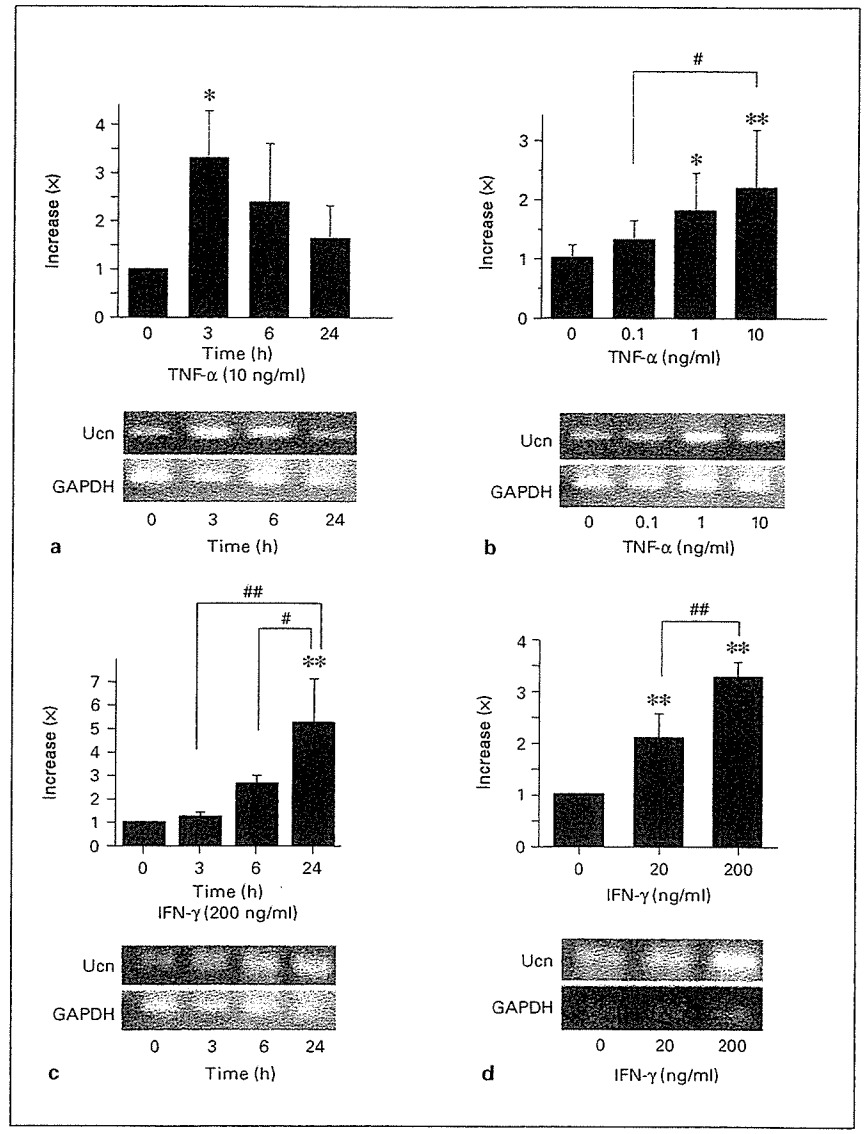


Fig. 4. Effects of TNF-α (**a**, **b**) or IFN-γ (**c**, **d**) on urocortin (Ucn) mRNA in HUVECs assessed by RT-PCR. **a**, **b** HUVECs were incubated with TNF-α (10 ng/ml) for the indicated time periods (**a**) or for 6 h with the indicated concentration of TNF-α (**b**). **c**, **d** HUVECs were incubated with IFN-γ (200 ng/ml) for the indicated time periods (**c**) or for 6 h with the indicated concentration of IFN-γ (**d**). After stimulation, mRNA levels of urocortin were assessed by RT-PCR. Densitometric analysis of five independent experiments. * $p<0.05$; ** $p<0.01$ vs. control; # $p<0.05$; ## $p<0.01$.

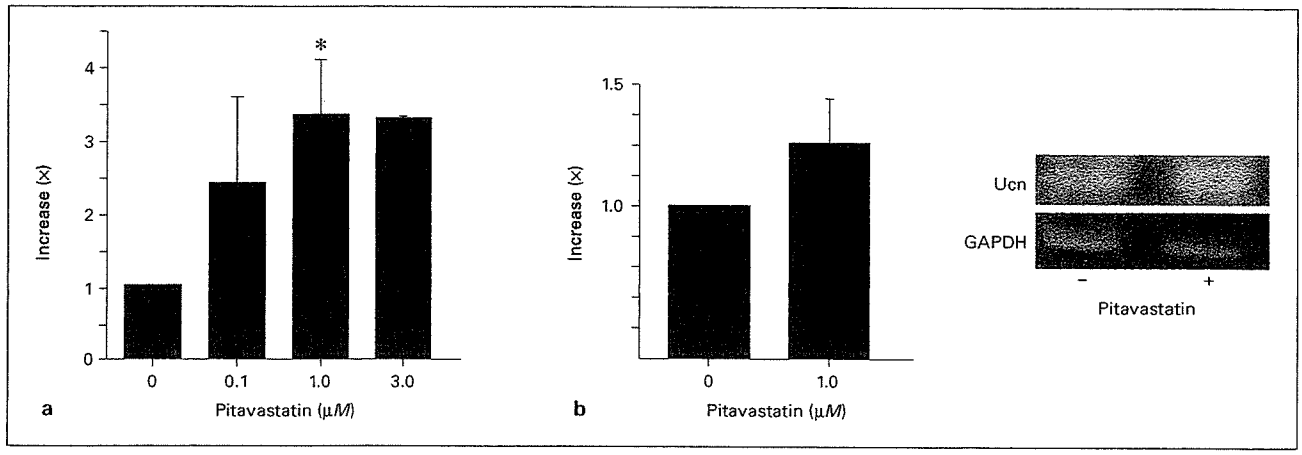
Expression of Endothelial Urocortin Was Increased by Inflammatory Cytokines and Pitavastatin

Because previous studies demonstrated that various cytokines modulate the expression of urocortin in neurons, the effects of inflammatory cytokines on expression of endothelial urocortin were examined by ELISA. Incubation with TNF-α (10 ng/ml) resulted in increased urocortin production in a time- and dose-dependent manner (fig. 3a, b). IFN-γ also increased the urocortin release from HUVECs (fig. 3c, d).

RT-PCR demonstrated that TNF-α and IFN-γ increased the steady state urocortin mRNA levels in HUVECs (fig. 4). Incubation with pitavastatin (0.1–3.0 μ*M*) potently increased the release of urocortin from HUVECs in a dose-dependent manner (fig. 5a). RT-PCR revealed modestly increased urocortin mRNA from HUVECs (fig. 5b).

Treatment with Pitavastatin Increases Serum Urocortin Levels in Humans

The findings using cultured HUVECs prompted us to examine the effects of treatment with pitavastatin on plasma urocortin levels in humans. Treatment with pitavastatin (2 mg/day) for 4 weeks decreased total cho-



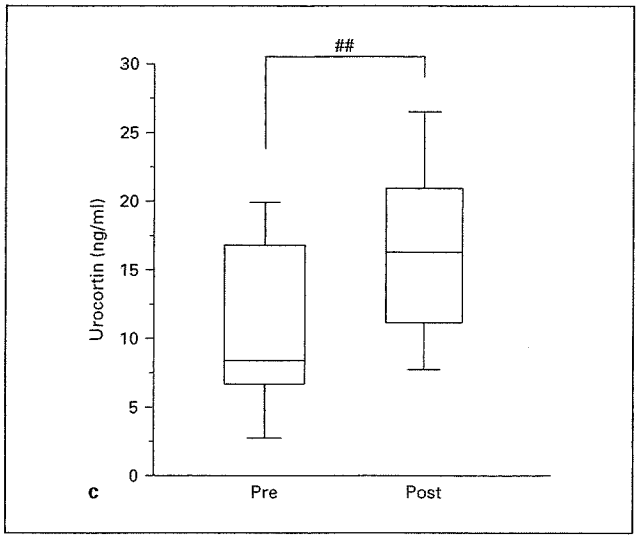


Fig. 5. a, b Effects of pitavastatin on urocortin release (**a**) and its mRNA (**b**) in HUVECs. HUVECs were incubated with pitavastatin with the indicated concentration for 24 h. After stimulation, the concentration of urocortin in the conditioned medium was assessed by ELISA (**a**). Data were plotted as mean ± SEM from three independent experiments, performed in duplicate. Pitavastatin-induced changes in urocortin mRNA were assessed by RT-PCR (**b**). Densitometric analysis of three independent experiments. * $p < 0.05$ vs. control. **c** Treatment with pitavastatin increases serum levels of urocortin in humans. Treatment with pitavastatin (2 mg/day) for 4 weeks increased urocortin levels in healthy male volunteers. ## $p < 0.01$.

Table 1. Change of serum levels of various parameters by pitavastatin

Charasteristics	Mean ± SD (pre)	Mean ± SD (post)
Blood glucose	96 ± 10.4	93 ± 6.5
Total cholesterol, mg/dl	192.8 ± 29.7	149.2 ± 27.4***
TG, mg/dl	88.4 ± 44.6	86.4 ± 40.3
HDL cholesterol, mg/dl	69.6 ± 15.1	67 ± 15.3
LDL cholesterol, mg/dl	100.0 ± 25.4	61.0 ± 22.5***
hsCRP, ng/ml	257.5 ± 235.9	254.1 ± 248.6
Serum urocortin, ng/ml	11.0 ± 6.5	16.4 ± 7.3**

Treatment with pitavastatin (2 mg/day) for 4 weeks significantly decreased total cholesterol and LDL cholesterol levels. Pitavastatin significantly increased serum urocortin levels. *** $p < 0.0001$ vs. pre. ** $p < 0.01$ vs. pre.

lesterol and LDL levels (table 1). Interestingly, pitavastatin increased urocortin levels from 11.0 ± 6.5 to 16.4 ± 7.3 ng/ml in healthy male volunteers (fig. 5c).

Discussion

Urocortin was originally identified in the central nervous system [1–3]; however, there is considerable evidence indicating that urocortin is also expressed in other organs, including the gastrointestinal [5], immune [6, 7] and cardiovascular systems [9]. There is no direct evidence, however, that urocortin is expressed in the vasculature. The present investigation is the first study to demonstrate that urocortin is expressed in endothelial cells.

Furthermore, urocortin (10^{-8} *M*) potently suppressed ROS generation induced by angiotensin II in HUVECs. TNF-α and IFN-γ increased urocortin mRNA level and its release from HUVECs. Interestingly, incubation with pitavastatin significantly increased urocortin mRNA levels and its release from HUVECs. Thus, endothelial urocortin is upregulated by inflammatory cytokines and pitavastatin, and it has anti-oxidative properties in endothelial cells. Furthermore, treatment with pitavastatin for 4 weeks increased the serum urocortin levels in healthy male volunteers.

