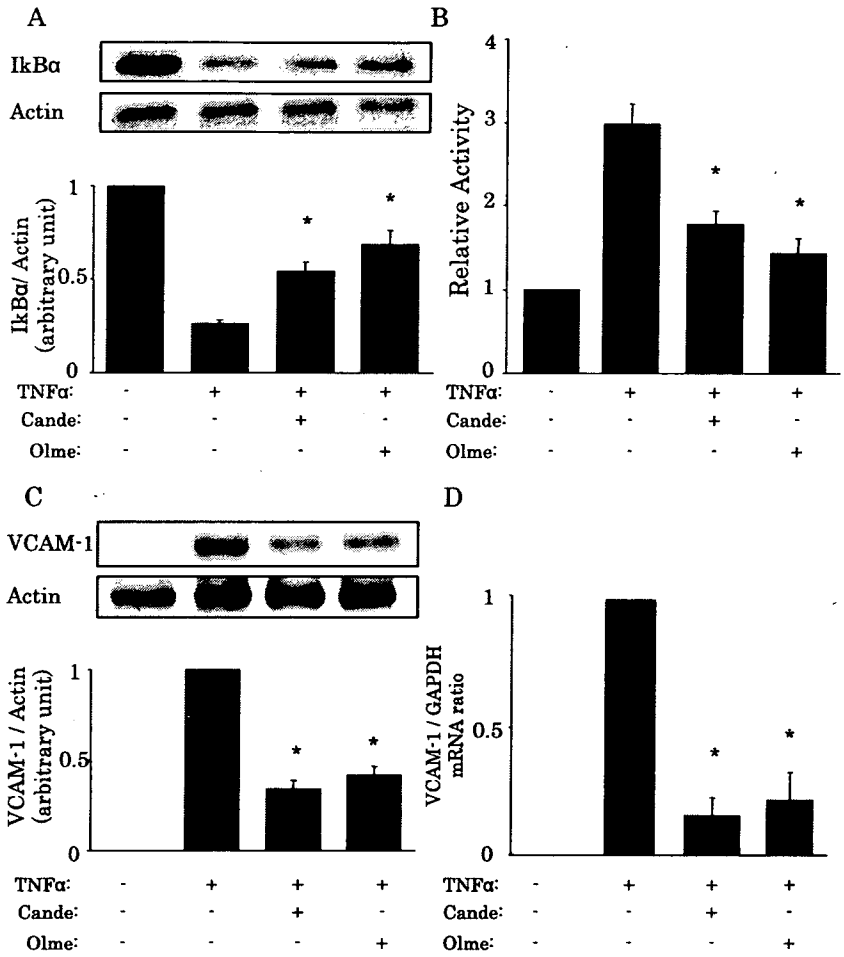

Figure 2. A, (upper panel) Representative Western blot analysis of IkBα proteins in HUVECs. (lower panel) Quantitative analysis of IkBα proteins by densitometry. Values are normalized to controls. B, The binding activity of NF-κB to the nucleus. C, Representative Western analysis of VCAM-1 proteins in HUVECs. D, Quantitative real-time RT-PCR measurement of VCAM-1 mRNA levels normalized GAPDH mRNA. *$P<0.05$ vs TNFα. Cande indicates candesartan; Olme, olmesartan.

olmesartan, on the expression of TNFα-induced RAGE protein. Treatment with olmesartan also reduced the expression of TNFα-induced RAGE protein with a maximal reduction at 10 nmol/L olmesartan (Figure 1B). Furthermore, to examine the decrease in endothelial RAGE protein expression by candesartan resulting from reduced mRNA expression, we investigated the RAGE mRNA levels by the quantitative real-time PCR. Treatment with candesartan significantly reduced the TNFα-induced RAGE mRNA expression (Figure 1C).

ARBs Inhibit the Binding of NF-κB to the RAGE Promoter

TNFα-induced endothelial RAGE expression is regulated by activation of the NF-κB–binding site (nucleotide number −686 to −678) in the RAGE promoter.[6,9] To examine whether ARBs may interact with this pathway, we performed ChIP assays to detect the binding of NF-κB to the RAGE gene promoter. As shown in Figure 1D, both candesartan (1 nmol/L) and olmesartan (10 nmol/L) inhibited the TNFα-induced binding of NF-κB to the RAGE promoter.

ARBs Reduced the Activation of NF-κB in Human Endothelial Cells

Either candesartan (1 nmol/L) or olmesartan(10 nmol/L) reduced the TNFα-induced activation of NF-κB detected by not only the degradation of IκB but also the transcriptional factor assay (Figure 2A and 2B).

ARB-Mediated Reduction of Endothelial RAGE Expression Decreases Proinflammatory Effects in Human Endothelial Cells

In human endothelial cells, activation of RAGE has previously been shown to upregulate the expression of proinflammatory mediators such as MCP-1 and VCAM-1.[3,4] To assess the functional relevance of reduced endothelial RAGE expression, human endothelial cells were stimulated with TNFα (25 ng/mL) in the presence or absence of candesartan (1 nmol/L) or olmesartan (10 nmol/L) for 12 hours before investigating VCAM-1 protein expression and for 4 hours before investigating mRNA expression. Stimulation of endothelial cells with TNFα (25 ng/mL) increased VCAM-1 protein and mRNA expressions as determined by Western blot analysis and quantitative real-time PCR, respectively. Treatment of cells with either candesartan or olmesartan significantly reduced TNFα-induced VCAM-1 protein and mRNA expressions (Figure 2C and 2D).

Gene Silencing of RAGE via RNA Interference

Adversely, to confirm the involvement of RAGE in the TNFα-mediated VCAM-1 expression, we performed gene silencing of RAGE via RNA interference. We checked the

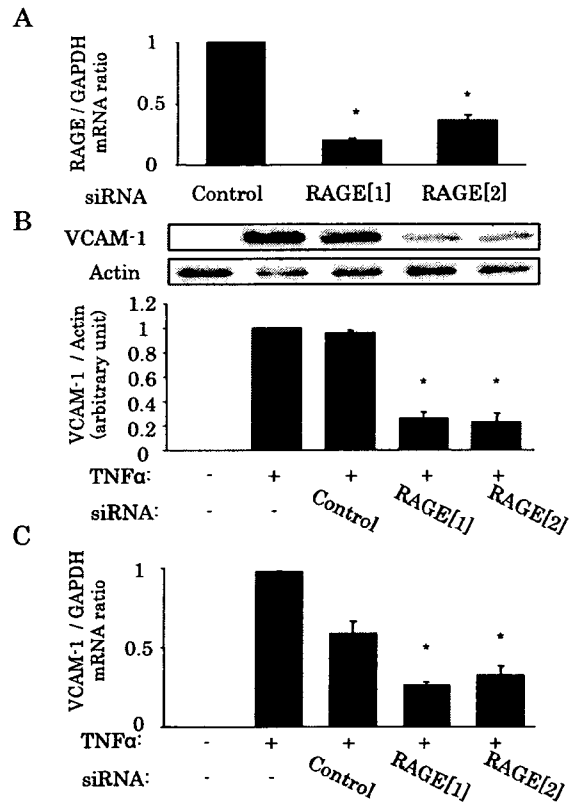

Figure 3. A, The effects of gene silencing of RAGE via RNA interference decreased RAGE mRNA levels determined by quantitative real-time PCR. We used 2 distinct siRNAs targeting the RAGE (RAGE siRNA, [1] [2]), all of which effectively decreased RAGE mRNA levels. B, (upper) Representative Western blot analysis of TNFα-induced VCAM-1 proteins in HUVECs transfected with RAGE siRNA, [1]. [2] (lower) Quantitative analysis of VCAM-1 proteins by densitometry. C; Quantitative real-time PCR measurement of VCAM-1 mRNA levels normalized GAPDH mRNA in HUVECs transfected with RAGE siRAGE, [1].[2] * indicates $P<0.05$ versus siControl.

inhibitory effects of siRNA on the expression of RAGE mRNA levels by quantitative real-time PCR. siRNA against RAGE [1] and [2] (siRAGE [1] and [2]) decreased the expression of RAGE in mRNA levels compared with that of siRNA control (18.8±1.2% and 32.4±2.6%, respectively; Figure 3A). In the transfected cells with siRNA against RAGE using siRAGE [1] and [2], the expression of TNFα-induced VCAM-1 protein were decreased compared with that of the nontransfected cells (Figure 3B). Furthermore, in the transfected human endothelial cells with siRAGE [1] and [2], TNFα-induced VCAM-1 mRNA expressions were also significantly decreased compared with the nontransfected cells (Figure 3C). These data suggest that RAGE is involved in the TNFα-induced VCAM-1 expression in both mRNA and protein levels.

Discussion

We showed here that angiotensin II receptors blockers (ARBs), such as candesartan or olmesartan, suppressed TNFα-induced RAGE protein and mRNA expressions at least partially through the inhibition of the binding of NF-κB to the RAGE gene promoter in human endothelial cells with subsequently reduced the expression of inflammatory mediators such as VCAM-1. Furthermore, we clearly showed that RAGE were involved in the process of TNFα-induced VCAM-1 mRNA and protein expression using RNA interference technique. These results may propose the novel mechanisms that ARBs exert the antiatherosclerotic effects.

RAGE Are Involved in TNFα-Induced Inflammatory Process of Atherogenesis

In the atheromatous plaque, TNFα is released from inflammatory cells.[10] TNFα activates NF-κB sites in the RAGE promoter to induce endothelial RAGE expression.[6] Stimulation of RAGE activates a key signal transduction and is believed to lead to a vicious circle that enhances atherosclerosis. Interestingly, blockade of RAGE with the soluble extracellular domain of RAGE completely suppressed atherosclerosis in diabetic mice.[11] Furthermore, blockade of RAGE in the established atherosclerotic plaques suppressed the further progression of atherosclerotic lesion area.[12] Taken together, in this study we focused on the TNFα-RAGE interaction in the vicious circles in the atherogenesis and we showed here that TNFα increased the RAGE expression in protein and mRNA levels in human endothelial cells, along with the activation of NF-κB and the increase in VCAM-1 protein and mRNA expression. Furthermore, we demonstrated that gene silencing of RAGE via RNA interference decreased the expression of VCAM-1 protein and mRNA induced by TNFα. These results suggested that TNFα-RAGE interaction was involved in the inflammatory process of atherogenesis.

