, using the protocol supplied by the manufacturer, and cDNA was produced using the ThermoScript RT-PCR Systems (Invitrogen, Carlsbad, CA). Real-time PCR was performed as described previously [22]. Transcript expression levels were determined as the number of transcripts relative to those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and normalized to the mean value from control hearts. Table 1 lists the primer sequences used in this study.

2.4. Histological Analysis. Heart sections were prepared and stained with hematoxylin and eosin (H&E) for overall morphology or Masson's trichrome (MT) to assess myocardial

Table 2: Body weight, blood pressure, heart rate, and serum CNP concentration in vehicle-and CNP-treated mice at 2 weeks after Ang II infusion.

	Saline		Ang II	
	Vehicle	CNP	Vehicle	CNP
Body weight (g)	24 ± 0.3	25 ± 2.1	21 ± 0.4*	22 ± 0.6*
SBP (mmHg)	97 ± 4	87 ± 3	136 ± 6*	130 ± 7*
HR (bpm)	690 ± 16	693 ± 14	706 ± 8	699 ± 14
CNP (ng/mL)	0.559 ± 0.093	0.783 ± 0.050*	0.510 ± 0.066	0.701 ± 0.060*

Data are mean ± SEM ($n = 4 - 9$ mice per experimental group).
*$P < 0.05$ versus saline-infused and vehicle-treated mice.

interstitial fibrosis [17]. At least 10 fields were selected randomly to determine myofiber size and myocardial interstitial fibrosis using Image *J* image analysis software, as described previously [17]. Dihydroethidium (DHE) was used to evaluate superoxide levels of cardiac tissue as described in detail previously [23]. DHE fluorescence of cardiac sections was quantified using Lumina Vision version 2.2 analysis software.

2.5. Statistical Analysis. All data are presented as mean ± SEM. Comparisons among groups were made by one-way ANOVA with Fisher's PLSD test. A level of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Effects of Chronic CNP Infusion on Hemodynamic Parameters. To examine the effect of exogenous CNP supplementation on Ang II-induced cardiac remodeling, Ang II- and saline-infused mice were treated with CNP or vehicle for 2 weeks (Table 2). Ang II infusion for 2 weeks significantly increased SBP compared with the saline-infused control group (136 ± 6 versus 97 ± 4 mmHg, resp.). Circulating CNP levels were significantly increased in CNP-treated mice compared with vehicle-treated controls. The dose of CNP used (0.05 μg/kg/min) had no additional effect on SBP in either Ang II- or saline-infused mice. HR was unaffected by Ang II infusion in both CNP- and vehicle-treated mice compared to saline-infused mice.

3.2. CNP Administration Attenuates Ang II-Induced Cardiac Dysfunction. Figure 1(a) shows representative LV M-mode echocardiographic recordings. As shown in Figure 1(b), IVS and PWT were significantly increased by Ang II infusion in vehicle-treated mice, and these changes were suppressed by CNP treatment (without affecting SBP, as stated). Echocardiographic analysis also revealed significantly increased LVEDD and %FS in vehicle-treated mice 2 weeks after Ang II infusion (Figure 1(c)); however, these effects were significantly attenuated by CNP treatment. In contrast, long-term infusion of CNP had no effect on echocardiographic parameters in saline-infused mice.

3.3. CNP Infusion Attenuates Ang II-Induced Cardiomyocyte Hypertrophy. Heart weight to tibial length (HW/TL) ratios were significantly increased by Ang II infusion in vehicle-treated mice (9.6 ± 0.5 versus 6.5 ± 0.2 mg/mm; Figure 2(b)), while treatment with CNP decreased this Ang II-induced cardiac hypertrophy by 16%. CNP had no effect on heart size in the saline-infused mice. Analysis of myocyte cross-sectional area in histological sections corroborated these findings (Figure 2(a)); the calculated area of myocytes was increased in vehicle-treated mice that underwent Ang II infusion, but this increase was largely blocked in mice given CNP (276 ± 16 versus 375 ± 11 μm^2; Figure 2(c)). CNP treatment of vehicle-treated mice had no effect on myocyte cross-sectional area. Consistent with this observation, Ang II infusion increased expression of the fetal-type cardiac genes ANP, BNP, and β-myosin heavy chain, and these upregulations were blocked by CNP treatment (Figure 2(e)). Two weeks after Ang II infusion, the lungs wet weight to TL ratios (LW/TL), a parameter used to assess pulmonary congestion, were significantly decreased in CNP-treated mice compared with vehicle-treated controls (8.1 ± 0.1 versus 10.6 ± 0.8 mg/g; Figure 2(d)).

3.4. CNP Infusion Reduces Ang II-Induced Myocardial Interstitial Fibrosis. To investigate the consequence of exogenous CNP administration on cardiac remodeling, we evaluated myocardial interstitial fibrosis (Figure 3(a)). Interstitial fibrosis was significantly increased in vehicle-treated mice 2 weeks after Ang II infusion (Figure 3(b)). However, treatment with CNP caused a significant decrease in this measure (4.0 ± 0.8 versus 9.4 ± 0.4%). In agreement with this observation, Ang II-induced gene upregulations of collagen I and III were significantly decreased by exogenous CNP treatment (Figure 3(c)). CNP infusion did not detectably affect fibrosis or collagen expression in saline-infused mice.

3.5. CNP Supplementation Diminishes ROS Production in Hearts Subjected to Ang II Infusion. Myocardial superoxide generation is a key mechanism underlying the Ang II-induced cardiac remodeling [24]. We therefore investigated whether CNP administration could attenuates Ang II-induced superoxide production in mice heart. Myocardial tissue sections were stained with DHE to determine superoxide production in vehicle- and CNP-treated mice 2 weeks after Ang II infusion (Figure 4(a)). Ang II infusion increased superoxide generation in vehicle-treated mice by a factor of 1.5, but treatment with CNP markedly diminished

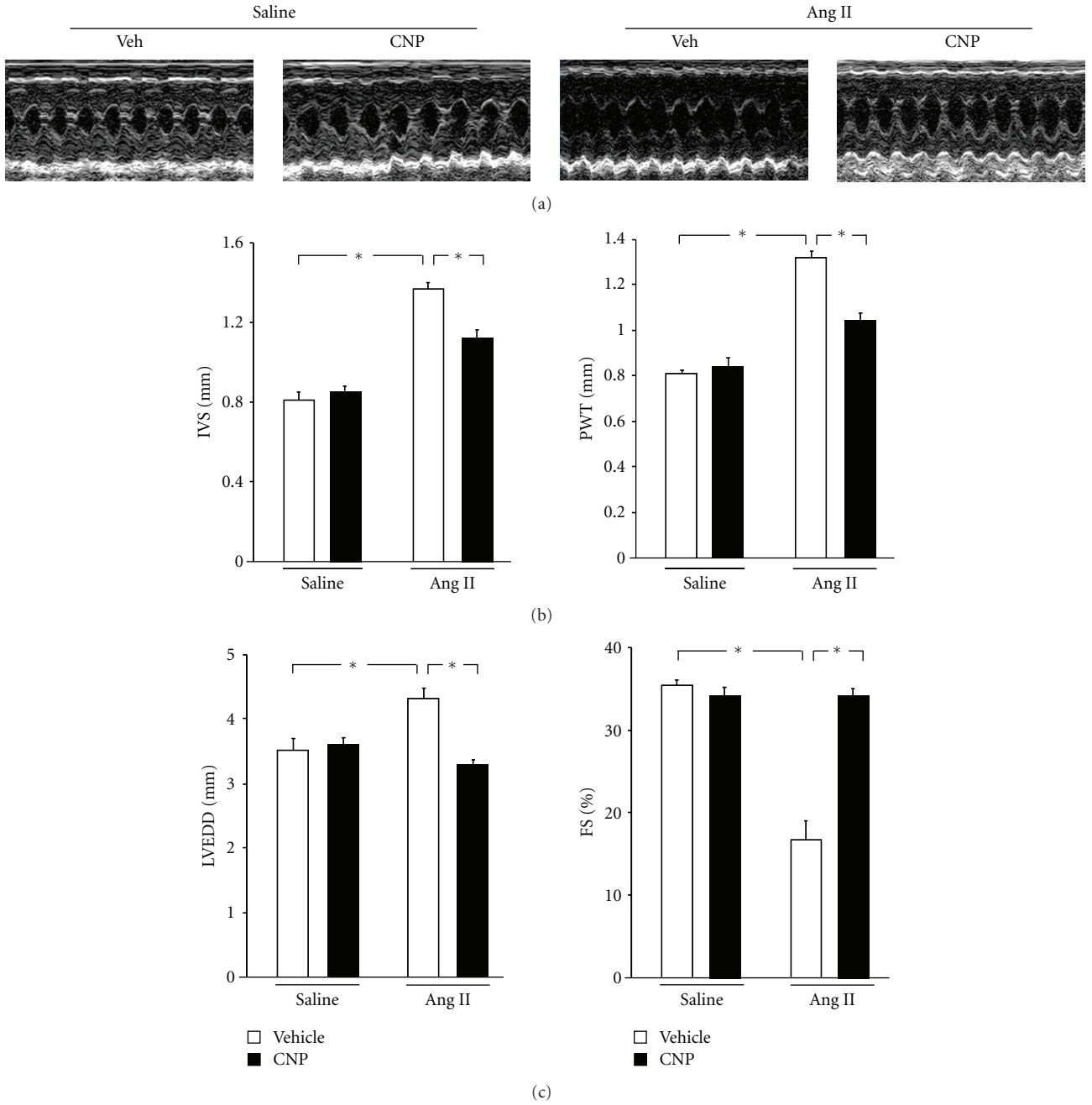

Figure 1: Effects of CNP on echocardiographic measurements. (a) Representative M-mode echocardiogram of vehicle- and CNP-treated mice 2 weeks after saline or Ang II infusion. (b) Interventricular septum (IVS) and posterior wall thickness (PWT) in vehicle- and CNP-treated mice 2 weeks after saline or Ang II infusion. (c) Left ventricular end-diastolic dimension (LVEDD) and fractional shortening (%FS) in vehicle- and CNP-treated mice 2 weeks after saline or Ang II infusion. Results are mean ± SEM ($n = 3$–6 each). $^*P < 0.05$.

Ang II-induced cardiac superoxide production (Figure 4(b)). Furthermore, Ang II induced the gene upregulation of NOX4, an NADPH oxidase subunit, and this was clearly suppressed by treatment with CNP (Figure 4(c)). In contrast, CNP treatment of vehicle-treated mice had no effect on myocardial superoxide production or NOX4 gene expression. Transcript expression levels of other NADPH oxidase subunits such as p22 and $p47^{phox}$ were not changed by CNP treatment.

4. Discussion

This study provides the first in vivo evidence that continuous infusion of exogenous CNP attenuated Ang II-induced myocardial superoxide production and cardiac dysfunction. Administration of CNP significantly prevented Ang II-induced cardiac hypertrophy and LV dilatation, thereby maintaining myocardial contractile function without affecting SBP. CNP reduced Ang II-induced increases in

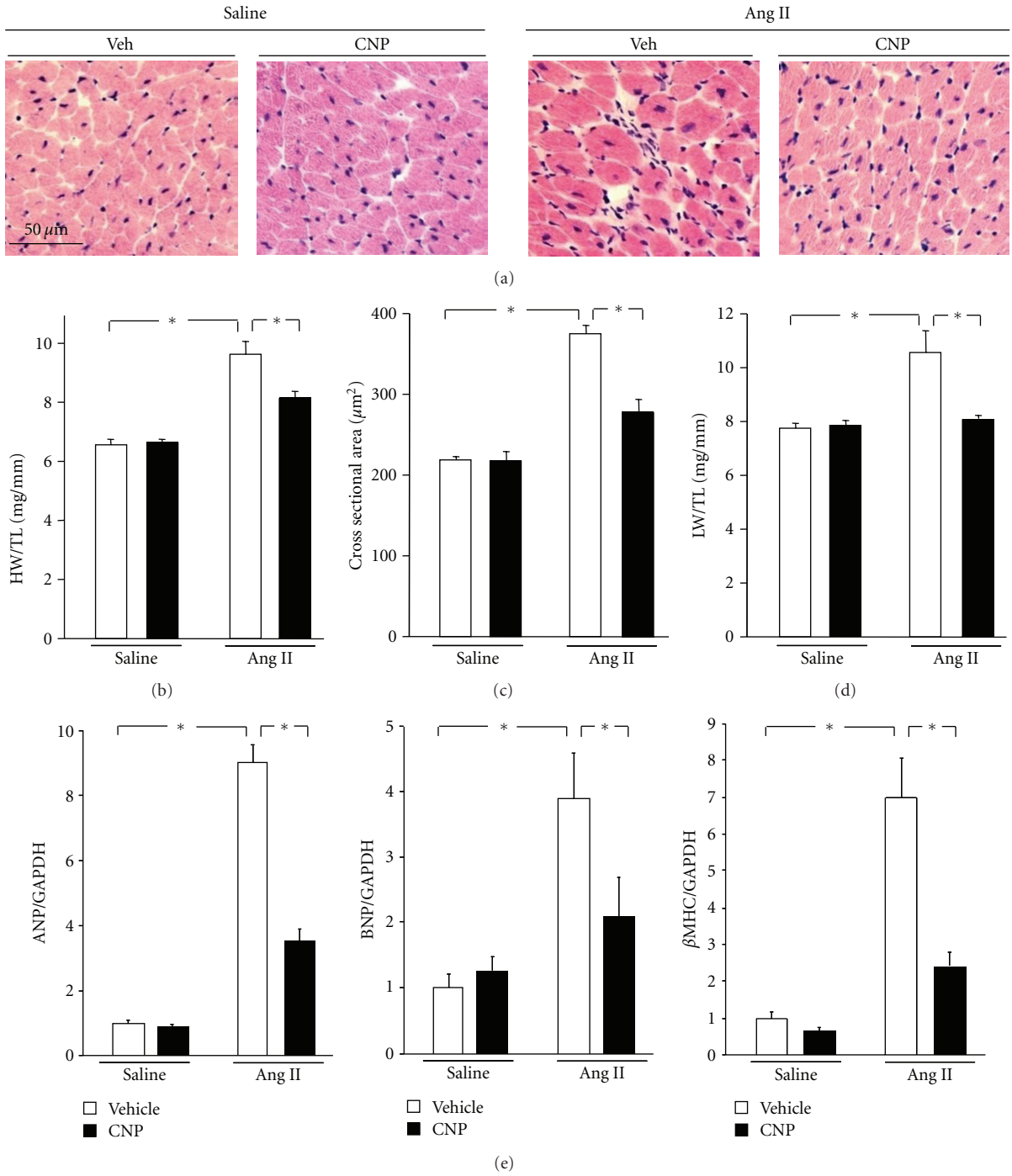

Figure 2: Effects of CNP on Ang II-induced cardiomyocyte hypertrophy. (a) Representative images of hematoxylin-eosin- (H&E-) stained heart sections. Scale bars: 50 μm. (b) Heart weight to tibial length ratio (HW/TL) in vehicle- and CNP-treated mice 2 weeks after saline or Ang II infusion. (c) Quantitative analysis of cardiac myocyte cross-sectional area in vehicle- and CNP-treated mice 2 weeks after saline or Ang II infusion. Data were obtained from analysis of H&E-stained heart sections. (d) Lung weight to tibial length ratio (LW/TL) in vehicle- and CNP-treated mice 2 weeks after saline or Ang II infusion. (e) Atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and β-myosin heavy chain (βMHC) transcript expression in vehicle- and CNP-treated mice 2 weeks after saline or Ang II infusion. Results are mean ± SEM (n = 4–9 each). $^*P < 0.05$.

cardiomyocyte size and interstitial fibrosis, and markedly suppressed hypertrophic and fibrotic gene expression. These beneficial effects were accompanied by reduced superoxide production and NOX4 gene expression in the Ang II-infused myocardium. Together, these findings suggested that CNP exerts antioxidant activity under conditions that promote cardiac hypertrophy and fibrosis, as a result, maintaining left ventricular systolic function.

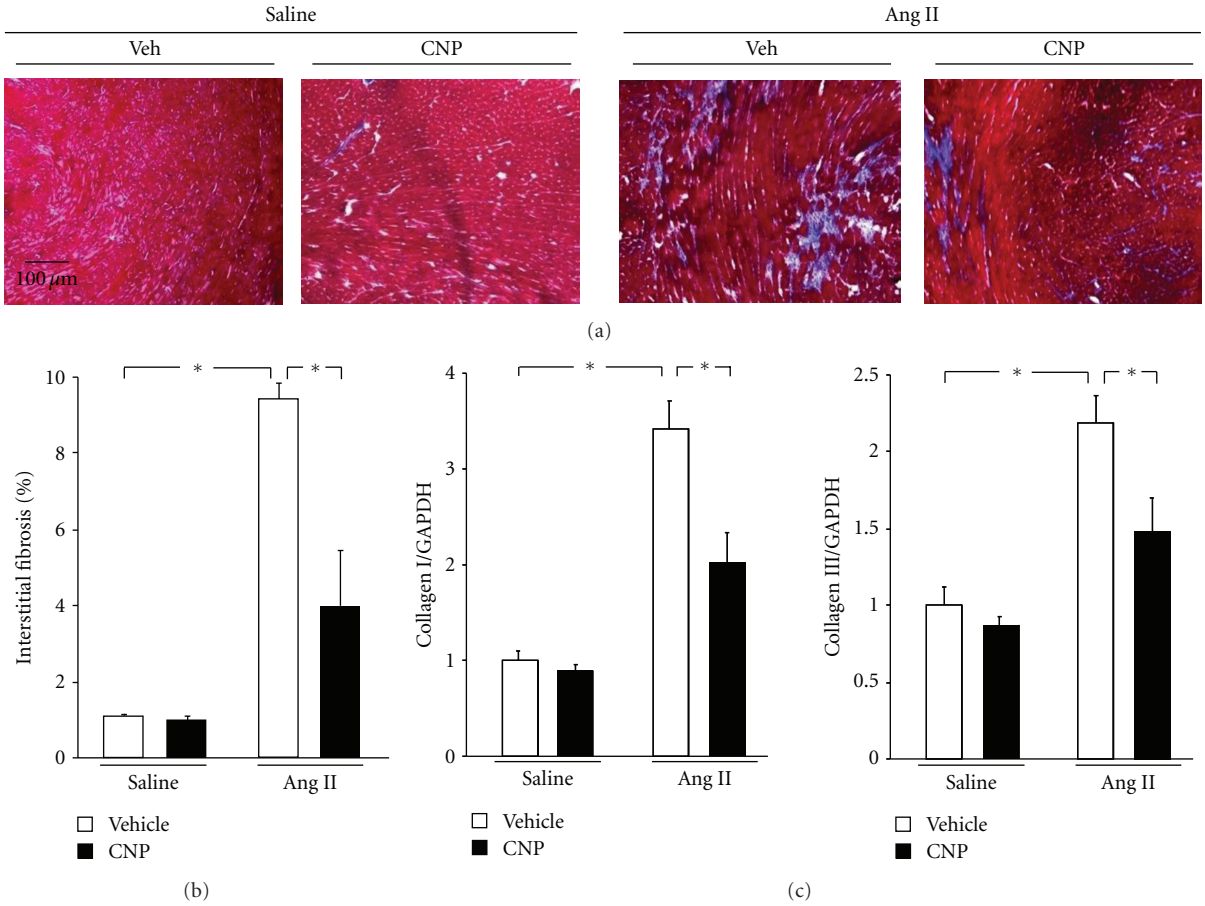

Figure 3: Effects of CNP on Ang II-induced myocardial interstitial fibrosis. (a) Representative images of Masson's trichrome (MT) staining of heart sections. Scale bars: 100 μm. (b) Quantitative analysis of myocardial interstitial fibrosis in vehicle- and CNP-treated mice 2 weeks after saline or Ang II infusion. (c) Collagen I and III transcript expression in vehicle- and CNP-treated mice 2 weeks after saline or Ang II infusion. Results are mean ± SEM (n = 4–9 each). $^*P < 0.05$.

Cardiac hypertrophy is initially an adaptive response designed to maintain cardiac function in response to hemodynamic or neurohumoral stress. However, a prolonged external load leads to decompensated cardiac hypertrophy characterized by ventricular dilation and loss of contractile function. Although the role of ANP and BNP in cardiac hypertrophy and remodeling has been investigated in several in vitro and in vivo studies, limited data are available on the role of CNP in this process [3]. We used an Ang II infusion model in this study because multiple lines of evidence established that Ang II induces not only hypertension, but also directly contributes to cardiac remodeling [15]. Contractile function following Ang II infusion to mice varies among report. For example, Essick et al. reported that contractile function was not impaired by same dose (3.2 mg/kg/day) and same duration (2 weeks) of Ang II infusion as our present study [25]. On the other hands, Fujita et al. reported that lower dose of Ang II infusion (1.2 mg/kg/day) for 2 weeks reduced contractile function [26]. The reason for the discrepancy was probably due to different reagents, equipments, or mice strain.

Ang II-induced ROS generation is recognized as a key mechanism regulating cardiovascular remodeling [24]. With regard to the cellular source of ROS, Ang II-induced upregulation of NADPH oxidase subunits has been documented in various cardiovascular cell types including cardiomyocytes, fibroblasts, and vascular smooth muscle cells (VSMC) [27–29]. One of these subunits, NOX4, was implicated in Ang II infusion and/or pressure overload-induced myocardial hypertrophy [30–32]. This study revealed that NOX4 gene upregulation was dramatically suppressed by CNP treatment. It has been demonstrated that CNP prevents agonist-induced cardiomyocyte hypertrophy via a cyclic GMP-dependent protein kinase-mediated mechanism in vitro [7, 13]. Thus, we speculate that the antihypertrophic effect of chronic CNP infusion observed in this study was mediated by cyclic GMP-dependent manner and inhibition of NOX4 production.

Another natriuretic peptide, ANP, can suppress ROS production in the cardiovascular system. For example, the antihypertrophic actions of ANP in vitro were accompanied by reduced levels of superoxide, mediated via the NPR-A/cGMP pathway [19]. We previously demonstrated that intravenous infusion of carperitide, the human form of ANP, significantly decreased serum ROS levels in patients with chronic heart failure and exogenous ANP exerted direct

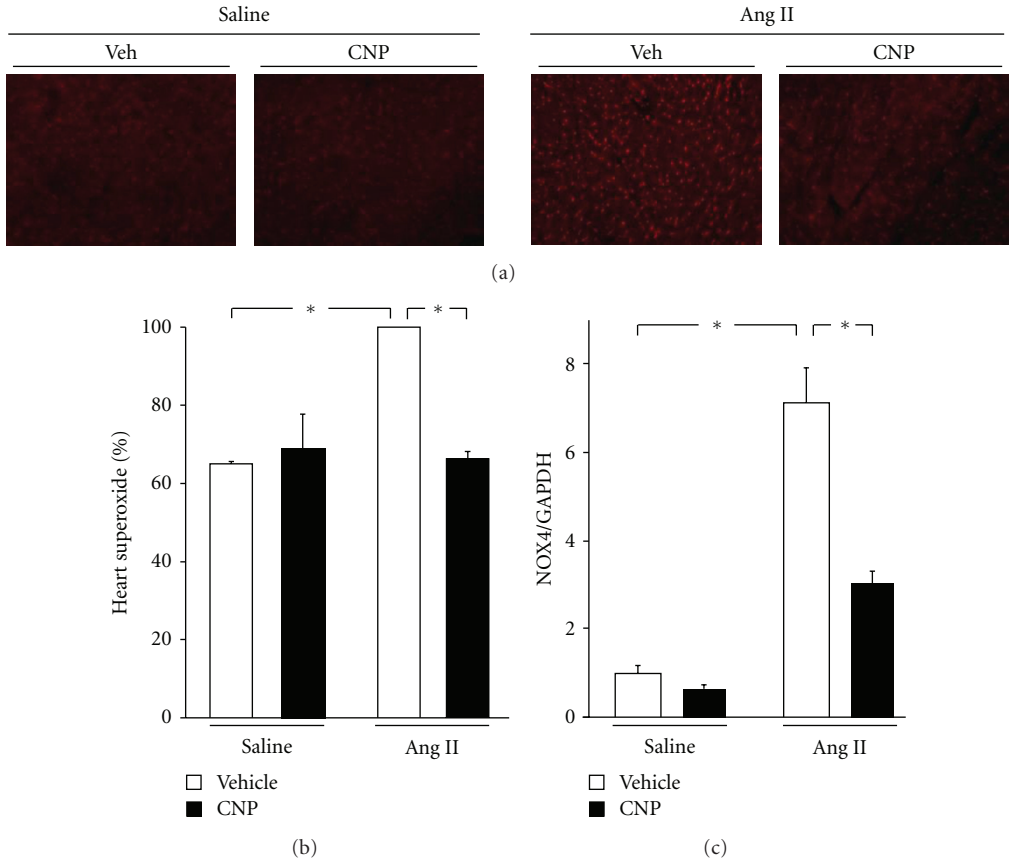

Figure 4: Effects of CNP on Ang II-induced ROS production. (a) Representative photomicrograph of myocardium stained with DHE. (b) DHE fluorescence was quantified to evaluate superoxide levels in situ and expressed relative to values obtained for vehicle-treated with Ang II infusion group (as 100%). (c) NOX4 transcript expression in vehicle- and CNP-treated mice 2 weeks after saline or Ang II infusion. Results are mean ± SEM ($n = 4$–9 each). $^*P < 0.05$.

antioxidant properties in cardiomyocytes in vitro [33]. In contrast to ANP and BNP, CNP binds to and activates its specific receptor, NPR-B [3]. Both NPR-A and NPR-B activate cGMP-dependent signaling upon binding of their specific ligands, suggesting that the antioxidative properties of CNP were mediated via the NPR-B/cGMP pathway. In addition, all natriuretic peptides bind to NPR-C. Recent reports indicated that stimulation of NPR-C decreases NADPH oxidase activity and production of superoxide by attenuating the expressions of NOX4 and $p47^{phox}$ subunits of NADPH oxidase in VSMC from spontaneous hypertensive rats [34]. We speculate that CNP inhibit the NOX4 expression by same mechanism in the present experiments.

The synthetic reagents for ANP and BNP are currently used in a clinical setting with reported antiremodeling effects [35]. However, due to their prompt vasodilatation and natriuretic actions, these agents cannot be used in patients with severe hypotension as sometimes seen in acute heart failure. Compared to ANP and BNP, CNP exerts lower hypotensive and natriuretic effects, and more potent antihypertrophic and antifibrotic properties [36]. These functional differences accounted for the relative abundance of NPR-B over NPR-A in cardiovascular tissue [6, 7]. The reduced hypotensive action of CNP also makes it usable in hemodynamically unstable patients. Furthermore, these hemodynamic and antiremodeling aspects are ideal for long-term administration, because cardiac remodeling is generally developed as a result of long-term external stimulus. In this study, chronic CNP infusion had little effect on SBP; however, Ang II-induced cardiac remodeling was markedly prevented. Our results thus support the notion that long-term CNP administration is potentially useful in anti-remodeling strategies for chronic heart diseases [10].

In conclusion, our data indicated that CNP protects against the development of Ang II-induced cardiac remodeling which was accompanied by reduced cardiac superoxide production. This novel mechanism of action makes CNP a potential additional anti-remodeling agent.