Urocortin is expressed in various immunocompetent cells such as macrophages and lymphocytes [7], and in inflammatory lesions. For example, Kohno et al. [6] reported that urocortin is expressed in the synovium of rheumatoid arthritis patients. The expression of immunoreactive urocortin in rheumatoid arthritis patients correlates with the extent of inflammatory infiltrates [6]. On the other hand, the concentration of immunoreactive urocortin was higher in gastric biopsies obtained from patients with active *Helicobacter pylori* gastritis than in normal controls [5]. After the apparent eradication of *H. pylori* infection, immunoreactive urocortin levels increased dramatically compared with pretreatment values. These findings strongly suggest that urocortin has a possible role in the pathogenesis of these inflammatory diseases. There remains controversy, however, as to whether urocortin exerts proinflammatory or anti-inflammatory effects. Urocortin increases the secretion of interleukin-6 from peripheral blood mononuclear cells [6], suggesting it has proinflammatory actions. In contrast, Agnello et al. [24] reported that urocortin significantly reduces lipopolysaccharide-induced serum TNF-α and interleukin-1β levels in mice. In the present investigation, urocortin potently suppressed ROS generation in HUVECs, whereas inflammatory cytokines increased urocortin expression and its release from HUVECs. Taken together, these findings suggest that endothelial urocortin acts as an anti-oxidative factor, and the upregulation of urocortin by inflammatory cytokines is part of a counter-regulatory mechanism against oxidative stress in inflammatory lesions.

Recently, a wide variety of pleiotropic effects of HMG-CoA reductase inhibitors was proposed, including improvement of endothelial function, stabilization of vulnerable plaque, anti-coagulation effects, anti-inflammatory effects, anti-oxidative effects, etc. [29, 30]. Treatment with HMG-CoA reductase inhibitors improves the functional classification of the New York Heart Association and left ventricular ejection fraction in patients with dilated cardiomyopathy [23]. Furthermore, plasma concentrations of TNF-α, interleukin-6, and brain natriuretic peptide are also significantly decreased by HMG-CoA reductase inhibitor treatment [23]. Thus, HMG-CoA reductase inhibitors likely induce potent cardioprotective effects. In the present study, pitavastatin increased the release of urocortin from HUVECs. There was no statistically significant increase in urocortin mRNA levels after incubation with pitavastatin, although mRNA levels tended to increase. Posttranscriptional regulation may mediate the increase in urocortin mRNA levels during incubation with pitavastatin. Furthermore, treatment with pitavastatin increased the serum urocortin levels in humans. Given its potent cardioprotective effects, urocortin might be partly involved in the mechanisms of the pleiotropic effects of HMG-CoA reductase inhibitors in the vasculature. The pitavastatin-induced increase in urocortin may originate from several possible sources, including the central and peripheral nervous systems, cardiomyocytes, and endothelial cells. Further investigation is needed to clarify the precise mechanisms whereby pitavastatin increases the serum urocortin levels.

In conclusion, urocortin was expressed in human endothelial cells. Endothelial urocotin is upregulated by inflammatory cytokines and pitavastatin, and it suppressed the production of ROS in endothelial cells. Treatment with pitavastatin increased the serum urocortin levels in human subjects. Endothelial urocortin might exert anti-oxidative effects in inflammatory lesions and this might partially explain the mechanisms of various pleiotropic effects of statins.

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Toll-like receptor 4 expressions on peripheral blood monocytes were enhanced in coronary artery disease even in patients with low C-reactive protein

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Abstract

Toll-like receptors (TLRs) play important roles in the pathogenesis of atherosclerosis. On the other hand, serum high sensitivity C-reactive protein (hsCRP) is known as an independent coronary risk factor, but cardiovascular events do occur even in low hsCRP levels. We investigated whether the TLR4 expression levels on human peripheral blood monocytes were associated with serum hsCRP levels or the occurrence of coronary artery diseases (CAD). One hundred CAD patients and 100 non-CAD subjects were enrolled. There were 72 non-CAD subjects and 53 CAD patients with low serum hsCRP levels. Among the low-hsCRP subjects, the TLR4 expression levels were higher in CAD patients than in non-CAD subjects ($P<0.05$, after being adjusted for other risk factors). Moreover, TLR4 expression levels in stable angina pectoris (SAP) patients were elevated compared with those in non-CAD subjects ($P<0.05$), and those in acute coronary syndrome patients were higher than SAP patients even in low-hsCRP subjects ($P<0.01$). In conclusion, the TLR4 expression levels on peripheral blood monocytes in CAD patients were higher than those in non-CAD subjects and correlated with disease activity, even in low-hsCRP subjects. The combined measurement of serum hsCRP and the TLR4 expression on peripheral blood monocytes, especially among low-hsCRP subjects, may become a new coronary risk marker.

Keywords: Inflammation; Immune system; Coronary artery disease

Introduction

Atherosclerosis is a chronic inflammatory disease (Ross, 1993). Vascular inflammation occurs in response to injury induced by various stimuli, such as oxidative stress (Madamanchi et al., 2005), shear stress (Shaaban and Duerinckx, 2000; Traub and Berk, 1998), and infection (Espinola-Klein et al., 2002; Danesh et al., 1997; Shor et al., 1992). The precise mechanisms whereby chronic infection causes cardiovascular disease, however, remain to be elucidated.

Toll-like receptors (TLRs) are pathogen-associated molecular pattern recognition molecules that play a crucial role in innate immunity as the first defense system against microbial infection (Akira and Hemmi, 2003). Eleven TLRs have been identified, and TLR1, TLR2, and TLR4 are intensely expressed in human atherosclerotic vessels (Edfeldt et al., 2002), and deletion of the TLR4 gene was shown to reduce atherosclerosis in apoprotein E knockout mice (Michelsen et al., 2004). We recently demonstrated that TLRs are expressed on human platelets and coronary thrombi of patients with acute coronary syndrome (Shiraki et al., 2004). These findings strongly suggest that the TLRs yield an important role in the pathogenesis of atherosclerotic vascular diseases. The association between TLRs and cardiovascular diseases is supported by the recent observations that a single nucleoside polymorphism of the TLR4 gene is closely related to a decreased

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risk of acute coronary events (Kiechl et al., 2002). Furthermore, very recently Methe et al. reported the enhanced expression of TLR4 in circulating monocytes in patients with unstable angina and acute myocardial infarction (Methe et al., 2005).

It is well recognized that there is an increase in serum C-reactive protein (CRP) levels in acute coronary syndrome, reflecting the presence of systemic inflammation in this pathological state. In addition, serum levels of CRP have been revealed as an independent predictor of cardiovascular events in patients with stable and unstable angina, high-risk individuals, and apparently healthy individuals. The Center for Disease Control and Prevention and the American Heart Association issued the first set of clinical guidelines for CRP as a part of global risk prediction and suggested that serum levels of high-sensitivity CRP (hsCRP) under 1 mg/L, 1 to 3 mg/L, and 3 or more mg/L represent low, moderate, and high vascular risk, respectively (Smith et al., 2004). Individuals with hsCRP under 1 mg/L are not, however, necessarily free of cardiovascular disease. Serum hsCRP levels are largely influenced by various factors including systemic inflammatory status. Further, hsCRP, which is a marker of systemic inflammation, may not always be associated with inflammatory changes occurring locally at the coronary vasculature.

In the present study, we investigated TLR4 expression levels on human peripheral blood monocytes in patients with coronary artery disease. Then we assessed whether TLR4 expression levels were related to serum hsCRP levels to develop a future coronary risk factor from the standpoint of inflammatory aspects of atherosclerotic vascular diseases.

Materials and methods

Subjects

The study protocol was approved by the Human Research Committee of Kobe University of Medicine and Kobe Steel Hospital, and written informed consent was obtained from all participants. CAD group included patients admitted to Kobe University Hospital for a diagnosis of stable angina pectoris (SAP) and acute coronary syndrome (ACS), which includes unstable angina (UAP) or acute myocardial infarction (AMI). UAP was defined according to the Braunwald's classification. SAP patients had clinical evidence of Canadian Cardiovascular Society class II and III, and coronary artery stenosis angiographically documented greater than 75% and perfusion scintigraphy showed myocardial ischemia.

The non-CAD subjects (non-CAD group) were inpatients and outpatients of Kobe University Hospital and Kobe Steel Hospital with diagnosis of hypertension, hyperlipidemia, diabetes, chest pain syndrome, or valvular heart disease. These subjects had neither symptoms of angina pectoris nor electrocardiographical abnormalities after exercise-loading test.

Patients with coronary interventions with 7 days, previous myocardial infarction within 6 months, heart failure (ejection fraction<40% or fractional shortening<20%), renal failure (serum creatinin levels>2.0 mg/dl), cancer, autoimmune disease, and infectious disease were excluded from the study. None of the patients were taking any anti-inflammatory agents other than aspirin (up to 100 mg daily).

Table 1

	Total population			Low CRP subjects		
	Non-CAD group (n=100)	CAD group (n=100)	P	Non-CAD group (n=72)	CAD group (n=49)	P
Age, y	63.6±12.4	68.0±8.4	0.03	62.9±12.6	67.8±7.9	n.s.
Male sex, %	59	77	0.01	56.9	77.6	0.032
BMI, kg/m^2	24.2±3.0	24.3±3.0	n.s	23.9±2.9	23.8±3.0	n.s
Hypertension, %	67	84		68	88	
Systolic blood pressure, mm Hg	131.5±16.6	132.3±20.0	n.s.	130.9±16.2	133.6±20.4	n.s.
Diastolic blood pressure, mm Hg	76.1±10.0	71.1±9.6	<0.001	76.3±10.6	72.2±10.6	n.s.
Hyperlipidemia, %	41	53		43	55	
Total cholesterol, mg/dl	203.3±30.1	184.8±29.3	<0.0001	199.9±29.5	183.4±28.6	0.003
HDL cholesterol, mg/dl	58.3±14.5	47.4±11.7	<0.0001	61.0±15.0	48.5±12.1	<0.0001
Triglycerides, mg/dl	144.2±72.0	133.3±55.1	n.s.	143.0±66.9	136.0±62.8	n.s.
Diabetes mellitus, %	14	52		10	53	
HbAlc, %	5.5±0.9	6.1±1.2	<0.0001	5.5±0.9	6.1±1.1	<0.0001
CRP, mg/L	0.51 (0.31–1.16)	0.93 (0.37–2.0)	<0.01	0.39 (0.22–0.61)	0.39 (0.24–0.63)	n.s.
Medications, %						
Aspirin	19	79	<0.0001	17	89	<0.0001
Renin–angiotensin inhibitors	36	56	0.001	39	64	0.013
β-blockers	16	48	<0.0001	17	56	<0.0001
Ca-blockers	50	36	n.s.	51	38	n.s.
Diuretics	7	15	n.s.	9	15	n.s.
Nitrates	5	59	<0.0001	7	63	<0.0001
Statins	29	50	<0.001	33	56	0.023
Oral antidiabetics and insulin	5	25	<0.0001	7	35	<0.001

Data are shown as mean±SD if normally distributed or otherwise by median with 25% and 75% percentiles.
Differences in baseline parameters were analyzed with the Mann–Whitney U test or the Pearson's correlation coefficiency where appropriate.
CAD = coronary artery disease. BMI = body mass index. HDL = high-density lipoprotein. CRP = C-reactive protein. n.s. = not significant.