ARBs Reduce the TNFα-Induced RAGE Protein and mRNA Expression

An ARB was reported to reduce RAGE expression in the kidney of diabetic KK/Ta mice.[13] However, to our knowledge, this is the first report that ARBs inhibit TNFα-induced RAGE expression in human endothelial cells, supporting that ARBs have antiatherogenic effects. TNFα-induced endothelial RAGE expression is regulated by the activation of the NF-κB site in the RAGE promoter.[6] In this study, we showed that both candesartan and olmesartan inhibited the binding of NF-κB to the RAGE gene promoter from ChIP assay. These results suggested that ARBs generally reduced TNFα-induced RAGE protein and mRNA expression via the inhibition of the binding of NF-κB to the RAGE gene promoter. Thus, we propose the novel mechanisms that ARBs have been demonstrated to attenuate the degree of atherosclerosis and suggest that the reduction of RAGE expression by ARBs might represent a novel strategy to limit RAGE-mediated inflammatory processes in the vessel wall.

Clinical Implications

Our findings might have important pathophysiological and clinical implications for the high-risk patients with atherosclerosis, because these patients have enhanced RAGE expression in the vasculature and TNFα-RAGE interaction leads to the expression of proatherogenic mediators, such as MCP-1 or VCAM-1. Although further investigation is needed to clarify the precise mechanisms by which ARB inhibit

RAGE expression, we believe that this study will throw light on the treatment of the patients with atherosclerosis.

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Disclosure(s)

None.

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Circ J 2006; **70:** 805–809

Assessment of Genetic Effects of Polymorphisms in the MCP-1 Gene on Serum MCP-1 Levels and Myocardial Infarction in Japanese

Naoharu Iwai, MD*,**; Kazuaki Kajimoto, PhD*; Yoshihiro Kokubo, MD†; Akira Okayama, MD†; Shunichi Miyazaki, MD**; Hiroshi Nonogi, MD**; Yoichi Goto, MD**; Hitonobu Tomoike, MD†

Background Recently, the Framingham Heart Study reported that genetic variations in *CCL2* influence serum levels of monocyte chemoattractant protein-1 (MCP-1) and the incidence of myocardial infarction (MI). The purpose of the present study was to investigate the possible involvement of *CCL2* in the pathogenesis of atherosclerosis and MI in Japanese.

Methods and Results Multiple regression analysis indicated that the MCP-1 levels were significantly influenced by various factors including age, body mass index, smoking, alcohol intake, high density lipoprotein-cholesterol, and systolic blood pressure. Moreover, the serum MCP-1 level was significantly correlated with intima–media thickness ($p<0.0001$). However, this association disappeared when other clinical confounding factors were included in the analyses. Comprehensive analysis of common polymorphisms of *CCL2* in a large community-based population and in subjects with MI found that the A(–2138)T polymorphism affected the serum MCP-1 level in a subgroup of subjects 65 years and older. However, no significant differences in the frequencies of any of the polymorphisms or haplotypes were found between subjects with and without MI. None of the polymorphisms in *CCL2* affected carotid atherosclerosis.

Conclusions The serum MCP-1 level was a good surrogate marker of atherosclerosis in the present study population. Although genetic variations in *CCL2* may have some influence on MCP-1 production, their influence does not seem to contribute appreciably to atherosclerosis in Japanese. The present results did not support the recently published findings from the Framingham Heart Study. The discrepancy between the 2 studies may be related to differences in confounding factors that contribute to MCP-1 levels and in the haplotype structure of the 2 populations. (*Circ J* 2006; **70:** 805–809)

Key Words: Atherosclerosis; Epidemiology; Monocyte chemoattractant protein-1; Myocardial infarction; Polymorphisms

Monocyte chemoattractant protein-1 (MCP-1; gene name *CCL2*) has been suggested to play an important role in the initiation of atherosclerosis by recruiting monocytes to sites of injured endothelium. MCP-1 promotes monocyte differentiation to lipid-laden macrophages, and also contributes to the proliferation of arterial smooth muscle cells[1–4]

In various murine models of atherosclerosis, deletion of *CCL2* has resulted in large reductions in atherosclerotic plaque size[5] but conversely, overexpression of MCP-1 in the leukocytes of susceptible mice resulted in increased plaque size[6]

Several human epidemiological studies have also suggested links between MCP-1 levels and atherosclerotic disease[7–10] Higher MCP-1 levels have been associated with increased risks of myocardial infarction (MI), sudden death, coronary angioplasty, and stent restenosis. Very recently, the Framingham Heart Study reported that *CCL2* polymor-

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**Department of Epidemiology, Divisions of **Cardiology and †Cardiovascular Preventive Medicine, Suita, Japan*

Mailing address: Naoharu Iwai, MD, Department of Epidemiology, Division of Cardiology, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita 565-8565, Japan. E-mail: iwai@ri.ncvc.go.jp

Table 1 Characteristics of the Study Population

	Suita	*MI*	*p value*
n	2,266	342	
M (%)	46.0	87.1	<0.0001
Age	65.2 (11.0)	57.9 (9.9)	<0.0001
BMI	22.8 (3.1)	23.9 (2.9)	<0.0001
HTN (%)	38.7	53.4	<0.0001
DM (%)	9.4	40.4	<0.0001
TG	107 (71)	125 (69)*	0.0007
TC	209 (33)	197 (37)*	<0.0001
HDL-C	60 (16)	43 (13)*	<0.0001
Smoking	16.3	61.1	<0.0001
MCP-1	243 (958)**	–	
log (MCP-1)	5.23 (0.42)**	–	
IMT	0.79 (0.13)***	–	
MI	34 (1.5%)	342 (100%)	

Values are expressed as mean (SD).
**n=235, **n=2,180, ***n=2,035.*
MI, myocardial infarction; M, male subjects; BMI, body mass index (kg/m²); HTN, hypertensive subjects; DM, diabetes mellitus; TG, triglycerides (mg/dl); TC, total cholesterol (mg/dl); HDL-C, high density lipoprotein cho-lesterol (mg/dl); Smoking, current smokers; MCP-1, serum MCP-1 level (ng/ml); log (MCP-1), logarithmic transformation of MCP-1 level; IMT, intima media thickness (mm).

Table 2 Probes and Primers in TaqMan

Polymorphisms	Probe		Primer	
	VIC	FAM	Forward	Reverse
G(−2581)A	acagctGtcactttc	agacagctAtcactttc	tttccactcacttctctcacgc	gacttggcctttgcatatatcaga
A(−2138)T	ctctctctaatcTgttagtgcat	ctctctctaatcAgttagtgcat	cccgaagcatgactggattat	cctaggccatctcacctcatct
A(−1811)G	aaatggccActccatag	aatggccGctccata	caaagcagggctcgagttg	cctgggactagacttgatgtctca
C(−972)G	ctttagctgtCtgcccat	ttagctgtGtgcccatt	gcctcctaactcataatgacttagcc	ctctgtcctcagcatcttccaa
G(−928)C	aagcaGgcaactagt	ccaagcacgCaacta	tggaagatgctgaggacagaga	ggaaacgtgtacaagtcctccaa
C(7320112)G	atgagctcttCtcttct	tgagctcttGtcttct	tgaggtataggcagaagcactgg	aagcaaaaggcaggcagga

Table 3 Summary of *CCL2* Polymorphisms

Polymorphism	Sequence	Region		Mi-AF
G(−2581)A	GACAGCT[G/A]TCACTTT	Promoter		0.332
G(−2411)C	CAAAGCT[G/C]GGAAGTT	Promoter		0.082
A(−2138)T	CACTAAC[T/A]GATTAGA	Promoter		0.049
A(−1811)T	AATGGCC[A/T]CTCCATA	Promoter		0.082
C(−972)G	TAGCTGT[C/G]TGCCCAT	Promoter		0.005
G(−928)C	CCAAGCA[G/C]GCAACTA	Promoter		0.049
C(−362)G	CGCTTCA[C/G]AGAAAGC	Promoter		0.332
C(7320112)G	GCTCTTT[C/G]TCTTCTC	Intron1		0.086
T(7320249)C	CCTGCTG[T/C]TATAACT	Exon2	Cys−>Cys	0.044
C(7320891)T	AGACACC[C/T]TGTTTTA	Exon3	3'−UTR	0.332

Mi-AF (minor allele frequency) was calculated based on the sequencing data of 93 subjects.

phisms are associated with serum MCP-1 levels and MI[11] In genetic association studies, validation in other study populations is very important to confirm that the observed effects are not statistical errors, so the purpose of the present study was to assess the genetic effects of *CCL2* polymorphisms on serum MCP-1 levels and atherosclerosis in Japanese subjects.

Methods

Study Population

The selection criteria and design of the Suita study have been described previously[12–14] The genotypes were determined in 2,266 subjects (including 34 MI subjects) recruited from the Suita study between September 2003 and March 2005. Serum MCP-1 levels were measured in 2,180 subjects. The MI group consisted of 342 randomly selected inpatients and outpatients with documented MI who were enrolled in the Division of Cardiology at the National Cardiovascular Center between May 2001 and April 2003[15,16] All the subjects enrolled in the present study gave written informed consent. The present study was approved by the Ethics Committee of the National Cardiovascular Center and by the Committee on Genetic Analysis and Gene Therapy of the National Cardiovascular Center. The characteristics of the study population are shown in Table 1. Subjects with systolic blood pressure (SBP) ≥140 mmHg, diastolic blood pressure ≥90 mmHg, and/or who were taking antihypertensive medication were categorized as having hypertension. Subject with fasting blood glucose ≥126 mg/dl, hemoglobin A1c ≥6.5%, and/or who were being treated for diabetes mellitus was categorized as having the disease.

Fasting serum samples were collected and stored at −80°C. MCP-1 levels were measured in duplicate with a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The inter- and intra-assay variabilities were 6.3% and 6.2%, respectively. Because the distribution of serum MCP-1 levels was skewed, the values were logarithmically transformed in the statistical analysis.

The details of the method used for the carotid ultrasonic examination have been reported previously[14] We used a high-resolution B-mode ultrasonic machine with 7.5-MHz transducers, which gave an axial resolution of 0.2 mm. The regions between 30 mm proximal from the beginning of the dilation of the bifurcation bulb and 15 mm distal from the flow divider of both common carotid arteries (CCAs) were scanned. All measurements were made at the time of scanning with the instrument's electronic caliper and were recorded as photocopies. The intima–media thickness (IMT) was measured on a longitudinal scan of the CCA at a point 10 mm proximal from the beginning of the dilation of the bulb.

DNA Study

The promoter (up to −2.8 kb) and exons 1, 2, and 3 (including 3'UTR) regions were sequenced in 93 subjects, which included the top 12 subjects with high serum MCP-1 levels and the bottom 12 subjects with low serum MCP-1 levels. The sequence primers will be provided on request. The genotypes were determined by the TaqMan method (Table 2). The success rate of genotyping was greater than 96%.