Acknowledgments

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Review Article

Anti-Inflammatory Effects of Epoxyeicosatrienoic Acids

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Epoxyeicosatrienoic acids (EETs) are generated by the activity of both selective and also more general cytochrome p450 (CYP) enzymes on arachidonic acid and inactivated largely by soluble epoxide hydrolase (sEH), which converts them to their corresponding dihydroxyeicosatrienoic acids (DHETs). EETs have been shown to have a diverse range of effects on the vasculature including relaxation of vascular tone, cellular proliferation, and angiogenesis as well as the migration of smooth muscle cells. This paper will highlight the growing evidence that EETs also mediate a number of anti-inflammatory effects in the cardiovascular system. In particular, numerous studies have demonstrated that potentiation of EET activity using different methods can inhibit inflammatory gene expression and signalling pathways in endothelial cells and monocytes and in models of cardiovascular diseases. The mechanisms by which EETs mediate their effects are largely unknown but may include direct binding to peroxisome proliferator-activated receptors (PPARs), G-protein coupled receptors (GPCRs), or transient receptor potential (TRP) channels, which initiate anti-inflammatory signalling cascades.

1. Introduction

Cardiovascular diseases such as atherosclerosis have a strong inflammatory component. Inflammation in the vascular wall may be initiated by endothelial dysfunction and the accumulation of toxic oxidised circulating lipids [1]. Inflammatory mediators such as TNFα and IL-1β secreted, which induces the upregulation of cell adhesion molecules, facilitates leukocyte recruitment in to the vascular wall [2, 3] and stimulates vascular smooth muscle cell migration and proliferation [4]. Circulating monocytes not only respond to inflammatory stimuli by producing large amounts of inflammatory mediators but they are also crucial for effective activation of lymphocytes and adaptive immunity. The hallmark of advanced unstable atherosclerotic lesions is that they are monocyte/macrophage rich and highly inflammatory.

Inflammatory responses are normally promptly terminated since excessive or prolonged inflammation can lead to chronic pathological conditions such as cardiovascular diseases, Crohn's disease, rheumatoid arthritis, or cancer. Although there have been many new treatments recently developed to combat inflammatory diseases, some of these treatments are either very expensive and/or not effective in subsets of patients. Therefore, it is important to continue to investigate mechanisms that regulate inflammatory responses as they may open up novel therapeutic targets. There is a growing list of evidence that the epoxygenase pathway of arachidonic acid metabolism, which generates epoxyeicosatrienoic acids (EETs), exerts anti-inflammatory effects that may be harnessed to treat disease. This paper will summarise that evidence and highlight outstanding questions that remain to be answered.

2. Overview of the Epoxygenase Pathway of Arachidonic Acid Metabolism

Arachidonic acid is an omega-6 polyunsaturated long chain fatty acid that contains 20 carbon atoms and four *cis*-double bonds and possesses a carboxyl group and a methyl group at respective ends of the molecule. The double bonds are located between carbons 5-6, 8-9, 11-12, and 14-15 relative to the carboxyl group. Therefore, its chemical name is *all-cis*-5,8,11,14-eicosatetraenoic acid and its lipid name

is 20 : 4 (n-6). The *cis*-configuration of the four double bonds causes the arachidonic acid backbone to significantly bend. In contrast, double bonds in the *trans*-configuration or saturated arachidonic acid result in structurally unbent or flexible backbones.

Experiments performed more than 30 years ago showed that incubations of radio-labelled arachidonic acid with microsomal preparations derived from a variety of tissues including liver [5, 6], kidney [7], hypothalamus [8], and anterior pituitary [9] resulted in the formation of EETs. This "epoxygenase" reaction required cytochrome p450 (CYP) enzymes and utilised NADPH and oxygen in a 1 : 1 stoichiometric ratio [5]. One atom of molecular oxygen is incorporated into one of the four double bonds of arachidonic acid retaining the *cis*-geometry and yielding four potential EETs, that is, 5,6-EET, 8,9-EET, 11,12-EET, or 14,15-EET, respectively. Furthermore, each EET can be present in either the S/R or R/S stereoconfiguration, thus eight potential EETs can be formed.

2.1. Epoxygenation of Arachidonic Acid Performed by Specific CYPs. CYP enzymes catalyze the oxidation of organic substances, as well as xenobiotics. Altogether, 57 putative CYP genes have been identified in man (by comparison mice have 103 and rat 89 CYP, resp.) that are divided into 15 subfamilies [10]. Attempts have been made to classify human CYP genes by substrate; however, a more systematic nomenclature is generally used since the true physiological roles of many of these genes are still unknown [11]. To date, at least 12 human CYP genes have been reported to possess epoxygenase activity, although most studies have been focussed on the CYP2C and CYP2J families, which are considered the major epoxygenase enzymes.

2.2. CYP2C. One of the earliest studies using recombinant human CYP compared the metabolic profiles of the CYP2C8 and CYP2C9 enzymes [12], which are 77% identical at the amino acid level. Despite their high similarity, CYP2C8 and CYP2C9 exhibit both regio- and stereoselective differences in their epoxygenation of arachidonic acid. For instance, CYP2C8 produced only 14,15-EET and 11,12-EET at a 1.25 : 1 ratio, which represented 68% of the total metabolites measured. By contrast, CYP2C9 produced 14,15-EET 11,12-EET and 8,9-EET at a ratio of 2.3 : 1 : 0.5, which represented 69% of total metabolites. Furthermore, with respect to stereoselectivity, CYP2C8 was 81% selective for the 11(R),12(S)-EET configuration, whereas CYP2C9 was 70% selective for the 11(S),12(R)-EET configuration [12]. These CYP enzymes also carry out other reactions including allylic hydroxylation on arachidonic acid and other fatty acids.

2.3. CYP2J2. Epoxygenase activity of human CYP2J2 was first demonstrated by the Zeldin lab, who initially cloned and characterised the gene [13]. Recombinant CYP2J2 metabolised arachidonic acid to all four potential epoxygenase products, with 14,15-EET being the predominant metabolite formed. CYP2J2 was found to be highly expressed in the heart, and EETs were produced in similar proportions as recombinant CYP2J2 suggesting that CYP2J2 played a major role in EET generation in the heart *in vivo* [13]. CYP2J2 expression is also seen in kidney, liver and muscle tissues [13], and, to a lesser extent, in the gut [14].

2.4. Other CYPs. A comprehensive comparison study by Rifkind and colleagues examined the epoxygenase activity of a panel of 10 CYP proteins by overexpressing them in HepG2 cells and measuring metabolic products. CYP 2C8, 2C9, 1A2, and 2E1 principally produced epoxygenase products. By contrast, CYP2D6 was inactive, while CYPs 2A6, 3A3, 3A4, and 3A5 had minimal epoxygenase activity [15]. CYP3A4 has also been shown to make the epoxygenase products 8,9-EET, 11,12-EET, and 14,15-EET, respectively, in several breast cancer cell lines [16]. Other CYPs that have been shown to possess epoxygenase activity include CYP1A, CYP2B1 and CYP2B2 [17] and CYP2B12 [18], CYP2C8, CYP2C9, CYP2D18 [19], CYP2N1 and CYP2N2 [20], and rat CYP4A2 and 4A3 [21]. The full extent of the epoxygenase activity of these enzymes and the physiological consequences of any activity is, however, poorly understood.

3. Soluble Epoxide Hydrolase

Once formed, EETs are unstable because they are rapidly metabolised. The main catabolic pathway is the conversion of EETs into dihydroxyeicosatrienoic acid (DHETs), catalysed by soluble epoxide hydrolase (sEH) [22]. DHETs are generally considered to be less active; however, they have been shown to exert vasodilatory effects on coronary arteries [23]. DHETS are far more polar than their corresponding EETs and quickly diffuse out of tissues as the 1, diols or conjugates of them. Other pathways of EET metabolism include chain elongation, β-oxidation, and ω-oxidation [24]. 5,6 EET and 8,9 EET are substrates for COX enzymes [25] and can also be incorporated into membrane phospholipids. DHETs have a lower binding affinity for phospholipids which may account for its relatively increased plasma levels [26].

Recently, the damaging cardiovascular risk factor homocysteine has been shown to upregulate sEH in endothelial cells and promote a proinflammatory environment [27]. In contrast, elevating the levels of endogenous CYP products by removing (sEH knockout mice) or inhibiting soluble epoxide hydrolase (sEH-1) has been shown to reduce neointima formation [28], atherosclerosis and abdominal aortic aneurysm development, dyslipidaemia in hyperlipidaemic mice [29], and reduce hypertension [30] and diabetes [31] in different mouse models. A number of sEH inhibitors have now been developed and are moving towards clinical trials for a variety of disorders related to cardiovascular disease.

4. Epoxygenases in Vascular and Inflammatory Cells

CYP2C mediated generation of 11,12 EET has also been documented in porcine coronary arteries [32], and CYP2C enzymes have been found expressed in endothelial cells [33],

and in primary human monocytes and M1 (CYP2C8) and M2 macrophages (CYP2C8 and CYP2C9) [34].

CYP2J2 immunoreactivity is seen in the endothelial and smooth muscle cell layers of human coronary arteries [35], as well as in the human monocytic cell lines THP-1 and U937, primary monocytes and M1 and M2 macrophages [34], and the endothelial cell line EA hy.926s [36]. Interestingly, neither CYP2J2 nor CYP2C8 mRNA expression was detected in human polymorphonuclear cells [34]. More recently, the increased risk of coronary artery disease was shown to be associated with a polymorphism in the promoter of *CYP2J2* gene in some populations, which decreases the expression of the enzyme [37].

5. Epoxygenases and EETs Suppress Inflammation

EETs have been shown to exert multiple biological effects on the vasculature including proproliferation and angiogenic effects [38]. EETs have also been hypothesized as endothelium-derived hyperpolarizing factors, as they hyperpolarize and relax vascular smooth muscle cells by activating calcium-activated potassium channels [32]. However, a number of the anti-inflammatory activities of EETs on inflammatory cells, as discussed below, appear independent of any cellular hyperpolarisation [35].

5.1. Endothelial Cells. Overexpression of CYP2J2 in human and bovine endothelial cells inhibits TNFα-induced VCAM-1 [39] and VCAM-1 promoter activity in reporter assays [35]. Treatment with the epoxygenase inhibitor SKF525A reversed the effects of CYP2J2 overexpression on VCAM-1 promoter activity [35]. Exogenous EETs also exert the same effects as CYP2J2 overexpression, although different EETs can have different selectivities. In human endothelial cells, 11,12-EET significantly inhibited VCAM-1 expression in response to TNFα, IL-1α, or LPS. By contrast, 14,15-EET had negligible effect, while 5,6-EET, 8,9-EET, and 11,12-DHET all inhibited to varying degrees but to a lesser extent than 11,12-EET. 11,12-EET also inhibited TNFα-induced E-selectin and ICAM-1 expression [35]. Mice engineered to overexpress the human epoxygenase genes *CYP2J2* or *CYP2C8,* respectively, were generated to investigate their roles in endothelial cells. Primary pulmonary endothelial cells derived from these mice showed reduced levels of LPS-induced adhesion molecule and chemokine gene expression. Furthermore, these anti-inflammatory effects were inhibited by treatment with the epoxygenase inhibitor MS-PPOH and a putative EET receptor antagonist 14,15-EEZE [40].

5.2. Monocytic Cells. Similar to endothelial cells, EET activity has also been shown to antagonise inflammatory signals in monocytic cells. Phorbol ester treatment of THP-1 led to a 4-fold increase in CYP2J2 expression between 3–7 days after stimulation, suggesting that endogenous expression of CYP2J2 may regulate inflammatory responses in these cells [36]. Addition of 8,9-EET or 11,12-EET inhibited basal TNF secretion from THP1 cells by about 90% and 40%, respectively [34]. Similarly, the epoxygenase inhibitor SKF525A led to a concentration-dependent superinduction of LPS-induced PGE_2 in rat monocytes and COX-2 in mouse and human monocytes [41]. Consistent with these findings, exogenous 11,12-EET dose dependently inhibited LPS-induced PGE_2 and attenuated SKF-mediated superinduction. 11,12-EET also inhibited LPS-induced COX-2 activity and expression [41]. EETs can, therefore, both compete with arachidonic acid for the binding site in COX enzymes as well as inhibit the inflammation induced induction of COX-2 expression. A study found that EETs were detected in human peritoneal macrophages under basal conditions, but not following zymosan treatment, which caused a shift to prostaglandin synthesis [42].

5.3. Leukocyte Endothelial Cell Interactions. Several studies have demonstrated that EETs can regulate functional interaction between leukocytes and endothelial cells. Treatment of endothelial cells with 14,15-EET significantly enhanced attachment of the monocytic cell line U937 [43]. Pretreatment of endothelial cells with EETs alone or in combination with PMA had negligible effects on adherence of PMNs. However, cotreatment of EETs and PMA led to a concentration-dependent decrease in adherence of PMNs when cocultured with endothelial cells [44]. 11,12-EET, but not 14,15-EET, was shown to inhibit adherence of monocytic cells in an *ex vivo* model. Mice were treated with TNFα alone or in combination with either 11,12-EET or 14,15-EET, and carotid arteries were removed and incubated with U937 cells. The level of inhibition of adherent cells was comparable to that of treatment with a blocking VCAM-1 antibody [35]. PBMCs derived from mice systemically overexpressing human CYP2J2 via *in vivo* gene delivery were significantly less adherent to TNFα-treated HUVECs compared to control PBMCs [39].

5.4. In Vivo Models. There have been conflicting reports on the effects of EETs in acute models of inflammation *in vivo*. Rats injected with TNFα showed elevated plasma levels of adhesion molecules and inflammatory cytokines, and decreased levels of the anti-inflammatory mediator IL-10. However, these effects were significantly reduced by systemic overexpression of human CYP2J2 [39], suggesting that EETs act as anti-inflammatory mediators. Similarly, TNFα-treated human bronchi also showed reduced inflammation when treated with 14,15-EET [45]. LPS responses of wild-type mice have also been compared to sEH$-/-$ null mice or mice that had endothelial-specific overexpression of the human CYP2J2 or CYP2C8. All three genetically modified mice had reduced levels of inflammatory gene expression and neutrophil recruitment in the lung following LPS injection. Moreover, these effects correlated with decreased activation of the key transcription factor NF-κB [40]. By contrast, another study found that to sEH$-/-$ null mice were not protected from LPS-induced inflammatory gene expression or neutrophil recruitment in the liver, and that treatment with the sEH inhibitor AUDA also had minimal effect liver inflammation, despite higher levels of endogenous EETs [46].

This suggests that either the effects of EETs are organ specific, or that liver is more susceptible to endotoxin shock.

6. Mechanisms of EET Action

6.1. NF-κB Inhibition. The mechanisms by which EETs mediate their anti-inflammatory effects remain ill-defined, but there are several reports that they can inhibit activation of NF-κB, a key transcription factor for inflammatory gene induction. In mammals NF-κB comprises five subunits, with the RelA (p65) subunit being expressed in most cell types. Under basal conditions, NF-κB dimers are localised in the cytoplasm due to interactions with IκB (inhibitor of NF-κB) proteins. Signalling cascades induced by inflammatory descend on the IKK (inhibitor of NF-κB kinase) complex, which phosphorylates IκB. This tags IκB for subsequent ubiquitination and degradation by the proteosome, which, in turn, facilitates NF-κB nuclear translocation where it binds to its cognate binding elements to activate transcription [47, 48].

11,12-EET inhibits NF-κB reporter activity in both HEK293 cells [34] and human endothelial cells [35] following stimulation. Furthermore, 11,12-EET also inhibited TNFα-induced RelA nuclear translocation, IκBα degradation, and IKKα activity, respectively [35], indicating that EET-mediated inhibition of NF-κB occurs upstream of IKK. Interestingly, 14,15-EET was also shown to inhibit the TNFα-induced degradation of IκBα in primary human lung tissue [45] but had no effect on NF-κB reporter activity in HEK293s, suggesting that 14,15-EET may act in a cell type-specific manner. Similarly, 8,9-EET and 11,12-EET inhibited NF-κB reporter gene activity in HEK293 cells [34]. In contrast to CYP2J2, CYP2C9 increased NF-κB activity in human vascular endothelium via superoxide generation, potentially giving this CYP a proinflammatory profile [49].

6.2. STAT3. EETs can also activate STAT3 in human breast cancer cell lines, with 14,15-EET promoting STAT3 tyrosine-705 phosphorylation and nuclear translocation [16]. Activation of STAT3 was shown to be dependent on cell proliferation, which led the authors to conclude that 14,15-EET may be involved in an autocrine/paracrine pathway driving cell growth. Interestingly, the anti-inflammatory effects of IL-10 in macrophages are also dependent on STAT3 tyrosine-705 phosphorylation [50]. Taken together, these results suggest that the anti-inflammatory effects of EETs may be mediated by activation of STAT3, in addition to the inhibition of NF-κB.

6.3. EETs as PPAR Agonists. PPARs are a subfamily of the nuclear receptor superfamily that comprises three ligand-activated transcription factors: PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3). Upon ligand binding, they form heterodimers with the retinoid X receptor and bind to specific response elements in gene promoters to upregulate gene transcription [51]. PPARs have been shown to regulate diverse physiological processes such as fatty acid and glucose metabolism, angiogenesis, and cellular proliferation and differentiation, in addition to inflammation. PPAR ligands include a variety of fatty acids, and there has been recent evidence that metabolites of the epoxygenase pathway can activate PPAR receptors.

The omega-alcohol of 14,15-EET, 20,14,15-HEET, or a 1 : 4 mixture of the omega-alcohols of 8,9- and 11,12-EETs activated human and mouse PPARα in transient transfection assays, suggesting a role for them as endogenous ligands for these orphan nuclear receptors [52]. Overexpression of human CYP2J2 in HEK293 cells resulted in a synergistic activation of PPARα, -β/δ and, -γ reporter gene activity. 8,9-EET and 11,12-EET, but not 14,15-EET, (in contrast to its hydroxy metabolite 20,14,15-HEET) were able to induce PPARα reporter activity [53]. Furthermore, IL-1β-induced NF-κB reporter activity and COX-2 mRNA induction in HEK293 cells was significantly inhibited cells expressing of CYP2J2 and PPARα.

Competition and direct binding assays subsequently revealed that EETs bind to the ligand-binding domain of PPARγ with K(d) in the μM range. In the presence of the sEH inhibitor AUDA, EETs increased PPARγ transcription activity in endothelial cells and 3T3-L1 preadipocytes. In endothelial cells, AUDA enhanced, but overexpression of sEH reduced laminar flow-induced PPARγ activity, EET generation, and the inhibition of VCAM-1 expression [54]. PPARs, therefore, represent a viable receptor target for the anti-inflammatory effects of EETs. However, it should be noted that AUDA may exert multiple effects in addition to sEH inhibition. It has been shown to act both as a PPAR agonist [55] and a EET mimetic [56]; therefore, results using AUDA should be cautiously interpreted.

6.4. GPCRs. For some time it has been suggested that EETs might mediate many of their effects via binding to a putative G-protein coupled receptor(s) (GPCRs). For example, 11,12-EET produced a 0.5- to 10-fold increase in the activity of the KCa channels in smooth muscle cells derived from bovine coronary arteries, which was dependent on the presence of GTP [57]. Furthermore, blocking antibodies against GSα, but not Gβγ or anti-Giα, were able to inhibit the activation induced by 11,12-EET [57]. Using radio-ligand binding, 14,15-EET has been shown to have a high affinity for a receptor expressed on guinea pig-derived mononuclear cells, which was purported to be a G-protein coupled receptor that stimulated cAMP production [58]. This putative GPCR-cAMP pathway remains elusive but may represent a novel anti-inflammatory pathway by which EETs act.

6.5. TRPV1 and EETs. TRPV4 is a cation channel of the "transient receptor potential" (TRP) family that functions as a Ca^{2+} entry channel, that is expressed in smooth muscle cells, endothelial cells, as well as in perivascular nerves. CYP-dependent generation of 5,6-EET can activate TRPV4 in murine endothelial cells and is a possible contributing mechanism to the hyperpolarising effects of EETs [59]. Additionally, 11,12-EET can activate TRPV4 channels in smooth muscle cells from rat cerebral arteries [60], and 5,6-EET and 8,9-EET can activate TRPV4 in human endothelial

cells [61]. Although activated by EETs, there is little evidence that activation of TRPV4 is anti-inflammatory, though it does lead to vasodilation via nitric oxide, prostacyclin, and intermediate/small conductance K+ channel-dependent pathways, and in vascular smooth muscle, large conductance K+ channel activation, and hyperpolarization [62].

7. Summary and Outlook

More than 100 metabolites derived from arachidonic acid have been described, with the best characterised coming from the COX and LOX pathways which generate prostanoids and leukotrienes, respectively [63]. Knowledge of these pathways has led to several important therapeutic breakthroughs such as COX inhibitors which are used to treat pain and inflammation and leukotriene antagonists that have been used to treat asthma. By contrast, much less is known about the epoxygenase pathway of arachidonic acid metabolism, although as outlined in this paper, EETs can exert a number of cardio-protective anti-inflammatory effects on vascular cells such as endothelial cells and monocytes. These include inhibition of proinflammatory mediators and cell adhesion molecules. Indeed, a recent study has measured epoxygenase products in atherosclerotic patients [64]. Compared to healthy volunteers, both obese and nonobese CAD patients had significantly higher plasma EETs [64], suggesting that this is a compensation mechanism to protect against ongoing vascular inflammation.

Although elevating epoxygenase products via sEH inhibition have been shown to be beneficial in a wide variety of animal models of cardiovascular disease, the mechanisms through which these effects are mediated are still largely unknown, although NF-κB and STAT3 have both been implicated. However, several fundamental question regarding the role of EETs in vascular inflammation remain unanswered. Firstly, it is clear that CYP epoxygenases can act on substrates other than arachidonic acid, such as cardioprotective fish oils. Eicosapentaenoic acid for example is an omega-3 long chain fatty acid that differs from arachidonic acid by the addition of one extra double bond at the 17-18 carbon position. Epoxygenation of eicosapentaenoic acid by CYP enzymes generates 17,18-epoxyeicosatrienoic acid, which has a hyperpolarising effect on bronchial smooth muscle cells *in vitro* and *in vivo* [65]. Similarly, linoleic acid, which is the major dietary fat, can be epoxygenated by CYP enzymes resulting in potent metabolites which are probably proinflammatory in nature. However, little is known regarding the function of many of these alternative "epoxygenase" metabolites have on the cardiovascular system during inflammation. Secondly, the full range of epoxygenase activity by CYP enzymes in healthy and diseased physiological settings is still not completely understood and remains a significant barrier to progress in the field. Thirdly, and probably most importantly, definitive identification of a specific receptor that mediates the activities of EETs is essential to fully understand the epoxygenase pathway, and will help to elucidate new therapies for cardiovascular diseases in the future.

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Research Article

A Model of Left Ventricular Dysfunction Complicated by CAWS Arteritis in DBA/2 Mice

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It was reported previously that a *Candida albicans* water-soluble fraction (CAWS), including a mannoprotein and β-glucan complex, has strong potency in inducing fatal necrotizing arteritis in DBA/2 mice. In this study, histopathological changes and cardiac function were investigated in this system. One mg/day of CAWS was given to DBA/2 mice via peritoneal injection for five days. The CAWS-treated DBA/2 mice were induced aortitis and died at an incidence of 100% within several weeks. Histological findings included stenosis in the left ventricular outflow tract (LVOT) and severe inflammatory changes of the aortic valve with fibrinoid necrosis. Cardiomegaly was observed and heart weight increased 1.62 fold ($P < 0.01$). Echocardiography revealed a severe reduction in contractility and dilatation of the cavity in the left ventricle (LV): LV fractional shortening (LVFS) decreased from 71% to 38% ($P < 0.01$), and the LV end-diastolic diameter (LVDd) increased from 2.21 mm to 3.26 mm ($P < 0.01$). The titer of BNP mRNA increased in the CAWS-treated group. Severe inflammatory changes resulting from CAWS brought about lethal LV dysfunction by aortic valve deformation with LVOT stenosis. This system is proposed as an easy and useful experimental model of heart failure because CAWS arteritis can be induced by CAWS injection alone.

1. Introduction

Cardiovascular disease is the major cause of death and one of the largest burdens on healthcare resources worldwide. Clinical guidelines for the treatment of cardiovascular disorders have been developed by the analysis of high quality Evidence-Based Medicine (EBM) methodology. However, it is considered that the existence of a superior animal model is indispensable for the development of new therapeutic agents and methods.

It was reported previously that pathogen-associated molecular patterns (PAMPs) [1] in the *Candida albicans* water-soluble fraction (CAWS) have a strong induction-potency for murine vasculitis and show acute lethal toxicity in certain mouse strains through various biological activities [2–5]. CAWS includes water-soluble mannoprotein and β-glucan complex obtained from the culture fluid of *C. albicans* strain NBRC 1385 [6]. On the other hand, CADS, which comprises *C. albicans* cell extract and originates from the stool sample of a Kawasaki disease patient, has been reported to induce murine vasculitis similar to Kawasaki disease coronary arteritis [7, 8]. Furthermore, in an additional study on *Candida*-derived agents, CAWS induced severe vasculitis at a higher rate of incidence than CADS in many mouse strains, and there are reports on this valuable animal model system in research on systemic immune responses and inflammatory vascular disease [3, 5, 9]. In particular, CAWS caused severe necrotizing coronary arteritis and aortitis in

DBA/2 mice with an incidence of 100% after intraperitoneal administration, and all the mice died within several weeks [10, 11].

The chronic mortality of CAWS arteritis is found only with DBA/2 mice, but CAWS arteritis is not fatal in other mouse strains that are sensitive to CAWS. Among mice that are susceptible to CAWS arteritis, the reason why only DBA/2 mice die is not clearly understood.

In this report, for the purpose of establishing the validity of DBA/2 mice as a heart failure model, the influence of CAWS arteritis on cardiac hypertrophy was investigated for its effect on cardiac function using echocardiography. Furthermore, cardiac function was analyzed by measuring type B natriuretic peptide (BNP) levels, which are used a humoral heart failure marker. From the present findings, this study proposes a simple and easy animal model of left ventricular dysfunction induced by CAWS.

2. Materials and Methods

2.1. Extraction and Preparation of CAWS. CAWS was prepared in accordance with the previous report [6]. *C. albicans* strain NRBC 1385 was cultured initially in complete synthetic culture medium (C-limiting medium) in acidic conditions at pH 5.2 C-limiting medium which contains sucrose 10 g, $(NH_4)_2SO_4$ 2 g, KH_2PO_4 2 g, $CaCl_2 \cdot 2H_2O$ 0.05 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $ZnSO_4 \cdot 7H_2O$ 1 mg, $CuSO_4 \cdot 5H_2O$ 1 mg, $FeSO_4 \cdot 7H_2O$ 0.01 g, and biotin 25 mg (per liter). Culture medium was maintained without pH adjustment while aerating with five litter/min room air at 27°C in a churning incubator at 400 rpm during the culture. After two days incubation, culture medium was mixed with an equal volume of ethanol and left overnight. The ethanol insoluble fraction was collected and water-soluble compounds were isolated by added distilled water to the precipitate. The water-soluble compounds were precipitated by added ethanol again and left overnight. CAWS was obtained from the precipitate dried with acetone. Each lot of CAWS used for this study was checked for the following physicochemical and biochemical parameters: endotoxin content, reactivity with anti-*Candida* monovalent antiserum, β-glucan content, elemental analysis for carbon, hydrogen, and nitrogen content, and acute fatal activity by the previous report had the same properties as the lots used in the present study.