Laboratory methods

Blood samples were drawn from each subject under standardized conditions and before coronary angiography if performed. Samples were centrifuged at 1700 g for 10 min, immediately divided into aliquots, and frozen until analysis. HsCRP was measured by the enzyme-linked immunosorbent assay.

Flow cytometry

Red blood cells in whole blood (1 mL) were lysed, and then centrifuged at 750 g for 10 min. The pellet was washed twice with phosphate-buffered saline, and fixed in 2% paraformaldehyde. The cells were then incubated with phycoerythrin (PE)-conjugated mouse anti-human TLR4 antibody (eBioscience, San Diego, CA). Isotype PE-conjugated IgG was used as a negative control. After washing with PBS, the cells were analyzed using the FACScan flow cytometer and CELLQuest^{R} software (BD Biosciences Clontech., Palo Alto, CA). To evaluate the relative surface expressions of TLR4 on peripheral blood monocytes, the mean fluorescent intensity (MFI) was determined, and then the ratio of TLR4 MFI to negative control MFI was calculated. As previously reported (Okumura et al., 2003), the relative surface expressions of TLR4 on peripheral blood monocytes were evaluated by use of the ratio of TLR4 MFI to negative control MFI.

Statistical analysis

Continuous variables are presented as mean±SD if normally distributed or otherwise by median with 25% and 75% percentiles. Differences in baseline parameters were analyzed with the Mann–Whitney U test or the Pearson's correlation coefficiency where appropriate. The data for coronary risk factors and medications regimen were included in a multiple logistic regression analysis. To compare the TLR4 expression levels on peripheral blood monocytes among clinical stages, the data were analyzed using the Wilcoxon's rank test and Kruskal–Wallis test where appropriate. A P value of less than 0.05 was considered statistically significant. For statistical analysis, StatView version 5.0 was used (Abacus Concepts, Berkeley, CA).

Results

Characteristics of the study population and hsCRP levels

The characteristics of the study population are shown in Table 1. One hundred patients with CAD (CAD group; 79 patients with SAP and 21 patients with ACS), and 100 non-CAD subjects (non-CAD group) were enrolled. All subjects were Japanese. As expected, coronary risk factors, including low HDL-cholesterol levels, diabetes mellitus, and taking medications regimen were significantly more frequent in the CAD group than in the non-CAD group. Total cholesterol levels and diastolic blood pressure were lower in the CAD group; however, this might have been due to the medication regimens (Table 1).

The hsCRP levels of the CAD group were significantly higher than those of the non-CAD group (0.93 [0.39–2.02] vs 0.51 [0.31–1.16] mg/L, $P<0.01$, Fig. 1A). The serum hsCRP levels of 53 CAD patients, however, were under 1 mg/L, indicating that subjects with low serum hsCRP levels are not necessarily free of CAD. In low-hsCRP subjects whose hsCRP levels were under 1 mg/L ($n=125$; CAD, $n=53$, non-CAD, $n=72$), there were no significant differences in hsCRP levels between the CAD and non-CAD group (0.42 [0.25–0.63] vs 0.39 [0.22–0.61] mg, $P=0.64$, Fig. 1B).

TLR4 expression levels on PBMCs were higher in CAD patients

The TLR4 expression levels on peripheral blood monocytes were analyzed by flow cytometry and these values were compared with the serum hsCRP levels. The expression of TLR4 on peripheral blood monocytes was confirmed with double staining of CD14 and TLR4 as shown in Fig. 2A and B. The TLR4 expression levels were significantly higher in the CAD group than in the non-CAD group (1.21 [1.13–1.47] vs 1.19 [1.10–1.37]; ratio of TLR4 MFI to negative control MFI,

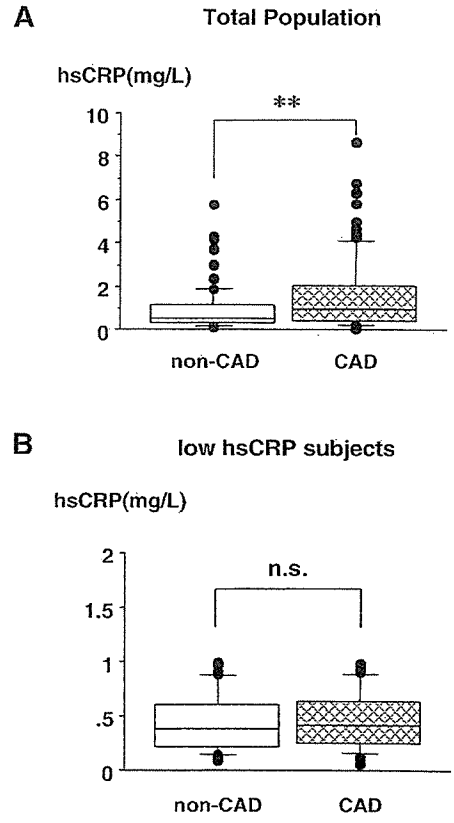

Fig. 1. Serum hsCRP levels of CAD patients and non-CAD subjects in the total population (A) ($n=200$; CAD $n=100$, non-CAD $n=100$) and in the low-hsCRP subjects (B) whose serum hsCRP levels were under 1 mg/L ($n=125$; CAD, $n=53$, non-CAD, $n=72$). Values are expressed as mean±25th percentile. **$P<0.01$, Mann–Whitney U test was used for statistical analysis.

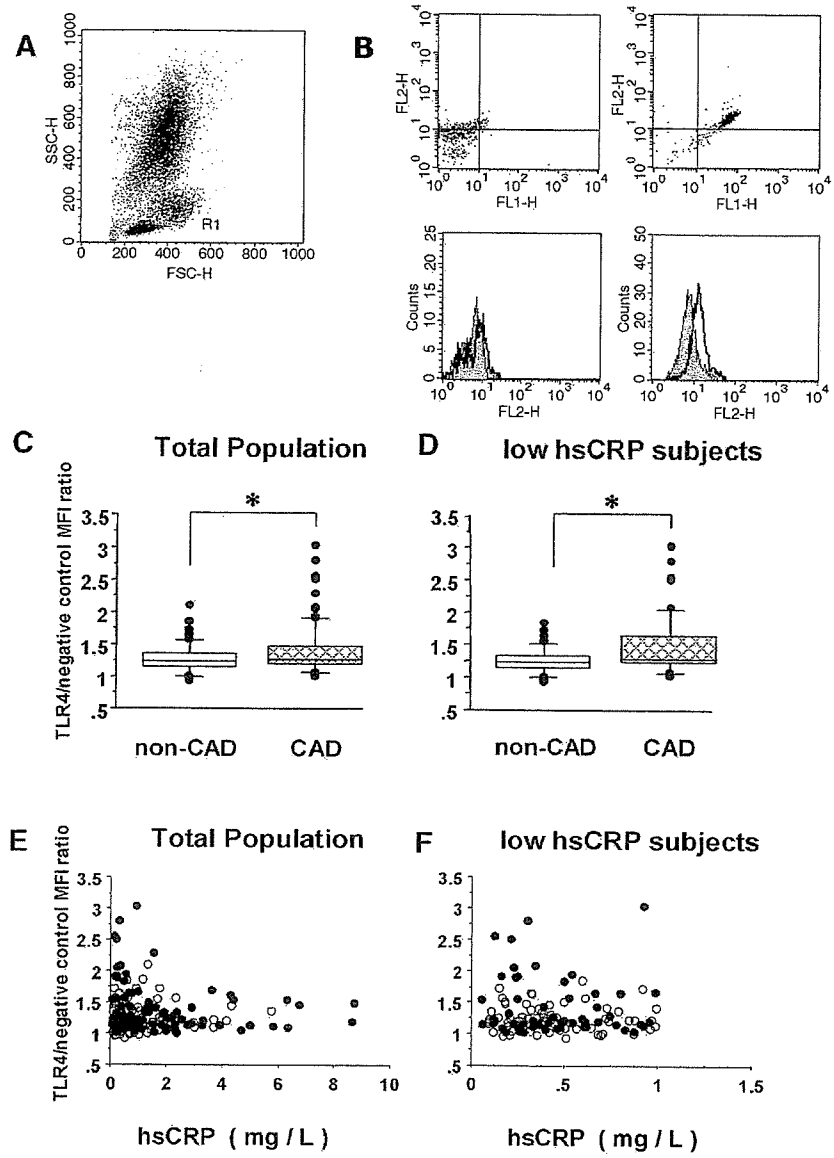

Fig. 2. The TLR4 expression levels on peripheral blood monocytes. (A) The FSC/SSC dot blot of peripheral blood cells from a CAD patient. We analyzed the gated area (R1). (B) The dot blots and histograms for CD14/TLR4 of the gated area R1. Upper figures: FL-1; CD14, FL-2, TLR4, lower figures: shaded area; isotype control, open area; TLR4. Upper left; isotype control, upper right; CD14/TLR4 double staining, lower left; non-CAD subject, lower right; CAD patient. The TLR4 expression levels on peripheral blood monocytes in the total population (C) and in the low-hsCRP subjects (D). Values are expressed as mean±25th percentile. *$P<0.05$, Mann–Whitney U test was used for statistical analysis. Relation between serum hsCRP levels and the TLR4 expression levels in the total population (E) and in the low-hsCRP subjects (F). Open circles; non-CAD group, closed circles; CAD group. Spearman's rank correlation coefficiency was used for statistical analysis.

$P<0.05$, after being adjusted for coronary risk factors, Fig. 2C, Table 2). Even in the low-hsCRP subjects, the TLR4 expression levels were also significantly higher in the CAD group than in the non-CAD group (1.22 [1.12–1.58] vs 1.20 [1.10–1.33]; ratio of TLR4 MFI to negative control MFI, $P<0.05$ after being adjusted for coronary risk factors, Fig. 2D, Table 2), though there were no differences in the serum hsCRP levels. TLR4 expression levels were affected by HDL-cholesterol levels, systolic blood pressure, and taking drugs of aspirin and nitrates. These parameters were selected by multiple regression analysis (Table 2), and TLR4 expression levels were significantly higher in CAD group even when these parameters were adjusted in analysis. Thus, both disease sensitivity and specificity of TLR4 expression were higher than those of hsCRP in low-hsCRP subjects. There was no correlation between the serum hsCRP levels and the TLR4 expression levels on peripheral blood monocytes ($P=0.17$, $\sigma=0.11$, Spearman rank correlation coefficient; Fig. 2E and F).