Statistical Analysis

Values are expressed as mean±standard deviation (SD). All statistical analyses were performed with the JMP statistical package (SAS Institute Inc, Cary, NC, USA). Multiple regression analysis was performed to obtain predictors of the serum MCP-1 level and to assess the contribution of polymorphisms of *CCL2* to the serum MCP-1 level. Multiple logistic analysis was performed to obtain predictors for MI. Residuals of the serum MCP-1 level and IMT were calculated by adjusting for appropriate confounding factors. R-square values between polymorphisms and haplotype frequencies in the control and MI groups were analyzed using the SNPAlyze Pro statistical package (version 3.2, Dynacom Inc). A statistical power calculation was per-

Table 4 Linkage Disequilibrium Among the Polymorphisms of *CCL2*

	G(−2581)A	A(−2138)T	A(−1811)T	C(−972)G	G(−928)C	C(7320112)G
G(−2581)A						
A(−2138)T	0.12356					
A(−1811)T	0.16035	0.00565				
C(−972)G	0.02467	0.00086	0.00034			
G(−928)C	0.12411	0.97084	0.00582	0.00089		
C(7320112)G	0.15605	0.00546	0.00716	0.00108	0.00562	

Linkage disequilibrium (LD) among the polymorphisms of CCL2 was calculated from the TaqMan data of the Suita subjects. R-square values between polymorphisms are shown. Tight LD was observed between the A(−2581)T and G(−928)C polymorphisms.

Table 5 Predictors of Serum MCP-1 Level

Predictor	t-ratio	p value
Age	7.9	<0.0001
BMI	−3.21	0.0014
SBP	2.42	0.0155
Alcohol	2.71	0.0067
Smoking	3.36	0.0008
HDL-C	−2.59	0.0096

Predictors of serum MCP-1 levels were identified by multiple regression analysis (n=2,180). Alcohol, ethanol consumption per day (g/day); Smoking, number of cigarettes per day X years.
SBP, systolic blood pressure. See Table 1 for other abbreviations.

Table 6 Predictors of Intima-Media Thickness

Predictor	t-ratio	p value
log (MCP-1)	0.13	0.7191
Age	353.82	<0.0001
SBP	29.67	<0.0001
Sex	33.21	<0.0001
BMI	83.45	<0.0001

n=2,034, F=128.197, p<0.0001.
The serum MCP-1 levels were assessed in 2,034 of the 2,035 subjects assessed by carotid sonography.
See Tables 1,5 for abbreviations.

Table 7 Influence of the Polymorphisms of *CCL2* on Serum MCP-1 Level

	AA	Aa	aa	p value
G(−2581)A	0.002 (0.399)	0.004 (0.418)	−0.020 (0.387)	0.692
n	936	961	270	
A(−2138)T	−0.006 (0.402)	0.049 (0.436)	−0.054 (0.211)	0.122 (0.052)
n	1,909	253	7	
A(−1811)T	0.006 (0.407)	−0.031 (0.406)	−0.079 (0.292)	0.268 (0.117)
n	1,839	313	13	
C(−972)G	−0.001 (0.406)	0.045 (0.404)	-	0.409
n	2,111	54		
G(−928)C	−0.006 (0.403)	0.048 (0.430)	−0.054 (0.211)	0.123 (0.052)
n	1,896	262	7	
C(7320112)G	0.004 (0.401)	−0.013 (0.023)	−0.163 (0.108)	0.259 (0.349)
	1,840	311	14	
A(−2138)T	−0.012 (0.351)	0.081 (0.462)	−0.051 (0.153)	0.0126 (0.0041)
Age ≥65 years	1,041	154	4	
G(−928)C	−0.012 (0.351)	0.081 (0.500)	−0.051 (0.153)	0.0124 (0.0040)
Age ≥65 years	1,035	156	4	

Residuals of log (MCP-1) were calculated by adjusting for Age, BMI, SBP, alcohol, smoking, and HDL-C. Values are expressed as mean (SD). p values calculated by grouping AA/Aa + aa are shown in parentheses. The effects of the A(−2138)T and G(−928)C polymorphisms on the MCP-1 level were more significant in subjects aged 65 years and older.
See Tables 1,5 for abbreviations.

formed with the statistical package SamplePower (version 2.0, SPSS, Chicago, IL, USA).

Results

Sequence Analysis of CCL2

Sequence analyses in 93 subjects revealed the existence of 10 polymorphisms (Table 3) of *CCL2*. The G(−2581)A was in almost complete linkage disequilibrium (LD) with the C(−362)G and C(7320891)T polymorphisms. The A(−1811)G polymorphism was in almost complete LD with the G(−2411)C polymorphism. Thus, the genotypes of the C(−362)G, C(7320891)T, and G(−2411)C polymorphisms were not determined in the present study. Because the polymorphism in exon 2 [T(7320249)C] was synonymous (Cys−>Cys), this polymorphism was also not determined in the present study. The genotypes of the remaining 6 polymorphisms were determined by the TaqMan method in a total of 2,570 subjects. The LD values calculated from R-square values among these SNPs are shown in Table 4.

Clinical Correlates of Serum MCP-1 Level

Multiple regression analysis indicated that the MCP-1 level was significantly influenced by various factors ($p<0.0001$, R-square=0.054) including age ($p<0.0001$), body mass index (BMI; $p=0.0014$), smoking ($p=0.0008$), alcohol intake ($p=0.0067$), high-density lipoprotein cholesterol ($p=0.0096$), and SBP ($p=0.0155$) (Table 5).

Many studies have reported that the serum MCP-1 level is an excellent indicator of atherosclerosis and in our study population the serum MCP-1 level significantly correlated with IMT ($p<0.0001$, R-square=0.009). However, this association disappeared when other clinical confounding factors were included in the multiple regression analyses (Table 6).

Table 8 *CCL2* **Polymorphisms and Incidence of MI**

	MI (−)			MI			p value
	AA	Aa	aa	AA	Aa	aa	
G(−2581)A (%)	946 (43.35)	966 (44.13)	274 (12.52)	149 (40.93)	176 (48.53)	39 (10.71)	0.2857
A(−2138)T (%)	1,931 (88.25)	250 (11.43)	7 (0.32)	218 (87.36)	45 (12.36)	1 (0.27)	0.8686 [0.6289]
A(−1811)T (%)	1,861 (85.02)	314 (14.34)	14 (0.64)	304 (83.29)	56 (15.34)	5 (1.37)	0.3337 [0.3999]
C(−972)G (%)	2,130 (97.53)	54 (2.47)		357 (98.08)	7 (1.92)		0.5548
G(−928)C (%)	1,918 (87.82)	259 (11.86)	7 (0.32)	319 (87.40)	45 (12.33)	1 (0.27)	0.9578 [0.8200]
C(7320112)G (%)	1,855 (84.94)	315 (14.42)	14 (0.64)	302 (82.74)	61 (16.71)	2 (0.55)	0.5229 [0.2880]

Genotype frequencies between subjects with and without MI are shown. p values calculated by grouping AA/Aa + aa are shown in square parentheses.
See Table 1 for abbreviation.

Table 9 Influence of *CCL2* Polymorphisms on IMT

	AA	Aa	aa	p value
G(−2581)A	−0.003 (0.104)	0.001 (0.105)	0.006 (0.115)	0.421
n	865	908	255	
A(−2138)T	0.000 (0.106)	−0.001 (0.103)	0.065 (0.118)	0.319
n	1,784	237	6	(0.958)
A(−1811)T	0.000 (0.105)	0.003 (0.112)	−0.049 (0.068)	0.227
n	1,717	294	12	(0.752)
C(−972)G	0.000 (0.106)	0.000 (0.113)	–	0.964
n	1,970	53		
G(−928)C	0.000 (0.106)	−0.003 (0.103)	0.065 (0.118)	0.291
n	1,771	246	6	(0.802)
C(7320112)G	−0.001 (0.106)	0.005 (0.101)	0.039 (0.159)	0.275 (0.278)

Residuals of IMT were calculated by adjusting for sex, age, BMI, and SBP. Values are expressed as mean (SD). p values calculated by grouping AA/Aa + aa are shown in parentheses.
See Tables 1,5 for abbreviations.

Table 10 Haplotype Analysis of the 2 Study Populations

Suita	Framingham	G(−2581)A	A(2138)T	A(−1811)G	G(−928)C	C7320112G	MI (−)	MI	Framingham
Haplo1	H1	G	A	A	G	C	65.2	65.0	27.0
Haplo2	H4 + H5	A	A	A	G	C	13.2	10.9	26.9
Haplo3	H6	A	A	G	G	C	7.8	9.0	4.2
Haplo4	–	A	A	A	G	G	7.5	8.7	–
Haplo5	–	A	T	A	C	C	6.1	6.5	–
–	H2	A	T	A	G	G	<0.01	<0.01	20.3
–	H3	A	A	A	C	C	<0.01	<0.01	18.6

Haplotype frequencies in the MI (−) and MI groups were calculated. Haplotype frequencies reported in the Framingham study are also shown for reference.
See Table 1 for abbreviation.

Thus, the serum MCP-1 level was only a surrogate marker of atherosclerosis in the present study population.

Influence of Polymorphisms on Serum MCP-1 Level

Next, we examined the influence of polymorphisms of *CCL2* on residuals of the MCP-1 level after adjusting for the above-mentioned confounding factors (Adj-MCP1) (Table 7). Two polymorphisms, A(−2138)T and G(−928)C, tended to affect Adj-MCP1. The A(−2138)T and G(−928)C polymorphisms were in tight LD (R-square=0.97084) in this study population (Table 4). Interestingly, the influence of these polymorphisms on Adj-MCP1 seemed to be exaggerated in subjects 65 years and older whose MCP-1 levels were significantly higher than those of younger subjects.

Association Study Between CCL2 Polymorphisms and MI

No significant difference was found in the frequencies of any of the polymorphisms between the cases and controls (Table 8). Multiple logistic analyses including age and BMI indicated that none of the polymorphisms contributed to MI. Moreover, none of them affected IMT after adjusting for sex, age, SBP, and BMI (Table 9).

Haplotype Analysis

We constructed haplotypes based on the G(−2581)A, A(−2138)T, A(−1811)T, G(−928)C, and C7320112G polymorphisms and identified 5 common haplotypes that accounted for 99.7% of all haplotypes. The C(−972)G polymorphism was not included because of its low frequency. No significant difference was observed in haplotype fre-

quencies between subjects with and without MI (Table 8).

The haplotype frequencies reported in the Framingham study[11] were significantly different from those in the present study population (Table 10). Although H2 and H3, which accounted for 20.3% and 18.6%, respectively, in the Framingham study, were very rare in this study population, Haplo4 and 5, which were rare in the Framingham study, were common.

Discussion

This report describes a comprehensive analysis of the common polymorphisms of *CCL2* in both a large community-based population and subjects with MI. No significant differences in the frequencies of any of the polymorphisms were found between cases and controls. Moreover, none of the polymorphisms of *CCL2* affected carotid atherosclerosis as assessed by IMT. However, the A(–2136)T and G(–928)C polymorphisms tended to affect the serum MCP-1 level. Although genetic variations in *CCL2* may have some influence on MCP-1 production, their does not seem to contribute appreciably to atherosclerosis in Japanese subjects. Thus, our findings do not support the recently published result from the Framingham Heart Study[11] that genetic variations in *CCL2* significantly influence serum MCP-1 levels and the incidence of MI.