2.2. Maintenance of Mice, Survival, and Histopathological Observation. The handling and treatment of laboratory animals conformed to the Tokyo University of Pharmacy and Life Science laboratory animal handling code. DBA/2 male mice of four and five weeks of age (provided from Japan SLC Co., Ltd.) were treated by intraperitoneal administration of CAWS at a rate of one mg/mouse for consecutive five days. Echocardiography was performed on a test bed maintained at a moderately warm temperature. After CAWS administration, mice were sacrificed every week by intravenous injection of KCl. Their organs were collected and sliced into serial sections for pathological observation using an optical or stereoscopic microscope. The pathological changes of the heart and vascular tissues slices were assessed by staining with Hematoxylin and Eosin (HE) stain. Tissue samples for electron microscopy were collected two weeks after CAWS injection and the images of transmission electron microscope (JEOL JEM-2000EX) were provided by the technical supplier (Hanaichi Co., Ltd.).

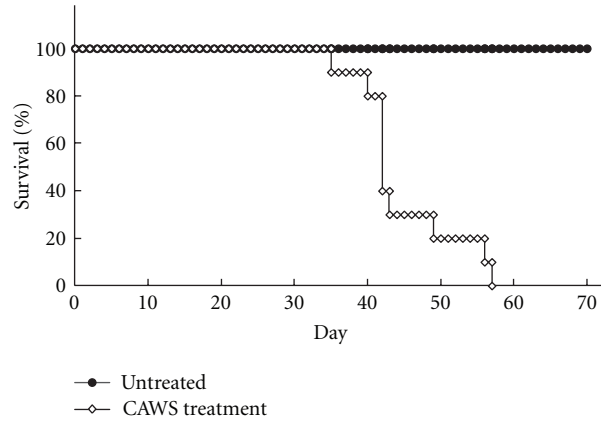

Figure 1: Kaplan-Meier analysis of CAWS arteritis in DBA/2 mice. CAWS treatment DBA/2 mice were administered with CAWS one mg/mouse via peritoneal injections for five consecutive days. DBA/2 mice at four or five weeks of age were maintained in specific pathogen free (SPF) conditions and observed every day. All CAWS treatment mice died between five and eight weeks after treatment. $P < 0.005$ for survival of untreated versus CAWS treatment mice at six weeks after the CAWS treatment; $n = 8$ (control) and $n = 10$ (CAWS treatment).

2.3. Echocardiography. A ProSound SSD-6500SV (ALOKA Co., Ltd.) linear transducer and neonatal probe were used for the determination of murine cardiac function. Mice were anesthetized using Nembutal 40 mg/kg by intraperitoneal administration, and ultrasonic permeable materials were sandwiched between a mouse and the probe to restrain the animal. Echocardiography was performed on a test bed maintained at a moderately warm temperature.

2.4. Extraction of Total RNA and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was obtained isolating a fixed protocol of chloroform extraction from myocardial tissue after homogenizing at low temperature (less than 4°C) in ISOGEN (Nippon GENE Co., Ltd.). The total RNA concentration and purity were measured using a NanoDrop ND-1000 UV Spectrophotometer (NanoDrop Technologies, Inc.). The reaction mixture was prepared from an RT-PCR Kit (Takara Bio Co., Ltd.) as follows for template RNA degeneration and the reverse transcription reaction. The PCR primers used for murine β-actin were sense 5′-GCCATGGATGACGATATCGCT-3′ and antisense 5′-TCATGAGGTAGTCTGTCAGGT-3′ (product size 574 bp), and for natriuretic peptide type B (BNP) sense 5′-ATGGATCTCCTGAAGGTGCTG-3′ and antisense 5′-GTGCTGCCTTGAGACCGAA-3′ (product size 241 bp) [12, 13]. The PCR reaction products were isolated by

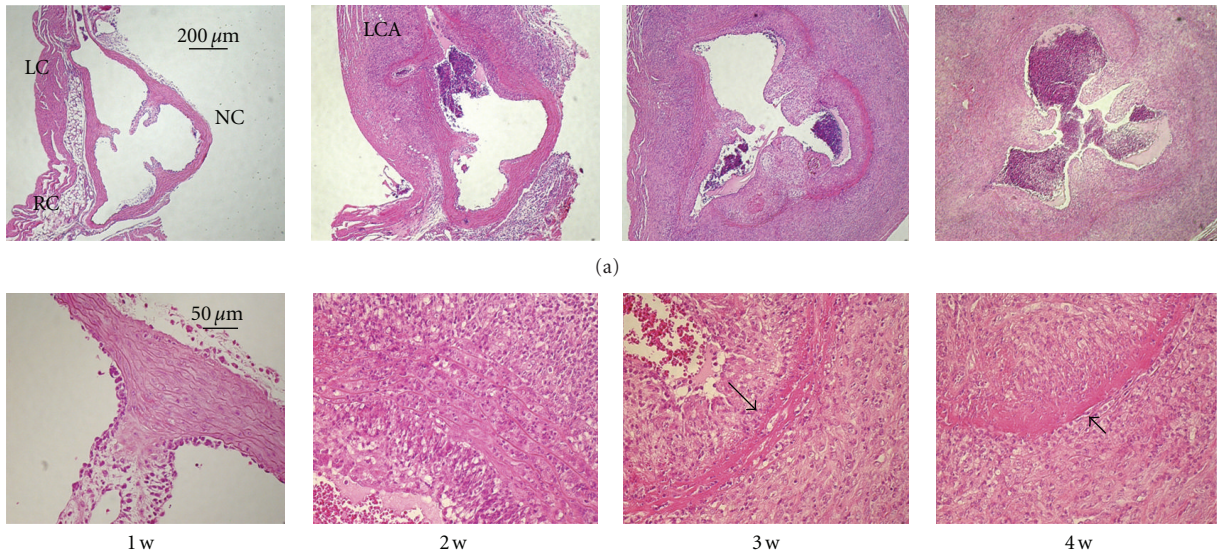

Figure 2: CAWS arteritis development and progress in DBA/2 mice. Cardiovascular tissue was sampled every week after CAWS administration, and induced aortitis was observed with an incidence of 100%. The first week panel (a, b—1w) shows slight aortic valve thickening and endocardial inflammation of the Valsalva sinuses; left coronary (LC) sinus, right coronary (RC) sinus, and noncoronary (NC) sinus. The second week panel (a, b—2w) shows expansion of the severe inflammatory lesion around the left coronary arteries (LCA). The third and fourth week panels (a, b—3w and 4w) show the features of the necrosis-related vasculitis. Inflammatory cells have spread to all the layers and sinuses with fibrinoid necrosis in the elastic lamina (arrows). Severe stenosis was found in coronary arteries because of inflammatory cells accumulation.

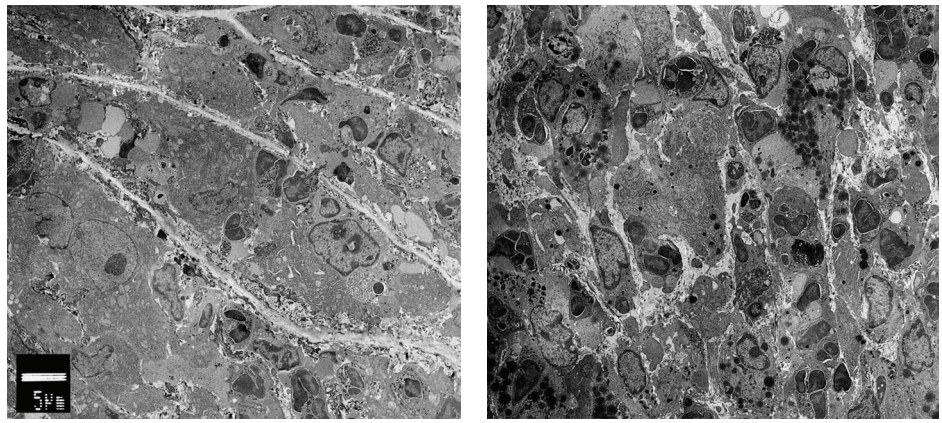

Figure 3: The tissue image of the CAWS arteritis by electron microscope. The images demonstrated direct invasion to the elastic fiber (a) and the activated inflammatory cells accumulation in the abscess-like lesion (b).

electrophoresis on a 1% agarose gel. The 574 bp band of β-actin and the 241 bp band of BNP were detected by ethidium bromide staining and UV irradiation. The band patterns were recorded digitally using a fluorescence intensimeter (IR LAS-1000, Fuji Film Co., Ltd.).

2.5. Statistical Analysis. Data are presented as mean ± SEM. The survival data were calculated by the Kaplan-Meier method and compared using Fisher's exact test between the untreated and CAWS treatment groups for the survival ratio at each time point. Parameters determined by echocardiography and of heart weight were examined using Student's t-test between untreated and CAWS treatment groups. A value of $P < 0.05$ was considered to be statistically significant. N.D. indicates no statistical difference.

3. Results

3.1. Survival of CAWS Treatment DBA/2 Mice. DBA/2 mice at four or five weeks of age were administered with CAWS at one mg/mouse via the peritoneum for five consecutive days

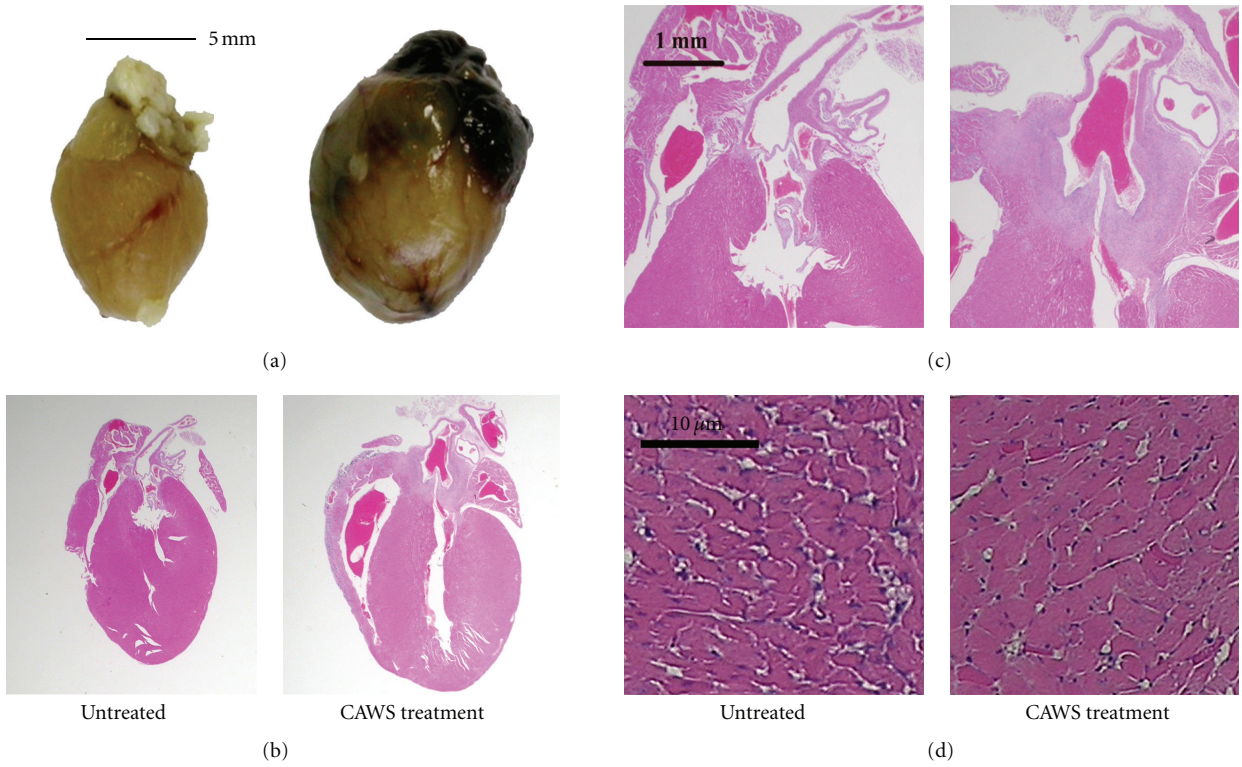

Figure 4: Cardiomegaly and histopathological changes of the aortic root due to CAWS arteritis. Macroscopic cardiomegaly and concentric left ventricular hypertrophy were observed in the CAWS treatment group (a, b). The panels (c) represent enlarged views of each aortic valve. Inflammatory cells were located mainly in the aortic valve and Valsalva sinus, and severe stenosis of the left ventricular outflow tract (LVOT) was found in CAWS arteritis. There was no evidence of necrosis or fibrosis to indicate broad infarctions in the left ventricular wall. The cross sectional area of CAWS-treated cardiomyocyte was increased by 1.14 fold (d).

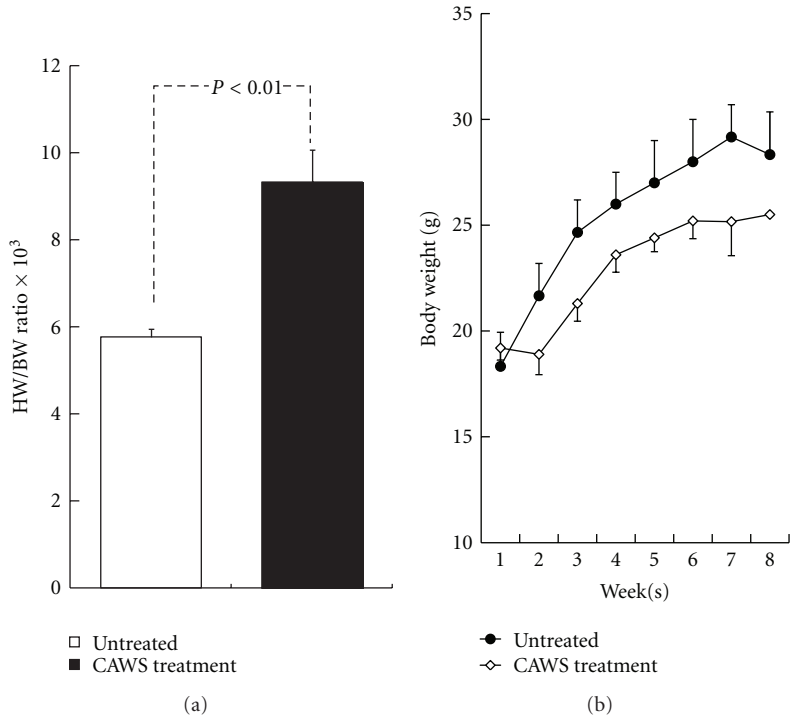

Figure 5: Heart weight and time course of body weight in CAWS arteritis. (a) The column graph represents the heart weight (HW)/body weight (BW) ratio, which increased significantly by 1.62-fold ($P < 0.01$); $n = 8$ (untreated) and $n = 7$ (CAWS treatment). (b) The time course of body weight with/without CAWS treatment DBA/2 mice.

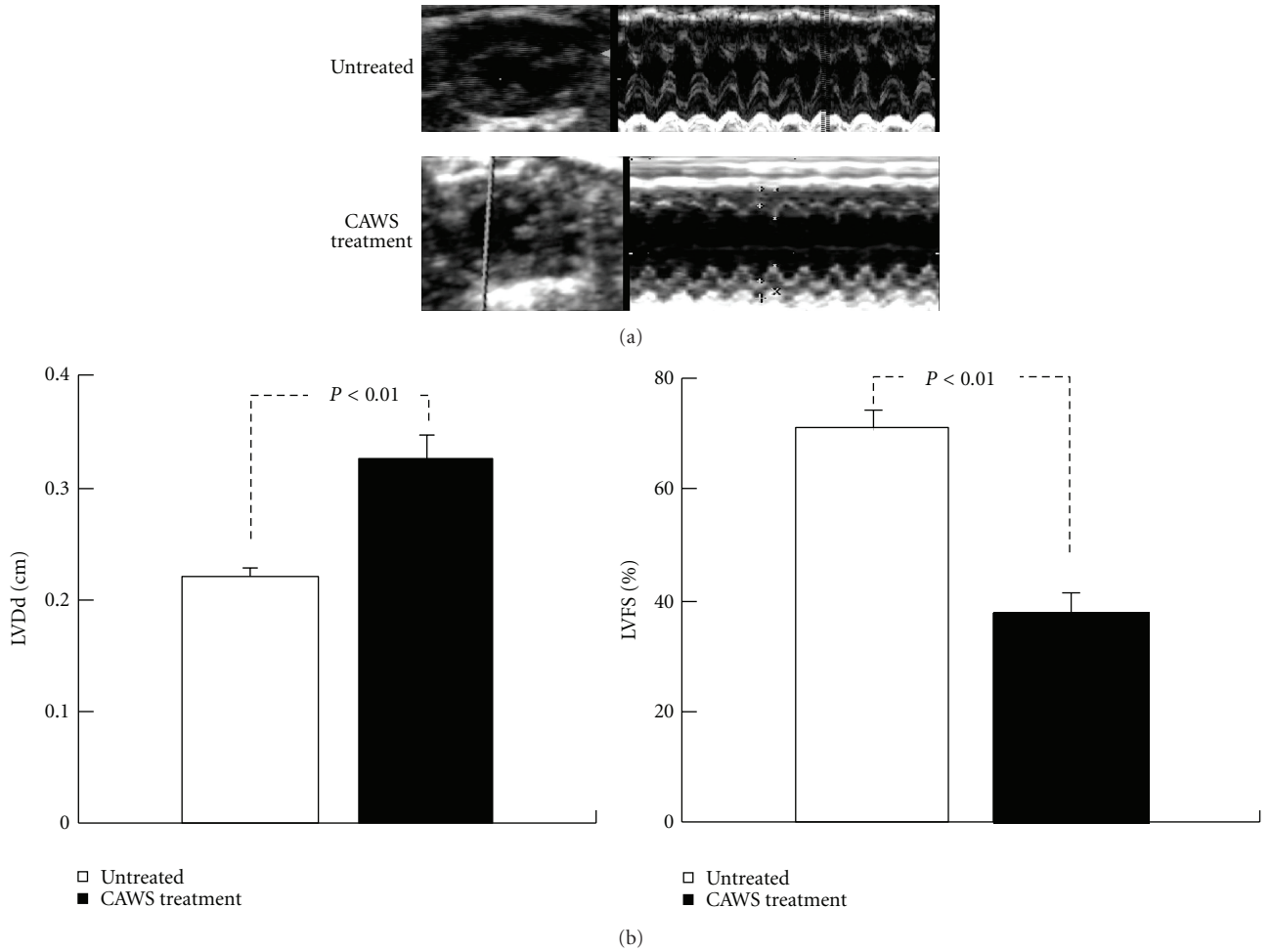

Figure 6: Comparison of the cardiac function measured by echocardiography in untreated and CAWS treatment DBA/2 mice. CAWS treatment DBA/2 mice developed left ventricular dilution and dysfunction between six and seven week after CAWS administration (a). Echocardiography revealed a diffuse, severe reduction of contractility in the left ventricle after CAWS treatment. Left ventricular fractional shortening (LVFS) decreased from 71% to 38% ($P < 0.01$), and dilation of the left ventricular diastolic dimension (LVDd) was observed from 2.21 mm to 3.26 mm ($P < 0.01$) (b); $n = 10$ (untreated) and $n = 6$ (CAWS treatment).

and maintained in specific pathogen free (SPF) conditions. All CAWS treatment DBA/2 mice died between five and eight weeks after CAWS administration (Figure 1). One mouse that died after a sudden convulsive seizure was found in the CAWS treatment group during the observation period.

3.2. Development and Progress of the CAWS Arteritis. The development and progress of CAWS arteritis took between three and four weeks. Initially, inflammatory cells invaded into the left coronary (LC) and right coronary (RC) sinus of Valsalva, and then the cells were delayed and invaded the noncoronary (NC) sinus (Figure 2—1w and 2w). These severe inflammatory lesions localized to the proximal coronary arteries and the Valsalva sinus including the aortic valve (Figure 2—2w). Inflammatory cells mainly including neutrophils invaded from the adventitia side to the media gradually, and cell accumulation progressed in the intima synchronously (Figure 2—2w and 3w). After the third week of CAWS administration, the inflammatory lesion spread to all layers of the aortic tissues and Valsalva sinuses with fibrinoid necrosis of the media (Figure 2—3w and 4w). The tissue image by the electron microscope demonstrated the inflammatory cells broke through the elastic fiber and infiltrated into the media side of the intima (Figure 3(a)),

Table 1: Cardiac parameter calculated from echocardiography.

	Untreated ($n = 10$)	CAWS treatment ($n = 6^*$)
LVDd (cm)	0.221 ± 0.008	0.326 ± 0.023†
LVDs (cm)	0.066 ± 0.009	0.204 ± 0.023†
LVEF (%)	96.438 ± 0.860	74.044 ± 5.132†
LVFS (%)	70.963 ± 3.760	37.928 ± 4.128†
HR (bpm)	317.648 ± 18.628	318.750 ± 18.414‡

*Four CAWS treatment mice died before and during examination, †: $P < 0.01$, ‡: N.D.
Left ventricle (LV): LVPWd; LV posterior wall thickness in diastole, LV end-diastolic diameter, LVDs; LV end-systolic diameter, LVEF; LV ejection fraction, LVFS; LV fractional shortening, HR; heart rate.

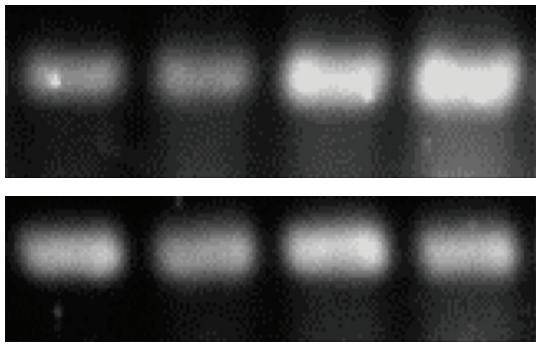

Figure 7: mRNA expression of murine BNP in CAWS arteritis. The cDNA levels of murine BNP were examined by RT-PCR and found to be increased.

and various inflammatory cells accumulated, but these were not classified in detail (Figure 3(b)).

3.3. Anatomical and Histopathological Features of CAWS Treatment Hearts. Significant increases in heart weight and cardiomegaly were observed in the CAWS treatment group at six weeks after CAWS administration (Figures 4(a) and 4(b), Figure 5(a)). On the other hand, the body weight of the CAWS treatment group tended to be about 10% lower than the untreated group (Figure 5(b)). A severe accumulation of inflammatory cells like an abscess was found in the Valsalva sinus. The left ventricular outflow tract (LVOT) stenosis and aortic valve deformation were found (Figure 4(c)). Scars of myocardial infarction were observed in the right ventricle wall, but abnormal changes were not found in other valves and there was no evidence of infarction in the left ventricular myocardial tissues. The cross sectional area of CAWS-treated cardiomyocyte was increased though there was no significant difference (Figure 4(d)).

3.4. Cardiac Function of CAWS Arteritis Detected by Echocardiography and BNP Induction. Ten DBA/2 mice each in the CAWS treatment and untreated groups were prepared for echocardiography. DBA/2 mice were tested after the sixth week from CAWS administration, but two mice had died in the CAWS treatment group before echocardiographic examination. Mice were anesthetized by intraperitoneal injection with Nembutal 40 mg/kg before the examination. All untreated mice awoke normally after the examination, but two mice in the CAWS treatment group with the severe cardiac dysfunction died because of cardiac arrest during echocardiographic examination (Table 1). The examination found a severe reduction in contractility and dilatation of the cavity in the left ventricle (Figure 6(a)). Dilation of left ventricular end-diastolic dimensions (LVDd) and a decrease in left ventricular fractional shortening (LVFS) were observed in the CAWS treatment group (Figure 6(b)). In this study, the determination of the exact aortic diameter and transmitral flow pattern of left ventricle were difficult to detect by echocardiography. The bloodstream and pressure increase were found in the LVOT and aortic valve, but were not able to show significant difference because only a few results were acquired (data not shown). Furthermore, BNP, which is a marker of heart failure, was measured by the luminous intensity of the band obtained by RT-PCR. The BNP bands showed remarkable enhancement in DBA/2 mice at six weeks after CAWS administration (Figure 7).

4. Discussion

The cause of chronic lethal toxicity of CAWS in DBA/2 mice has not been determined sufficiently in previous reports that examined this model only from the viewpoint of arteritis [4, 10, 11]. From previous histopathological reports with high dosage of CAWS, the features of CAWS arteritis were described as necrotizing angiitis, because of the accumulation of various inflammatory cells accompanied by fibrinoid necrosis in the aortic valve and Valsalva sinuses, but other significant abnormalities in CAWS-treated heart were not found [11]. Moreover, severe changes that could be the cause of death due to CAWS were not found in any other main internal organs (digestive system, respiratory system, or cerebral nervous system) previously. It was considered that the weight loss of CAWS-treated group was related to inflammatory cytokines because a lot of inflammatory cytokines production was induced by the CAWS treatment in vitro.

In this histopathological study, inflammatory cells accumulation was located in the aortic root, but there was no evidence of the left ventricular myocarditis or infarction by CAWS treatment. Therefore, the concentric-like left ventricular hypertrophy strongly suggested that the LVOT stenosis and aortic valve deformity brought about left ventricular pressure overload after development of CAWS arteritis in several weeks. The direct measurement of the pressure gradient by using microcatheter was extremely difficult because very practiced technique was needed for operation in the murine aorta of less than one millimeter. By the echocardiography, the bloodstream and pressure increase were found in the LVOT and aortic valve of CAWS-treated mice though there were only a few data. However these data strongly suggested the existence of LVOT stenosis. On the other hand, the results of echocardiography revealed decreasing left ventricular contractility and eccentric-like left ventricular hypertrophy just before death in the CAWS-treated mice. The reasons for this process considered that the left ventricular concentric hypertrophy progressed to dilated phase. In addition, because the two mice caused cardiac arrest during echocardiography test, it was suggested that CAWS-treated mice developed severe cardiac dysfunction of the terminal stage.

The cross-sectional area of CAWS-treated cardiomyocyte was increased, but there was no evidence of fibrosis because only HE stain was performed in this experiment. Furthermore, in the previous report [11], interstitial fibrosis was not found in the limited area around inflammatory lesion with Elastica van Gieson (EVG) stain. Therefore it was considered further data needed to prove fibrosis of the myocardium.

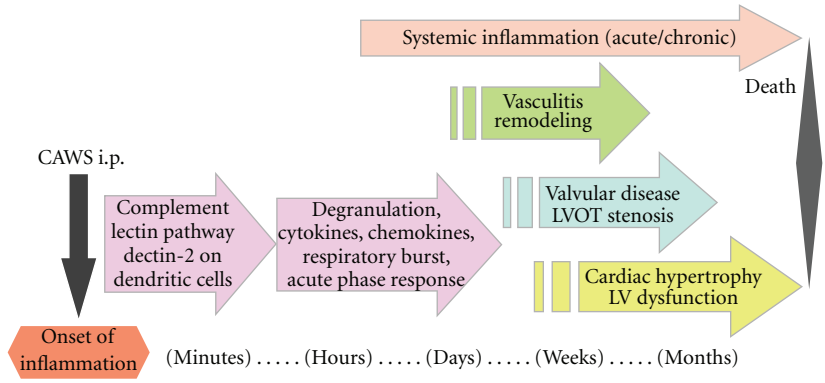

Figure 8: Summarized time course of CAWS-induced systemic inflammation and vasculitis.