Disease activity and TLR4 expression on peripheral blood monocytes

We then investigated the relation between the serum hsCRP levels or the TLR4 expression levels on peripheral blood

Table 2
Logistic multiple regression analysis of various parameters TLR4 expression on peripheral blood monocytes

	Standardized coefficient of correlation	P value
Total population		
Systolic blood pressure	0.269	0.013
HDL cholesterol	−0.3	0.020
Taking aspirin	0.2	0.066
Taking nitrates	0.202	0.082
Low CRP subjects		
Systolic blood pressure	0.426	0.007
HDL cholesterol	−0.47	0.004

The parameters that P values were lower than 0.1 were shown.

monocytes and the clinical profile of CAD. The representative histograms of TLR4 expression are shown in Fig. 3A. In the total population, hsCRP levels in SAP or ACS patients were significantly higher than those in the non-CAD group ($P<0.01$, $P<0.01$, respectively), however, there were no differences in serum hsCRP levels between SAP and ACS patients (Fig. 3B). In the low-hsCRP subjects, as expected, there were no differences in serum hsCRP levels among the three groups (Fig. 3C).

On the other hand, the TLR4 expression levels on peripheral blood monocytes in both SAP and ACS patients were significantly higher than in the non-CAD group ($P<0.05$, $P<0.01$, respectively). Importantly, the TLR4 expression levels on peripheral blood monocytes in ACS patients were significantly higher than those in SAP patients ($P<0.01$), and these results were observed even in the low-hsCRP subjects (Fig. 3D and E).

TLR4 expression on peripheral blood monocytes changed in association with disease activity

In some patients with ACS, we examined the association of TLR4 expression with disease activity. All patients examined underwent PCI after blood sampling for TLR expression measurement and thereafter treated by conventional medications including aspirin, statins and angiotensin converting enzyme (ACE) inhibitors or angiotensin type 1 receptor blockers (ARBs). At 6 months after admission for ACS, patients were free of cardiac symptoms. Expression levels of TLR4 on peripheral blood monocytes were markedly decreased, but serum hsCRP levels remained unchanged (Fig. 4).

Discussion

In the present study, we examined the association of the TLR4 expression levels on PBMCs with coronary artery disease. Subjects with a serum hsCRP levels under 1 mg/L are considered to be at low coronary risk; however, 49% of the CAD group had a serum hsCRP levels under 1 mg/L in the present investigation. In these low-hsCRP subjects, the TLR4 expression levels on peripheral blood monocytes in the CAD group were significantly higher than those in the non-CAD group. The TLR4 expression levels on peripheral blood monocytes were particularly elevated in patients with ACS irrespective of their CRP levels.

It is well recognized that CRP is elevated in the serum of patients with CAD, suggesting that inflammation is closely involved in the pathophysiology of CAD.

Recent studies suggest that CRP itself may act to promote local inflammation. Immunohistochemical analysis demonstrated that CRP is expressed in atherosclerotic coronary arteries, and CRP localized at the vascular wall may enhance the generation of superoxide (Kobayashi et al., 2003). CRP in the bloodstream is, however, generated by hepatocytes in response to systemic inflammation, and the serum CRP levels do not reflect localized inflammation in the vasculature. Indeed, 49% of patients had low serum hsCRP levels in the present investigation, confirming the limitation of hsCRP as a marker of localized vascular inflammation. This nature of CRP may limit the usefulness of hsCRP as a sensitive coronary risk factor.

There was no correlation between serum hsCRP levels and TLR4 expression levels on peripheral blood monocytes, particularly in the low-CRP range in our study (Fig. 2E and F). The results of these figures suggest that the elevated TLR4 expression levels in the absence of elevated hsCRP implied the presence of mechanisms that regulate TLR4 expression independently of the regulation of hsCRP expression. It seems that expression levels of TLR4 on peripheral blood monocytes reflect more sensitively the local atherosclerotic events than serum hsCRP levels. The elevation of TLR4 expression provides new insight into pathophysiology of coronary artery disease from the standpoint of inflammatory and immune responses in the pathogenesis of atherosclerotic disease. Recent investigations revealed that microbial antigens such as lipopolysaccharides and bacterial heat shock proteins interact with the extracellular domain of TLRs and subsequently activate multiple intracellular signaling pathways leading to induction of inflammatory responses (Shor et al., 1992; Akira and Hemmi, 2003). For example, Sasu et al. demonstrated that Chlamydial heat shock protein 60 stimulated the proliferation of vascular smooth muscle cells via TLRs (Sasu et al., 2001). Furthermore, TLRs recognize not only microbial antigens but also endogenous factors (Akira and Hemmi, 2003). Thus, pro-atherogenic mechanisms by various atherogenic factors seem to be mediated through TLRs.

Recent studies reveal that TLRs have an important role in the differentiation of macrophages (Karlsson et al., 2004), which is one of the essential processes in atherogenesis. Furthermore, stimulation through TLR4 is important for differentiation of phagosomes (Blander and Medzhitov, 2004), or osteoclasts (Kikuchi et al., 2001). TLRs are up-regulated by several inflammatory cytokines such as interferon-gamma and tumor necrosis factor alpha (Miettinen et al., 2001; Wolfs et al., 2002), and, therefore, these inflammatory cytokines might promote differentiation and activation of macrophages, leading to the progression of atherosclerosis, via

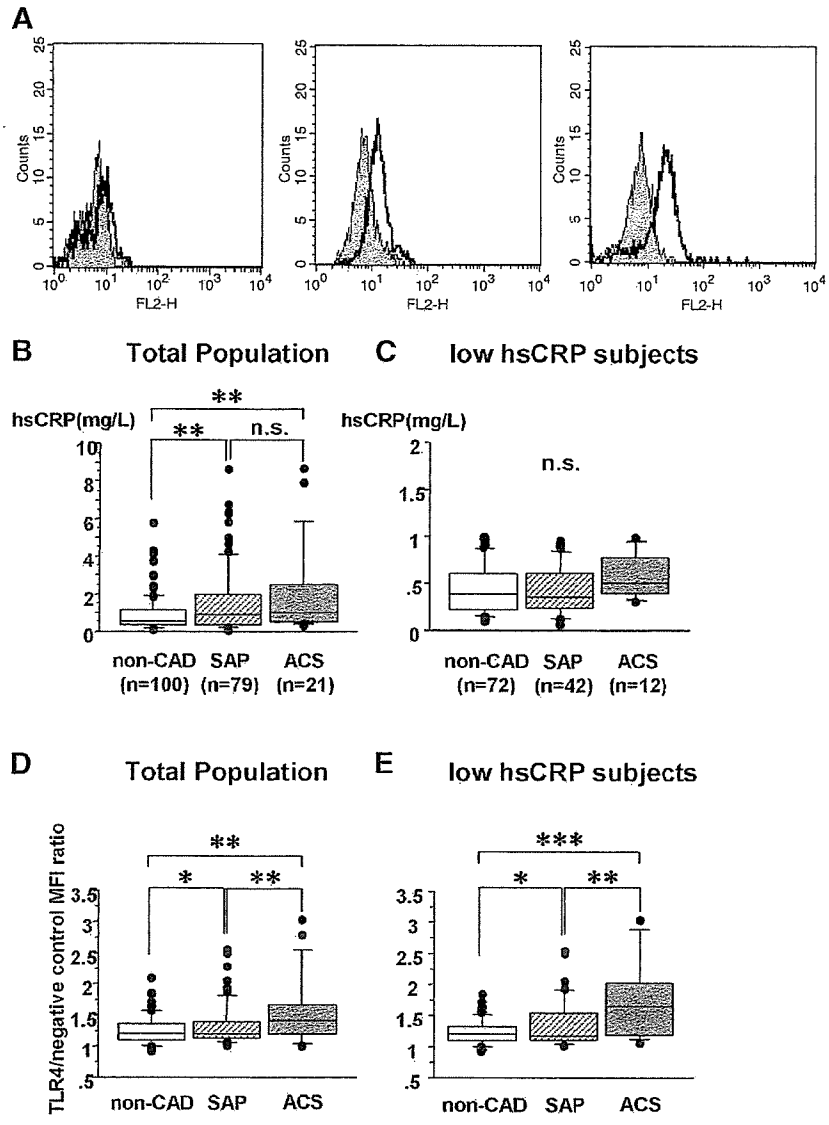

Fig. 3. Disease activity and TLR4 expression. (A) The representative TLR4-histograms of non-CAD subject (left), SAP patient (middle), and ACS patient (right). Relations between serum hsCRP levels (B and C) or TLR4 expression levels on peripheral blood monocytes (D and E) and the clinical profile. In the total population; B and D, in the low-hsCRP subjects; C and E. Values are expressed as mean±25th percentile $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, Kruskal–Wallis rank test was used for statistical analysis.

TLRs. The finding that TLR4 expression on peripheral blood monocytes was elevated in patients with stable angina may reflect not only that stimuli for TLR4 expression were increased even in stable angina but also that the up-regulated TLR4-mediated signals lead to progression of atherosclerotic lesion formation.

In previous reports, individuals carrying Gly alleles at 299 of the TLR4 gene had less responsibility to lipopolysaccharides and lower serum CRP levels compared with wild type (Arbour et al., 2000). Since the report that the Asp299Gly polymorphism of the TLR4 gene decreased the risk of atherosclerotic disease (Kiechl et al., 2002), other reports have been published with conflicting results (Ameziane et al., 2003; Yang et al., 2003; Reismann et al., 2004). Thus, a controversy still exists with regard to the association between TLR4 Asp299Gly polymorphism and cardiovascular diseases. The present study showed the close association of TLR4 expression levels on peripheral blood monocytes with the occurrence of cardiovascular diseases, however none of the patients carried Gly alleles at 299 in the TLR4 gene (data not shown). It might be due to the ethnical background. Indeed, it was reported that there were few Asp299Gly polymorphisms of TLR4 gene in Japanese population (Okayama et al., 2002), however, the population of this study is too small to evaluate this important point unfortunately. Taken together, these findings suggest that the Asp299Gly polymorphism of TLR4 gene did not influence the results of the present study.