There may be several reasons for this discrepancy. The MCP-1 levels in the Framingham Heart Study were approximately 1.4-fold higher than those in the present study population. Genetic variation might well have an influence under a stimulated state. MCP-1 levels are influenced by various factors, as described in Table 5. It is conceivable that subjects in the Framingham Heart Study may have had higher MCP-1 levels because of stimulation by atherogenic factors that may be more prevalent in Caucasians. Indeed, the influence of genetic variations was more evident in the present study population when the analysis was limited to older subjects who had higher MCP-1 levels (Table 7).

In the Framingham Heart Study, the haplotype H2 was reported to contribute to higher MCP-1 levels, and the frequency of this haplotype was 20.3%.[11] It is defined by the (–2138)T and (77320112)G genotypes, and although the A(–2138)T and G(7320112)C polymorphisms were observed in the present study population, the H2 haplotype was not ($p<0.01\%$). This difference in the haplotype structure between Caucasians and Japanese might also contribute to the discrepancy between the 2 studies.

The reported positive association between the A(–2581)T polymorphism and MI in the Framingham Heart Study was based on 1,797 study subjects, including just 107 MI subjects,[11] which was insufficient statistical power ($p<0.50$) to conclude that there was a positive association between the genotype and MI. Moreover, although the H2 haplotype was reported to be associated with the serum MCP-1 level, the H1 haplotype but not the H2 haplotype, was reported to be associated with MI. This inconsistency might also indicate that the Framingham study had insufficient statistical power.

Although the serum MCP-1 level is an excellent indicator of atherosclerosis,[7–10] MCP-1 itself appears to make only a slight contribution to atherosclerosis (Table 6). Thus, it is unlikely that genetic polymorphisms that may only slightly influence the serum MCP-1 level will contribute significantly to the occurrence of MI and atherosclerosis. Our present findings suggest that, although genetic variations in *CCL2* may have some influence on MCP-1 production, their influence on the incidence of MI is not appreciable in Japanese. The present study also indicates the importance of clarifying the haplotype structure for comparing genetic association studies involving different ethnic backgrounds.

Acknowledgement

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The Antagonism of Aldosterone Receptor Prevents the Development of Hypertensive Heart Failure Induced by Chronic Inhibition of Nitric Oxide Synthesis in Rats

Osamu Tsukamoto[1], Tetsuo Minamino[1], Shoji Sanada[1], Ken-ichiro Okada[1], Akio Hirata[1], Masashi Fujita[1], Yasunori Shintani[1], Liao Yulin[1], Yoshihiro Asano[1], Seiji Takashima[1], Satoru Yamasaki[2], Hitonobu Tomoike[2], Masatsugu Hori[1], and Masafumi Kitakaze[2]

[1] *Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita Osaka 565-0871, Japan;*
[2] *Departments of Cardiovascular Medicine, National Cardiovascular Center, Suita, Osaka 565-8565, Japan*

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Summary. **Aldosterone promotes cardiovascular inflammation and remodeling, both of which are characteristic changes in hypertensive and failing hearts. Since chronic inhibition of nitric oxide (NO) synthase with N^{ω}-nitro-L-arginine methyl ester (L-NAME) induces systemic hypertension associated with cardiovascular inflammation and remodeling, we examined the potential role of aldosterone in this process using eplerenone, a selective aldosterone receptor antagonist. Ten-week-old male Wistar-Kyoto rats were randomly divided into 3 groups: the control group (no treatment), the L-NAME group (received L-NAME 1 g/L in drinking water), and the L-NAME + Eplerenone group (L-NAME plus eplerenone at 100 mg/kg/day). After 8 weeks of the treatment, the L-NAME group showed significantly higher systolic blood pressure than the control group (198 ± 7 vs. 141 ± 3 mmHg, $P < 0.05$). Eplerenone did not affect the increase in blood pressure caused by L-NAME (189 ± 12 mmHg). Chronic inhibition of NO synthesis increased the plasma aldosterone concentration and CYP11B2 mRNA in adrenal glands. Cardiac inflammation and fibrosis were detected in the L-NAME group, while both changes were completely prevented by eplerenone. Cardiac hypertrophy was induced in L-NAME group, but was partially prevented by eplerenone. In the L-NAME group, left ventricular fractional shortening (LVFS: 27 ± 2 vs. 38 ± 1%) and E/A ratio (1.7 ± 0.1 vs. 2.1 ± 0.1) were significantly lower and LV end-diastolic pressure (LVEDP) was higher (4.9 ± 0.6 vs. 13.9 ± 0.5 mmHg) without LV enlargement, compared with those in the control group ($P < 0.05$). Eplerenone completely normalized LVFS (36 ± 2%), E/A ratio (2.2 ± 0.1), and LVEDP (6.2 ± 0.7 mmHg). These results suggest that chronic inhibition of NO synthesis induces cardiac inflammation and dysfunction via an aldosterone receptor-dependent mechanism.**

Key words. **aldosterone, nitric oxide, inflammation, hypertension, heart failure**

Introduction

The renin-angiotensin system (RAS) is excessively activated in patients with either hypertension or chronic heart failure, while recent clinical data have clearly shown the beneficial effects of RAS suppression by the treatment with an angiotensin-converting enzyme (ACE) inhibitor or an angiotensin type 1 receptor blocker (ARB) [1–3]. However, either ACE inhibitor or the ARB therapy does not afford protection from the effects of high circulating aldosterone levels, a well-documented phenomenon known as 'aldosterone escape' [4]. There is compelling evidence that aldosterone had various deleterious effects on the cardiovascular system via aldosterone receptors in the heart, brain and blood vessel [5,6]. These effects include vascular inflammation and damage [7], endothelial dysfunction [8], and myocardial fibrosis [9]. Eplerenone is a selective aldosterone receptor antagonist that has been shown to protect against target organ damage in various models of experimental cardiovascular diseases [10]. In addition, recent clinical trials demonstrated that an addition of an aldosterone receptor blocker to conventional therapy including either ACE inhibitors or ARBs reduces the incidence of mortality and morbidity in patients with chronic heart failure [11,12] and reduces the degree of the target organ damage in patients with hypertension [13,14]. These findings indicate that the blockade of aldosterone receptors by eplerenone has significant therapeutic promises for protecting patients with hypertension and chronic

Address for correspondence: Tetsuo Minamino, MD, PhD, Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan. E-mail: minamino@medone.med.osaka-u.ac.jp

heart failure from the deleterious cardiovascular effects of aldosterone [10].

Endothelial dysfunction has been documented in the peripheral and coronary arteries of patients with chronic heart failure and hypertension [15–17], which is mainly attributable to a decrease in bioavailability of nitric oxide (NO) [18,19]. Recently, we and others have reported that chronic inhibition of NO synthesis with N^{ω}-nitro-L-arginine methyl ester (L-NAME) induces systemic hypertension along with vascular inflammation *via a local auto/paracrine RAS activation* [20] and increased expression of inflammatory cytokines, resulting in medial thickening, perivascular and cardiac interstitial fibrosis [21], and cardiac hypertrophy [20,22]. Interestingly, aldosterone also induces inflammation, fibrosis, and hypertrophy in the heart [7,23,24]. These pathological changes are often observed in patients with either hypertension or chronic heart failure and may contribute to the pathophysiological development of both diseases [25,26]. Thus, we hypothesized that reduced NO bioactivity induces cardiac inflammation, fibrosis, and hypertrophy through an *aldosterone receptor-dependent mechanism.* To test this hypothesis, we examined the effects of aldosterone receptor blockade on cardiovascular pathophysiological changes and cardiac function in the chronic NO inhibition model of rats.

Methods

Materials

Eplerenone was provided from Pfizer Co. Ltd. and the other drugs were obtained from Shigma Chemical Co. All procedures were performed in conformity with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 93-23, revised 1985).

Animal model of chronic inhibition of NO synthesis

Ten-week-old male Wistar-Kyoto rats (Charles River, Kanagawa, Japan) were randomly divided into 3 groups. The control group (n = 11) received no treatment. The L-NAME group (n = 11) received L-NAME at 1 g/L in drinking water. The L-NAME + Eplerenone group (n = 11) received L-NAME plus eplerenone (100 mg/kg per day) mixed with normal salt diet. We have preliminary confirmed that the dose of eplerenone used in the present study (100 mg/kg per day for 4 weeks) did not significantly change either heart rate (318 ± 9 vs. 326 ± 10 bpm, n = 3) or systolic blood pressure (SBP) (127 ± 3 vs. 122 ± 4 mmHg, n = 3). Body weight (BW) was measured at 1 week before and 8 weeks after the initiation of treatment. SBP was measured by the tail-cuff method at 1 week before and 1, 2, 4, and 8 weeks after starting the experiments. Both daily urine outputs and urinary Na^+ concentrations were measured for 3 days in succession at 8 weeks of the treatment. All rats were housed, treated, and subjected to euthanasia as described previously [27].

Echocardiographic studies

Transthoracic echocardiopraphy was performed using a machine equipped with a 7.5-MHz transducer (ALOKA Co. Ltd) after 8 weeks of treatment. Rats were anesthetized intraperitoneally with ketamine (15 mg/kg) and xylamine (5 mg/kg) and subjected to echocardiographic study. The method of anesthetization used minimally affects the rat hemodynamics [28]. Left ventricular (LV) end-diastolic dimension (LVDd), LV end-systolic dimension (LVDs), LV fractional shortening (LVFS), and ratio of early to late filling wave (E/A) of transmitral pulse-wave Doppler velocity were measured.

Hemodynamic measurement

Rats were anesthetized intraperitoneally by pentobarbital (15 mg/kg) after 8 weeks of the treatment. LV end-diastolic pressure (LVEDP) was measured using a 2-French micromanometer-tipped catheter (Millar Instruments).