BNP was measured as the most sensitive marker of heart failure in clinical settings and secreted by mainly extended ventricular cardiomyocytes [14]. Accordingly, high titer of BNP with cardiomyocyte hypertrophy suggested left ventricular overload by LVOT stenosis and aortic valve disease.

In experimental coronary artery ligation models and atherosclerosis progression models by apolipoprotein-E deficiency, left ventricular myocardial infarction, and fibrosis were often found [15, 16]. However, left ventricular necrosis and fibrosis to indicate myocardial infarction were not found in CAWS arteritis. Furthermore, echocardiography revealed diffuse wall hypokinesis in the left ventricle, but akinesia in a specific segment of wall was not found. It was suggested that the influence of the ischemia did not cause infarction of the left ventricle despite of the ostial coronary lesion in CAWS arteritis. On the other hand, the myocardial infarction of the right ventricle was found in the CAWS-treated group. DBA/2 mouse is a strain that has severe pericarditis with calcification in wild type. Especially remarkable change is observed in right ventricle pericardium. It is difficult to explain the reason why myocardial infarction was developed only in the right ventricle, but it was suspected that the native severe pericardium calcification and the direct invasion of the inflammatory cells were causes of the necrosis.

In the previous studies, CBA/J mice were found to be resistant to CAWS by immunosuppressive IL-10 induction [17], but significant inflammatory cytokines production and neutrophil activation were observed in sensitive murine strains, such as DBA/2, ICR, C57BL/6, BALBc, C3H/HeN, and C3H/HeJ [3–5, 18]. CAWS-sensitive mice, except for strain DBA/2, did not die despite developing arteritis. One of the reasons for the lethal cardiac toxicity is that DBA/2 mice show the most severe progression of arteritis among CAWS-sensitive murine strains. In addition, wild-type DBA/2 mice were found to have spontaneous extensive calcification of the epicardium and myocarditis of the right ventricle [19]. The origin of the epicardial calcification has been explained as the necrosis and mineralization of subepicardial myocytes, which may indicate epicardial inflammation and dystrophic calcinosis in DBA/2 mice [20]. In the present study it was not possible to determine ventricular dysfunction in wild-type DBA/2 mouse compared with other less-susceptible strains.

In the report of CADS-vasculitis [21], abscess and necrosis were observed in the renal artery, lymph nodes and the liver, but the lesions of CAWS arteritis were localized to the aortic root including ostial coronary arteries and aortic valve.

An antiaortic antibody (autoantibody with collagen reactivity) and antineutrophil cytoplasmic antibody (ANCA) have been suggested to participate in human vasculitis [22]. An anticardiac myosin autoantibody was reported in a Kawasaki disease patient [23], though there seems to be no myocarditis in CAWS arteritis. In this study, it was not possible to prove the existence of autoantibodies or to clarify the reason why severe lesions were limited to proximal cardiovascular tissues, but further studies are proceeding to examine the involvement of autoantibodies.

Recently, there have been many reports suggesting a relationship with microbial infection or antigens and vascular disease. There are reports to suggest the participation of ANCA-associated vasculitis and infection [24]. In addition, research into the mechanisms of innate immunity in response to infectious diseases has advanced rapidly. In particular, immunostimulatory agents of microbial origin, such as the lipopolysaccharide (LPS), have been identified as pathogen-associated molecular patterns (PAMPs) [1]. It has been recognized that pattern recognition receptors (PRRs), including toll-like receptors (TLRs), play an important role in the biological activities of PAMPs [25]. TLR 2 and MyD 88 are related to the murine Kawasaki disease model caused by *Lactobacillus casei* cell wall extract (LCCWE) but no evidences of cooperating with TLR 4 in LCCWE arteritis [26]. Furthermore, there is a report of increased expression of CD180, which is regarded as a B-cell surface TLRs homologue, in viral infection and a Kawasaki disease patient [27]. Although TLR 2 and/or TLR 4 are associated with cardiovascular remodeling, atherosclerosis, and *Candida* derived mannan activity [28, 29], at least TLR 4 seems not to be required in CAWS arteritis because it was induced even in C3H/HeJ mice, which is a TLR 4-mutant strain and shows a weak response to LPS [2, 5]. Lectin receptor is related to the signaling of PAMPs, and Dectin-1 and -2 are known as lectin receptors of β-glucan and α-mannan, those are representative PAMPs of fungal origin [30, 31].

In a recent study, *Candida* mannan and glucan require the cooperation of lectin and TLRs to induce immune responses [32]. It was speculated that CAWS activates the human complement system strongly through a lectin pathway when lectin receptor participates in the vasculitis initiation activity of CAWS [10]. Therefore, it seems that cooperation with various receptors and immune molecules is involved in the biological activity of CADS and CAWS. Infection with *C. albicans* does not lead to the direct induction of vasculitis, and the activity of CAWS depends on its higher structure. In particular, it is recognized that the structure of mannan is influenced by the culture conditions of *C. albicans* [3, 6, 10].

In conclusion, these inflammatory changes of CAWS arteritis bring about aortic valvular disease and LVOT stenosis. It was suggested that the progression of CAWS arteritis is classified into the following three stages, the first stage involves the development of vasculitis, the second stage includes complicated myocardial remodeling with hypertrophy by overload, and the last stage is fatal severe left ventricular dysfunction and sudden death (Figure 8). It is proposed that the easy and unique heart failure model induced by CAWS arteritis, without the requirement for specific techniques such as a minute surgical procedure or virus infection, constitutes a valuable model system. Furthermore, large numbers of study animals can be induced to develop angiitis at the same time through simple intraperitoneal injections of CAWS. CAWS arteritis and the complications of fatal heart failure form a simple and useful experimental model of cardiovascular diseases for preclinical investigations.

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Clinical Study

Expression and Function of Ephrin-B1 and Its Cognate Receptor EphB2 in Human Abdominal Aortic Aneurysm

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We examined the expression of ephrin-B1 and its cognate receptor EphB2, key regulators of angiogenesis and embryogenesis, in human abdominal aortic aneurysm (AAA) and analyzed their functional roles in cell migration. From 10 patients (9 males and 1 female; age, 68.5 ± 2.4) who underwent vascular surgery for AAA, we obtained AAA and adjacent control tissues. Using real-time RT-PCR, we analyzed expression of ephrin-B1 and EphB2. We also histologically localized these molecules in AAA tissues. Finally, effects of ephrin-B1 and EphB2 on inflammatory cell chemotaxis were examined by cell migration assay. Expression levels of ephrin-B1 (0.410 ± 0.046 versus 1.198 ± 0.252, $P = 0.027$) and EphB2 (0.764 ± 0.212 versus 1.272 ± 0.137, $P = 0.594$) were higher in AAA than normal control. Both ephrin-B1 and EphB2 were expressed in macrophages, T lymphocytes, and endothelial cells within AAA. In chemotaxis assay, ephrin-B1 and EphB2 inhibited mononuclear-cell chemotaxis induced by stromal derived factor-1 down to 54.7 ± 12.7% ($P = 0.01$) and 50.7 ± 13.1% ($P = 0.01$), respectively. These data suggest that ephrin-B1 and EphB2 might be functional in human adult inflammatory cells and involved in the pathogenesis of AAA, although specific roles of these molecules should further be sought.

1. Introduction

Abdominal aortic aneurysm (AAA) has high risk for aortic rupture and constitutes one of the major causes of elderly death [1], sometimes being associated with coronary ectasia [2]. Compared to occlusive atherosclerosis such as carotid atheroma, AAA affects much more extensive layers of blood vessels but shares some pathogenic aspects such as inflammatory cell accumulation [3]. Genetically engineered mouse models for AAA have elucidated key molecules for the pathogenesis of AAA [4]. For example, some matrix metalloproteinases (MMPs) are upregulated and expressed in macrophages within AAA, which is likely to cause medial degeneration in AAA [5] with or without physiological stress such as hypoxia [6]. However, our understanding on the molecular and cellular pathogenesis of AAA is still limited, especially in cases of humans.

Recently, we have found that ephrin-B1 and its cognate receptor EphB2, the key regulators of angiogenesis and embryogenesis, are upregulated and predominantly expressed in macrophages and T-lymphocytes in human carotid atherosclerotic plaque [6]. Ephrin-B1 and EphB2 belong to ephrin and Eph family of genes consisting 21 members, which are expressed ubiquitously in embryonic tissues and involved in morphogenesis by regulating cell adhesion and migration [7, 8]. Therefore, we hypothesized that ephrin-B1 and EphB2 might be also involved in the pathogenesis of AAA and set out to analyze the expression of these molecules in human AAA and their modulatory effects on chemotaxis of inflammatory cells.

2. Methods

2.1. Patients. The experimental protocol complied with the principles of the Declaration of Helsinki and was approved by the ethical committee of National Cerebral and Cardiovascular Center. Written informed consent was obtained from all the 10 patients who underwent elective graft replacement surgery for AAA (9 males and 1 female; age, 68.5 ± 2.4) [9]. None of the AAA patients suffered from clinically unstable state such as aortic rupture before surgery. The diameter of AAA measured by computed tomography ranged from 38 to 80 mm (55.3 ± 3.5 mm). The prevalence of risk factors for AAA was as follows: hypertension in 9, hyperlipidemia in 8, smoking in 7, and diabetes mellitus in 2 out of 10 patients.

2.2. Aortic Tissue Sampling. During graft replacement for AAA, a strip of aortic wall that contained the dilated region and lacked mural thrombus was carefully excised. An infrarenal aortic strip which contained minimal atherosclerotic changes without dilation was also obtained from five patients as control. All the samples were quickly frozen in liquid nitrogen and stored at −80°C until extraction of RNA. A part of the tissue was fixed with non-formalin-fixative solution, Histochoice (Amresco, Solon, OH), and embedded in paraffin.

2.3. Antibodies and Reagents. For immunohistochemistry, we used the following mouse monoclonal antibodies: anti-CD68 (clone KP1) (Dako, Glostrup, Denmark) and anti-CD8 (clone 4B11) (Novocastra, Newcastle, UK). The following polyclonal antibodies were employed: rabbit anti-ephrin-B1 (Santa Cruz, Santa Cruz, CA); goat anti-EphB2 (Sigma, St. Louis, MO); rabbit anti-von Willebrand Factor (vWF) (Dako); rabbit anti-CXCR-4 (Sigma-Aldrich, St. Louis, MO). Biotinylated swine anti-goat IgG (Dako) was also used. The following recombinant proteins were used: human stromal-derived factor-1 (SDF-1) (PeproTech, London, UK); human monocyte chemotactic protein-1 (MCP-1) (BioLegend, San Diego, CA); mouse ephrin-B1-IgG-Fc chimera and mouse EphB2-IgG-Fc chimera (R&D Systems, Minneapolis, MN); IgG-Fc (Athens Research & Technology, Athens, GA).

2.4. Quantitative Reverse Transcription-Polymerase Chain Reaction (Quantitative RT-PCR). Total RNA was isolated from the aortic specimens using Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions [10]. Concentration of the RNA was measured with spectrophotometer and its integrity was confirmed visually on 1.2% denaturing agarose gel electrophoresis stained with SYBR Green dye (Invitrogen, Carsbad, CA). Then, 10 μg of total RNA was treated with DNA-free agent (Ambion, Austin, TX) and converted to cDNA using random primers and SuperScript II reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed using TaqMan reagents on ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Assay-on-Demand cocktails (PE Applied Biosystems) were used for ephrin-B1 (Hs00270004_m1) and EphB2 (Hs00362096_m1). For GAPDH, predeveloped TaqMan assay reagent was used. The expression levels of ephrin-B1 or EphB2 were normalized to those of GAPDH as described previously [5, 11].

2.5. Immunohistochemistry. The paraffin-embedded specimens of AAA were sectioned in 3 μm thickness. These sections were deparaffinized with Neo-Clear (Merk, Darmsdat, Germany). For histopathological evaluation, the sections were stained with hematoxylin and eosin (H&E). For double immunohistochemical staining of ephrin-B1 and CD68, ephrin-B1 and CD8, ephrin-B1 and vWF, or ephrin-B1 and CXCR-4, the sections were first incubated with anti-ephrin-B1 antibody (1 : 300 dilution) and indirectly stained as brown precipitates using peroxidase-coupled Envision system (PO-Envision, Dako) and 3, 3′-diaminobenzidine (DAB) as substrate. Subsequently, the same sections were incubated with anti-CD68 (1 : 300 dilution), anti-CD8 (1 : 40 dilution), anti-vWF (1 : 200 dilution), or anti-CXCR4 (1 : 100 dilution) antibodies and stained as red precipitates using alkaline phosphatase-coupled Envision system (AP-Envision, Dako) and New Fuchsin (Dako) as substrate.

For double immunostaining of EphB2 and CD68, EphB2 and CD8, EphB2 and vWF, or EphB2 and CXCR-4, sections were first incubated with goat anti-EphB2 antibody (1 : 50 dilution) and processed with peroxidase-coupled Histofine MAX-(G) system (Nichirei, Tokyo, Japan) and DAB. The same sections were then incubated with anti-CD68, anti-CD8, anti-vWF, or anti-CXCR4 antibodies and processed with AP-Envision and New Fuchsin. Before the double immunohistochemistry for ephrin-B1 and CD8 or EphB2 and CD8, the sections had been autoclaved in 0.01 mol/L citrate buffer (pH 6.0) for 5 minutes at 121°C to unmask CD8 antigen. For double immunostaining of CD68 and CXCR-4, the sections were first incubated with anti-CD68 antibody and processed with PO-Envision and DAB. The same sections were then incubated with anti-CXCR-4 antibody and processed with AP-Envision and New Fuchsin. All these preparations were counterstained with hematoxylin, mounted with Neo-Mount (Merk), and examined with Axiophot2 light microscope equipped with AxioCam CCD camera (Carl Zeiss, Hallbergmoos, Germany).

2.6. RT-PCR for Ephrin-B1 and EphB2 in Peripheral Blood Mononuclear Cells. Human peripheral blood mononuclear cells (PBMC) were prepared from forearm venous blood of healthy adult volunteers by density-gradient centrifugation through Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). From the human PBMC, total RNA was isolated with Isogen reagent, which was cleared of con-taminating genomic DNA and reversely transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). For RT-PCR, the forward and reverse primers were designed within a single exon of ephrin-B1 (exon 5) or EphB2 (exon 6): ephrin-B1, 5′-GTCCTACTACTGAAGCTACG-3′/5′-CTCTTGGACGATGTAGACAG-3′; EphB2, 5′-GCAGTGTCCATCATGCATC-3′/5′-AGTACTGCAGCT-CATAGTCC-3′.

As negative and positive controls, the DNA-cleared RNA without reverse transcription and human genomic DNA (50 nM; Clontech, Palo Alto, CA) were used, respectively. The reaction mixture was assembled to a total volume of 10 μL as follows: 6.65 μL water, 1.0 μL 10 × Ex Taq buffer, 0.8 μL dNTP mixture (comprising 2.5 mM of each nucleotide), 1.0 μL forward and reverse primer mixture (5 μM of each primer), 0.5 μL template, and 0.05 μL Ex Taq polymerase (TaKaRa, Tokyo, Japan). The PCR was carried out with preheating (94°C for 2 min) and 35 cycles of amplification (94°C for 20 s, 60°C for 30 s and 72°C for 40 s). The PCR products were subjected to acrylamide gel electrophoresis and stained with Ethidium bromide. The experiments were performed at least three times and the representative data are shown.

2.7. Cell Migration Assay. Boyden chamber cell migration assay was performed using 24-well Transwell plates which contain, in each well, a chamber with 5 μm porous polycarbonate membrane separating the well into upper and lower parts (Corning, Corning, NY). The chamber membranes were precoated with ephrin-B1-Fc, EphB2-Fc, or IgG-Fc as control (5 μg/mL, overnight). Then the chambers were inserted into Transwell plates containing 600 μL of assay buffer (RPMI1640 medium with 0.1% heat-inactivated BSA) with or without chemokines (SDF-1 or MCP-1 at 0.1 μg/mL). Human PBMC (1×10^5 cells in 100 μL of the assay buffer) were loaded into the chamber and allowed to migrate down to the lower part of the well for 2 hours in a humidified incubator (37°C; 5% CO_2). After the migration period, the PBMC that had passed down through the membrane were collected and counted using Z2 Coulter counter (Beckman Coulter, Fullerton, CA). Under the same condition, we also tried to use soluble recombinant ephrin-B1 and EphB2 diluted into the medium to assess the cell migration, because there were some reports that demonstrated differences in results of cell migration [12].

2.8. Statistical Analysis. The results of Quantitative RT-PCR and Boyden chamber assay were expressed as mean ± SEM of two independent experiments done in triplicate. All statistical analyses were performed by using StatView Ver.5.0 software (SAS Institute Inc, Cary, NC). A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Expression of Ephrin-B1 and EphB2 in Human AAA. All the total RNA isolated from AAA or control aortas maintained robust integrity on agarose gel electrophoresis, ensuring reliable analysis of gene expression. Quantitative real-time RT-PCR showed that ephrin-B1 was significantly upregulated in AAA than in control (Figure 1). As for EphB2, a trend toward higher expression in AAA was observed, although the data did not reach statistical significance.

3.2. Immunohistochemical Analysis of Ephrin-B1 and EphB2 in Human AAA Lesions. Each of the tissue specimens used in this study showed typical structures of AAA. All the layers of aorta were affected and the tunica media were severely damaged (Figure 2(a)). Prominent fatty streaks and inflammatory cell infiltration were observed in the intima (Figure 2(b)). Under these conditions, the presence of these inflammatory cells was apparently greater in AAA tissues than those in controls. We performed double immunohistochemical staining to clarify the precise cell types expressing ephrin-B1 or EphB2 therein, because we found that ephrin-B1 and EphB2 were expressed in various types of cells within human AAA with single immunostaining.

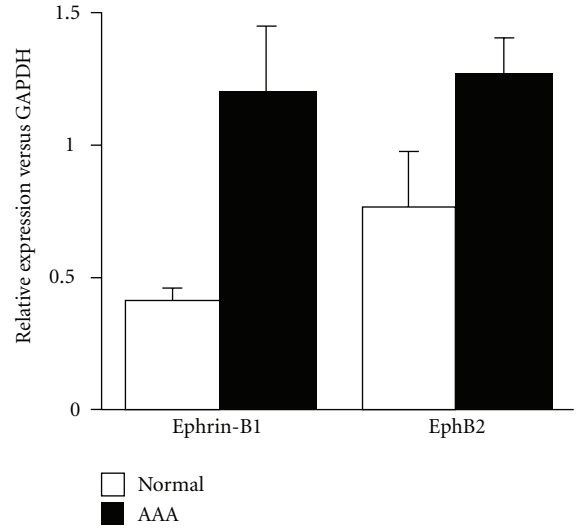

Figure 1: Quantitative real-time RT-PCR analysis of ephrin-B1 and EphB2 in human AAA. The results are mean ± SEM ($n = 10$) and were analysed by an unpaired Student's t-test.

Immunoreactivity of ephrin-B1 was found in the following cell types: macrophages positive for CD68 [13] (Figure 2(c)); lymphocytes positive for CD8 [14] (Figure 2(d)); endothelial cells positive for vWF [15] (Figure 2(e)). Similarly, EphB2 was detected in macrophages positive for CD68 (Figure 3(a)), lymphocytes positive for CD8 (Figure 3(b)), and endothelial cells positive for vWF (Figure 3(c)).

To investigate functional significance of ephrin-B1 and EphB2 on chemotaxis of macrophages or lymphocytes, we examined coexpression of these molecules with CXCR4, a receptor for SDF-1 [16] by double immunohistochemistry. Many macrophage- or lymphocyte-like cells were found to express both ephrin-B1 and CXCR4 (Figure 2(f)) or both EphB2 and CXCR4 (Figure 3(d)).

3.3. Functional Expression of Ephrin-B1 and EphB2 in Peripheral Blood Mononuclear Cells. To confirm the expression at mRNA levels of ephrin-B1 and EphB2 on human adult inflammatory cells, we performed RT-PCR. In human PBMC, both ephrin-B1and EphB2 were robustly expressed (Figure 4). We then tested whether ephrin-B1 and EphB2 have functional importance in human PBMC, by examining the effects of ephrin-B1 and EphB2 on their chemotaxis. In Boyden chamber assay, membrane-coated ephrin-B1-Fc or EphB2-Fc chimeric proteins did not affect spontaneous

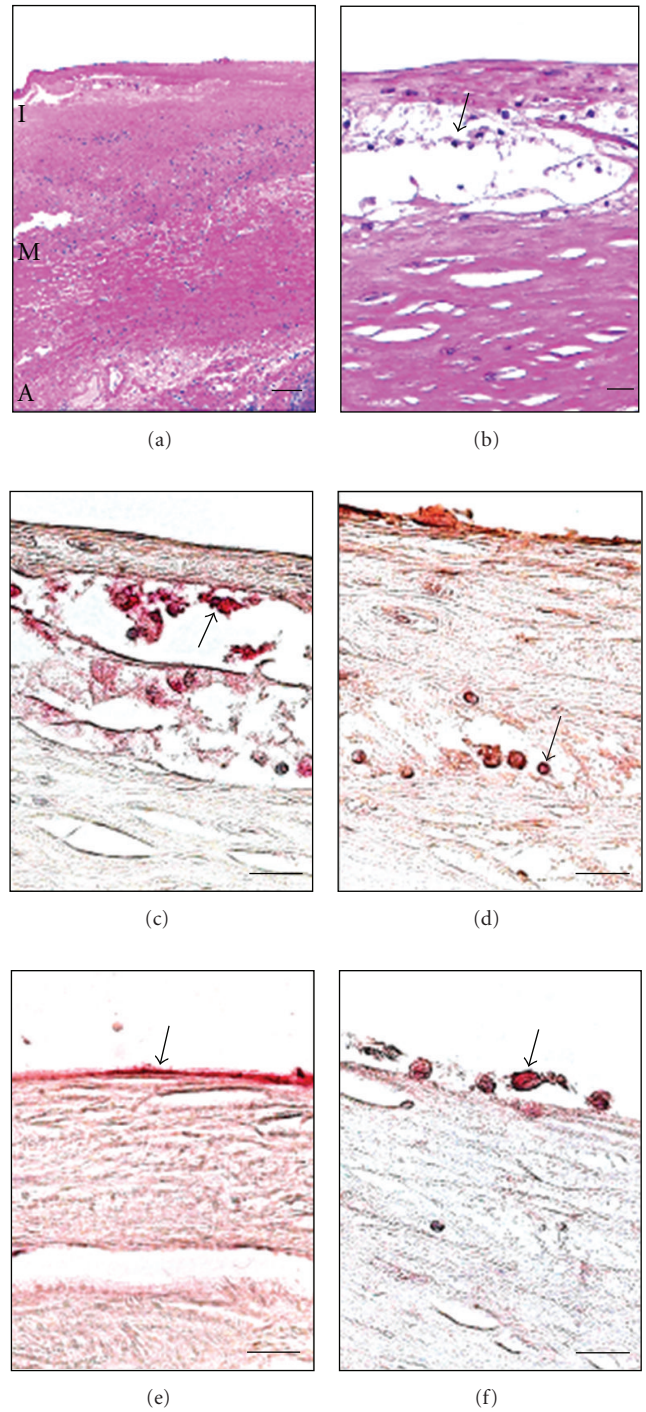

Figure 2: Histology of human AAA and double immunostaining of ephrin-B1 and others therein. (a) I, intima; M, tunica media; A, adventitia. Note severely destructed tunica media. Bars, 200 μm. (b) Arrow indicates fatty streak in intima. Bars, 20 μm. (c) and (d) Double immunostaining. Ephrin-B1 was stained with DAB (brown), the others with New Fuchsin (red). Bars, 20 μm.

migration of PBMC (Figure 5(a)). However, the chemotaxis of PBMC induced by SDF-1 was significantly inhibited by ephrin-B1-IgG-Fc and EphB2-IgG-Fc down to 54.7 ± 12.7% ($P = 0.01$) and 50.7 ± 13.1% ($P = 0.01$), respectively (Figure 5(b)). Also, MCP-1-induced chemotaxis of PBMC was significantly inhibited by ephrin-B1-IgG-Fc and EphB2-IgG-Fc down to 53.8 ± 10.9% ($P = 0.01$) and 51.3 ± 11.6% ($P = 0.01$), respectively, although no significant increases or decreases of cell migration were observed in cases of soluble ephrin-B1 and EphB2.

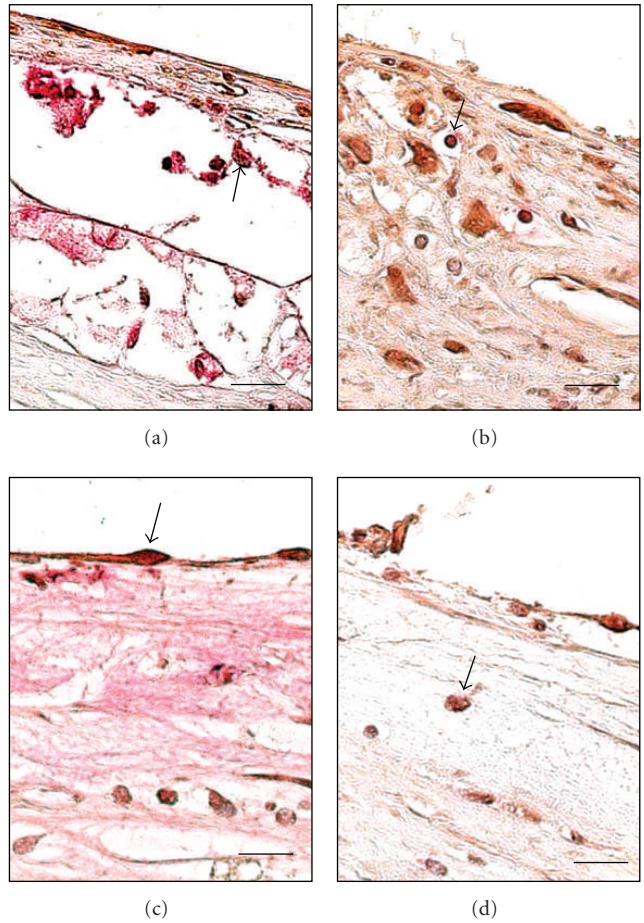

Figure 3: Double immunostaining of EphB2 and others in human AAA. EphB2 was stained with DAB (brown), the others with New Fuchsin (red). Bars, 20 μm.

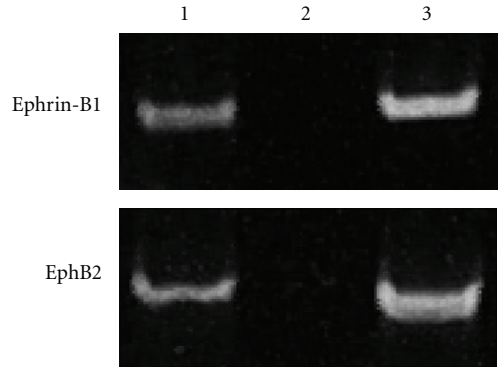

Figure 4: RT-PCR analysis for ephrin-B1 and EphB2 in human PBMC. Three kinds of templates were used to validate the results: lane 1, cDNA; lane 2, DNA-cleared RNA; lane 3; genomic DNA.