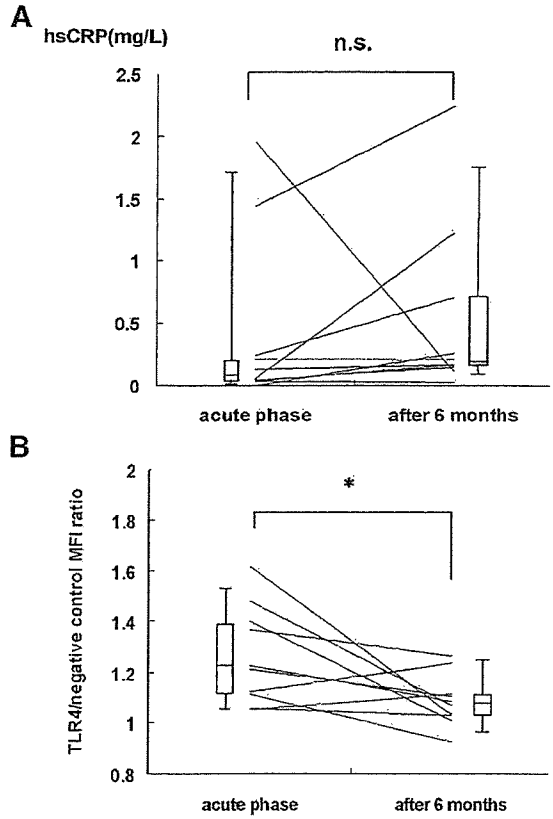

Fig. 4. The association of TLR4 expression levels on peripheral blood monocytes with the disease activity. The levels of hsCRP and TLR4 expression on peripheral monocytes were evaluated in eleven patients with ACS at acute phase and 6 months after the onset. HsCRP levels were not changed (A), however, TLR4 expressional levels on peripheral blood monocytes decreased at 6 months after the onset (B). $^{*}P<0.05$, Wilcoxon's rank test was used for statistical analysis.

Very recently, Methe et al. demonstrated that TLR4 expression on circulating monocytes and serum heat shock protein 60 levels was elevated in patients with ACS (Methe et al., 2005). They also showed that serum from patients with ACS activated TLR4-transfected Chinese hamster ovary cells through TLR4 (Methe et al., 2005). Their work was the first report in humans on the association of TLR4 on circulating monocytes and coronary artery disease. In their study the relation between serum hsCRP levels and TLR4 expression levels was not assessed, and no difference was found in TLR4 expression levels between patients with stable angina and control subjects. We speculate that these different results may be due to difference of methodology in the TLR4 expression level measurement. Indeed, the examination of the level of mRNA would provide more information, however, we did not examine it due to several reasons. Our investigation is a population study, and it is impossible to isolate mRNA of peripheral blood monocytes from all subjects including patients with acute coronary syndrome in our institute. Nevertheless, we think that the protein levels of TLR reflect the function more precisely than the levels of mRNA.

Study limitations

This research is a case-control study; therefore, the usefulness of the obtained results in the primary and secondary prevention of coronary events is not known. To confirm the TLR4 expression level on peripheral blood monocytes as a coronary risk, it is necessary to perform a prospective investigation in a large population.

Conclusion

Even in the low-hsCRP subjects, the TLR4 expression levels on peripheral blood monocytes of the CAD group were significantly higher than those of the control group. Moreover, the TLR4 expression levels on peripheral blood monocytes are likely a better marker for the CAD activity than serum hsCRP levels. The combined measurement of serum hsCRP and TLR4 expression on peripheral blood monocytes, especially among low-hsCRP subjects, might serve as a new marker that reflects coronary risk and vulnerable patients more precisely than serum hsCRP levels alone.

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Angiotensin II type 1 receptor blocker telmisartan suppresses superoxide production and reduces atherosclerotic lesion formation in apolipoprotein E-deficient mice

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Abstract

Angiotensin II is involved in the process of atherosclerosis and stimulates superoxide production from cardiovascular cells. We examined the effect of telmisartan, an angiotensin II type 1 receptor blocker, on atherosclerosis. We chronically treated apolipoprotein E-deficient mice with two different doses of telmisartan dissolved in drinking water (0.3 and 3 mg/kg) starting from 4 weeks of age for 12 weeks. Lipid contents were not different in both telmisartan-treated groups compared with control group. Systolic blood pressure was significantly reduced with 3 mg/kg, but unchanged with 0.3 mg/kg. The total atherosclerotic lesion size at the aortic sinus was reduced with 0.3 mg/kg compared with control, and additional reduction was proved with 3 mg/kg. The fibrotic change was not different among three groups, but MOMA-2-, malondialdehyde-, 4-hydroxy-2-nonenal-immunostained areas were reduced by telmisartan. As the mechanism, we revealed that both doses of telmisartan markedly reduced superoxide production from in situ vessels assessed by lucigenin-enhanced chemiluminescence and dihydroethidium staining. And NAD(P)H dependent oxidase activity in vessels was reduced by telmisartan. Further, 8-*iso*-prostaglandin F2α level, a systemic oxidative stress marker, obtained from urine and plasma samples were significantly reduced by telmisartan. Telmisartan reduced atherosclerosis in apolipoprotein E-deficient mice at least partly via the suppression of oxidative stress.

Keywords: Angiotensin II type 1 receptor blocker; Atherosclerosis; Oxidative stress; Apolipoprotein E-deficient mouse

1. Introduction

The renin-angiotensin system (RAS) plays important roles in the regulation of not only blood pressure but also vascular structure. The main component of RAS is angiotensin II (Ang II), which is a potent vasoconstrictor and elevates blood pressure. Ang II generates aldosterone and activates sympathetic nervous system, leading to blood pressure elevation.

Besides its effect on blood pressure, a number of evidence revealed that Ang II is involved in atherogenesis. In animal models, chronic infusion of Ang II promotes atherosclerotic lesion formation [1]. It is shown that Ang II promotes atherogenesis via direct effects on vascular beds independent of hypertensive effects. Among them, the effect as the inducer of oxidative stress is recently attracting attention. In atherosclerosis, there is augmented production of reactive oxygen species (ROS) from various cell types including endothelial cells, vascular smooth muscle cells and monocytes/macrophages, and Ang II plays a pivotal role in their production [2–4]. There are increased expressions of angiotensin converting enzyme (ACE) and Ang II type 1 receptor in atherosclerotic arteries, indicating the presence of augmented local RAS activation [5,6]. Ang II increases superoxide production from vessel wall by activating NAD(P)H oxidase [7]. ROS are closely implicated

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in atherogenesis, by damaging and activating the endothelium, oxidizing low-density lipoprotein (LDL) cholesterol, and promoting proliferation of vascular smooth muscle cells. They also induce various genes such as those of adhesion molecules and chemokines, which play important roles in the initiation and progression of atherosclerotic lesion formation [8,9].

ACE inhibitors (ACE-I) and Ang II type 1 receptor blockers (ARB) are widely used for treatment of hypertension to prevent organ damages. These drugs have been already reported to prevent atherosclerosis in several studies using animal models [10–13]. Losartan reduced atherosclerotic lesion formation without changing blood pressure in cynomologous monkeys [10] and apolipoprotein E-deficient (apoE-KO) mice [11]. Olmesartan was reported to reduce atherosclerosis in association with suppressions of serum macrophage-colony stimulating factor, transforming growth factor-beta 1 and intracellular adhesion molecule-1 in monkeys fed a high cholesterol chow [13].

There has been little information whether ARB can show anti-atherogenic effects by the mechanisms related to suppression of oxidative stress. In the present study, we investigated the effects of telmisartan, an ARB, on atherosclerotic lesion formation in apoE-KO mice. Particularly, we examined whether the effects of telmisartan on atherogenesis were independent of its effect on blood pressure and associated with changes in oxidative stress.

2. Materials and methods

2.1. Materials and animal preparation

Telmisartan was obtained from Boehringer Ingelheim Inc. (Germany). All other commercial drugs used in this study were purchased from Sigma Chemical Co. (MO). ApoE-KO mice on a C57BL/6 genetic background at 4 weeks of age were assigned to control group and two telmisartan-treatment groups given different dosages. Drug treatment consisted of 0.3 and 3 mg/kg body weight per day of telmisartan dissolved in drinking water. Mice were fed a standard chow and supplemented with telmisartan for next 12 weeks and sacrificed at 16 weeks of age. Animals were provided the chow and water ad libitium and maintained on a 12 h light/dark cycle. All animal experiments were conducted according to the guidelines for animal experiments at Kobe University Graduate School of Medicine.

2.2. Plasma analysis

After overnight fasting, blood was collected by the cardiac puncture into heparin-coated tubes under anesthetic condition using pentobarbital sodium (80 mg/kg intraperitoneal injection). Plasma was obtained through centrifugation of the blood for 10 min at 5500 × *g* at 4 °C and stored at −80 °C until each assay. Concentrations of plasma total cholesterol and triglyceride were determined by use of an automated clinical chemistry analyzer. High-density lipoprotein cholesterol levels were quantified by enzymatic reaction using a commercially available kit (Wako, Japan). Glucose levels were determined by glucometer (Sanwa Kagaku, Japan) and insulin levels were determined with a commercially available kit (LINCO Research Inc., MO).

2.3. Hemodynamic analysis

Heart rate and systolic blood pressure of apoE-KO mice were measured at 16 weeks of age using the tail-cuff method without heating. The mouse tail was placed into a device with a rubber cuff and a photoelectric sensor, and heart rate and systolic blood pressure were measured using MK-2000 (Muromachi Kikai, Japan). All measurements were repeated six times for each mouse.

2.4. Atherosclerotic lesion assessment at the aortic sinus

After 12 weeks of telmisartan treatment, both gender mice (16 weeks of age) were anesthetized as above and the aorta was perfused with normal saline containing 10 U/ml heparin. Then the aorta sample was dissected from the middle of the left ventricle to the aortic arch, and fixed with 4% paraformaldehyde for overnight. The sample was cut in the ascending aorta, and the proximal sample containing the aortic sinus was embedded in OCT compounds (Tissue-Tek, CA). Five consecutive sections (10 μm thickness), spanning 550 μm of the aortic sinus, were collected from each mouse and stained with Sudan III and Masson's trichrome. For quantitative analysis of atherosclerosis, the average lesion area of five separate sections from each mouse was obtained with the use of the Image J (National Institutes of Health, MD) according to the method described by Paigen et al. [14].

2.5. Immunohistochemistry

Immunohistochemical staining with MOMA-2 (BMA Biomedicals AG, Switzerland; 1:500 dilution), malondialdehyde (MDA) (Alpha Diagnostic International Inc., TX; 1:100 dilution) and 4-hydroxy-2-nonenal (HNE) (Alpha Diagnostic International Inc., TX; 1:100 dilution) of atherosclerotic lesions at the aortic sinus was performed by the labeled streptaviadin biotin method as previously reported [15]. Quantitative analysis of MOMA-2-immunostaining was evaluated as a ratio of the positive-stained area to total plaque area in the atherosclerotic lesion at the aortic sinus.