Histopathology and immunohistochemistry

Five rats in each group were sacrificed after 1 week of treatment and the remainders were sacrificed after 8 weeks for blood sampling, morphometric, immunohistochemical, and biochemical analyses. Excised hearts were weighed, cut, stained with hematoxylin-eosin (HE) solution and Masson's trichrome solution, and carefully scanned as described previously [27]. Wall thickening was measured in small coronary arteries (internal diameters <200 μm) and morphometry was done on LV cardiomyocytes. Also, the wall-to-lumen ratio (the ratio of medial thickness to internal diameter) and the cross-sectional area were calculated according to the previous report [22]. Perivascular fibrosis of small coronary arteries and myocardial interstitial fibrosis were also assessed according to the previous report [20]. Each section was scanned at 100× magnification. For immunohistochemistry, paraffin sections (5 μm thick) were preincubated with 3% skim milk to decrease nonspecific binding. Then the sections were incubated overnight at 4°C with a mouse anti-human proliferation cell nuclear antigen (PCNA) antibody (Dako), a rat anti-monocyte/macrophage antibody (ED1, Serotec), α-smooth muscle actin (α-SMA) antibody (Dako), a mouse anti-human transforming growth factor-β1 (TGF-β1) antibody (1 μg/mL, AHG0051, Biosource), a gout anti-human TGF-β1 latency-associated peptide (TGF-β1LAP) antibody (10 μg/mL, AB-246NA, R&D), and nonimmune mouse or rabbit IgG (Zymed Laboratory Inc). Coronal sections of kidney were cut at 3–4 mm and at least three or four of these were prepared as paraffin-embedded blocks. Histological sections were stained with periodic acid-Schiff reagent and examined by light microscopy. Renal arterial and arteriolar damage was categorizes as the presence of fibrinoid necrosis of vascular wall [7]. The number of injured vessels per section was divided by the number of glomeruli in the same section to normalize for the amount of tissue examined.

Renal vascular lesions were expressed as the number of injured vessels per 100 glomeruli.

NFκB p50/p65 transcription factor assay

Five rats in each group were used. The activation of NFκB binding to the nucleus at 1 week of the treatment was determined using the non-radioactive NFκB p50/p65 transcription factor assay kit (Chemicon Internationanl, Inc.) according to instructions provided. Nuclear protein extracts were prepared from the hearts as previously described [29].

Western blot analysis

Cytosolic fractions were extracted form hearts at 1 week of the treatment as previously described [29]. Using an IκBα-specific polyclonal antibody (Santa Cruz Biotechnology Inc.), Western blot analysis was performed as previously described [30].

Northern blot analysis

Northern blotting was performed according to the previous report [9]. The following oligonucleotide primers were used for monocyte chemoattractant protein-1 (MCP-1) and GAPDH were as follows: a rat MCP-1, sense: 5′-TAT GCA GGT CTC TGT CAC GC-3′; antisense: 5′-AAG TGT TGA ACC AGG ATT CAC A-3′; a mouse GAPDH, sense: 5′-TAT GCA GGT CTC TGT CAC GC-3′; antisense: 5′-AGA TCC ACA ACG GAT ACA TT-3′. Relative amount of MCP-1 was normalized against the amount of GAPDH mRNA.

Real-time quantitative RT-PCR

RT-PCR of rat myocardial samples was performed according to the Omniscript Reverse Transcription Handbook (QIAGEN Inc.). The rat primers and probes used for quantification of CYP11B2 and GAPDH (internal control) were all designed according to the previous report [31]. Real-time quantitative RT-PCR was performed with an ABI PRISM7700 Sequence Detection System (Applied Biosystems) by the relative standard curve method according to the previous report [31]. The target amount was determined from the relative standard curves constructed with serial dilutions of the control total RNA.

Measurement of aldosterone

Plasma aldosterone concentration was determined by radioimmunoassay (SRL Co Ltd).

Statistical analysis

Data are expressed as the mean ± SEM. Heart weight (HW), BW, hemodynamic variables, vascular wall-to-lumen ratio, perivascular fibrosis, myocyte surface area, and the numbers of renal vascular lesions were compared using one-way ANOVA followed by Bonferroni's test for multiple comparisons. Comparison between the control and L-NAME groups for the plasma aldosterone concentration and the mRNA levels of CYP11B2 were performed by Student's unpaired t test. Comparisons of the changes of BP among the groups over time were performed by two-way repeated-measures ANOVA followed by Bonferroni's correction and $P < 0.05$ was considered to be statistically significant.

Table 1. *Changes in systolic blood pressure and heart rate*

	n	Weeks	Control	L-NAME	L-NAME + Eplerenone
SBP, mmHg	11	0	129 ± 4	130 ± 3	130 ± 3
	11	1	136 ± 6	158 ± 6	150 ± 7
	6	2	140 ± 2	177 ± 10*	166 ± 5*
	6	4	142 ± 3	185 ± 7*	199 ± 6*
	6	8	141 ± 3	198 ± 7*	189 ± 12*
HR, bpm	11	0	321 ± 11	334 ± 9	335 ± 8
	6	8	404 ± 23	387 ± 14	355 ± 10

SBP indicates systolic blood pressure; HR, heart rate.
Data are mean ± SEM. *$P < 0.05$ vs. the control group.

Results

Hemodynamic variables and serum parameters

The hemodynamic variables are presented in Table 1. SBP was comparable among the 3 groups before the study. In the L-NAME and L-NAME + Eplerenone groups, SBP increased progressively and became higher than in the control group from 2 weeks of the treatment onwards. The increase of SBP produced by L-NAME was not affected by 100 mg/kg/day of eplerenone. Heart rate was comparable among the 3 groups and did not change throughout the study. Serum Na^+ levels were not affected by the treatment with either L-NAME or eplerenone, or both (control, L-NAME, and L-NAME + Eplerenone: 144 ± 0.3, 144 ± 0.6, and 144 ± 0.1 mmol/L). Serum K^+ levels increased in the L-NAME + Eplerenone group compared with the control group (control, L-NAME, and L-NAME + Eplerenone groups: 4.1 ± 0.1, 4.4 ± 0.1, and 4.6 ± 0.2 mmol/L, respectively, $P < 0.05$).

Effects of aldosterone receptor blockade on cardiovascular inflammation

The plasma aldosterone concentration was increased in the L-NAME group compared with the control group (Fig. 1(A)). Consistent with these data, the expression of CYP11B2 mRNA in adrenal gland of rats treated with L-NAME increased (Fig. 1(B)). The L-NAME-induced increase in either level of plasma aldosterone or adrenal CYP11B2 mRNA was not altered by eplerenone. One week after the start of experimental protocol, there was no evidence of cardiac inflammation in the control group (Fig. 2(A)). In contrast, there was an inflammatory process in the L-NAME group that included the infiltration of α-SMA positive cells (myofibroblast-like cells)

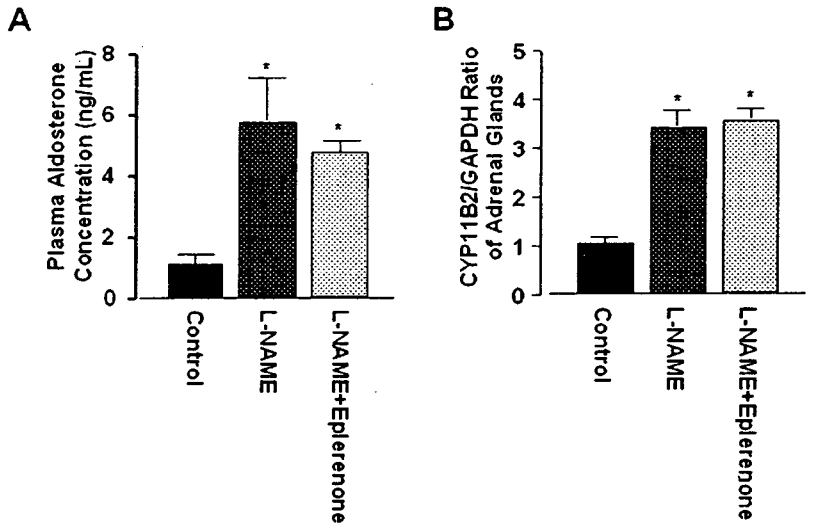

Fig. 1. *Effects of the chronic NOS inhibition on aldosterone production. (A) Plasma aldosterone concentration. (B) mRNA levels of CYP11B2 normalized by GAPDH. *P < 0.05 vs. the control group.*

and ED1-positive monocytes around the coronary arteries and into the myocardial interstitial spaces. Nuclear staining with PCNA antibody was observed in some endothelial cells, vascular smooth muscle cells in the media, monocytes, and myofibroblast-like cells. There was weak immunoreactivity for TGF-β1 and TGF-β1LAP in areas that normally contain collagen, such as the perivascular and myocardial interstitial spaces in the control group. In the L-NAME group, both TGF-β1 and TGF-β1LAP immunoreactivity were prominent in monocytes and/or spindle-shaped fibroblast-like cells. These inflammatory and proliferative changes were markedly attenuated by the treatment with eplerenone.

Then, we examined the possible involvement of oxidative stress in cardiac inflammation by observing the activation of NFκB and the expression of MCP-1 mRNA 1 week after the treatment. The binding activity of NFκB to the nucleus was increased and the cytosolic protein level of IκBα was decreased in the heart from the L-NAME group compared with the control group (Fig. 2(B) and (C)), indicating the activation of NFκB in the L-NAME group. These changes were prevented by eplerenone (Fig. 2(B) and (C)). The myocardial level of MCP-1 mRNA was also markedly increased in the L-NAME group, while this increase of MCP-1 expressions was significantly prevented by eplerenone (Fig. 2(D)).

Effects of aldosterone receptor blockade on cardiovascular remodeling

The wall-to-lumen ratio and perivascular fibrosis of coronary arteries in the L-NAME group were significantly greater than that in the control group, but these vascular structural changes were also prevented by eplerenone (Fig. 3(A)–(C)). Renal arteriolar damage by the chronic inhibition of NO synthesis was attenuated by eplerenone (Fig. 3(D) and (E)).

The extent of cardiac interstitial fibrosis was examined 8 weeks after the treatment with L-NAME (Fig. 4(A) and (C)). Cardiac interstitial fibrosis in the L-NAME group was greater than those in the control group (Fig. 4(A) and (C)). These structural changes were prevented by eplerenone (Fig. 4(A) and (C)). We also examined the cross-sectional areas of LV cardiomyocytes (Fig. 4(B) and (E)) and the LV weight to BW ratio (LVW/BW) (Fig. 4(D)). Both parameters were increased in the L-NAME group compared with those in the control group. The increases of both LVW/BW and cross-sectional area were partially, but significantly, attenuated by eplerenone.

Effects of aldosterone receptor blockade on cardiac function

Echocardiographic and hemodynamic studies were performed at 8 weeks of the treatment in each group (Fig. 5(A) and (B)). Quantitative echocardiographic data are presented in Table 2. In the L-NAME group, LVFS was decreased significantly compared with the control, indicating systolic dysfunction was induced when NO synthesis was chronically inhibited. Eplerenone completely prevented the reduction of LVFS induced by the treatment with L-NAME. In addition, although LVDd/BW ratio was comparable among the 3 groups, the E/A ratio was significantly decreased and the LVEDP was significantly elevated in the L-NAME group compared with the control, indicating that diastolic dysfunction was also induced when NO synthesis was chronically inhibited. Eplerenone completely prevented both E/A reduction and LVEDP elevation produced by L-NAME without changing the LVDd/BW.