4. Discussion

We found that both ephrin-B1 and EphB2 exhibited higher expression in human AAA and were expressed in macrophages, T lymphocytes, and endothelial cells therein. Furthermore, we found that membrane-bound ephrin-B1 and EphB2 inhibited chemotaxis of human PBMC in Boyden chamber assay. Ephrins and their cognate receptor Ephs are well-known regulators in embryogenesis [7, 8] but their expressions and pathogenic roles in AAA remain unclear. Therefore, the findings of this study would give us a new clue to investigate the molecular pathogenesis of human AAA.

One major cause of higher expression of ephrin-B1 and EphB2 in AAA is probably the infiltration therein of macrophages and T-lymphocytes expressing these two genes. However, gene expression levels of ephrin-B1 and EphB2 in monocytes/macrophages, T-lymphocytes, and endothelial cells in AAA might be upregulated, which remains to be elucidated. Taken together with the fact that ephrin-A5 is upregulated in human AAA [17], wide spectrum of ephrins and Ephs [7] might be upregulated in human AAA. We further tested whether ephrin-B1 and EphB2 are functional or nonfunctional on human adult inflammatory cells. For this purpose, we examined the effects of ephrin-B1 and EphB2 on chemotaxis, because these molecules are well known to regulate migration of embryonic cells. In Boyden chamber assay, the membrane-fixed ephrin-B1 inhibited chemotaxis of human PBMC induced by SDF-1 or MCP-1, which is in line with the previous reports that ephrin-B1 inhibits SDF-1-induced chemotaxis of Jurkat T-lymphocytic [18] and THP-1 monocytic cell lines [6].

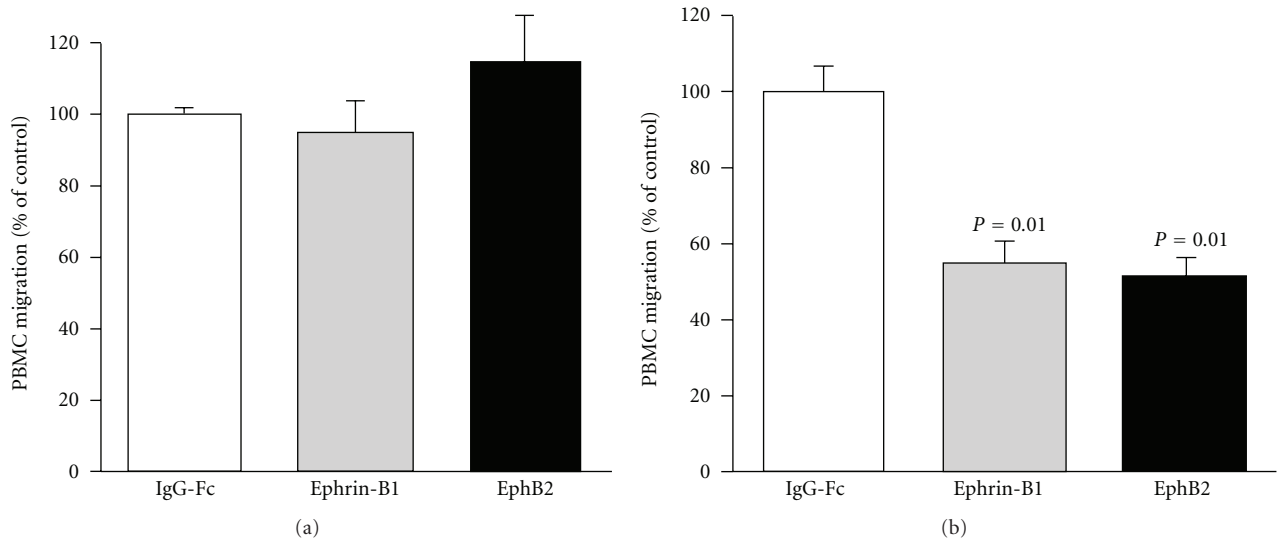

Figure 5: Modulatory effects of ephrin-B1 and EphB2 on transmigration of human PBMC by SDF-1. Boyden chamber assay was performed. (a) Effect of ephrin-B1 or EphB2 on spontaneous transmigration. The number of cells migrating into the well without SDF-1 was considered spontaneous transmigration. (b) Effect of ephrin-B1 or EphB2 on SDF-1-induced transmigration. With SDF-1, the increased number of cells migrating into the well was due to SDF-1-dependent transmigration. The level of PBMC migration with IgG-Fc used as control, which was 3.6% (a) and 18.5% (b) of inputs (1×10^5 cells), was set at 100% for analysing the effect of ephrin-B1 or EphB2. Ephrin-B1, ephrin-B1-IgG-Fc; EphB2, EphB2-IgG-Fc. The results are mean ± SEM ($n = 6$) and were analysed by one factor ANOVA and Dunnett's test compared with IgG-Fc (control).

The chemotaxis of human PBMC induced by SDF-1 or MCP-1 was also inhibited by the membrane-fixed EphB2, which usually functions as receptor. This ligand-like action by EphB2 is considered so-called reverse signalling [8], like EphB2 inhibiting SDF-1-dependent migration of cerebellar granule cell of mouse embryo [19]. In addition, there are several reports which show ephrin signalling interacts with inflammatory molecules such as Rho family GTPases [20] and VCAM-1 [21]. Thus, our findings from Boyden chamber assay suggest that ephrin-B1 and EphB2 on human PBMC are functional.

Importantly, these inhibitory effects of ephrin-B1 and EphB2 were not clearly observed when we used soluble agents. This may be partially explained by a dependence of Eph receptor activation on lateral movement across the cell membrane for complete activity [12]. Pfaff et al. [22] demonstrated lateral movement of endothelial ephrinB2 from the apical surface to cell-cell junctions preceding degradation. Additionally, one might speculate that ephrins/eph receptors fixed to transwell membranes may not induce proper spatial distribution of counter-receptors/ligands for chemotaxis. However, the present data demonstrate at least the inhibitory effects of cell migration induced by SDF-1 *in vitro*.

Including cytokines and chemokines, most of the known factors that regulate the activities of macrophages or T-lymphocytes are soluble molecules which are widely spread [23]. In contrast, ephrins and Ephs are membrane-bound molecules which exert their functions locally through cell-to-cell interaction with their neighbouring cells [24]. During the pathological courses of many inflammatory diseases including AAA [25–29], various types of cell-to-cell interaction occur: macrophages-to-macrophages, macrophages-to-T-lymphocytes, macrophages-to-endothelial cells, and so on. Ephrin-B1 and EphB2, or more generally ephrins and Ephs, might locally and precisely modulate the activities of macrophages and T-lymphocytes conditioned by diffusing factors such as cytokines and chemokines. Understanding the effect of ephrinB1 or EphB2 activation on the inflammatory condition, perhaps to MMP expression or function, may make clear the role of ephrinB1 or EphB2 in this context [30]. Interestingly, we recently found that ephrin and Eph family are widely expressed in atherosclerosis-related cells in human [31]. Further study will elucidate the *in vivo* roles of ephrins and Ephs in the development of atherosclerosis, both dilated and occlusive, and other inflammatory diseases.

5. Conclusions

This study suggests that ephrin-B1 and EphB2, the key embryogenic regulators, might be involved in the molecular and cellular pathogenesis of human AAA. Through cell-to-cell interactions, ephrin-B1 and EphB2 might locally modulate the inflammatory cell activities conditioned by soluble mediators such as cytokines. Ephrin/Eph system, the largest receptor tyrosine kinase system in the human genome, may give us a clue to unravel yet unknown pathogenesis of AAA and to devise novel therapeutic strategies for AAA and other inflammatory diseases, although specific roles of these molecules in the development of AAA should further be sought.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Effects of Statins on Cardiorenal Syndrome

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Cardiovascular disease and renal disease have a close relationship that forms a vicious cycle as a cardiorenal syndrome (CRS). Oxidative stress, endothelial dysfunction, and vascular inflammation could be therapeutic targets when the renin-angiotensin-aldosterone system is activated by accumulation of conventional cardiovascular risk factors; however, a strategy for management of CRS has not been established yet. Statins, HMG-CoA reductase inhibitors, have not only cholesterol-lowering effects but also pleiotropic effects on cardiovascular systems, including anti-inflammatory and antioxidant effects and improvement of nitric oxide bioavailability. Since recent studies have indicated that statins have beneficial effects on chronic kidney disease and heart failure as well as coronary artery disease in cholesterol-lowering-dependent/independent manners, treatment with statins might be a successful strategy for preventing deterioration of CRS.

1. Introduction

It is well known that metabolic syndrome contributes directly to the occurrence of cardiovascular events. Accumulation of cardiovascular risk factors, including dyslipidemia, hypertension, and diabetes, activates the renin-angiotensin-aldosterone system (RAAS), leading to not only ischemic cardiovascular disease (CVD) but also left ventricular (LV) dysfunction and chronic kidney disease (CKD) [1]. It is also known that LV dysfunction and renal dysfunction frequently coexist in the same individual. Therefore, it has been advocated that cardiovascular disease and renal disease are closely related to each other as a cardiorenal syndrome (CRS). Disorders of these two organs are co activated and co regulated by various lifestyle-related problems, falling into a vicious cycle of cardiorenal diseases; however, a strategy for management of this syndrome has not been established yet. Therefore, identification of the key therapeutic targets of CRS is needed to break the vicious cycle development of cardiac disease and renal disease.

Statins, HMG-CoA reductase inhibitors, have not only cholesterol-lowering effects but also pleiotropic effects on cardiovascular systems, including anti-inflammatory and antioxidant effects and improvement of nitric oxide (NO) bioavailability [2–5]. In this paper, we focus on the effects of statins on CRS.

2. CRS Is Based on a Close Network between Heart and Kidney Disorders

CRS is defined as "disorders of the heart and kidneys whereby acute or chronic dysfunction in one organ may induce acute or chronic dysfunction of the other" [6]. Since low cardiac output due to heart failure (HF) leads to decrease in renal blood flow, HF exacerbates renal function.

In fact, a 25% decrease in cardiac output leads to a 50% decrease in renal blood flow, and 25% to 40% of patients with decompensated HF experience deterioration in renal function [7, 8]. Renal perfusion is strictly regulated by an autoregulation system to maintain intraglomerular pressure at a constant level; however, prolonged HF evokes glomerular endothelial dysfunction and breakdown of the glomerular autoregulation system. Decrease in renal perfusion due to HF activates the RAAS, leading to cardiorenal damage. It has been reported that patients with CKD, especially elderly patients, are at high risk for major CVD morbidity and mortality. Indeed, the mortality rate in a 2-year interval after acute myocardial infarction is around 50% in patients with stage 5 CKD. In addition, it is noteworthy that patients with CKD have a 10- to 20-fold increased risk of cardiac death [9, 10]. Albuminuria, an early manifestation of CKD, has been shown to be a risk factor for not only the development of CKD but also cardiovascular disease even at stage 1 of CKD [11]. Therefore, CKD is characterized as intraglomerular hypertension and glomerular leakage from albuminuria to overt proteinuria, leading to decrease in glomerular filtration rate (GFR) and ultimately end-stage kidney disease.

Taken together, the heart and kidneys form a tight network in both physiological and pathological conditions, and homeostatic failure of these organs exacerbates clinical states of CKD and CVD in a synergistic manner.

3. Mechanisms of the Development of CRS

The mechanisms of the development of CVD evoked by CKD have not been fully clarified; however, a number of previous studies suggested that several biomarkers yield clues for understanding the mechanisms of the development of CRS. Under normal physiological conditions, oxidative stress and NO bioavailability are each countervailed to maintain vascular homeostasis; however, under pathological conditions such as excessive RAAS activation, this balance is disrupted by an increase of oxidative stress and decrease of NO bioavailability, and disruption of the balance evokes vascular inflammation, leading to cardiovascular remodeling and CKD and eventually to cardiovascular events (Figure 1). Therefore, oxidative stress and NO bioavailability and vascular inflammation could be therapeutic targets for preventing cardiovascular events in addition to conventional risk factors of CVD such as hypertension, diabetes, dyslipidemia, and smoking.

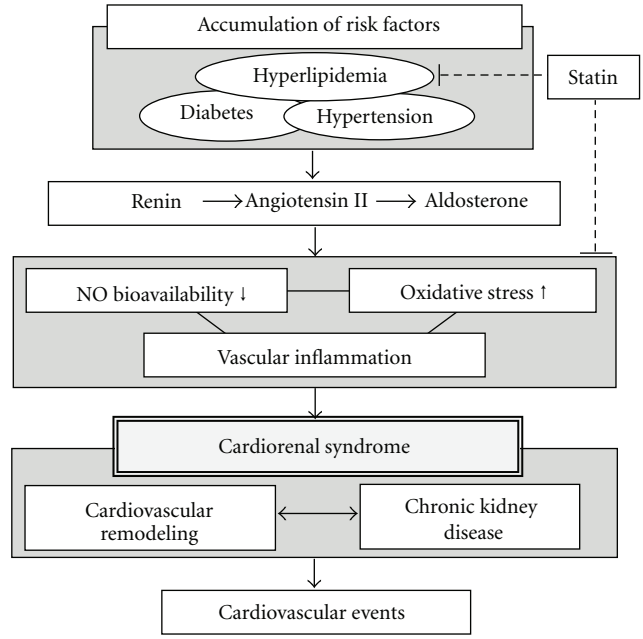

Figure 1: Activation of the renin-angiotensin-aldosterone system due to accumulation of cardiovascular risk factors causes imbalance between nitric oxide (NO) bioavailability and oxidative stress, leading to vascular inflammation. This upstream signaling exacerbates cardiorenal syndrome and evokes cardiovascular events. Statins ameliorate not only the serum lipid profile but also oxidative stress, NO bioavailability, and vascular inflammation by their pleiotropic effects.

4. Statins Improve Endothelial Function

Endothelial function is important for maintaining vascular homeostasis. Endothelium-derived NO mediates vascular relaxation and inhibits platelet aggregation, vascular smooth muscle proliferation, and endothelium-leukocyte interactions and it protects the cardiovascular system from remodeling [12–14]. On the other hand, accumulation of cardiovascular risk factors impairs endothelial function, which is the earliest manifestation of atherosclerosis. It has been recognized that improvement of endothelial function by statins was observed before a significant reduction in serum cholesterol level, suggesting that these preferable effects of statins on endothelial function are cholesterol-lowering-independent actions [15, 16]. It has been shown that statins increase NO bioavailability by activation and up regulation of endothelial NO synthase (eNOS) through inhibition of Rho/ROCK signaling, activation of the PI3kinase/Akt pathway, and amelioration of eNOS uncoupling [4, 17–21].

5. Statins Decrease Oxidative Stress

Another potential mechanism by which statins may improve endothelial function is their antioxidant effects. Reactive oxygen species (ROS) as a major source of oxidative stress are mainly produced by NADPH oxidase in the cardiovascular system, and Rac-1, a small G protein, has recently been shown to be a key molecule for the assembly and function of NADPH oxidase components [22]. Therefore, NADPH oxidase components, especially Rac-1, could be targets of statin therapy to reduce oxidative stress. In fact, statins attenuate angiotensin II-induced oxidative stress in cardiac myocytes and vascular smooth muscle cells by inhibiting Rac-1-mediated NADH oxidase activity [22, 23]. Statins also

reduce mRNA expression of $p22^{phox}$ and Nox1, NADPH oxidase subunits [3].

6. Statins Ameliorate Vascular Inflammation

It is well known that vascular inflammation plays a central role in the pathogenesis of cardiovascular disease. In addition, it is a widely accepted view that atherosclerosis is a chronic inflammatory disease that is initiated by endothelial cell dysfunction at the vascular surface [24]. Inflammatory cytokines secreted from mononuclear cells such as macrophages and T lymphocytes promote endothelial dysfunction, smooth muscle cell proliferation, and thrombosis. An early step in atherogenesis involves monocyte adhesion to the endothelium and penetration into the subendothelial space. It has been shown that statins reduce expression levels of proinflammatory cytokines and adhesion molecules, including tumor necrosis factor- (TNF)-α, interleukin (IL)-1β and IL-6, soluble vascular cell adhesion molecule-1 (sVCAM-1), and von Willebrand factor, and that statins reduce the number of inflammatory cells in atherosclerotic plaques [2, 25–27]. More recently, it has been shown that statins inhibit endothelial exocytosis of Weibel-Palade bodies, endothelial cell granules containing adhesion molecules, in an eNOS-dependent manner and that statins attenuate T cell-mediated myocardial inflammation in a KLF2-dependent manner in a mouse model [27, 28]. The inhibitory effects on endothelial exocytosis and modulation of T-cell function in the immune system may explain the mechanisms of the anti-inflammatory effects of statins in addition to improvement of imbalance between NO bioavailability, and oxidative stress.

Taken together, the results indicate that administration of statins could be a sound strategy for CRS patients with excessive oxidative stress, decreased NO bioavailability and vascular inflammation.

7. Statins for CVD

As for primary prevention against CVD, initial trials of lipid-lowering with clofibrate, cholestyramine, and gemfibrozil failed to reduce coronary mortality [29–31]; however, WOSCOPS [32], a landmark trial, showed that pravastatin reduced coronary mortality and the following AFCAPS/TexCAPS trial revealed that lovastatin reduced cardiovascular events [33]. In addition, the MEGA trial showed that Japanese hypercholesterolemia patients with no history of coronary disease or stroke, relatively low-risk group for CVD, could benefit from pravastatin for primary prevention against CVD [34]. On the other hand, the 4S and LIPID trials demonstrated that statin therapy improved total mortality as well as morbidity as secondary prevention of CVD [35–37].

The ASTEROID and REVERSAL trials showed that intensive statin therapy in patients with CVD reduced coronary atherosclerotic volume dependent on levels of LDL-C reduction [38, 39]. The PROVE-IT and TNT trials indicated that intensive statin therapy prominently decreased cardiovascular events compared with standard statin therapy in patients with acute or stable CVD, respectively [40, 41].

8. Statins for Systolic HF

The end-stage manifestation of cardiac remodeling due to ischemic and non ischemic heart diseases leads to systolic HF, and it had been expected that statins would be effective for preventing systolic HF. Experimental studies and a meta-analysis showed that a statin ameliorates cardiac remodeling and systolic function [42, 43]; however, two major randomized trials, CORONA and GISSI-HF, failed to reveal beneficial effects of rosuvastatin in patients with systolic HF [44, 45]. These two studies included patients of relatively advanced age who had severe systolic heart failure with/without ischemic heart disease. The CORONA study included patients with ischemic systolic heart failure (mean age of 73 y.o, NYHA class II to IV, mean LV ejection fraction of 31%), and the GISSI-HF study included over 60% of subjects with non-ischemic heart disease (mean age of 68 y.o. NYHA class II to IV, mean LV ejection fraction of 33%), suggesting that a statin is not effective for severe systolic HF in patients with both ischemic and non-ischemic heart diseases. Post-hoc analysis of the results of these studies showed that a statin was effective with patients in a low N terminal-pro B type natriuretic peptide (NT-proBNP) group (<103 pmol/L: 868 pg/mL) and in a high-sensitivity CRP (hs-CRP) group (> or = 2.0 mg/L) [46, 47]. These results indicate that statin treatment is effective in patients with early-stage cardiac injury but not in patients with advanced-stage cardiac injury and that statin therapy is more beneficial in HF patients with increased hs-CRP.

9. Statins for Diastolic HF

LV diastolic dysfunction is an early manifestation of cardiac remodeling, and statins prevent cardiac remodeling including cardiac fibrosis and hypertrophy. Therefore, there is a possibility that statins improve LV diastolic function. Several experimental and observational studies showed the effectiveness of a statin for LV diastolic function [48–51]. In addition, a randomized controlled small trial of statin therapy in patients with diastolic HF (statin group, $n = 81$; no statin group, $n = 189$) showed that a statin improved survival in patients with diastolic HF during a 5-year follow-up period [52]. Since other antioxidant agents such as RAAS inhibitors have some beneficial effects on cardiac diastolic function, there is a possibility that statins might work for improving cardiac diastolic function via attenuation of oxidative stress.

10. Statins for Atrial Function

Elevated cardiac end-diastolic pressure due to cardiac diastolic dysfunction plays a central role in atrial remodeling, leading to atrial fibrillation (AF) and cardiogenic embolism. Although antiarrhythmic and anticoagulant agents are used for these pathological conditions, these drugs are not able

to ameliorate atrial remodeling. It has been reported that structural and electrophysiological changes in the atrium are associated with inflammation and oxidative stress [53]. It has also been reported that statins reduce the incidence of AF through attenuation of atrial remodeling [54]. A meta-analysis suggested an antiarrhythmic effect of statins against AF, especially under conditions of increased inflammation such as post-operative cardiac surgery and acute coronary syndromes [54]. These studies indicate that statin therapy prevents not only LV remodeling but also atrial structural and electrical remodeling, leading to decrease in the incidence of AF and cardiogenic embolism.

11. Statins for CVD in Patients with CKD

Mild to moderate CKD is associated with increased cardiovascular risk. Therefore, strict management of serum lipid profile by statins also seems to be effective for reducing cardiovascular events in patients with mild to moderate CKD. A meta-analysis showed that statins reduce all-cause mortality, cardiovascular death, and non fatal cardiovascular events in mild to moderate CKD patients [55]. Post-hoc subgroup analysis of the Treating to New Targets (TNT) trial showed that a high dose of atorvastatin (80 mg) reduced cardiovascular events more than did a low dose of atorvastatin (10 mg) [56].

Although the SHARP trial, a randomized large-scale trial, has directly shown that simvastatin plus ezetimibe reduced the incidence of major vascular events in patients with CKD compared with placebo, the 4-D, AURORA, and SHARP trials showed no definite clinical benefit of statin monotherapy in hemodialysis patients regardless of significant reductions in LDL-C [57, 58]. These findings indicate that a statin is no longer able to reduce cardiovascular events in end-stage CKD, and statin therapy should therefore be recommended to CKD patients with mild to moderate CKD.

12. Statins for Renal Function

Experimental studies showed that hyperlipidemia is a risk factor for renal disease: cholesterol loading enhances glomerular injury and the decrease in serum lipid level by statins slows the rate of renal injury progression [59, 60]. Deposition of lipids in glomeruli and activation of the RAAS by accumulation of CVD risk factors cause glomerular endothelial dysfunction and increase intraglomerular pressure, leading to tangible albuminuria, an early manifestation of proteinuria, and decrease in glomerular filtration rate (GFR). Since albuminuria also directly injures glomeruli by itself, reduction of albuminuria might be essential to prevent progression of CKD. Animal studies have shown renal protective actions of statins [61, 62]; however, there are conflicting data concerning the effects of statins on renal function. In a clinical setting, two meta-analyses have shown that statins decrease albuminuria [63, 64]; whereas, other studies have shown that there were no significant effects of statins on albuminuria [65–67]. Additional evidence is needed to apply statin administration for treatment of CKD.

13. Beneficial Effects of Pitavastatin, a New Lipophilic Strong Statin

It has not been established whether the beneficial effects of statins on cardiorenal tissues are class effects; however, lipophilic statins seem to have direct effects on cardiovascular organs. The newly developed drug pitavastatin, a lipophilic and a strong statin, is effective for lowering LDL-cholesterol and increasing high-density lipoprotein cholesterol (HDL-C), which are preferable in the serum lipid profile for preventing CVD. In terms of tissue distribution of statins, pharmacokinetic profile before and after liver metabolism is an important consideration. Most of pitavastatin is absorbed following ingestion (80%), and its level of protein binding is extremely high (>95%) [68]. In addition, pitavastatin is hardly metabolized by cytochrome p450 (CYP), and most of the bioavailable pitavastatin is excreted in an unchanged form in bile and enters enterohepatic circulation by reabsorption in the small intestine [69]. Therefore, bioavailable pitavastatin is able to be distributed directly to cardiovascular and renal tissues directly and exerts cardiorenal protective effects. From these facts, pitavastatin has been expected to have stronger pleiotropic effects than those of other classical statins. In fact, it has been reported that pitavastatin has anti-oxidant and anti-inflammatory effects and that it improves NO bioavailability, leading to improvement of cardiorenal function [70–78]. Minimal interaction with CYP suggests that pitavastatin is safer than other statins regardless of lipophilicity.

14. Pitavastatin Ameliorates CRS

Our previous experimental data showed that pitavastatin treatment improved angiotensin II-induced LV remodeling, renal insufficiency, atrial remodeling, incidence of AF, and atrial prothrombotic condition in eNOS knockout mice [79, 80]. In that study, we concluded that pitavastatin exerts eNOS-independent protective actions against angiotensin II-induced cardiovascular remodeling and renal insufficiency through inhibition of the transforming growth factor-(TGF)-β 1-Smad 2/3 signaling pathway by suppression of oxidative stress [79, 80]. In addition, our clinical study showed that a low dose (1 mg/day) of pitavastatin improved LV diastolic function and reduced albuminuria [81]. In that study, statistical analysis demonstrated that the effect of pitavastatin on cardiorenal protection was associated with reduction of oxidative stress but not reduction of LDL-C [81].

Pitavastatin might have more overt pleiotropic effects, especially on reduction of oxidative stress, than those of other statins. A large clinical trial is needed to confirm the effects of pitavastatin on CRS.

15. Conclusion

Accumulation of CVD risk factors leads to activation of the RAAS, which exacerbates CRS with inflammation and eventually evokes cardiovascular events. Statin therapy plays

an important role in prevention of these cardiovascular and renal events. Early use of statins is recommended in patients with multiple cardiovascular risk factors before the development of irreversible cardiorenal remodeling.

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Research Article

Involvement of Inflammation and Adverse Vascular Remodelling in the Blood Pressure Raising Effect of Repeatedly Heated Palm Oil in Rats

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Oil thermoxidation during deep frying generates harmful oxidative free radicals that induce inflammation and increase the risk of hypertension. This study aimed to investigate the effect of repeatedly heated palm oil on blood pressure, aortic morphometry, and vascular cell adhesion molecule-1 (VCAM-1) expression in rats. Male Sprague-Dawley rats were divided into five groups: control, fresh palm oil (FPO), one-time-heated palm oil (1HPO), five-time-heated palm oil (5HPO), or ten-time-heated palm oil (10HPO). Feeding duration was six months. Blood pressure was measured at baseline and monthly using tail-cuff method. After six months, the rats were sacrificed and the aortic arches were dissected for morphometric and immunohistochemical analyses. FPO group showed significantly lower blood pressure than all other groups. Blood pressure was increased significantly in 5HPO and 10HPO groups. The aortae of 5HPO and 10HPO groups showed significantly increased thickness and area of intima-media, circumferential wall tension, and VCAM-1 than other groups. Elastic lamellae were disorganised and fragmented in 5HPO- and 10HPO-treated rats. VCAM-1 expression showed a significant positive correlation with blood pressure. In conclusion, prolonged consumption of repeatedly heated palm oil causes blood pressure elevation, adverse remodelling, and increased VCAM-1, which suggests a possible involvement of inflammation.

1. Introduction

The practice of reusing vegetable oils several times for deep frying before disposing them is quite common among Malaysians. It is thought to be a way to cut the expense. Such practice might be detrimental. However, general public awareness about this is only at moderate level [1]. Deep fried foods have been becoming more popular in daily diet, especially in this modern fast-paced lifestyle. Heating the vegetable oils to a high level of temperature, that is, approximately 160–180°C, also exposes them to the air and moisture at the same time, in which the oils will undergo a complex series of physical and chemical deterioration known as oil thermoxidation. This oxidative deterioration affects the chemical compositions of the vegetable oils by saturating its fatty acids and generating reactive oxygen species (ROS) which are potential in causing deleterious effects on the normal function of endothelial cells [2] and increasing risk of hypertension [3, 4].