2.6. Measurement of superoxide production from aortas

After euthanization of mice, the aorta was cut out from the aortic arch to the bifurcation of iliac arteries and the tissues around the vessel were cleaned. Then the aorta were cut into four pieces (approximately 5 mm length per each pieces) and

these aortic rings were incubated with the Cu–Zn superoxide dismutase inhibitor for 30 min at 37 °C, and vascular superoxide production levels were measured by chemiluminescence (CL) with 10 μM lucigenin (bis-*N*-methylacridinium nitrate). The final volume of lucigenin solution was 1 mL. The light reaction between superoxide and lucigenin was detected with a BLR-201 CL reader (ALOKA, Japan) and photon emission was continuously recorded for 15 min. The CL signal was expressed as the average count per minute (C.P.M.) for 15 min periods and the counts were corrected by vessel dry weights.

2.7. Measurement of NAD(P)H dependent oxidase activity of aorta homogenates

The aorta was cut out from each mouse as described above, and the aortic segments (almost 2 cm length) were placed in a chilled modified 50 mM HEPES/PSS buffer and homogenized on ice with a motor-driven tissue homogenizerfor 1 min in 200 μL homogenate buffer, which contained 0.01 mM EDTA. The homogenates were centrifuged at 1000 × *g* for 10 min. The pellet was discarded and the supernatant was stored on ice until use. Protein concentration of aorta homogenate was measured by the method of Bradford [16]. The assay solution contained 50 mM HEPES/PSS (pH 7.4), 1 mM EDTA, 6.5 mM $MgCl_2$, 83 mM sucrose, and 250 μM lucigenin as the electron acceptor and 100 μM NADH or 100 μM NADPH as the electron donor [17]. After pre-incubation at 37 °C for 20 min, the reaction was started by adding 20 μL of aorta homogenates. All CL data were evaluated after subtracting the CL counts obtained in the absence of homogenates. Each count was corrected by protein levels of aorta homogenate.

In some experiments, we examined the effects of 100 μM diphenylene iodonium (DPI), an inhibitor of all flavoenzymes, and 500 μM apocynin, an inhibitor of NAD(P)H oxidase, on superoxide production after stimulation of homogenates with NAD(P)H. The aorta homogenates were pre-incubated with each agent for 15 min before CL measurement.

2.8. In situ detection of superoxide production in aortas and endothelial cells

To evaluate in situ superoxide production from vessels, unfixed frozen cross sections of aortas were stained with dihydroethidium (DHE; Molecular Probe, OR) according to the previously validated method [18]. In the presence of superoxide, DHE is converted to the fluorescent molecule ethidium, which can then label nuclei by intercalating with DNA. Briefly, the unfixed frozen tissues were cut into 10 μm thick sections, and incubated with 10 μM DHE at 37 °C for 30 min in a light-protected humidified chamber. The images were obtained with a laser scanning confocal microscope (Carl ZEISS, Germany). Superoxide production was demonstrated by red fluorescence labeling.

For quantification of ethidium fluorescence from endothelial cells, fluorescence (intensity × area) was measured only on the luminal side of the internal elastic lamina using the Image J in high-power (100×) images [19]. For each vessel, total fluorescence was calculated from three separate high-power fields taken in each section of the vessel to produce $n = 1$.

2.9. Measurement of 8-iso-prostaglandin F2α and serum amyloid A levels

Urine samples were collected from mice at the age of 12–16 weeks, and stored at −80 °C after addition of butyrated hydroxytoluene (BHT) at a final concentration of 0.01%. After purification using C18 reverse phase extraction column (Waters Corporation, MA), urine 8-*iso*-prostaglandin (PG) F2α levels were measured with EIA kits (Assay Designs Inc., MI) according to the manufacturer's instructions, and data were corrected by urine creatinine levels. Plasma samples were collected as above and stored at −80 °C after addition of BHT at a final concentration of 0.01%. We measured direct 8-*iso*-PGF2α levels from plasma samples with EIA kits (Assay Designs Inc., MI) according to the manufacturer's instructions.

We collected plasma from each mouse as shown in Section 2 and measured serum amyloid A (SAA) levels with mouse SAA ELISA kit (BioSource International Inc., CA) according to the manufacturer's instructions.

2.10. Statistical analysis

Data were expressed as mean ± S.E.M. One-way ANOVA was used to compare the differences among three groups with Fisher's PLSD test for post hoc analysis. Values of $P < 0.05$ were considered statistically significant.

Table 1
Effect of telmisartan on body weight, lipid contents, glucose and insulin levels, and vital signs

	Control	0.3 mg/kg	3 mg/kg
Female body weight (g)	21.8 ± 0.5	21.2 ± 0.3	21.2 ± 0.5
Male body weight (g)	26.9 ± 0.6	27.0 ± 0.4	26.7 ± 1.5
Total cholesterol (mg/dl)	478.6 ± 28.8	463.4 ± 19.1	464.9 ± 22.8
Triglyceride (mg/dl)	53.5 ± 6.8	50.0 ± 9.7	57.8 ± 8.1
HDL cholesterol (mg/dl)	9.4 ± 0.8	9.3 ± 0.9	9.0 ± 0.7
Glucose (mg/dl)	108.4 ± 8.2	116.1 ± 7.1	112.5 ± 5.5
Insulin (ng/ml)	0.24 ± 0.12	0.18 ± 0.06	0.24 ± 0.05
Heart rate (min^{-1})	543.9 ± 50.8	536.3 ± 89.7	540.5 ± 76.7
Systolic BP (mmHg)	107.4 ± 1.7	106.9 ± 2.1	90.1 ± 1.8*

Mice were fed a standard chow for 16 weeks and body weight of each mouse was measured. Mice were fasted for at least 12 h and bled, and plasma total cholesterol, triglyceride, high-density lipoprotein cholesterol, glucose and insulin levels were determined as described in Section 2 ($n = 8$ per group). Heart rate and systolic blood pressure were measured with the use of the tail-cuff method ($n = 15$ per group). Results were expressed as mean ± S.E.M.; BP, blood pressure.

* $P < 0.0001$ vs. control.

3. Results

3.1. The effects of telmisartan on blood pressure and plasma lipid levels

Body weight was not significantly different among three groups of each gender (Table 1). Neither plasma total cholesterol, triglyceride, nor high-density lipoprotein cholesterol levels were affected by the treatment with telmisartan. And neither plasma glucose, nor insulin levels were affected by the treatment with telmisartan. Although heart rate was not affected, 3 mg/kg telmisartan significantly reduced systolic blood pressure compared with that of control group (Table 1). In contrast, 0.3 mg/kg telmisartan did not change systolic blood pressure.

3.2. Atherosclerotic lesion formation at the aortic sinus

After feeding a standard chow for 16 weeks, the atherosclerotic lesion formation was assessed at the aortic sinus. Representative photographs of each mouse were shown in Fig. 1A. In quantitative analysis of histological examination with Sudan III staining, the atherosclerotic lesion formation of both 0.3 and 3 mg/kg groups were markedly reduced compared with control group in both gender (Fig. 1B). About plaque contents, scattered small fibrotic area was distributed in the plaque lesions of both control and telmisartan treatment groups when evaluated by Masson's trichrome staining (Fig. 2). MOMA-2-immunostained area was significantly reduced with telmisartan, but a ratio of positive-stained

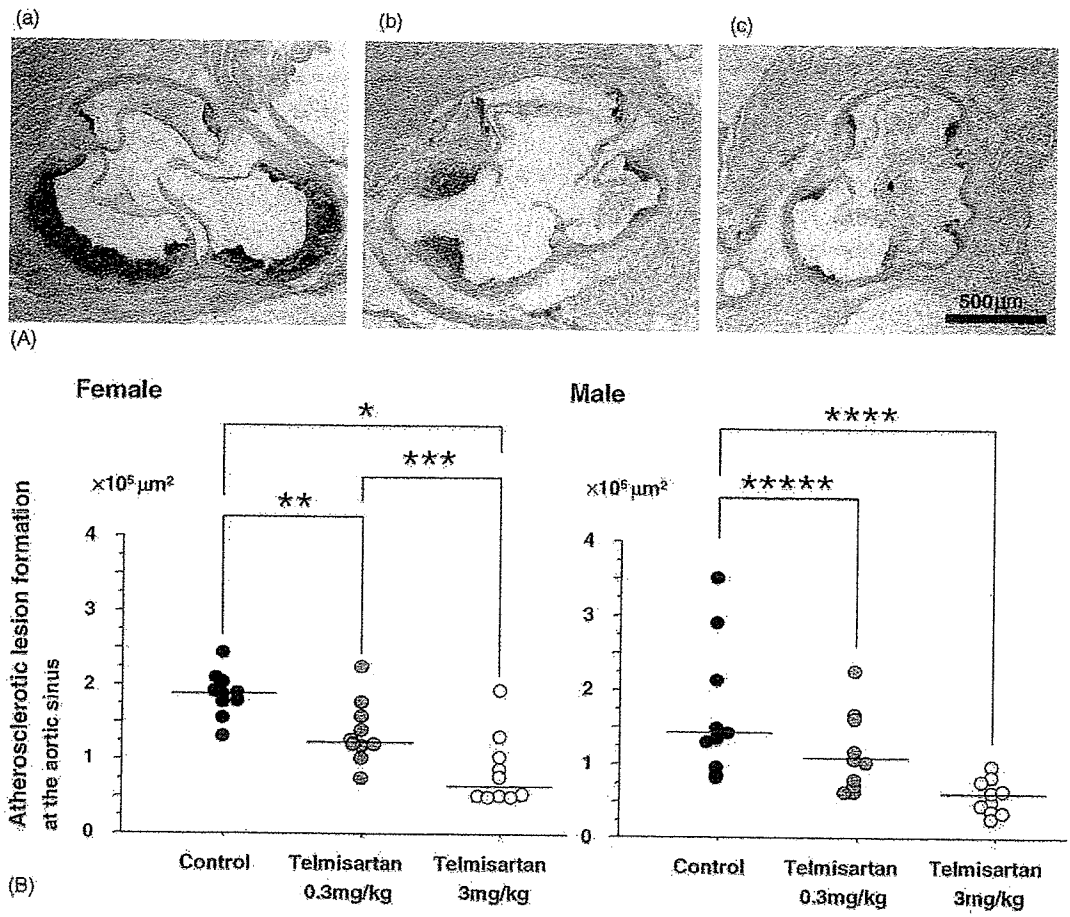

Fig. 1. (A) Representative photographs of atherosclerotic lesion formation at the aortic sinus of each mouse. Panel (a) through (c) are representative photographs of the atherosclerotic lesion formation at the aortic sinus of mice fed a standard chow in control group (a), that treated with 0.3 mg/kg telmisartan (b) and that treated with 3 mg/kg telmisartan (c), respectively. Sections were taken at the same level of aortic sinus and stained with Sudan III staining as described in Section 2. Original magnifications were 40×. A black bar on photomicrograph represents 500 μm. (B) Quantitative analysis of atherosclerotic lesion formation at the aortic sinus in both gender mice. The average lesion area of five sections at the aortic sinus from each mouse was quantified morphometrically as described in Section 2. Each symbol represents the average lesion area in each mouse, with the mean per group indicated by a horizontal line. After 12 weeks telmisartan treatment, the atherosclerotic lesion formation was significantly reduced in both 0.3 and 3 mg/kg telmisartan groups compared with control ($n = 10$ per group). $^{*}P < 0.0001$ vs. control; $^{**}P < 0.01$ vs. control; $^{***}P < 0.01$ vs. telmisartan 0.3 mg/kg; $^{****}P < 0.001$ vs. control; $^{*****}P < 0.05$ vs. control.