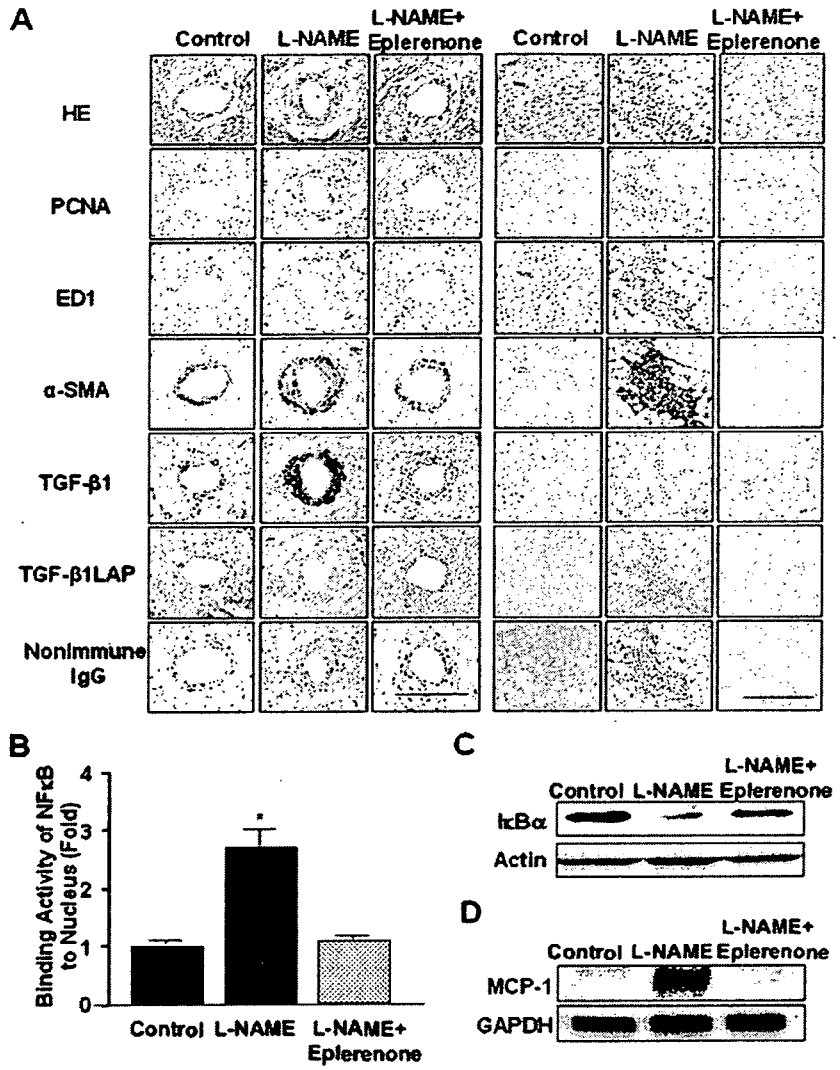

Fig. 2. *Effects of the aldosterone receptor blockade on cardiovascular inflammation 1 week after the treatment. (A) Representative histopathological and immunohistochemical changes of the coronary arteries (left panels) and myocardium (right panels). Sections were stained with HE and were immunostained for PCNA, ED1, α-SMA, TGF-β1, TGF-βLAP, and nonimmune IgG (negative control). Each bar indicates 100 μm. (B) The binding activity of NFκB to the nucleus. Binding activity in L-NAME treated groups was expressed as relative to that in control group. *P < 0.05 vs. the control group. (C) Western blot analysis of cytosolic IκBα protein from hearts. (D) Representative myocardial expression of MCP-1 and GAPDH mRNA. Abbreviations were described in the text.*

No significant differences were observed between the L-NAME and L-NAME + Eplerenone groups in urine output (27 ± 3 vs. 28 ± 4 mL/day, $n = 6$ each) and urinary sodium excretion (1.23 ± 0.255 vs. 1.63 ± 0.432 mmol/day, $n = 6$ each) at 8 weeks after the onset of the treatment.

Discussion

This study demonstrated that aldosterone receptor blockade could almost completely prevent cardiovascular inflammation, coronary and renal artery remodeling, and cardiac fibrosis, and partially, but significantly, prevented cardiac hypertrophy after the treatment with a NO synthesis inhibitor. Chronic inhibition of NO synthesis also induced systolic and diastolic dysfunction, while the aldosterone receptor blockade prevented the progression of LV systolic and diastolic dysfunction in this model.

Consistent with the previous report [32], the present study demonstrated that the chronic inhibition of NO synthesis increased both plasma aldosterone concentration and expression of CYP11B2 mRNA in adrenal gland of rats. In experimental model, the aldosterone administration with excess salt intake induces oxidative stress and inflammation that cause cardiovascular damages [33–35]. Consistent with these studies, Delcayre

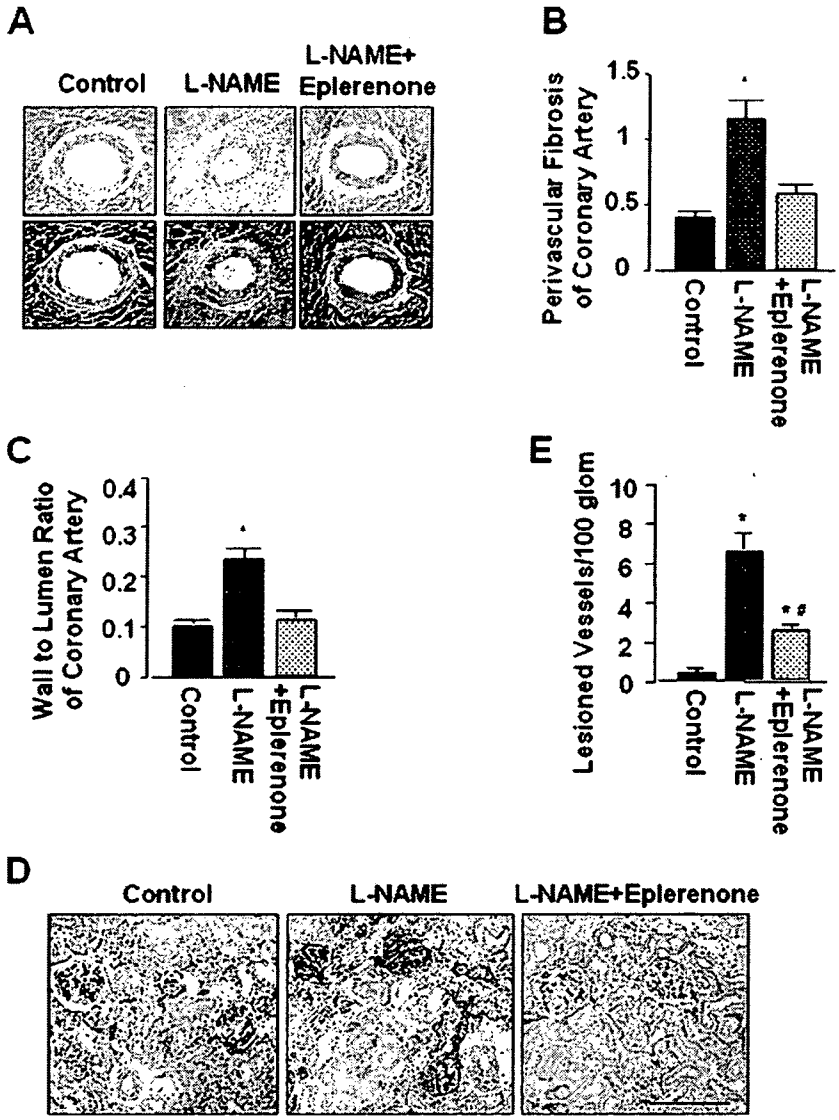

*Fig. 3. Effects of the aldosterone receptor blockade on both perivascular and myocardial interstitial fibrosis 8 weeks after the treatment. (A) Representative changes of coronary arteries with HE stain (upper panels) and Masson's trichrome stain (lower panels). Bar indicates 100 μm. (B) Perivascular fibrosis of coronary artery. (C) Wall-to-lumen ratio of coronary arteries. (D) Representative changes of kidney with periodic acid-Schiff stain. Bar indicates 100 μm. (E) Histopathological scores for renal vascular injury. *P < 0.05 vs. the control group. #P < 0.05 vs. the L-NAME group.*

et al. [36] demonstrated that increased cardiac aldosterone production in mice was not associated with vascular inflammation or myocardial fibrosis, suggesting that aldosterone alone does not induce cardiovascular structural remodeling. However, our model of rats treated with L-NAME and normal salt diet demonstrated increased plasma aldosterone level, perivascular and interstitial cardiac fibrosis, and cardiac hypertrophy. Since NO *per se* has anti-inflammatory and anti-hypertrophic effects, it is likely that the coexisting pathological conditions such as decreased NO production or excess salt intake would be required to augment the aldosterone-induced cardiovascular remodeling.

Aldosterone has been reported to increase ROS generation [37,38] and activate NFκB [39]. Chronic inhibition of NO synthesis in rats has been also reported to increase oxidative stress [40] and activate NFκB [41]. We also demonstrated that chronic inhibition of NO synthesis in rats activates NFκB and induce the expression of inflammatory cytokine such as MCP-1. The aldosterone receptor blockade inhibited the activation of NFκB, the up-regulation of MCP-1 mRNA, and reduced

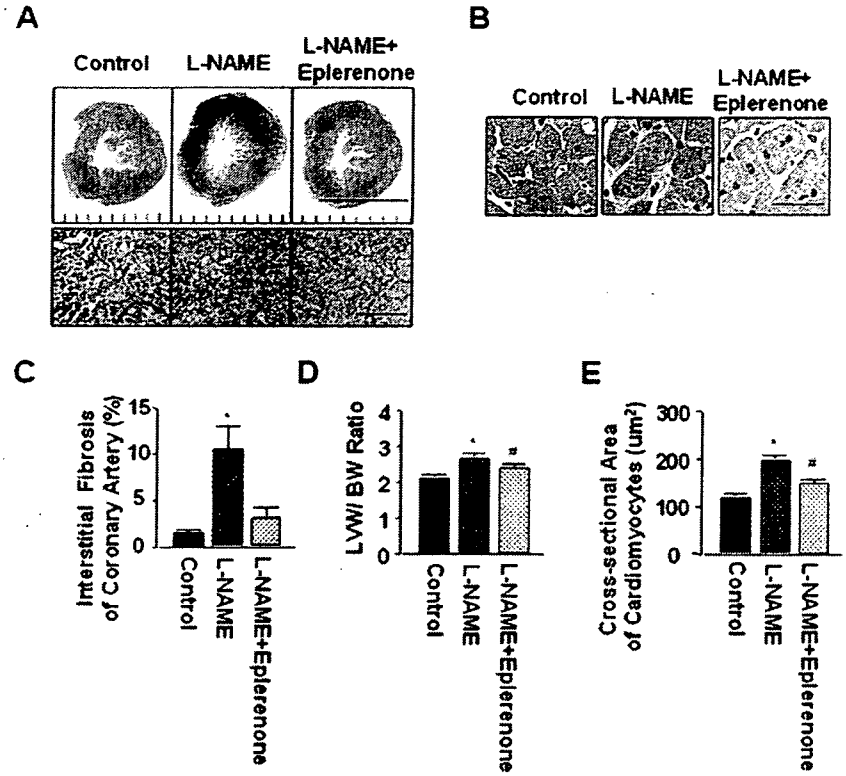

Fig. 4. *Effects of aldosterone receptor blockade on cardiac hypertrophy 8 weeks after the treatment. (A) Representative histological findings of the myocardium with Masson's trichrome stain. Upper and lower bars indicated 5 mm and 100 μm, respectively. (B) Representative histological findings of cardiomyocytes. A bar indicates 30 μm. (C) Myocardial interstitial fibrosis. (D) LV weight and BW ratio. (E) Cross-sectional area of cardiomyocytes. *P < 0.05 vs. the control group. #P < 0.05 vs. the L-NAME group.*