Due to their unpaired shell electron, ROS are highly damaging to cells and therefore recognised to be a major cause of endothelial dysfunction and vascular inflammation [5–7]. Pathogenesis of hypertension might be attributed to inflammation [8]. Several reports documented that inflammation may play a pivotal role in the initiation as well as progression of hypertension [9, 10]. Endothelial cells which line the intimal surface of blood vessel and maintain the integrity of the vascular system are the primary target of immunological attack in inflammatory diseases. Endothelial dysfunction is manifested by altered anti-inflammatory

properties of the endothelium, impaired modulation of vascular growth, leukocyte adhesion, dysregulation of vasomotion, and smooth muscle cell proliferation [11–13], which may play a major role in the development of high blood pressure. Vascular cell adhesion molecule-1 (VCAM-1) is one of the endothelial cell adhesion molecules that mediate leukocytes binding. The increased expression of VCAM-1 on endothelial cells is a common process in response to inflammation [14], and it is recognised as an important cardiovascular risk marker [15, 16]. Nevertheless, ROS also stimulate expression of adhesion and chemotactic molecules, which promote uptake of inflammatory cells into the vessel wall [5]. Previous works found elevated level of soluble VCAM-1 in hypertensive subjects [17–19].

Palm oil, which contains both saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) at almost similar levels [20], is popular in the food industry as well as in family kitchen due to its oxidative stability. It is a commonly used vegetable oil in Malaysia. It has been previously demonstrated that consumption of repeatedly heated palm oil causes a significant elevation in blood pressure [4]. We believe that the ROS and other harmful oxidation products present in the repeatedly heated vegetable oils may induce inflammation in vascular system. The present study aimed to investigate the possible role of inflammation in blood pressure elevation after the prolonged intake of repeatedly heated palm oil in blood vessel as well as the vascular morphometric alterations.

2. Materials and Methods

2.1. Experimental Design. Thirty adult male Sprague-Dawley rats ($n = 30$) aged three months, weighing 200–280 g were obtained from the Laboratory Animal Resource Unit, Universiti Kebangsaan Malaysia. The handling and experimental protocols were approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee. The animals were housed in stainless-steel cages and kept at room temperature of 27°C ± 2°C with a 12-hour light cycle at the Pharmacology Department Animal House. All rats had free access to food and tap water throughout the experiment. The animals were acclimatised for one week, prior to administration of test diets. The rats were divided into five groups comprising six animals each and given the following course of diet: (i) basal diet without any addition of oil (as control) or basal diet fortified with 15% weight/weight (w/w), (ii) fresh palm oil (FPO) as described earlier by Owu et al. [21], (iii) one-time-heated palm oil (1HPO), (iv) five-time-heated palm oil (5HPO), or (v) ten-time-heated palm oil (10HPO) for six months. Body weight and blood pressure were determined before the treatment and at monthly intervals. At the end of the study, rats were sacrificed and aortic arches were excised and processed according to the routine histological procedures for histological and immunohistochemical examination.

2.2. Preparation of Palm Oil Diets. Commercially purchased palm oil (Cap Buruh, Lam Soon Edible Oil, Kuala Lumpur, Malaysia) was used in fresh state or heated once, five times, and ten times, according to the modified method of Owu et al. [21]. The heating process involved using 2.5 L of the oil to fry 1 kg of sweet potatoes in a stainless-steel wok at about 180°C for 10 min. The heated oil was cooled for five hours, and then the entire frying process was repeated with a fresh batch of sweet potatoes. The process was repeated four, and nine times to obtain the five- and ten-times-heated-oil respectively. No replenishment of fresh oil was done between batches to make up for the loss due to uptake of the oil by the frying material. Standard rat chow (Gold Coin, Kepong, Malaysia) was ground and formulated by mixing 15% (w/w) of respective oils prepared. The pellets were reformed and dried in an oven at 80°C overnight.

2.3. Measurement of Blood Pressure. Systolic blood pressure of rats was measured by the tail-cuff method using PowerLab data acquisition systems (ADI Instruments, NSW, Australia) after warming the rats for 10 minutes. Five readings were obtained from each rat and then averaged.

2.4. Aortic Morphometry. Aortic arches were embedded in Paraplast Plus (Sigma-Aldrich, St. Louis, MO, USA), and 5 μm cuts were accomplished (LEICA RM2235, Walldorf, Germany). Aortic sections were stained with Verhoeff-Van Gieson to identify elastic fibres and smooth muscle cells. Digital images of aortic sections were acquired (JPEG format, 24-bit colour, 2560 × 1920 pixels) with a MicroPublisher 5.0 RTV camera (Q Imaging, Surrey, BC, Canada) and a Nikon Eclipse 80i microscope (Nikon Corporation, Tokyo, Japan) and analysed with the software Image-Pro Plus version 7.0 (Media Cybernetics, Silver Spring, MD, USA). Morphometric measurements, which included intima-media thickness (IMT), intima-media area (IMA), lumen diameter, lamellar units, circumferential wall tension (CWT), and tensile stress (TS), were done according to the method described by Fernandes-Santos et al. [22].

Briefly, four measurements of IMT per image were obtained at 0°, 90°, 180°, and 270° by drawing a line across the tunica intima and media. The measurements were averaged to get the value corresponding to the single image. Lumen area (a) was estimated by drawing a line over the circle delimited by the inner face of the intima layer. Then by using the values of a, the lumen diameter (d) was calculated as $d = (2\sqrt{a})/\pi$, where a is expressed in mm^2 and π is 3.14. The mean cross-sectional area of the tunica intima and tunica media (intima-media area, IMA) was calculated as IMA $= [\pi(d/2 + \text{IMT})^2] - [\pi(d/2)^2]$. The number of elastic fibres lamellae (lamellar units) in the tunica media was counted. CWT was calculated as CWT = MSBP × $(d/2)$, where CWT was expressed in dyne/cm, MSBP (mean systolic blood pressure) as dynes/cm^2, and d (lumen diameter) in cm. TS was calculated as TS = CWT/IMT. It was expressed in dyne/cm^2 and IMT in cm.

2.5. Immunohistochemical Study of VCAM-1. Aortic sections (5 μm) cuts were accomplished and adhered to polylysine glass slides (Polysine, Thermo Scientific, Braunschweig,

Table 1: Body weight and food intake for all the experimental groups.

	Groups				
	Control	FPO	1HPO	5HPO	10HPO
Food intake (g/week)	163.67 ± 4.60	151.42 ± 4.94	159.18 ± 5.01	153.61 ± 4.031	152.42 ± 4.11
Initial body weight (g)	252.50 ± 7.09	230.67 ± 4.52	230.17 ± 11.65	244.33 ± 10.48	245.33 ± 7.14
Final body weight (g)	485.83 ± 34.25*	477.50 ± 20.35*	440.67 ± 16.96*	503.67 ± 28.23*	504.00 ± 30.66*
Weight gain (g)	233.33 ± 36.38	246.83 ± 20.74	210.50 ± 27.46	259.33 ± 36.75	258.67 ± 36.56

Data are expressed as mean ± SEM. FPO: fresh palm oil; 1HPO: one-time-heated palm oil; 5HPO: five-time-heated palm oil; 10HPO: ten-time-heated palm oil.
*$P < 0.05$ versus initial body weight for the same group.

Germany). After deparaffinised and hydrated gradually, the sections were rinsed and subjected to microwave antigen retrieval in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). After blocking endogenous peroxidase and nonspecific background staining, the aortic sections were then incubated with anti-VCAM-1 antibody (1 : 100, sc-8304, Santa Cruz Biotechnology, CA, USA) at room temperature for an hour. After washing, the reaction was amplified with a micropolymeric labelling technology (UltraVision Quanto Detection System HRP DAB, Thermo Fisher Scientific, Fremont, CA, USA). Antibody binding was visualised with diaminobenzidine. Sections were then counterstained with haematoxylin.

VCAM-1 immunostaining was quantified as described by Moraes-Teixeira et al. [23]. Briefly, tunica intima boundary was delimited by drawing a line over it using an irregular "area of interest" tool. Inside the delimited tunica intima, VCAM-1 immunostaining was selected and segmented into a new binary image, where white colour represented immunostaining and black colour represented unstained area. The percentage of area that was occupied by white colour was quantified using the image histogram tool [24]. VCAM-1 immunostaining was expressed as the percentage of tunica intima area (%). Measurements were obtained from five nonconsecutive aortic sections from each animal.

2.6. Statistical Analysis. All results were expressed as mean ± SEM. Normality of data was determined using Kolmogorov-Smirnov test. Paired Student's *t*-test was used to compare pre- and posttreatment data. The data among groups were analysed using one-way analysis of variances (ANOVA) followed by Tukey's Honestly Significant Differences (HSD) post-hoc test. Correlation between blood pressure and VCAM-1 density was analysed using Pearson's correlation test for all the animals irrespective of treatment groups. A value of $P < 0.05$ was considered as statistically significant. All statistical analyses were performed using the SPSS version 14.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Body Weight and Food Intake. There was a significant increase ($P < 0.05$) in body weight at the end of this study in all groups. However, the body weight gain and final body weight did not significantly differ among the groups. There was no significant difference in the weekly food intake in all study groups as well (Table 1).

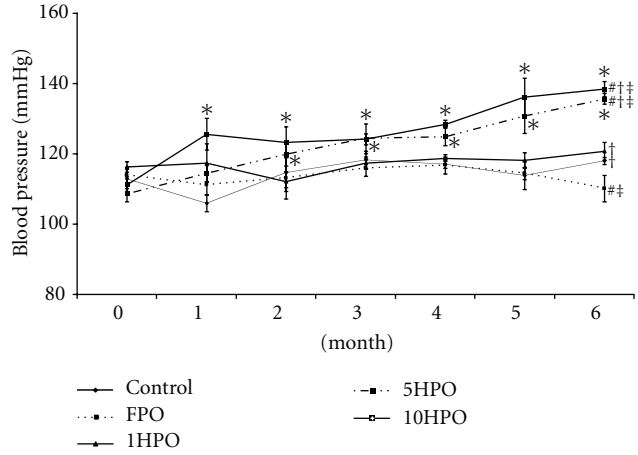

Figure 1: Blood pressure in all groups during the study period. Data are expressed as mean ± SEM. FPO, fresh palm oil: 1HPO, one-times-heated palm oil: 5HPO five-time-heated palm oil; 10HPO ten-time-heated palm oil. *$P < 0.05$ between pre- and posttreatment values for the same group; #$P < 0.05$ versus control; †$P < 0.05$ versus FPO; ‡$P < 0.05$ versus 1HPO.

3.2. Blood Pressure. By the end of the study, there was a significant increase ($P < 0.05$) in blood pressure in rats fed 5HPO or 10HPO along and at the end of the study, which was observed as early as after the first month of diet administration. Rats fed 5HPO or 10HPO showed a significant increase ($P < 0.05$) in blood pressure compared to the control, FPO, and 1HPO groups. On the other hand, the blood pressure of the rats fed basal diet (control), FPO, or 1HPO did not change significantly throughout the experiment. However, we found that the rats fed FPO showed significantly lower blood pressure at the final month compared to all experimental groups (Figure 1).

3.3. Aortic Morphometry. Aortic sections from rats fed 5HPO or 10HPO showed significant increase ($P < 0.05$) in IMT compared to control, FPO, and 1HPO groups. Aortic IMA from 5HPO and 10HPO groups were also significantly greater ($P < 0.05$) than the control, FPO, and 1HPO groups. However, lumen diameter and elastic lamellar units did not

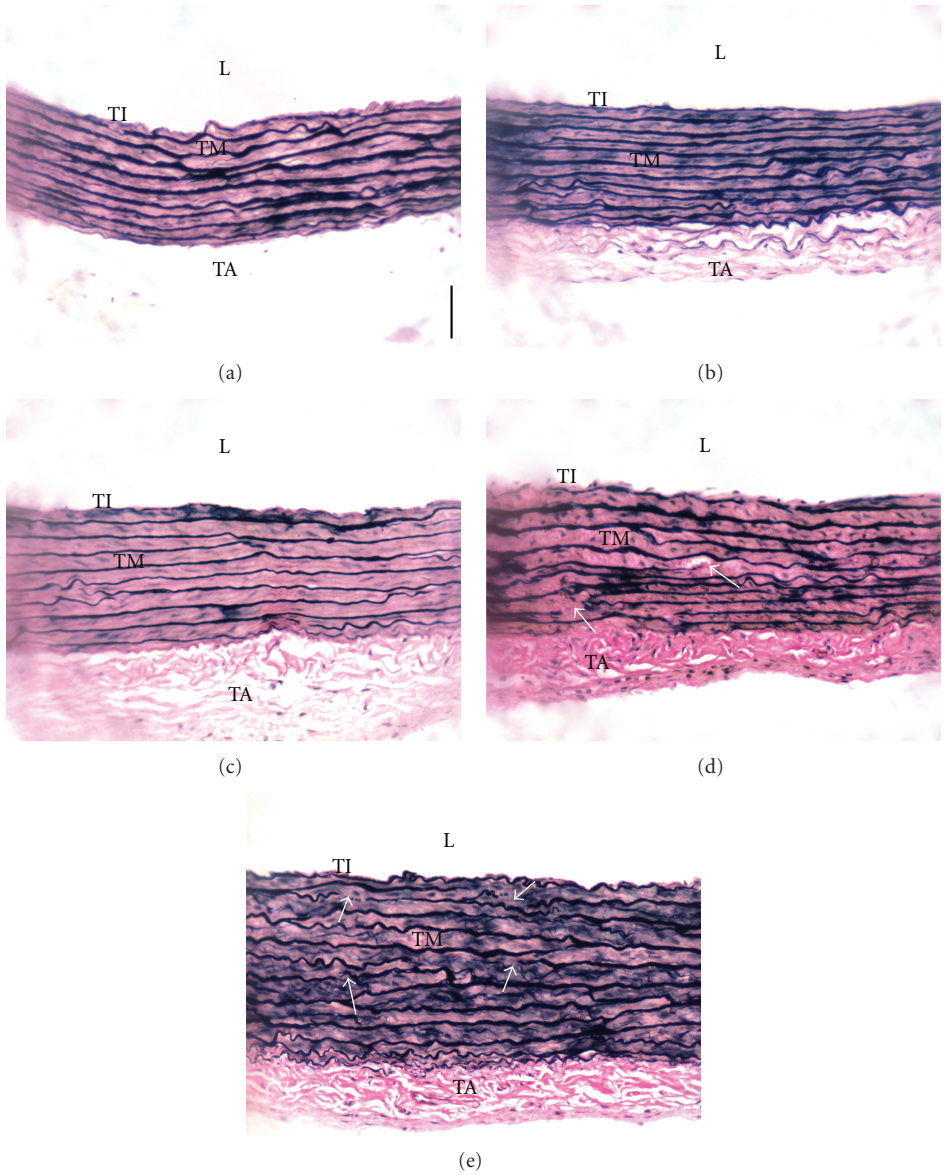

Figure 2: Photomicrographs of aortic sections stained with Verhoeff-Van Gieson. Groups are as follows: (a) control rats; rats fed, (b) fresh (FPO), (c) one-time-heated (1HPO), (d) five-time-heated (5HPO), or (e) ten-time-heated palm oil (10HPO). Thickened tunica media is observed in 5HPO and 10HPO groups [(d) and (e)], with an increased interlamellar space when compared to the control and FPO groups [(a) and (b)]. Disorganisation and fragmentation of the elastic lamellae were also observed in 5HPO and 10HPO (arrow, (d) and (e)). L: lumen; TI: tunica intima; TM: tunica media; TA: tunica adventitia. Same magnification is applied to all pictures (×200). Calibration bar = 50 μm.

differ significantly among the groups. With increased IMT and IMA but unaltered lumen diameter, a hypertrophic outward remodelling was indicated in the 5HPO and 10HPO groups. CWT was increased significantly ($P < 0.05$) in rats fed 5HPO or 10HPO compared to the control, FPO, and 1HPO groups. We did not observe significant difference in CWT between the control, FPO, and 1HPO groups. There were no significant differences in TS among the groups (Table 2).

Aortic architecture in rats fed 5HPO or 10HPO were observed and characterised by an increase in interlamellar space in the tunica media when compared to the control and FPO groups. In addition, the elastic lamellae in 5HPO and 10HPO groups were observed to be disorganised and fragmented (arrow, Figures 2(d) and 2(e)). On the other hand, the aortic structure in FPO, and 1HPO groups did not show much remarkable difference than the control (Figures 2(a)–2(c)).

3.4. Expression of VCAM-1. Positive immunostaining for VCAM-1 was observed in the endothelial cells. The aortic VCAM-1 expression was found to be significantly higher ($P < 0.05$) in rats fed 5HPO or 10HPO than the control, FPO,

Table 2: Aortic morphometric measurements.

	Group				
	Control	FPO	1HPO	5HPO	10HPO
Intima-media thickness (μm)	105.26 ± 2.18	107.87 ± 1.38	112.00 ± 5.51	134.54 ± 1.71*	143.09 ± 3.83*
Lumen diameter (mm)	1.33 ± 0.03	1.27 ± 0.04	1.33 ± 0.04	1.39 ± 0.04	1.35 ± 0.04
Intima-media area (mm^2)	0.48 ± 0.02	0.47 ± 0.01	0.51 ± 0.04	0.65 ± 0.02*	0.67 ± 0.02*
Lamellar units	10.33 ± 0.67	9.67 ± 0.51	10.07 ± 0.28	9.89 ± 0.71	10.58 ± 0.56
CWT (10^4 dyne/cm)	1.05 ± 0.06	0.97 ± 0.03	1.06 ± 0.04	1.25 ± 0.03*	1.27 ± 0.05*
Tensile stress (10^4 dyne/cm^2)	99.88 ± 5.65	89.93 ± 3.04	95.73 ± 3.80	92.88 ± 3.37	89.08 ± 5.05

Data are expressed as mean ± SEM. FPO: fresh palm oil; 1HPO: one-time-heated palm oil; 5HPO: five-time-heated palm oil; 10HPO: ten-time-heated palm oil; CWT, circumferential wall tension.
*$P < 0.05$ versus control, FPO, and 1HPO.

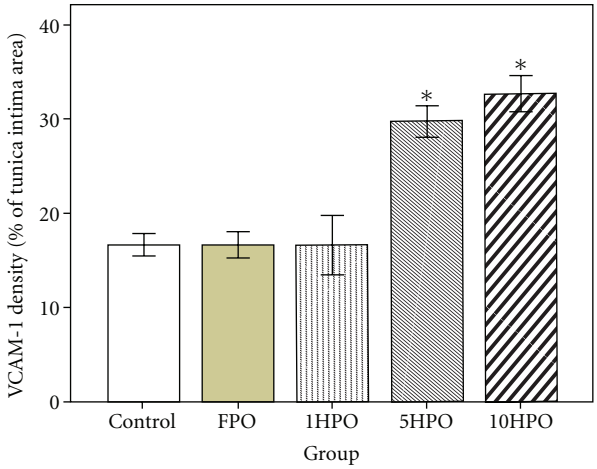

Figure 3: Endothelial VCAM-1 expression in rats. Data are expressed as mean ± SEM. FPO, fresh palm oil; 1HPO, one-time-heated palm oil; 5HPO, five-time-heated palm oil; 10HPO, ten-time-heated palm oil. *$P < 0.05$ versus control, FPO, and 1HPO.

and 1HPO groups (Figure 3). As shown in Figures 4(a)–4(c), little VCAM-1 immunostaining was observed in the aortic sections of rats fed FPO or 1HPO when compared to the control. On the other hand, the aortic VCAM-1 expression on tunica intima was distinctly denser in the rats that fed 5HPO or 10HPO when compared to the control (Figures 4(d) and 4(e)).

3.5. Correlation between Blood Pressure and VCAM-1 Expression. There was a significant positive relationship ($r = 0.757$, $P < 0.001$) between systolic blood pressure and aortic VCAM-1 expression (Figure 5).

4. Discussion

This study was carried out to ascertain the involvement of inflammation in blood pressure elevation after consumption of heated palm oil We postulated that heating the palm oil repeatedly would generate harmful ROS and hence induce inflammation and endothelial dysfunction.

In the present study, we observed a significant increase in blood pressure in the rats fed 5HPO or 10HPO compared to the control and rats fed FPO or 1HPO. This significant increase in blood pressure was in agreement with a previous study [4] showing prolonged intake of repeatedly heated palm oil increased blood pressure. Osim et al. [25] also reported that the oxidised oil-fed group had a greater rise in blood pressure than the fresh oil-fed group. A study carried out by Soriguer et al. [3] showed that the risk of hypertension was positively correlated with the consumption of polar compounds that were present in the cooking oil. Repeatedly heating makes the oil more susceptible to lipid peroxidation [26], which also reduces the vitamin E constituents such as α-tocopherol, α-tocotrienol, and γ-tocotrienol in palm and soy oils [27]. Consumption of repeatedly heated palm oil in postmenopausal state may contribute to the development of atherosclerosis because of increased lipid peroxidation [28]. Therefore, the deleterious effect of prolonged intake of 5HPO and 10HPO on blood pressure that we observed might be contributed by the overproduction of ROS that causes vascular inflammation and impairs the endothelial function. Oxidative stress, due to over-production of ROS, exerts endothelial dysfunction which plays a key role in pathogenesis of hypertension. A study done by Chan et al. [29] demonstrated that the increased levels of ROS may contribute to oxidative stress and hypertension in rats.

Blood pressure in the rats fed FPO or 1HPO did not show any remarkable change throughout the experiment. In fact, interestingly, we found that rats fed FPO even showed a significantly lower blood pressure after six months compared to the control. This shows that palm oil did not only prevent the increase of blood pressure but also had a tendency to lower it. Palm oil is rich in natural antioxidants like tocotrienol which have beneficial effect on oxidative stress associated with hypertension [30]. Medeiros et al. [31] also demonstrated that the long-term intake of palm oil had beneficial effect in reducing blood pressure in spontaneously hypertensive rats. Free radical scavenging antioxidants in palm oil serve to protect endothelial cells against oxidative injury and thus improve endothelial functions [32]. Furthermore, the blood pressure lowering mechanism of palm oil may also involve the beneficial alteration in endothelium-derived factors [33]. The present finding provides further support to the cardiovascular protective effect of palm oil.

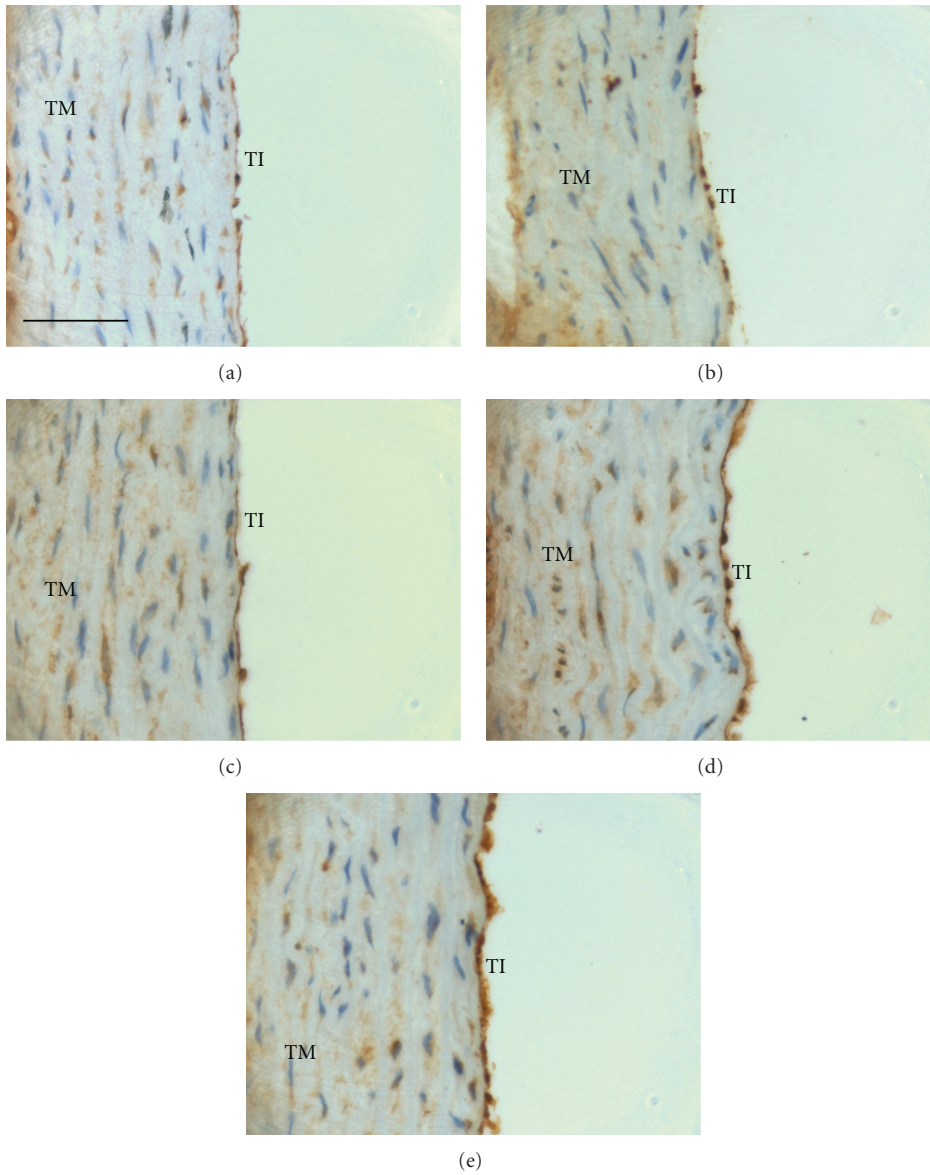

Figure 4: Photomicrographs of aortic sections showing immunostaining for VCAM-1. Groups are as follows: (a) control rats; rats fed (b) fresh (FPO), (c) one-time-heated (1HPO), (d) five-time-heated (5HPO), or (e) ten-time-heated palm oil (10HPO). Aortic sections from rats fed 5HPO and 10HPO showed an intense staining of VCAM-1 on the tunica intima [(d) and (e)]. In contrast, aortic sections from rats fed FPO and 1HPO showed relatively little staining of VCAM-1 [(b) and (c)] compared to control (a). TI: tunica intima; TM: tunica media. Same magnification is applied to all pictures (×400). Calibration bar = 50 μm.

From morphometric aspects, we observed that prolonged consumption of 5HPO or 10HPO resulted in significantly increased thickness (IMT) and area (IMA) of intima-media of the aortic wall in rats compared to those fed basal diet, FPO, or 1HPO. This finding suggests that prolonged consumption of heated oils causes vascular hypertrophic remodelling. IMT thickening and vascular architectural changes are commonly associated with high blood pressure [34]. Therefore, IMT is a marker of coronary disease [35]. Albeit increased IMT, the number of elastic lamellae did not differ among the groups, which might suggest that the IMT thickening is due to vascular smooth muscle cells hypertrophy, as indicated by increased interlamellar space. We believe that the ROS present in the heated oils may play a role in this remodelling. Previously, we observed a reduction in nitric oxide (NO) level following administration of repeatedly heated oil in rats [36]. Increased ROS generation may contribute to endothelial dysfunction by decreasing the bioavailability of NO which functions to reduce cellular proliferation. Hypertension is accompanied by an altered biochemical environment between the factors that act as cell growth promoters and the factors that reduce cellular proliferation [37]. This may lead to an imbalance between the rate of growth and death, causing wall hypertrophy.