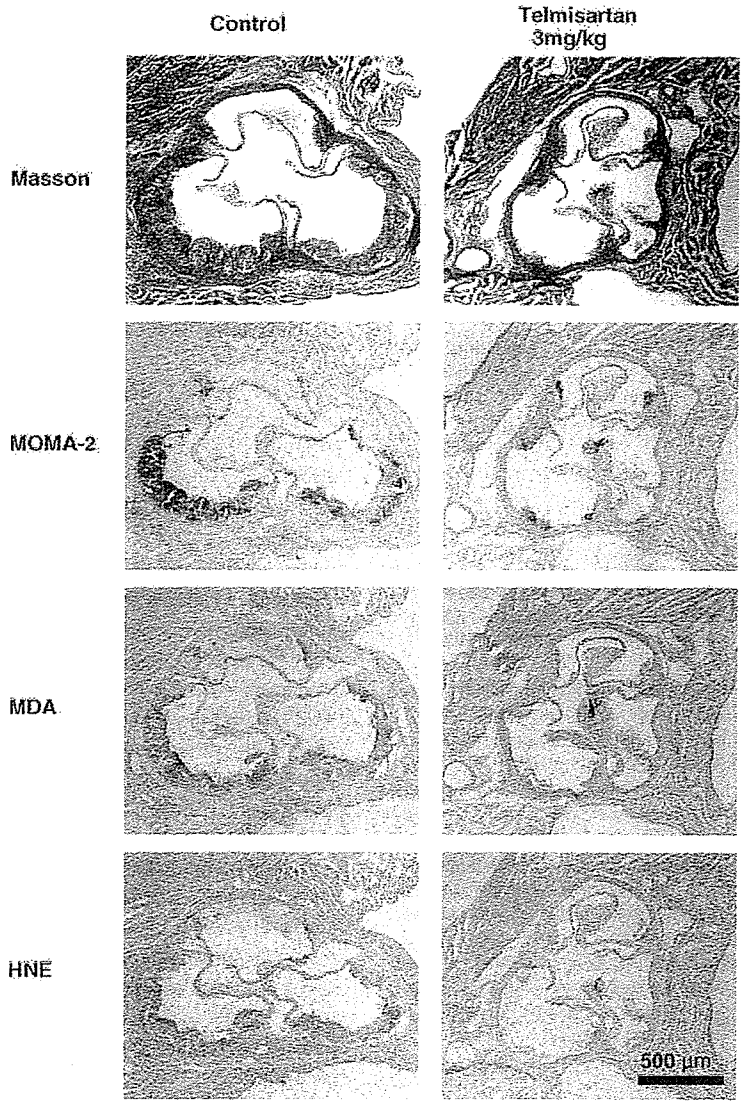

Fig. 2. Effect of telmisartan on plaque contents and the distribution of oxidative stress in atherosclerotic lesion at the aortic sinus. Panels are representative photographs of Masson trichrome staining and immunostained with MOMA-2, MDA and HNE in the atherosclerotic lesions from control group and 3 mg/kg telmisartan group (original magnification were 40×). Scattered fibrosis was partly distributed in the plaque lesions of both groups. On the whole, telmisartan reduced MOMA-2-, MDA- and HNE-immunostained areas compared with control group. A black bar on photomicrograph represents 500 μm.

area to total plaque area was not significantly different among three groups (data not shown). MDA-, HNE-immunostained areas were also reduced with telmisartan (Fig. 2).

3.3. Superoxide production from aortas

To investigate the effect of telmisartan on superoxide production in the aortic vessel wall, we measured superoxide production using the lucigenin-enhanced CL. By treatment with 0.3 and 3 mg/kg telmisartan, superoxide production was significantly decreased compared with control group (Fig. 3A).

3.4. NAD(P)H dependent oxidase activity of aorta homogenates

NAD(P)H dependent oxidase activity in aorta homogenates stimulated with 100 μM NADH or 100 μM NADPH was measured by use of the lucigenin-enhanced CL. Telmisartan significantly decreased NAD(P)H dependent oxidase activity by more than 60% in both 3 and 0.3 mg/kg groups compared with control group (Fig. 3B). Furthermore, aorta homogenates were incubated with either 100 μM DPI or 500 μM apocynin for 15 min to abolish the increment of NAD(P)H dependent oxidase activity. The addition of NAD(P)H oxidase inhibitors significantly reduced

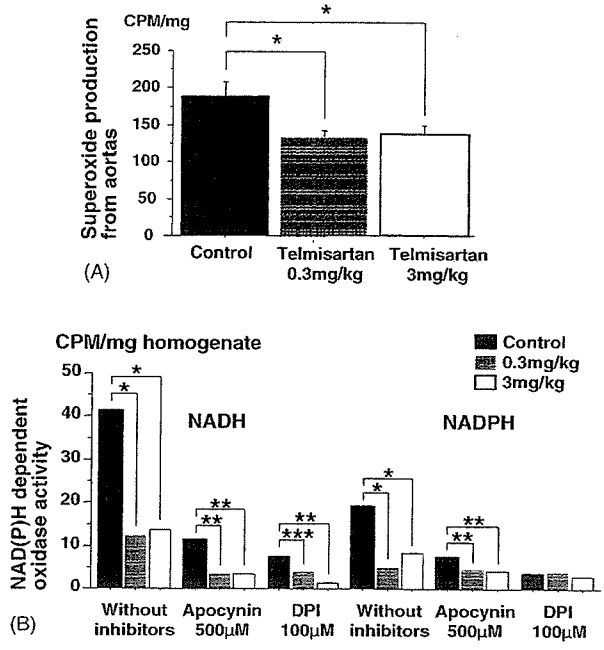

Fig. 3. (A) Effect of telmisartan on superoxide production from whole aortas using lucigenin-enhanced chemiluminescence. Aortic rings were incubated with the Cu–Zn superoxide dismutase inhibitor and vascular superoxide levels were measured by chemiluminescence with 10 μM lucigenin as described in Section 2. The counts by a luminometer were corrected by vessel dry weights. Results were expressed as mean ± S.E.M. ($n = 8$ per group). $^{*}P < 0.05$ vs. control. (B) Effect of telmisartan on NAD(P)H dependent oxidase activity of aorta homogenates using lucigenin-enhanced chemiluminescence. Aorta was homogenated and vascular NAD(P)H dependent oxidase activity was measured by use of chemiluminescence with 250 μM lucigenin in the presence of 100 μM NAD(P)H. NAD(P)H dependent oxidase activity was measured with 500 μM apocynin or 100 μM diphenylene iodinium (DPI) as described in Section 2. Results are expressed as mean ± S.E.M. of counts by luminometer in each group ($n = 8$ per group). $^{*}P < 0.0001$ vs. control; $^{**}P < 0.001$ vs. control; $^{***}P < 0.05$ vs. control.

lucigenin-enhanced CL in both control and telmisartan treatment groups.

3.5. *In situ superoxide production in the vessel wall of aorta*

In situ superoxide production was measured using DHE oxidative fluorescent microtopography. Ethidium fluorescence was detected throughout all layers of the vessel wall and both doses (0.3 and 3 mg/kg) of telmisartan significantly suppressed the staining (Fig. 4A). We next focused on the vascular superoxide production in the endothelial cells by measuring the ethidium fluorescence particularly on the luminal side of the internal elastic lamina. Endothelial ethidium fluorescence in 0.3 mg/kg group was decreased by 30% compared with control group and by 40% in 3 mg/kg group (Fig. 4B). These results indicated that telmisartan decreased superoxide production from the vessel wall, particularly from endothelial cells.

3.6. *8-iso-PGF2α and SAA levels*

8-*iso*-PGF2α level was measured as an indicative marker of systemic oxidative stress. 8-*iso*-PGF2α levels from both urine (Fig. 5A) and plasma samples (Fig. 5B) were significantly decreased with telmisartan treatment compared with control group. On the other hand, SAA levels did not change by telmisartan (Fig. 5C).

4. Discussion

In the present study, we demonstrated that telmisartan suppressed the atherosclerotic lesion formation in apoE-KO mice. The suppressive effect was detected by 0.3 mg/kg telmisartan, which did not change systolic blood pressure, and further suppression occurred by 3 mg/kg telmisartan. As the mechanism of the drug's anti-atherogenic action, we focused on the effects for oxidative states in vivo and in vitro. Telmisartan reduced MDA- and HNE-immunostained areas compared with control group. Telmisartan suppressed superoxide production from the vessel wall via reducing NAD(P)H dependent oxidase activity. Telmisartan also reduced 8-*iso*-PGF2α levels in urine and plasma samples, which are one of indices of systemic oxidative stress. These inhibitory effects on oxidative stress were associated with suppression of atherosclerotic lesion formation.

Several animal studies demonstrated that ARB showed anti-atherogenic effects besides its effect on blood pressure [10–13]. Our finding is in agreement with the results of Hayak et al. and Dol et al., in which ARB reduced atherosclerotic lesion formation in apoE-KO mice via decreased chemokine expression and macrophage accumulation, and the inhibition of LDL oxidation [11,12]. In the present study, we demonstrated that telmisartan reduced atherosclerotic lesion formation in association with the suppression of oxidative stress via the inhibition of NAD(P)H oxidase activity.

Ang II stimulation has been reported to produce ROS from various vascular cell types [2–4]. ROS from the vessel wall are thought to play critical roles in atherogenesis. ROS induce the expression of adhesion molecules and chemokines, accelerate the formation of atherosclerotic plaque, increase matrix metalloprotease production and cause the vulnerable changes of fibrous cap [20].