TGF-β1 immunoreactivity, all of which may contribute to the dramatic prevention of myocardial and vascular inflammation and the reduction of myocardial fibrosis. These findings suggest that chronic inhibition of NO synthesis may induce oxidative stress and cardiovascular inflammation via an aldosterone receptor-dependent mechanism. One of possible mechanisms by which aldosterone induces oxidative stress and cardiac inflammation is a fall in cytosolic $[Mg^{2+}]_I$ with Ca^{2+} loading of peripheral blood mononuclear cells by inappropriate chronic elevations in plasma aldosterone level (relative to dietary Na^+), which results in H_2O_2 production in these cells and contributes to cardiovascular inflammatory phenotype [38,42]. Furthermore, since NO has anti-inflammatory effects, we must consider that chronic inhibition of NO synthesis augments aldosterone-induced oxidative and inflammatory changes in our model.

In contrast to complete prevention of inflammation and fibrosis, an aldosterone receptor blockade only achieved partial prevention of the cardiomyocyte hypertrophy induced by chronic inhibition of NO synthesis. Consistent with these data, left ventricular hypertrophy was also partially prevented by the aldosterone receptor blockade. Although it remains controversial that aldosterone causes cardiac hypertrophy directly [24,36], there are 2 possible mechanisms by which aldosterone receptor blockade could attenuate cardiac hypertrophy. One is the attenuation of inflammation,which has also

Table 2. *Echocardiographic data*

	Control (n = 6)	L-NAME (n = 6)	L-NAME + Eplerenone (n = 6)
LVDd/BW, mm/mg	22 ± 1	25 ± 1	23 ± 1
LVDs/BW, mm/mg	14 ± 1#	18 ± 1	15 ± 1#
LVFS, %	37.5 ± 0.6#	27.4 ± 2.4	36.0 ± 2.0#
E/A ratio	2.16 ± 0.08#	1.67 ± 0.11	2.15 ± 0.08#

LVDd indicates left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension, LVFS, left ventricular fractional shortening; BW, body weight. Data are mean ± SEM.

#P < 0.05 vs. the L-NAME group.

receptor blockade may contributable to the improvement of diastolic function [45,46]. Rats in the L-NAME group also showed a decrease of LVFS, indicating that systolic dysfunction was induced by the chronic inhibition of NO synthesis. Systolic dysfunction may be caused by loss of cardiomyocytes due to myocardial inflammation and fibrosis and/or by the increase of proinflammatory cytokines which have cardiodepressive and proapoptotic effect [25]. The anti-inflammatory and antifibrotic actions of aldosterone receptor blockade may have contributed to the prevention of systolic dysfunction caused by chronic inhibition of NO synthesis. Since we used eplerenone at the dose that did not affect SBP and urinary output and sodium excretion, the beneficial effects of aldosterone receptor blockade on cardiovascular remodeling were independent of anti-hypertensive or diuretic effects.

Patients with hypertension and chronic heart failure show endothelial dysfunction that may be largely attributable to the reduced NO bioavailability [15–17]. Since endothelial-derived NO is recognized to have an anti-inflammatory effect [47,48], the reduced NO bioavailability may cause activation of inflammatory responses. Indeed, there are several reports showing the activation of immunologic and inflammatory responses that may play a pathogenic role in the development of chronic heart failure [25,26]. In present study, we demonstrated that chronic inhibition of NO synthesis led to increased aldosterone synthesis. Interestingly, spironolactone improves endothelial dysfunction and increases NO bioavailability in patients with chronic heart failure [8], suggesting that elevated aldosterone may decrease NO bioactivity in patients with hypertension and chronic heart failure. Thus, in addition to the direct blockade of aldosterone's effects, aldosterone antagonism may prevent a vicious cycle between the activation of renin-angiotensin-aldosterone system and reduced NO bioavailability in such patients.

In conclusion, chronic inhibition of NO synthesis induces cardiovascular inflammation that is followed by cardiovascular remodeling as well as LV systolic and diastolic dysfunction via an aldosterone receptor-dependent mechanism.

Acknowledgment

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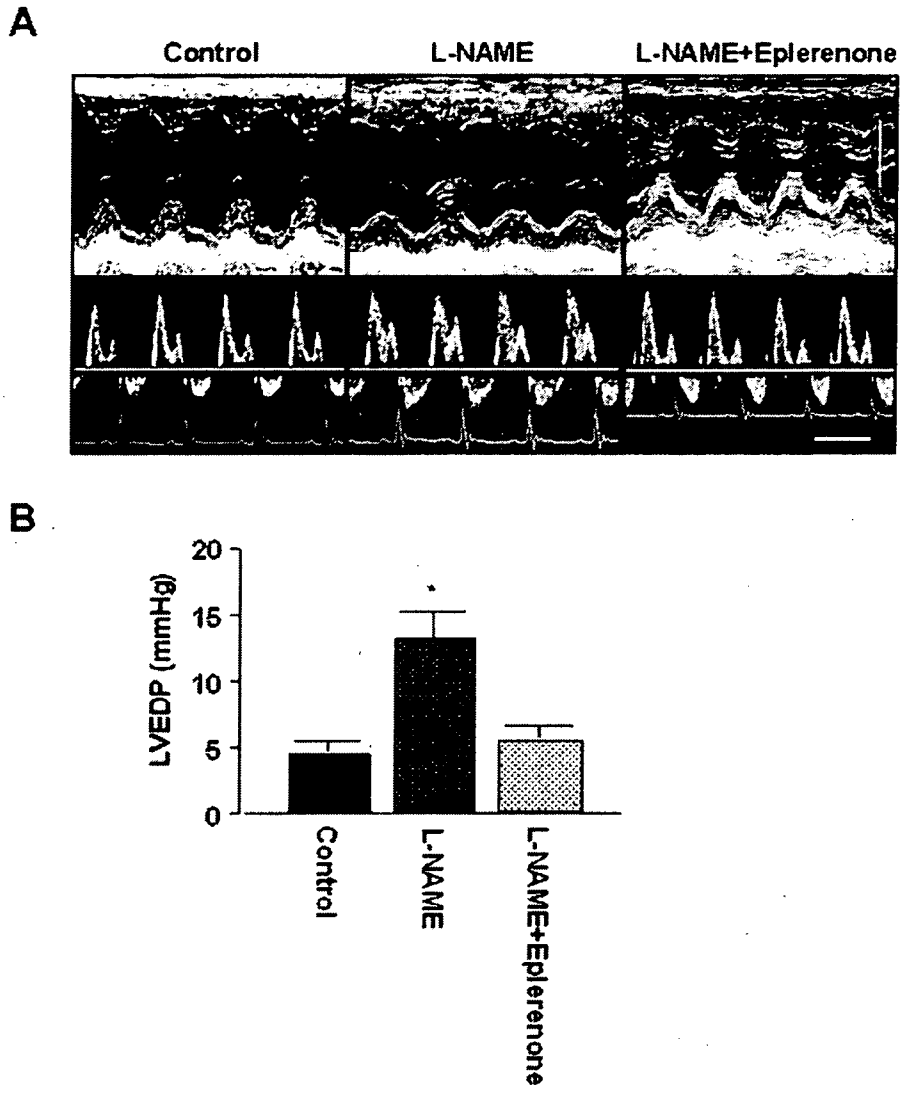

*Fig. 5. Effects of aldosterone receptor blockade on cardiac function 8 weeks after the treatment. (A) Representative M-mode echocardiogram (top) and transmitral inflow Doppler patterns (bottom). Vertical and horizontal bars indicates 5 mm and 200 msec, respectively. (B) LVEDP. *P < 0.05 vs. the control group.*

been reported to provoke cardiac hypertrophy [26,43]. The other is the inhibition of positive feedback from aldosterone to ACE in the local cardiac renin-angiotensin-aldosterone system [44]. In the previous studies, the pathways that involve angiotensin II are activated in the chronic NO synthesis inhibition model [20]. Since angiotensin II can directly induce cardiac hypertrophy, it is likely that the complete inhibition of cardiac hypertrophy was not obtained by eplerenone alone. Further investigations will be needed to clarify the contribution of aldosterone to the development of cardiac hypertrophy when NO synthesis was chronically inhibited. Since aldosterone breakthrough during ACE inhibitor or ARB therapy has been reported [4], the combination of a selective aldosterone receptor blocker and an ACE inhibitor or an ARB would be useful for the prevention of cardiovascular inflammation and fibrosis, and renal damage in patients with hypertension and chronic heart failure.

Aldosterone receptor blockade completely prevented systolic and diastolic dysfunctions induced by chronic inhibition of NO synthesis. The rats in the L-NAME group showed a decreased E/A ratio and elevated LVEDP in the absence of LVDd/BW enlargement, which indicates diastolic dysfunction. One of possible mechanisms by which chronic inhibition of NO synthesis induced diastolic dysfunction may be increased myocardial stiffness caused by fibrosis and hypertrophy [45,46]. Thus, it is likely that the prevention of myocardial fibrosis and attenuation of cardiac hypertrophy by aldosterone

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Human atrial natriuretic peptide and nicorandil as adjuncts to reperfusion treatment for acute myocardial infarction (J-WIND): two randomised trials

*Masafumi Kitakaze, Masanori Asakura, Jiyoong Kim, Yasunori Shintani, Hiroshi Asanuma, Toshimitsu Hamasaki, Osamu Seguchi, Masafumi Myoishi, Tetsuo Minamino, Takahiro Ohara, Yoshiyuki Nagai, Shinsuke Nanto, Kouki Watanabe, Shigeru Fukuzawa, Atsushi Hirayama, Natsuki Nakamura, Kazuo Kimura, Kenshi Fujii, Masaharu Ishihara, Yoshihiko Saito, Hitonobu Tomoike, Soichiro Kitamura, and the J-WIND investigators**

Summary

Background Patients who have acute myocardial infarction remain at major risk of cardiovascular events. We aimed to assess the effects of either human atrial natriuretic peptide or nicorandil on infarct size and cardiovascular outcome.