Nakaki and Kato [38] also reported that vascular remodelling could be due to decreased NO and high blood pressure. Further, TS (which is the tension per unit of thickness and acts perpendicularly to the wall) did not differ among the groups, maybe due to the IMT thickening. On the other hand, there was no significant difference of lumen diameter among the groups. This may suggest that the increased CWT that we observed in rats fed 5HPO or 10HPO could be due to the elevated blood pressure in them. CWT is the force that acts in longitudinal and circumferential directions to oppose the distending effects of blood pressure. High blood pressure increases CWT, in which further predisposing the vascular wall to damage and impairing its normal functions [39].

Prolonged consumption of 5HPO or 10HPO significantly increased the aortic endothelial expression of VCAM-1 in rats when compared to the control, FPO, and 1HPO groups. FPO that did not induce VCAM-1 expression might be due to its rich antioxidant contents that maintain the endothelial oxidative status [30]. VCAM-1 is induced on endothelial inflammatory sites [40] and this finding suggests that 5HPO and 10HPO might induce vascular inflammation. The repeated deep frying process is deleterious on the oxidative stability and biochemical characteristics of the oil, in which it generates ROS and other lipid oxidation products. Our earlier experiment [28] reported that heated palm oil increased lipid peroxidation as indicated by a significant increase in serum thiobarbituric acid reactive substance (TBARS). Moreover, the effect of deep frying process on oil thermoxidation has been documented by previous studies [41–44]. ROS in the diet are absorbed into the blood circulation and their increased presence can overwhelm the intercellular antioxidant defence, leading to oxidative stress which can induce endothelial injury [2, 45]. Vascular endothelial cells, which provide a physical barrier for the underlying smooth muscle cells and play a pivotal role in maintaining cardiovascular homeostasis, are sensitive to disturbances in the redox steady state [46]. Previous reports have demonstrated the association between the disturbance in the redox steady state with modulated endothelial function and inflammation [47–50]. Therefore, prolonged intake of 5HPO and 10HPO may induce inflammation, hence endothelial cells respond by expressing VCAM-1.

VCAM-1 is a member of the immunoglobulin gen superfamily that participates in the adhesion and migration of leukocytes into tissues during immune response. We measured the aortic expression of VCAM-1 in rats because VCAM-1 is expressed on endothelial cells in inflammatory sites. Although VCAM-1 has been well documented to be associated with the development of atherosclerosis [51–53], several studies also demonstrated that the expression of VCAM-1 was increased in patients with hypertension [17–19, 54]. A study performed by Parissis et al. [17] showed that hypertensive patients exhibited higher plasma levels of VCAM-1. We found that there was a significant positive correlation between blood pressure and VCAM-1 density on endothelial cells. This suggests that augmented VCAM-1 expression may reflect inflammation and endothelial dysfunction and hence impairing the regulation of blood pressure. This is because a significant reduction in the cell adhesion molecules expression may indicate a better preservation of endothelial function [55]. Besides, we do believe that the increased VCAM-1 (that is inflammation) may play a role, at least in part, in causing adverse remodelling. A previous study documented that increased inflammatory mediator aggravated vascular remodelling [56]. Remodelling impairs the compliance of the blood vessel and leads to pathogenesis of raising blood pressure [57].

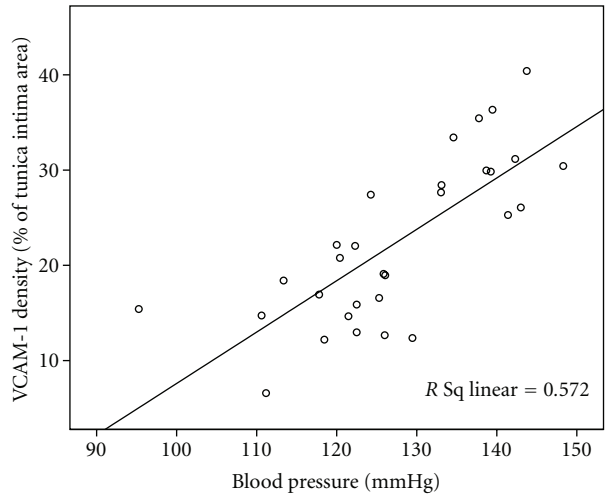

Figure 5: Correlation between blood pressure and endothelial VCAM-1 expression in rats.

In addition, our results suggest that intake of 5HPO and 10HPO is detrimental to vascular morphometry as well as the regulation of blood pressure. Intake of 5HPO and 10HPO increased blood pressure, morphometric alterations, and expression of VCAM-1 significantly than 1HPO. In fact, we found that rats fed 1HPO did not exhibit significant change when we compared to the control and FPO groups. Palm oil contains saturated fatty acids and unsaturated fatty acids at almost equal ratio [58], in which saturated fatty acids are more resistant to thermoxidation. Though being heated once, the palm oil might still retain abundant antioxidants and generate less ROS than 5HPO and 10HPO and hence protects the vascular endothelium from inflammation. Therefore, it suggests that intake of palm oil heated once might not cause remarkable harmful effect in rats. However, when the oil is reused repeatedly, deleterious effects ensue.

This study provides the hints about the pivotal role of inflammation in the blood pressure raising effect of repeatedly heated palm oil. Repeatedly heated palm oil induces inflammation and vascular remodelling in the rats, which subsequently leads to an increase in blood pressure. Further research need to focus on more inflammatory biomarkers such as intercellular adhesion molecule-1 (ICAM-1), endothelin-1, and others as well as their pathways to understand thepagebreak possible molecular mechanisms involved in the inducing of hypertension by heated palm oil.

5. Summary

In conclusion, prolonged intake of repeatedly heated palm oil appears to increase blood pressure in rats, which might

be mediated by inflammation and endothelial dysfunction, as reflected by the adverse vascular remodelling and the induction of VCAM-1 expression on endothelial cells.

Acknowledgments

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Research Article

Imaging-Based Biomarkers: Characterization of Post-Kawasaki Vasculitis in Infants and Hypertension Phenotype in Rat Model

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Background. Investigating the mechanical properties of the arteries is essential in cardiovascular diseases. Recent imaging modalities allow mapping mechanical properties within the arterial wall. *Aims*. We report the potential of *imaging-based biomarker* (ImBioMark) to investigate the effect of aging on the rat. We also present preliminary data with ImBioMark characterizing vascular sequelae of Kawasaki disease (KD) in young humans. *Methods*. We investigated *in vivo* the effect of aging on male Brown Norway (BN) rats' ($n = 5$) carotid stiffness. In a second experiment, the impact of KD on the ascending aorta (AA) was examined in KD children ($n = 2$) aged 13 ± 1.41 years old compared to KD-free children ($n = 5$) aged 13.13 ± 0.18 years old. *Results*. The stiffness of BN's carotid artery was three times stiffer in the old rats, with a turning point at 40 weeks old ($P = 0.001$). KD had a very significant impact on the AA stiffness with strain estimates of $2.39 \pm 0.51\%$ versus $4.24 \pm 0.65\%$ in controls ($P < 0.001$). *Conclusion*. ImBioMark phenotypes hypertension in rat models noninvasively *in vivo* without resorting to euthanasia. Quantifying aortic wall remodeling is also feasible in humans. Future investigations target human cardiovascular disease.

1. Introduction

Age-related stiffening of large arteries reduces their function of transforming a pulsatile to a more even blood flow; this may be the most serious change affecting the cardiovascular system [1, 2]. Accordingly, arterial stiffness has been reported to be an independent predictor of cardiovascular mortality [3]. Provided there is progressive fibrosis of large arteries that reduce their elasticity, aging is known to be the dominant process altering arterial stiffness. This stiffness alteration with age is accelerated in hypertension and amplified by the association with other cardiovascular diseases.

On the other hand, Kawasaki disease (KD) is an immune-mediated pathology that mostly affects children under 5 years old, and in which multiorgan vasculitis affects large and mid size arteries. KD was described in 1967 by Tomisaku Kawasaki as a self-resolving disease causing acute vasculitis [4, 5]. The prognosis of the disease largely depends on the extent of coronary artery (CA) complications that may lead to myocardial infarction and possibly to death [6, 7]. Nevertheless, the reported dilatation of the ascending aorta (AA) along with aneurismal formation in major arteries, such as the axillary, carotid, renal, celiac, and femoral arteries to name a few [8], compelled us to address potential remodelling of the AA. Our initial experience involved 64 KD patients, among whom 15 had CA aneurysms, and 154 healthy children aged 9.7 ± 6.1 and 7.9 ± 5.1 years, respectively ($P = 0.39$) [9]. The methodology that was used in that work utilized equations conceived for invasive measurements and adapted for noninvasive ultrasound derived data [10]. Accordingly, significant alterations were observed in the elastic pressure strain modulus, the β resistance index, as well as

the distensibility. On this basis, we are planning to perform direct measurements from the AA wall motion and characteristics. The methodology we expose in the current paper represents the working template of our group.

In addition, mechanical properties of biological soft tissues can be used as a diagnostic tool and potentially as an element for prognostic estimates as well. With this regards, Non-Invasive Vascular Elastography (NIVE) was proposed to characterize superficial arteries [11]. We recently addressed the feasibility and reproducibility of NIVE for the purpose of *in vivo* applications in human carotid arteries [12]. In that clinical trial, we derived an elastic modulus calculation that, on average, exhibited less than 100 kPa for the population free of vascular disease. Data were reported for the left and right common and internal carotid arteries, respectively.

NIVE has been extended to investigate small vessel walls in the order 150 μm in rodent models of hypertension, which was labelled as MicroNIVE [13]. In a very recent study, we observed that female recombinant inbred hypertensive rat model (RI-17) carotid stiffness increased with age by a factor of about 2 between the age of 30 and 80 weeks old [14]. In that same paper, we also reported preliminary data on the impact of various salt diets on the carotid stiffness in different hypertensive rat models. Accordingly, a high salt diet induces an increase in BP, which could subsequently produce the carotid artery wall remodeling only in a mid- or long-term perspective. MicroNIVE can, therefore, provide reliable mechanical markers for vascular tissue investigations. We herein refer to the technique as an Imaging-based BioMarker (ImBioMark).

This paper reports additional data of ImBioMark that confirm the effect of aging on the rat carotid artery stiffness. The common carotid artery of male Brown Norway (BN) rats ($n = 5$) was investigated *in vivo* and throughout the animals' lifetime, that is, from 15 up until 105 weeks old. We also report preliminary feasibility data supporting the potential of ImBioMark to investigate human vascular sequalae as we study the impact of KD on the AA.

2. Methodology

2.1. Hypertension Phenotype in Rat Models

2.1.1. Animal Models. The present investigation conforms to Canadian Council on Animal Care (CCAC) guidelines as well as the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). All procedures were approved by the institutional Animal Care Committee of the *Centre hospitalier de l'Université de Montréal* (CHUM).

In this experiment, the left and right common carotid arteries of 5 male BN rats were studied *in vivo* and throughout animals' lifetime, that is, from 15 up until 105 weeks old. They were anaesthetized by inhalation of 1.5% isofluorane during ultrasound scanning. Body temperature was maintained at 37 ± 1°C by a heated surface and monitored with a rectal probe (Thermalert TH-5, Physitemp Instruments, Clifton, NJ, USA). Systolic blood pressure (SBP) was measured with a tail-cuff monitoring system (Model XBP-1000, Kent Scientific, Torrington, CT, USA); such pressure is indicative of mean systemic pressure of the animals [15]. Hair over the neck was shaved and further removed with depilatory cream (Nair, Church & Dwight Co., USA) to facilitate ultrasound recordings with a coupling gel.

2.1.2. Experimental Setup. Time sequences, that is, dynamic radio-frequency (RF) images, were recorded, for typically 7 cardiac cycles (CC), over longitudinal segments of the arteries with the *Vevo 660* ultrasonic biomicroscope (Visualsonics, Toronto, ON, Canada). The *Vevo* was equipped with an encapsulated oscillating single element transducer. A 40-MHz central frequency probe (f number = 2, diameter = 3 mm, focal length = 6 mm, and fractional bandwidth at −6 dB = 110%) was used. The frame rate was 60 images/s. All data were digitized with an acquisition board (Model 8500 CS, Gagescope, Montreal, QC, Canada) installed in a personal computer. Sampling frequency was 500 MHz in an 8-bit format.

2.1.3. Motion Estimation. The motion estimation method that is used to compute mechanical parameters from BN rats' carotid RF data is described in detail elsewhere [13]. In summary, it required partitioning the RF data into small measurement-windows (MW) in which the tissue motion is assumed to be affine between two consecutive images. More explicitly, let us consider an MW at time "t" ($I(x(t), y(t))$) and the same MW at time "$t + \delta t$" ($I(x(t + \delta t), y(t + \delta t))$). The method consists of computing the affine transformation that allows the best match between MW at "t" and "$t + \delta t$", that is

$$\underset{\Delta}{\mathrm{MIN}} \| I(x(t), y(t)) - I(x(t + \delta t) - [T_1], y(t + \delta t) - [T_2]) \|^2. \tag{1}$$

As a first step of the algorithm, lateral (T_1) and axial (T_2) translations are computed using cross-correlation technique. Following that, the 2D-deformation matrix (Δ) is then assessed through solving a non linear minimization problem. Δ can be expressed as

$$\Delta(t) = \begin{bmatrix} \Delta_{xx}(t) & \Delta_{xy}(t) \\ \Delta_{yx}(t) & \Delta_{yy}(t) \end{bmatrix}, \tag{2}$$

where Δ_{xx} is the lateral strain, Δ_{yy} is the axial strain, and Δ_{xy} and Δ_{yx} are lateral and axial shear parameters, respectively. Provided the axial strain is a relative measure of stiffness, data are reported for only Δ_{yy} in the context of this paper.

Δ_{yy} was computed for each pair of consecutive RF images using MW of $108 \times 312\ \mu\mathrm{m}^2$ with 90% axial and lateral overlaps. A wall mean strain value ($\bar{\Delta}_{yy}$) was calculated from each Δ_{yy}. Because of RF echo attenuation with depth that reduces the signal-to-noise ratio (SNR), strain measurements were performed only at the near wall.

2.2. Characterization of Post-KD Vasculitis

2.2.1. The Pediatric Population. Data from a small sample ($n = 2$) of children diagnosed with KD during the preschool age were compared to samples obtained from healthy children ($n = 5$) of similar age at the time of this investigation (13 ± 1.41 years old versus 13.13 ± 0.18 years old, resp.). B-mode echocardiography video sequences were acquired in the left parasternal acoustic window at the second or third intercostal space using either a iE33 Philips (Philips, Eindhoven, the Netherlands) or a GE Vivid 8 (GE Healthcare, Louisville, Kentucky) echocardiograph. Images were focused on the ascending aorta at the point of intersection with the right pulmonary artery. Three loops of 4 to 5 beats were recorded serially with simultaneous electrocardiographic signal recordings and blood pressure measurements with an automatic sphygmomanometer (Welch Allyn, Inc., Skaneateles Falls, NY). The acquired images were obtained in the context of the noninvasive clinical research project cited above, which obtained approval from our institutional ethics board.

2.2.2. Motion Estimation. The motion estimation method that was used to compute mechanical parameters from the AA is similar to the one described elsewhere for B-mode data [14]. However, since the proximity of the AA with the heart and the lungs induces very complex artifactual movements of this artery, a previous rigid registration procedure was then implemented prior to tissue motion estimation.

2.3. Statistical Analyses. "SigmaStat" (Systat Software Inc., ver. 3.11.0, 2004) was used for statistical analyses. Data on the effect of aging on the carotid stiffness in rat models of hypertension (HT) were computed with One-Way Analysis of Variance (ANOVA) Kruskal-Wallis' and Dunnett's tests to identify the turning point where the increase in stiffness mainly takes place. Otherwise, One-Way ANOVA Tukey test was used as well to investigate physiological parameters in rat models of HT as to compare post-KD sequelae at the AA level.

3. Results

3.1. ImBioMark Implementation in Rat Models' Carotid

3.1.1. Stiffness Quantification. Since a high frequency ultrasound transducer (40 MHz) was used, the carotid far wall is most of the times a lot less identifiable than the near one, that is because of signal attenuation with depth. Owing to that, the strain elastograms were analyzed with respect to the near wall. Figure 1(a) displays a typical RF image of BN's carotid, whereas, for illustrative purposes, Figure 1(b) presents the equivalent B-mode image. Figures 1(c) and 1(d), respectively, exhibit typical systolic and diastolic axial strain distribution images (Δ_{yy}), also said elastograms, which superimpose the B-mode. In this display, the elastograms were segmented manually and postprocessed with a 5×5-pixel median filter. Color bars give strain in percentages. In this configuration, blue is associated with positive strain values, while yellow is indicative of negative values. Systolic axial strain elastograms are characterized by positive strain values, indicating vessel wall compression. Inversely, diastolic strain elastograms are characterized by negative values within the wall, indicating dilation.

As previously described [14], mean axial strain (MAS) was calculated for a 5×9-pixel (axial lateral) region-of-interest (ROI) within the near wall of each axial strain elastogram to provide the MAS curve. Such a MAS curve is plotted in Figure 1(e) over 7 consecutive CC. As can be observed in Figure 1(e), peak systolic strain (PSS) and peak diastolic strain (PDS) values are very stable and reproducible. We then propose PSS, which averages PSS values over 3 CC, and PDS, which averages PDS values over 3 CC, as stiffness parameters.

3.1.2. Stiffness as a Function of Aging. One-way ANOVA indicated no significant statistical difference between left and right carotids' strain values, with $\Delta_{yy} = 6.52 \pm 3.51\%$ and $\Delta_{yy} = 6.52 \pm 3.76\%$, respectively ($P = 0.681$). *Pooling* data for sides (left/right), no significant statistical difference was found between PDS and PSS, in absolute values, with $6.08 \pm 3.06\%$ and $6.93 \pm 4.05\%$ strain values, respectively ($P = 0.189$). The mean strain value that is reported to evaluate BN's carotid stiffness as a function of aging was obtained by *pooling* sides (left and right) and CC phase (systole and diastole); the animal population was then virtually increased by a factor of 4.

Figure 2 plots the interrelationships between BN's carotid stiffness (axial strain) and aging. The strain values stand from $12.24 \pm 3.84\%$ at 15 weeks old down to $3.91 \pm 0.95\%$ at 105 weeks old. In addition, Dunnett's test indicated that such an increase in the carotid stiffness mainly takes place around 40 weeks of age.

3.1.3. Physiological Parameters. Complementary to ImBioMark, we here report some physiological data. All those measurements, which also took place from animals' 15 up until 105 weeks of age, were recorded along with MicroNIVE data.

Figure 3 plots systolic blood pressure (SBP) follow-up as a function of aging. However, SBP monitored at 56 and 99 weeks of age were relatively high with respect to the other measurements. Provided that was basically experimental artefacts, we removed those two experiment SBP values from the statistical analysis. One-way ANOVA then indicated no significant change in SBP along time with an average of 95 ± 18 mmHg, $P = 0.108$. It is to note that BN's SBP usually sets around 120 mmHg. However, because of physiological effects of anaesthesia on the cardiovascular system, such an underestimation was quite expectable.

As plotted in Figure 4, we also investigated the follow-up of heart rate (HR) as a function of aging. One-way ANOVA indicated no significant change in HR along time with an average of 351 ± 26 heart-beats/min, $P = 0.075$. Similar to SBP, HR was likely slowed down because of anaesthesia.

To complete the physiological part of this longitudinal study, Figure 5 plots BN's weight as a function of aging.

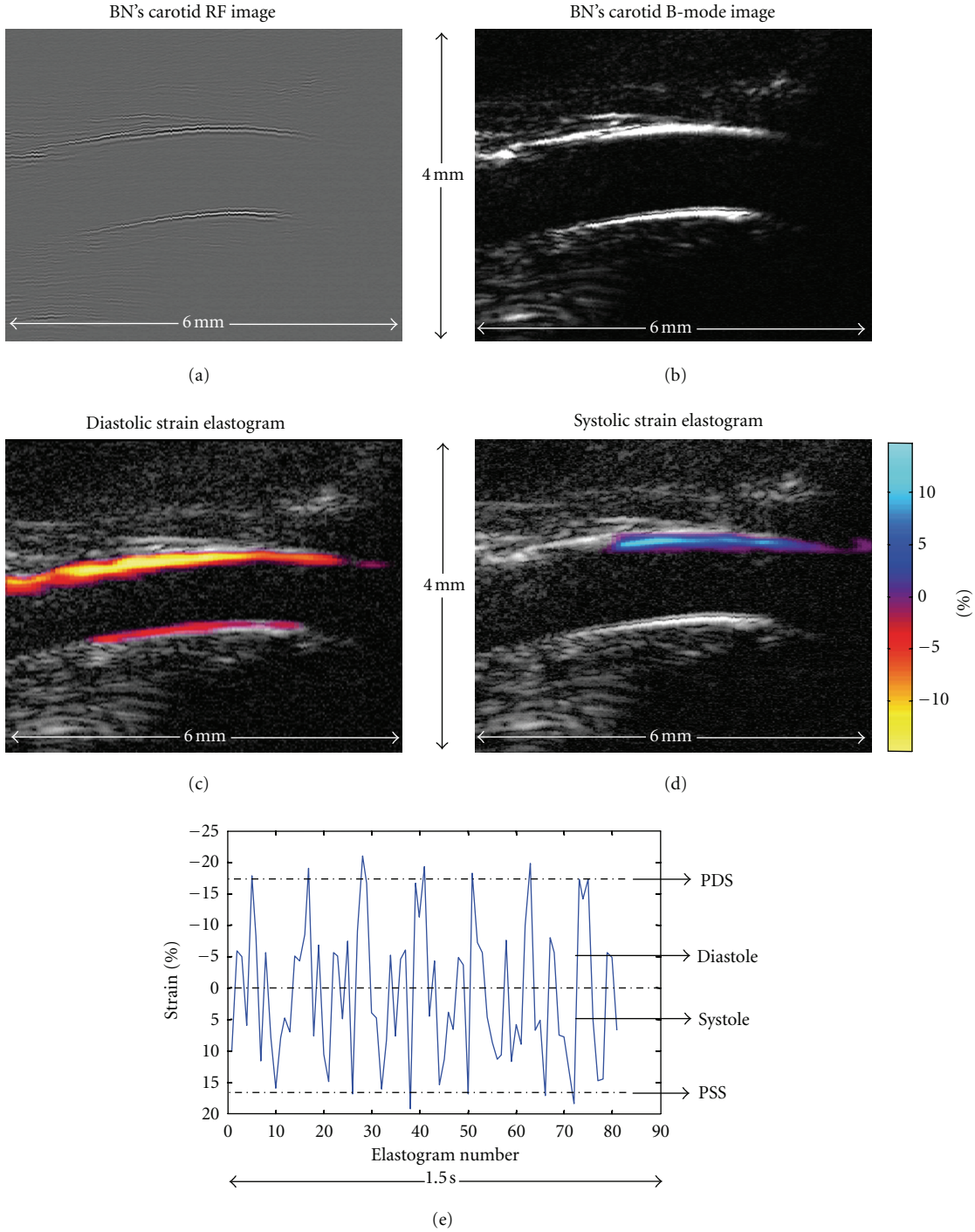

Figure 1: (a) BN's carotid RF image, (b) BN's carotid B-mode image, (c) diastolic strain elastogram that superimposes the B-mode image, (d) systolic strain elastogram that superimposes the B-mode image, (e) MAS curve showing the PDS and PSS parameters that were used as relative measures of stiffness.

As could be expected, the weight significantly increased with age, standing from 278 ± 13 g at 15 weeks old up to 482 ± 22 g at 105 weeks old, $P < 0.001$. Interestingly, the weight was observed to stabilize from around 60 weeks old, $P = 0.081$.

3.2. ImBioMark Implementation to Evaluate Post-KD Vasculitis. Figure 6(a) displays a typical B-mode image of a KD subject's AA. The artery stiffness was assessed within the near and far walls, respectively, for a 3 × 9-pixel (axial × lateral) ROI. Figure 6(b) plots the MAS curve that was obtained for the far wall of the AA given in Figure 6(a). In this case, we computed mean systolic and mean diastolic strains (MSS and MDS, resp.). Provided there sometimes is an offset between systolic and diastolic strains, a mean strain that is an average

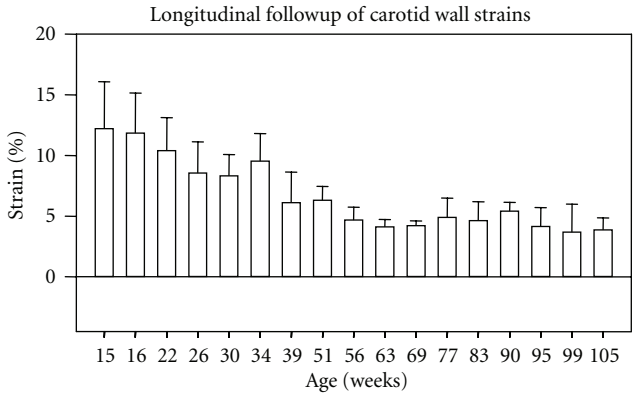

Figure 2: BN's carotid stiffness as a function of aging. Dunnett's test indicated that the increase in stiffness mainly takes place around 40 weeks of age.

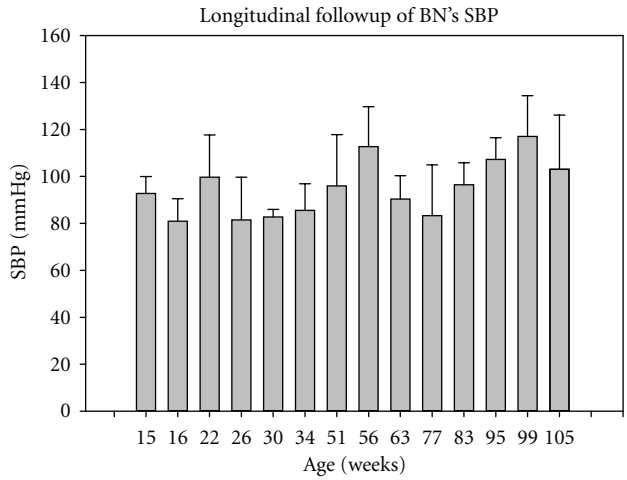

Figure 3: Follow-up of BN's SBP as a function of aging. By removing SBP values at 56 and 99 weeks old, no significant change in SBP was observed, $P = 0.108$.

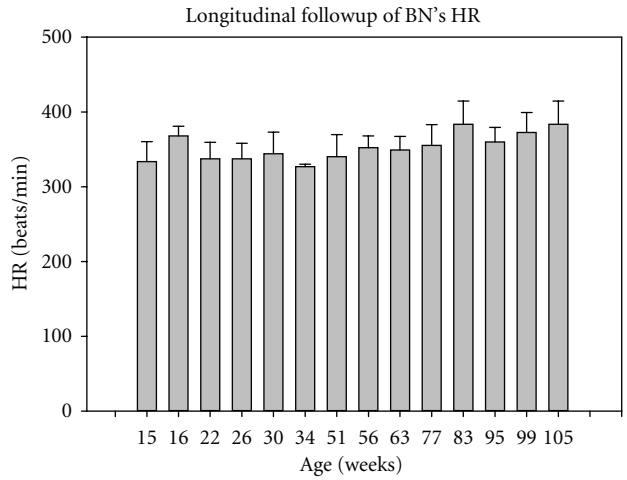

Figure 4: Follow-up of BN's heart rate (HR) as a function of aging. No significant change in HR was observed, $P = 0.075$.