In the present study, we clearly demonstrated that telmisartan suppressed superoxide production from the vessel wall assessed by lucigenin-enhanced CL method. This action was independent of the blood pressure lowering effect. We also revealed the inhibitory action of telmisartan on superoxide production by DHE staining. Telmisartan suppressed superoxide signals in all layers of aortas, particularly in the endothelium. We next focused on NAD(P)H oxidase to clarify the mechanisms of suppression of superoxide production. Superoxide anion is produced via the activation of NAD(P)H oxidase in vessel wall cells and plays an important role as the intracellular transmission factor in the Ang

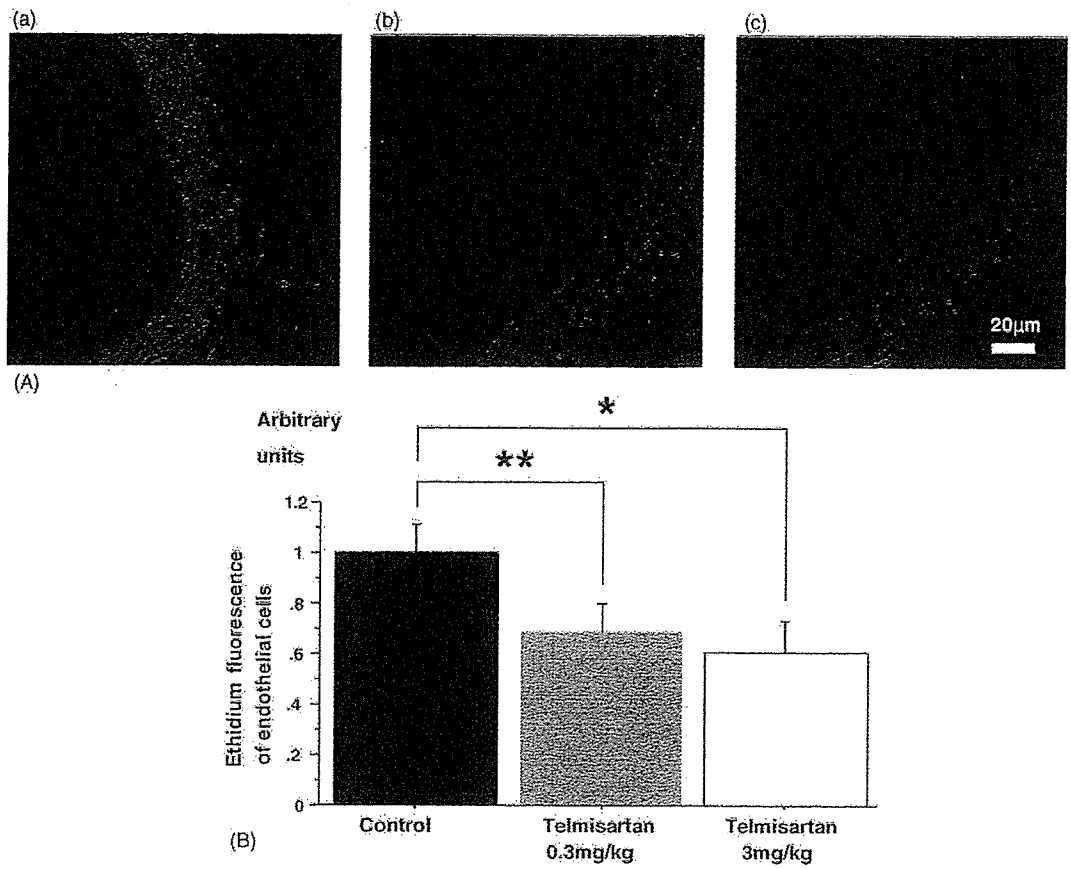

Fig. 4. (A) Representative photographs of in situ superoxide production in aortic vessel wall using dihydroethidium staining. Panel (a) through (c) are representative photographs of aortic vessel wall from each mouse in control group (a), that treated with 0.3 mg/kg telmisartan (b) and that treated with 3 mg/kg telmisartan (c), respectively. Sections were stained with dihydroethidium as described in Section 2. Original magnification were 200×. A white bar represents 20 μm. (B) Quantitative analysis of in situ superoxide production in aorta endothelial cells using dihydroethidium staining. For quantification of ethidium fluorescence from the endothelial cells in high-power (200×) images, fluorescence (intensity × area) was measured only on the luminal side of the internal elastic lamina using the Image J as described in Section 2 and expressed in arbitrary units. Results were expressed as mean ± S.E.M. in each group ($n = 10$ per group). $^{*}P < 0.05$ vs. control; $^{**}P = 0.07$ vs. control.

II-signaling system [21]. Ang II-mediated hypertension is associated with the increased superoxide production and NAD(P)H oxidase activity [7]. Other than hypertension, the increased superoxide production due to NAD(P)H oxidase activation was demonstrated in the rabbit model of atherosclerosis [22]. We examined lucigenin-enhanced CL with NAD(P)H as the substrates and revealed that telmisartan suppressed NAD(P)H dependent oxidase activity with the dosage that did not change blood pressure. This effect was reduced by DPI, an inhibitor of all flavoenzymes and also by apocynin, a more specific inhibitor for NAD(P)H oxidase. These results indicated that the increment of superoxide production was reduced by inactivation of NAD(P)H dependent oxidase activity due to Ang II type 1 receptor blockade with telmisartan.

Our findings are in accordance with the study of Warnholtz et al., who showed that Bay 10-6734, an ARB, reduced plaque formation in association with reductions of vascular superoxide production and NAD(P)H oxidase activity in the rabbit model of atherosclerosis [22]. They did not show whether the anti-atherogenic effects of Bay 10-6734 were independent of the blood pressure lowering effect. In the present study, we clearly demonstrated that telmisartan, a clinically used ARB, reduced atherosclerotic lesion formation without changing blood pressure in apoE-KO mice. In most animal studies showing the blood pressure-independent anti-atherogenic actions of ARB, the far-high dosages have been used compared with those applied clinically [10–13]. In the present study, we showed that the anti-atherogenic action of telmisartan was detected with 0.3 mg/kg, which is lower than the clinically relevant dose. We also showed that telmisartan reduced not only vascular superoxide production but also the marker of systemic oxidative status. 8-*iso*-PGF2α has been recognized as a marker of systemic oxidative stress [23] and revealed as a risk marker in patients with coronary heart disease in matched case-control studies [24]. In this study, telmisartan suppressed 8-*iso*-PGF2α levels in both urine and plasma with the non-blood pressure lowing dosage.

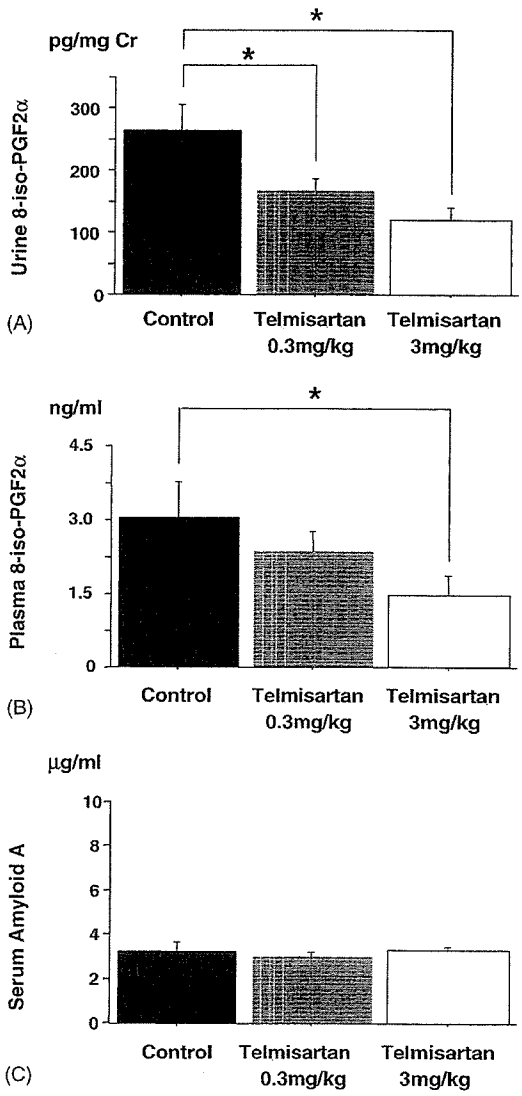

Fig. 5. (A) and (B) Effect of telmisartan on 8-*iso*-PGF2α levels of urine and plasma samples 8-*iso*-PGF2α from urine (A) and plasma (B) samples were measured as described in Section 2. Urine data are corrected by urine creatinine levels. Results were expressed as mean ± S.E.M. ($n = 8$ per group). $^{*}P < 0.05$ vs. control. (C) Effect of telmisartan on SAA levels. SAA levels were measured as described in Section 2. Results were expressed as mean ± S.E.M. ($n = 8$ per group).

The association of suppressed oxidative stress and reduction of atherosclerotic lesion formation does not necessarily mean the cause-effect relation. Both occurred independently of changes in blood pressure, and the well-known roles of oxidative stress in the initiation and progression of atherosclerosis strongly suggest that the anti-oxidative effects are at least partly responsible for the suppression of atherosclerotic lesion formation by telmisartan. Telmisartan might, however, reduce atherosclerotic lesion size by mechanisms other than its effects on oxidative stress, such as anti-inflammatory effects and effects on peroxisome proliferator-activated receptor-gamma (PPAR-g) activity. It is reported that telmisartan induced PPAR-g activation [25,26], but in the present study, telmisartan did not change plasma glucose, triglyceride or insulin levels. Therefore the effect of telmisartan on PPAR-g seemed to play a minimum role in the present study. Further, Ang II increases expression of lectin-like oxidized LDL receptor of macrophage and accelerates the foam cell formation and the deposition of oxidized lipid to the plaque [27]. Further studies are needed to clarify how inhibition of those actions of Ang II by telmisartan is related to its anti-atherogenic action.

As a limitation of the present study, we quantified superoxide production from aorta homogenates by use of 250 μm lucigenin. The validity of data on superoxide has been questioned when the relatively high dose of lucigenin is applied. In the present study, however, the levels of oxidative stress were relatively low, and we could not detect any fluorescence signals when we used 5 μm lucigenin, which was revealed not to produce superoxide by itself. In the report of Warnholtz et al., they compared the data on superoxide productions measured by 5 μM lucigenin with those by 250 μM lucigenin and confirmed the validity of the data obtained by the latter concentration [22].

In conclusion, we for the first time reported that in apoE-KO mice clinically relevant doses of telmisartan reduced atherosclerosis in association with suppressions in vascular oxidative stress and vascular systemic oxidative state. Our results suggest that telmisartan is beneficial not only for hypertension but also for atherosclerosis and imply that this drug may work as an anti-oxidant in various organs, although additional experiments will be needed.

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