Methods We enrolled 1216 patients who had acute myocardial infarction and were undergoing reperfusion treatment in two prospective, single-blind trials at 65 hospitals in Japan. We randomly assigned 277 patients to receive intravenous atrial natriuretic peptide (0·025 μg/kg per min for 3 days) and 292 the same dose of placebo. 276 patients were assigned to receive intravenous nicorandil (0·067 mg/kg as a bolus, followed by 1·67 μg/kg per min as a 24-h continuous infusion), and 269 the same dose of placebo. Median follow-up was 2·7 (IQR 1·5–3·6) years for patients in the atrial natriuretic peptide trial and 2·5 (1·5–3·7) years for those in the nicorandil trial. Primary endpoints were infarct size (estimated from creatine kinase) and left ventricular ejection fraction (gauged by angiography of the left ventricle).

Findings 43 patients withdrew consent after randomisation, and 59 did not have acute myocardial infarction. We did not assess infarct size in 50 patients for whom we had fewer than six samples of blood. We did not have angiographs of left ventricles in 383 patients. Total creatine kinase was 66459·9 IU/mL per h in patients given atrial natriuretic peptide, compared with 77878·9 IU/mL per h in controls, with a ratio of 0·85 between these groups (95% CI 0·75–0·97, p=0·016), which indicated a reduction of 14·7% in infarct size (95% CI 3·0–24·9%). The left ventricular ejection fraction at 6–12 months increased in the atrial natriuretic peptide group (ratio 1·05, 95% CI 1·01–1·10, p=0·024). Total activity of creatine kinase did not differ between patients given nicorandil (70520·5 IU/mL per h) and controls (70852·7 IU/mL per h) (ratio 0·995, 95% CI 0·878–1·138, p=0·94). Intravenous nicorandil did not affect the size of the left ventricular ejection fraction, although oral administration of nicorandil during follow-up increased the left ventricular ejection fraction between the chronic and acute phases. 29 patients in the atrial natriuretic peptide group had severe hypotension, compared with one in the corresponding placebo group.

Interpretation Patients with acute myocardial infarction who were given atrial natriuretic peptide had lower infarct size, fewer reperfusion injuries, and better outcomes than controls. We believe that atrial natriuretic peptide could be a safe and effective adjunctive treatment in patients with acute myocardial infarction who receive percutaneous coronary intervention.

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See **Comment** page 1461

*Other investigators listed at end of study

Cardiovascular Division of Medicine, National Cardiovascular Centre, Suita, Osaka, Japan (Prof M Kitakaze MD, M Asakura MD, J Kim MD, O Seguchi MD, M Myoishi MD, T Ohara MD, Prof H Tomoike MD, Prof S Kitamura MD); **Department of Clinical Research and Development, National Cardiovascular Centre, Suita, Osaka, Japan** (M Kitakaze MD, M Asakura MD); **Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan** (Y Shintani MD, T Minamino MD); **Research Institute, National Cardiovascular Centre, Suita, Osaka, Japan** (H Asanuma MD); **Department of Biomedical Statistics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan** (T Hamasaki PhD); **Heart Centre Department of Cardiology, Rinku General Medical Centre, Izumisano, Osaka, Japan** (Y Nagai MD); **Cardiovascular Division, Kansai Rosai Hospital, Amagasaki, Hyogo, Japan** (Prof S Nanto MD); **Department of Cardiology, Uwajima-City Hospital, Uwajima, Ehime, Japan** (K Watanabe MD); **Division of Cardiology, Funabashi Municipal Medical Centre, Funabashi, Chiba, Japan** (S Fukuzawa MD); **Cardiovascular Division, Osaka Police Hospital, Osaka, Osaka, Japan** (Prof A Hirayama MD); **Department of Cardiology, Shinbeppu Hospital, Beppu, Oita, Japan** (N Nakamura MD); **Division of Cardiology, Yokohama City University Medical Centre, Yokohama,**

Introduction

Despite availability of effective medical treatments, chronic heart failure remains a major cause of morbidity and mortality worldwide.[1–3] Ischaemic heart disease, in turn, is one of the main causes of chronic heart failure.[4] The most important treatment objectives are prevention of acute myocardial infarction, and, in individuals who have an acute myocardial infarction, reduction in infarct size and ischaemia or reperfusion injury.[5] Only a few medications have been shown to decrease ischaemia or reperfusion injury.[6–8]

Reperfusion of ischaemic myocardium reduces infarct size and improves left ventricular function, both of which contribute to better clinical outcomes in patients with acute myocardial infarction.[9–11] However, reperfusion can also cause tissue damage.[12] Several drugs have been trialled for the prevention or amelioration of such injuries, but results have not been consistently satisfactory.[13–15] Recently, human atrial natriuretic peptide and nicorandil have both been shown to be effective for reduction of myocardial damage after acute myocardial infarction in basic and clinical studies.[16–25] Atrial natriuretic peptide is a candidate for adjunctive treatment after acute myocardial infarction, because it has been shown to suppress the renin–angiotensin–aldosterone system and endothelin-1, both of which which modulate infarct size and cardiac remodelling.[19] Nicorandil is a combined adenosine triphosphate (ATP)-sensitive potassium channel opener and nitrate preparation that has also shown promise as an adjunctive treatment for acute myocardial infarction. In the clinical setting, however,

Kanagawa, Japan (Prof K Kimura MD); Division of Cardiology, Sakurabashi Watanabe Hospital, Osaka, Osaka, Japan (K Fujii MD); Department of Cardiology, Hiroshima City Hospital, Hiroshima, Hiroshima, Japan (M Ishihara MD); and First Department of Medicine, Nara Medical University, Kashihara, Nara, Japan (Prof Y Saito MD)

Correspondence to: Professor Masafumi Kitakaze, Cardiovascular Division of Medicine, National Cardiovascular Centre Suita, Osaka 565-8565, Japan
kitakaze@hsp.ncvc.go.jp

the beneficial effects of atrial natriuretic peptide and nicorandil have only been tested in single-centre studies with small sample sizes.[20–25] The Japan working group studies on acute myocardial infarction for the reduction of necrotic damage by human atrial natriuretic peptide or nicorandil (J-WIND-ANP and J-WIND-KATP, respectively) aimed to assess the value of these drugs as adjuncts to percutaneous coronary intervention for patients with acute myocardial infarction.

Methods

Patients

We have described the protocols for the two trials previously.[26,27] In brief, we recruited patients to two independent, investigator-initiated, investigator-led, multicentre, prospective, randomised, single-blind, controlled trials at 65 hospitals. 27 hospitals participated in the atrial natriuretic peptide trial, and 38 separate hospitals in the nicorandil trial (table 1); the two studies were completely independent. We initially planned to include fewer hospitals, but we increased the number to promote enrolment of sufficient patients.

Eligibility criteria were age between 20 and 79 years; chest pain for more than 30 min; at least 0·1 mV of ST segment elevation in two adjacent ECG leads; admission to hospital within 12 h of the onset of symptoms; and one instance of acute myocardial infarction. Exclusion criteria were a history of myocardial infarction; left main trunk stenosis; severe liver or kidney dysfunction or both; suspected aortic dissection; previous coronary artery bypass grafting; and a history of drug allergy.

All patients gave written informed consent immediately after admission to hospital, and were asked to sign the same consent form again after 2 weeks when they had more time to decide. This system was applied on the recommendation of the institutional review boards. Only one patient, who was in the nicorandil group, withdrew their consent at their second opportunity. We enrolled patients from Oct 24, 2001, to Dec 13, 2005. The study protocol was approved by the institutional review boards and ethics committees of all participating hospitals, and was in accordance with the Declaration of Helsinki.

Procedures

An independent statistician generated our randomisation lists with a computer, by the permuted-block method. Within each centre, the block length was eight. Treatment allocations were concealed in opaque sealed envelopes until patients were enrolled. Physicians were not aware of the random assignments of patients until the follow-up stage; patients and those who analysed the data were unaware of the treatment assignment for the duration of the study. Both trials were designed as single-blind studies.

277 patients who were enrolled in the atrial natriuretic peptide trial were randomly assigned to receive an intravenous infusion of this drug after reperfusion treatment, at 0·025 μg/kg per min for 3 days, and 292 a placebo of 5% glucose solution by the same method. 276 patients in the other trial were randomly assigned to intravenous nicorandil, infused at 1·67 μg/kg per min for 24 h after bolus injection of nicorandil at a dose of 0·067 mg/kg, and 269 were assigned to 0·9% saline solution, by the same method. Previous studies have shown substantial cardiovascular protection with atrial natriuretic peptide and nicorandil at these doses.[20,22] Of the 276 patients assigned to receive nicorandil, 61 were given nicorandil orally, at the discretion of individual investigators, during the follow-up period.

We planned to stop the administration of treatment drugs in case of severe hypotension, which was defined as systolic blood pressure of less than 90 mm Hg, because of the vasodilator effect of these drugs. The study protocol did not restrict or specify any other diagnostic or therapeutic methods in the acute phase (2–8 weeks after acute myocardial infarction) or chronic phase (6–12 months).

We obtained data on baseline characteristics, emergent catheterisation, and medication at discharge after 1 month; data on follow-up catheterisation and medication after 6 months; and data on medication after 24 months. We also followed up all patients for cardiovascular events (ie, cardiac death, readmission to hospital due to heart failure, new onset of acute coronary syndrome, or revascularisation of new lesions) until the end of August, 2006. We took blood samples to measure concentrations of creatine kinase at a central laboratory, before the procedure and at 1, 3, 6, 9, 12, 18, 24, 36, 48, and 72 h after the onset of reperfusion.[14] We analysed total creatine kinase for all patients with at least six blood samples. We obtained right anterior oblique views with angiography of the left ventricle once in the acute phase (2–8 weeks), and once in the chronic phase (6–12 months).

Our primary endpoints were infarct size (which was estimated as the area under the concentration versus time curve for creatine kinase)[14] and ventricular ejection fraction (which was assessed by angiography of the left ventricle at 6–12 months after hospital admission).[15] The prespecified secondary endpoints were survival rate; cardiovascular events (such as cardiac death, readmission to hospital for heart failure, new onset of acute coronary syndrome, or revascularisation of new lesions); incidence of cardiac death or readmission to hospital for

	J-WIND-ANP study	J-WIND-KATP study
1–4 patients	7 hospitals	9 hospitals
5–9 patients	3 hospitals	13 hospitals
10–19 patients	7 hospitals	6 hospitals
More than 20 patients	10 hospitals	10 hospitals

Table 1: **Distribution of patients between participating hospitals**