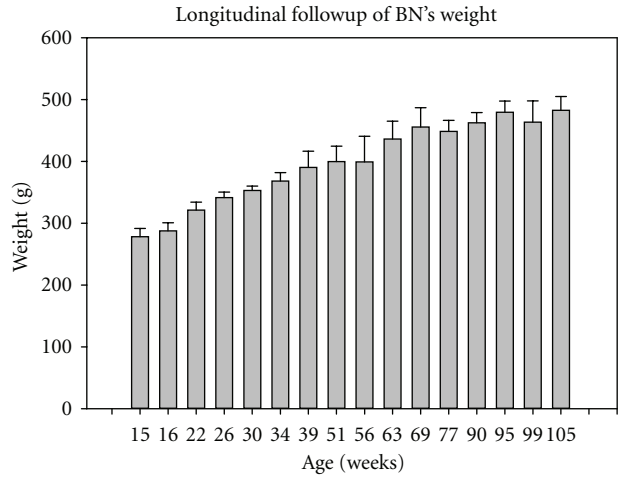

Figure 5: Follow-up of BN's weight as a function of aging. Standing from 278 ± 13 g up to 482 ± 22 g, it was observed to significantly increase with age ($P < 0.001$), but was observed to stabilize from around 60 weeks old ($P = 0.081$).

of MSS and MDS in absolute values was used to compare KD and KD-free subjects' AA stiffness.

One-way ANOVA indicated no significant statistical difference between near and far walls' strain values, with $\Delta_{yy} = 3.64 \pm 0.88\%$ and $\Delta_{yy} = 3.80 \pm 1.19\%$, respectively ($P = 0.635$). The mean strain value that is reported to compare KD and KD-free subjects' AA stiffness was obtained by *pooling* near and far walls. Provided three sets of data were recorded for each subject, we then *virtually* increased our population by a factor of 6. Figure 7 exhibits a histogram comparing KD and KD-free subjects' AA stiffness. One-way ANOVA indicated that there is a statistical difference between the two groups, with $\Delta_{yy} = 4.24 \pm 0.65\%$ (KD-free) and $\Delta_{yy} = 2.39 \pm 0.51\%$ (KD), $P < 0.001$.

4. Discussion

4.1. Hypertension Phenotype with ImBioMark. In a previous paper [14], we have reported data on the effect of salt diet on the carotid artery stiffness of rat models of HT. In the current work, we have investigated the interrelationships between carotid stiffness and aging. We also report some physiological data. We observed that BN's carotid strain decreases with age by a factor close to 3, but with no significant change in SBP.

Provided that the *in vivo* strain measurements were not influenced by physiological parameters (blood pressures and heart rate) and that strain is inversely proportional to stiffness, these results confirm that the carotid artery becomes stiffer with aging. Very interestingly, it is also suggested that this increase in stiffness may take place around a nominal age, that is, 40 weeks old for BN.

The main innovative relevance of MicroNIVE stems from the fact that it would allow *in vivo* investigation of HT, noninvasively and throughout an animal's lifetime. Indeed, despite impressive progress in engineering animal models, such longitudinal studies were not possible before MicroNIVE. To

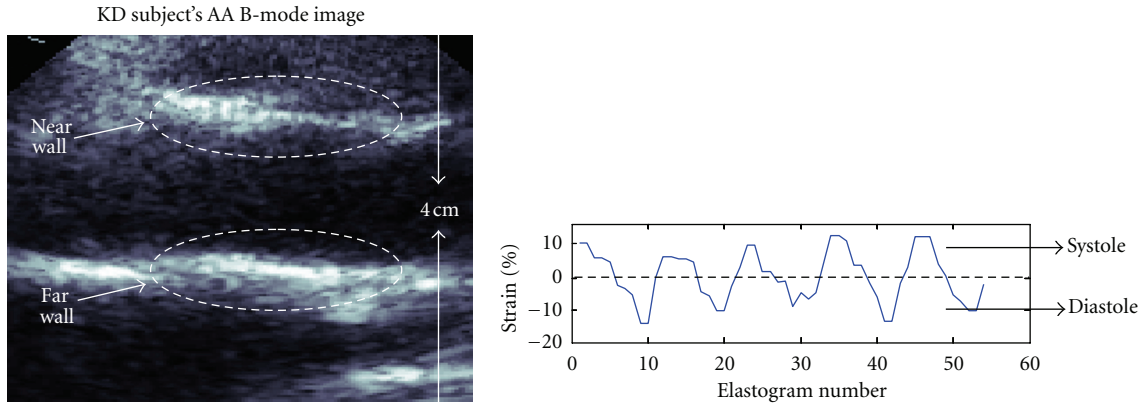

Figure 6: (a) Ascending aorta B-mode image of a KD subject and (b) MAS curve clearly delineating the systolic phase (with positive strain values) and diastolic phase (with negative strain values).

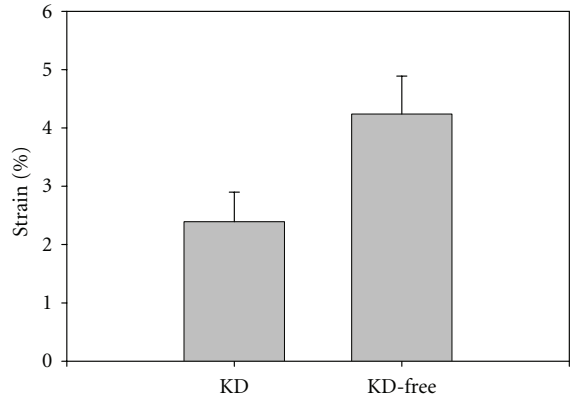

Figure 7: Comparison between KD and KD-free subjects' ascending aorta stiffness. The two groups were found statistically different, $P < 0.001$.

study the pathophysiology of HT, groups of animals are usually sacrificed for tissue or molecular analysis, which eliminates the possibility of understanding regulation *in vivo* in real time as a function of aging, and it also requires a large number of animals.

4.2. ImBioMark to Evaluate Post-KD Vasculitis. In this paper, we also report preliminary data on the feasibility and potential of ImBioMark to characterize post-KD vasculitis in humans at the pediatric age. The ascending aorta (AA) was investigated. Whereas the study was limited to 2 KD and to 5 KD-free subjects, data were recorded three times for each subject and were, respectively, analyzed for near, far walls and for systolic, diastolic phases of the cardiac cycle (CC) as to *virtually* increase the data set. In this context, Figure 6 dictates that ImBioMark is reproducible along several consecutive CC. The reproducibility between near and far wall measurements was also statistically validated, $P = 0.635$. In summary, ImBioMark clearly allowed dissociating AA stiffness of KD subjects ($\Delta_{yy} = 2.39 \pm 0.51\%$) versus KD-free ($\Delta_{yy} = 4.24 \pm 0.65\%$), $P < 0.001$.

5. Conclusion

In this paper, an imaging-based biomarker (ImBioMark) approach was introduced. The first application reported here addressed hypertension phenotype in rat models. Data reported in a previous study indicated that, in a mean term perspective, high-salt diet induces high blood pressure without remodelling of the carotid artery wall. In the current investigation, we observed that the carotid stiffness increases with aging, but without significant change in blood pressure. These results suggest that an increase in artery stiffness does not necessarily induce high blood pressure or hypertension. However, in return, high blood pressure may, in a long-term perspective, induce arterial wall fatigue thus leading to its remodelling.

Although BN rats are normotensive, we are currently investigating interrelationships between the carotid stiffness and aging in hypertensive rats, namely, salt hypertensive and recombinant inbred models. Because ImBioMark is noninvasive and allows *in vivo* follow-up all along complete animal's lifetime, it seems logical to promote this tool for future investigations in the pathophysiology of hypertension.

In addition, we also reported preliminary data on the potential of ImBioMark to evaluate post-KD vasculitis in humans at the pediatric age. In the light of the very promising results, we plan to further investigate prospectively newly diagnosed KD patients in an observational prospective study. The physiology of the ascending aorta and the related stiffness may lead to new risk stratification of the disease. Furthermore, with the potential of ImBioMark to fully evaluate such a remodelling in the peripheral arteries, we also intend to extend this investigation to larger KD populations studying post-KD vasculitis at the carotid artery level.

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Research Article

Selective Gene Expression Analysis of Muscular and Vascular Components in Hearts Using Laser Microdissection Method

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Background. The heart consists of various kinds of cell components. However, it has not been feasible to separately analyze the gene expression of individual components. The laser microdissection (LMD) method, a new technology to collect target cells from the microscopic regions, has been used for malignancies. We sought to establish a method to selectively collect the muscular and vascular regions from the heart sections and to compare the marker gene expressions with this method. *Methods and Results*. Frozen left ventricle sections were obtained from Wistar-Kyoto rats (WKY) and stroke-prone spontaneously hypertensive rats (SHR-SP) at 24 weeks of age. Using the LMD method, the muscular and vascular regions were selectively collected under microscopic guidance. Real-time RT-PCR analysis showed that brain-type natriuretic peptide (BNP), a marker of cardiac myocytes, was expressed in the muscular samples, but not in the vascular samples, whereas α-smooth muscle actin, a marker of smooth muscle cells, was detected only in the vascular samples. Moreover, SHR-SP had significantly greater BNP upregulation than WKY ($P < 0.05$) in the muscular samples. *Conclusions*. The LMD method enabled us to separately collect the muscular and vascular samples from myocardial sections and to selectively evaluate mRNA expressions of the individual tissue component.

1. Introduction

The heart consists of the cardiac muscle, vasculature, and to a lesser extent interstitial infiltrating cells. It is considered that the gene expressions are separately regulated in these tissue components under physiological and diseased conditions [1, 2]. Although the mRNA expression analysis has been established in the whole myocardium, it is difficult to investigate the expression level of each tissue component.

Recently, the laser microdissection (LMD) method has been developed to isolate specific microscopic regions from tissue samples and separately collect the specimens of interest, which enables us to selectively evaluate the mRNA expression levels in targeted cell clusters in the tissues, especially in malignant tissues [3]. The regions of interest are marked on the monitor of a vertical microscope and cut out by the laser beam under computer control. The isolated samples fall down into collecting tubes, which are subjected to quantitative real-time reverse-transcribed polymerase chain reaction (RT-PCR) or gene-chip/microarray assay. However, the LMD method has not been applied to the heart. Thus, we sought to establish a method to selectively collect the muscular region and arterial region in myocardial sections using the LMD method. The mRNA expression levels of maker genes specific for cardiac myocytes or vascular smooth muscle cells (VSMCs) were analyzed in the muscular and vascular samples, respectively, obtained from rat heart sections.

2. Methods

The study protocol was reviewed and approved by the Animal Care and Treatment Committee of Kurume University.

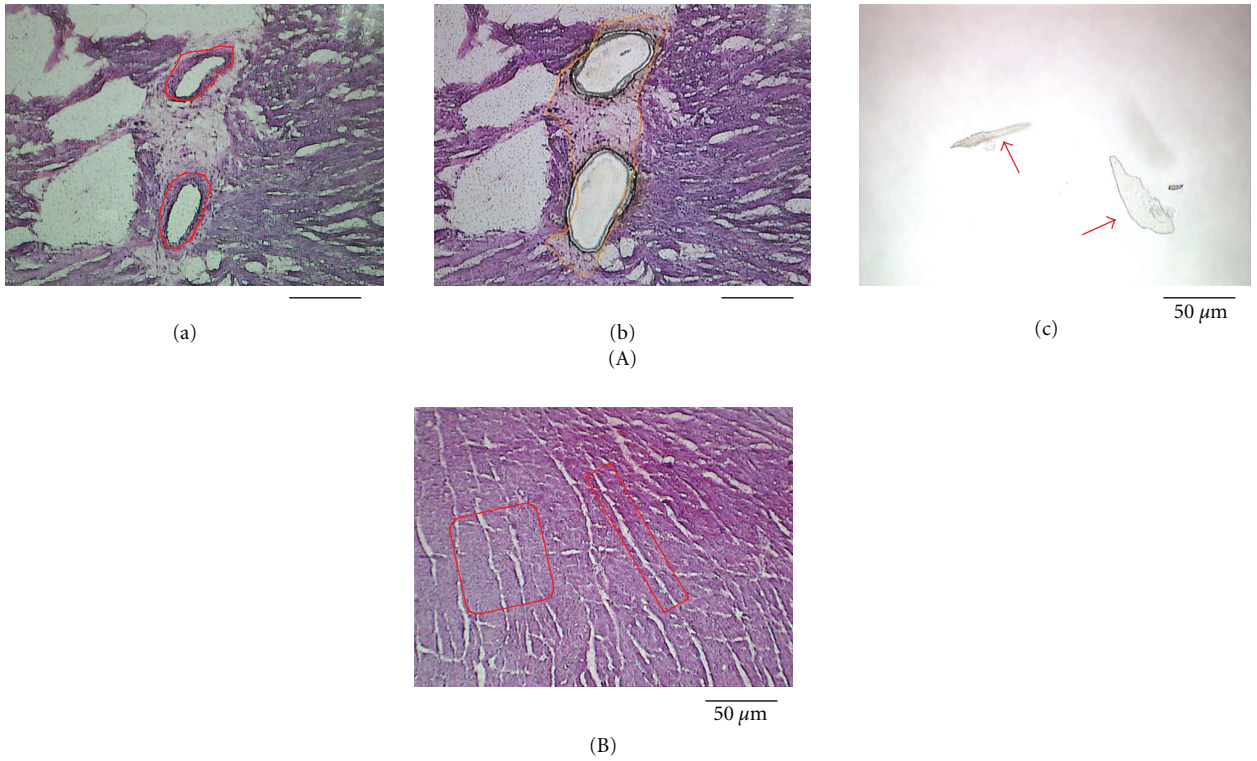

Figure 1: (A) Representative microphotographs demonstrating the sampling of vascular area of the myocardial section obtained from SHR-SP using LMD method. (a) Laser cut lines (red lines) were placed on the outside border of the medial smooth muscle layer. (b) Myocardial section after vascular samples were cut off by laser dissection shown. (c) After laser dissection, isolated vascular fragments (red arrows) fell down into the caps of Eppendorf tubes containing TRIzol reagent. The vascular fragments were being lysed in the TRIzol reagent. (B) For sampling myocardial area, laser cut lines were placed not to include microscopically visible vasculatures and infiltrating cells in the area of interest.

Male Wistar-Kyoto rats (WKY) and stroke-prone spontaneously hypertensive rats (SHR-SP) were purchased from SLC (Shizuoka, Japan) and housed under standard conditions of humidity, room temperature, and a 12 : 12-hour dark-light cycles. They were provided with free access to tap water and chow.

2.1. Animals. At 24 weeks, blood pressure was measured using a tail-cuff sphygmomanometer (MK-2000ST, Muromachi, Tokyo, Japan), as described previously [4]. Thereafter, rats were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (10 mg/kg). Percentage of left ventricular fractional shortening was measured using an echocardiography equipped with a 10 MHz transducer (Aloka, Tokyo, Japan) [5–8]. The next day, rats were euthanized with an overdose of pentobarbital (100 mg/kg, intraperitoneally). After the rats were perfused with ice-cold saline (4°C) at 100 mmHg, the heart was removed. The left ventricle was snap-frozen in isopentane/dry ice, embedded in OCT compound, and sectioned with cryostat. The cryosections (7 μm in thickness) were mounted on ice-cold PEN-slides (Leica Microsystems, Wetzlar, Germany).

2.2. Laser Microdissection Method. Fresh cryosections were fixed in RNase-free-ethyl acetate (acetic acid : ethanol = 1 : 19) and stained with 0.05% toluidine blue dissolved in RNase-free distilled water. The regions of the cardiac muscle and intramyocardial arteries were identified based on microscopic observation and were separately isolated from the section using LMD6000 system (Leica Microsystems). To collect vascular samples, we consistently placed the laser cut line on the outside border of the medial VSMC layer (Figure 1(A)). The isolated fragments were collected in the cap of an Eppendorf tube (Eppendorf Japan, Tokyo, Japan) containing TRIzol reagent (Life Technologies Japan, Tokyo, Japan). Muscular samples were dissected from the myocardium area without microscopically visible vasculatures and infiltrating cells (Figure 1(B)). The vascular sample included 40 cross-sections of the intramyocardial arteries for each animal. The muscular fragments with a total of 6×10^4 μm^2 were collected in each animal.

2.3. Quantitative Real-Time RT-PCR. Total RNA was purified using RNeasy micro (Qiagen, Valencia, CA) according to the manufacturer's instructions. Electropherogram exhibited clear peaks for 18S and 28S ribosomal RNAs in the purified RNA samples (Figure 2(a)). RNA was reverse transcribed using a High Capacity RNA-to-cDNA kit (GE Health Care, Waukesha, WI). Equal amount of the resulting cDNA was subjected to real-time PCR using the TaqMan Universal PCR Master Mix and a Sequence Detection System model 7700 (Life Technologies Japan) [5, 9, 10]. Primer pairs and TaqMan probes for rat type B-natriuretic peptide (BNP), α-smooth muscle actin (α-SMA), and β-actin were

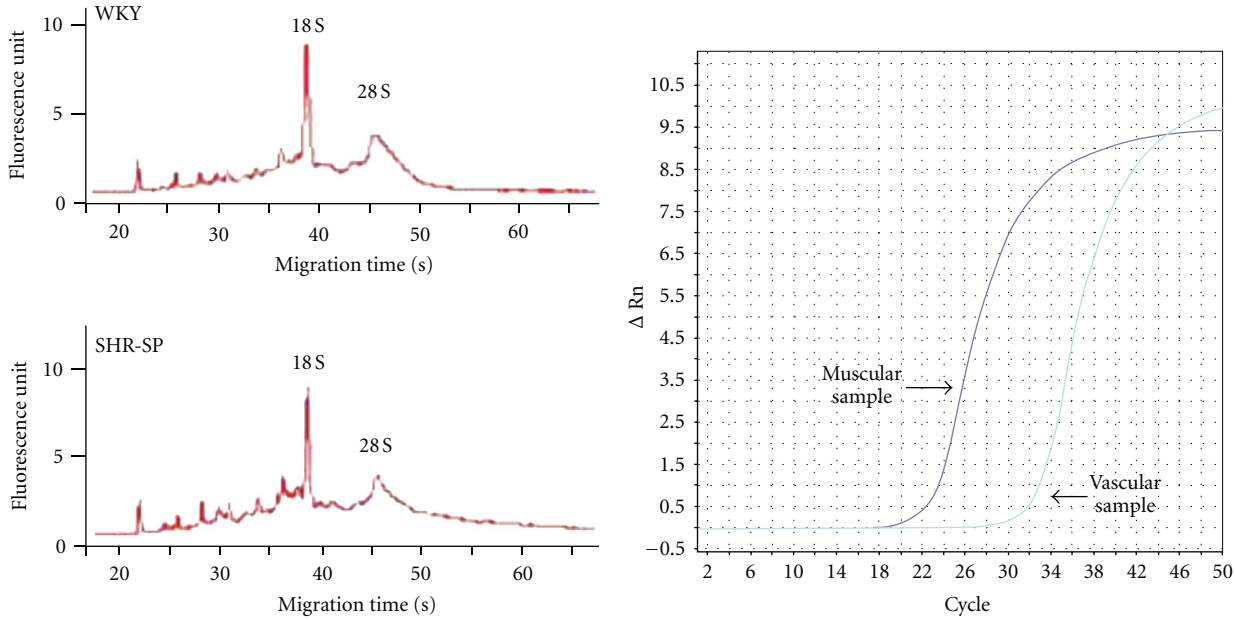

Figure 2: (a) Representative RNA electrophoregrams of the muscular samples of WKY (top) and SHR-SP (bottom). (b) Representative amplification plots of BNP mRNA in the muscular and vascular samples of WKY.

obtained from GE Health Care. Good amplification plots for target genes were shown in the muscular and vascular samples (Figure 2(b)). Expression level of the target gene was normalized by β-actin level in each sample.

2.4. Statistical Analysis. Data were expressed as mean ± SD. Unpaired t-test was performed for comparison between two groups.

3. Results

At 24 weeks of age, systolic blood pressure was 120.5 ± 12.2 mmHg in WKY ($n = 5$) and 252.5 ± 16.7 mmHg in SHR-SP ($n = 3$) ($P < 0.001$). Percentage of Left ventricular fractional shortening was 26.8 ± 4.1% in WKY ($n = 5$) and 29.8 ± 5.2% in SHR-SP ($n = 3$) (no significance). We present one example of real-time RT-PCR analysis in WKY, which showed BNP mRNA expression in the muscular samples, but not in the vascular samples (Figure 3). In contrast, α-SMA expression was found exclusively in the vascular samples. Next, we compared the expression levels of BNP, a molecular marker of cardiac myocyte hypertrophy, between WKY and SHR-SP (Figure 4). SHR-SP had a significantly greater BNP expression than WKY in the muscular samples. BNP mRNA was not expressed in the vascular samples of WKY and SHR-SP.

4. Discussion

The present study demonstrated that the LMD method enabled us to selectively collect myocardium and intramyocardial arteries from the heart section. Selective sampling was verified because the expression of cardiac myocyte-specific gene marker, BNP, was detected exclusively in the muscular samples, whereas the VSMC-specific marker, α-SMA, was expressed only in the vascular samples. Moreover, hypertrophic gene upregulation, as assessed by BNP expression, was significantly greater in the muscular samples obtained from SHR-SP than from WKY.

Recently, it has been reported that the LMD method is useful for selective sampling of the arterial component from the surrounding tissues, for example, the isolation of the arterial lesions in the lung specimen of patients with familiar pulmonary hypertension [11] and the isolation of the collateral vessels in the ischemic hindlimb in mice [12]. Also, the LMD method was used for selective sampling of the intimal plaques in the human atherosclerotic lesions [13, 14]. However, there have been few studies using the LMD method for gene expression analysis of the heart [15]. Thus, we sought to establish the method to selectively collect muscular and vascular samples from the heart sections using the LMD method.

As shown in Figure 1(A), the intramyocardial arterioles were surrounded by thin loose connective tissue separating from the cardiac muscle tissue. Thus, the microscopic guidance allows us to easily isolate vascular samples. In this study, we successfully collected and analyzed the intramyocardial arteries with a diameter of approximately 50 μm. This finding was in line with the previous studies demonstrating that relatively small vascular samples, such as intrapulmonary arteries at a size between 50 and 200 μm and intrarenal arterioles with a diameter of approximately 100 μm, were selectively isolated using the LMD method [16, 17]. In contrast, it was necessary that we consistently took a great care in isolating muscular samples by avoiding

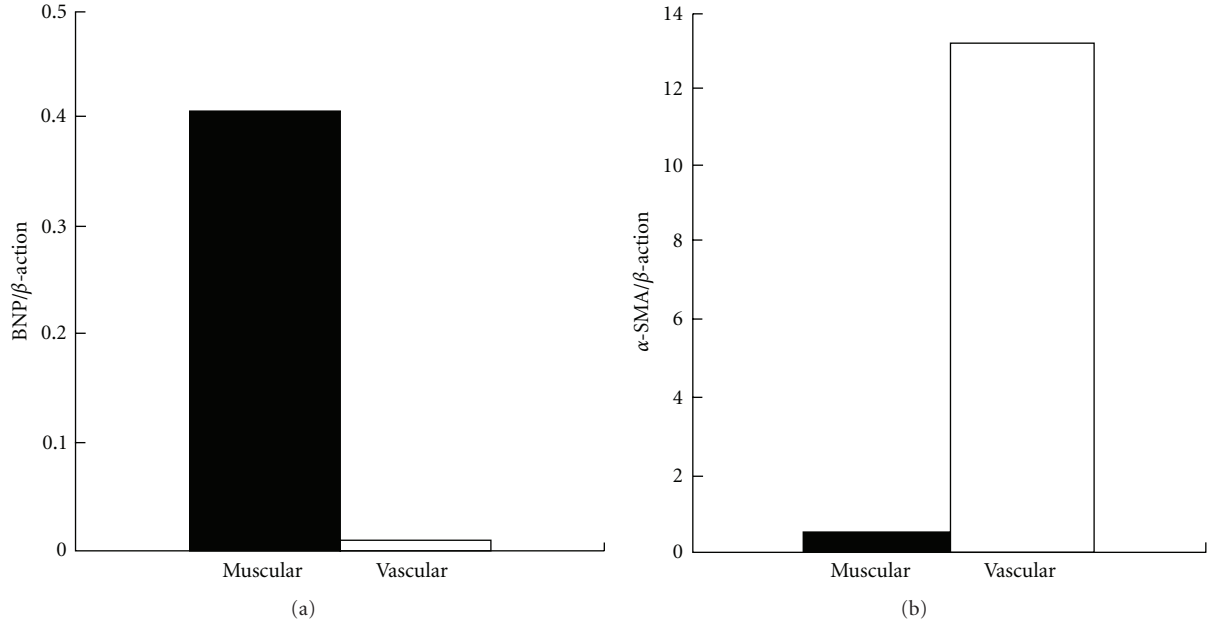

Figure 3: BNP and α-SMA mRNA expression in the muscular (closed column) and vascular (open column) samples obtained from the heart of WKY ($n = 2$). Expression level of the target gene was normalized by β-actin level in each sample.

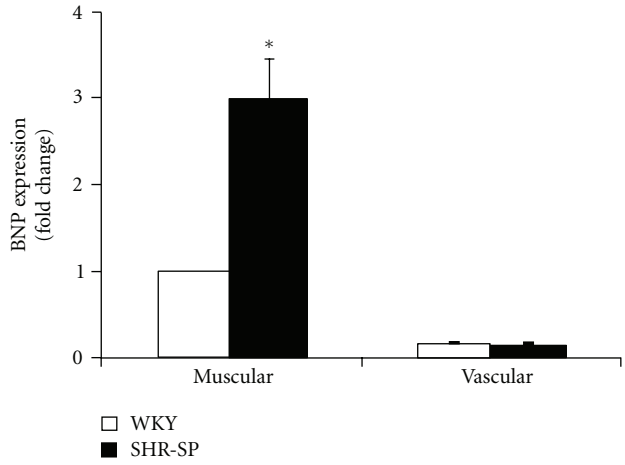

Figure 4: BNP mRNA expression in the muscular and vascular samples obtained from the heart of WKY (open column) and SHR-SP (closed column). BNP expression levels were expressed as fold change from the muscular sample of WKY. Unpaired t-test was used for comparison of initial data before expression as fold changes. Bar = 1 × SD ($n = 3$). $^*P < 0.05$ versus WKY.

microscopically visible vessels and infiltrating cells. The mRNA expressions of BNP and α-SMA were not detected in the vascular and muscular samples, respectively (Figure 3). Moreover, the BNP mRNA upregulation associated with cardiac hypertrophy was documented specifically in the muscular samples in SHR-SP (Figure 4). These findings suggested that the contamination of cardiac myocytes in the vascular samples or VSMCs in the muscular samples was negligible.

In conclusion, the LMD method enabled us to separately collect the muscular and vascular samples from the myocardial sections and to selectively evaluate the mRNA expression changes in individual tissue component.

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