International Journal of Cardiology xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Letter to the Editor

Increased serum neopterin in patients with nonrheumatic aortic valve stenosis

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ARTICLE INFO

Article history:
Received 11 December 2009
Accepted 13 February 2010
Available online xxxx

Keywords:
Aortic valve stenosis
Inflammation
Neopterin

ABSTRACT

Background: Inflammation and immunity play an intrinsic role in the pathogenesis of NR-AS. Neopterin is mainly produced by activated macrophages.
Methods: We examined serum neopterin levels in 51 patients undergoing valve replacement surgery [37 NR-AS and 14 aortic regurgitation (AR)].
Results: Serum neopterin levels were significantly higher in patients with NR-AS than in those with AR and age matched controls, whereas serum high-sensitivity C-reactive protein (hs-CRP) levels did not differ among three groups. Moreover, serum neopterin levels were not different between NR-AS patients with and without coronary artery disease.
Conclusions: Serum neopterin levels appear to be a useful maker in patients with NR-AS.

1. Introduction

Inflammation and immunity play an important role in the pathogenesis of not only atherosclerosis but also nonrheumatic aortic valve stenosis (NR-AS) [1,2]. Atherosclerotic inflammation triggers osteogenic bone transformation in NR-AS. Since current pharmacotherapy seems to be not entirely effective completed calcified lesion in NR-AS valves, it is important to detect the early development of inflammation on aortic valves before calcified lesions are completed and to start a prompt pharmacotherapy against the progression of NR-AS. In this regards, circulating biomarkers may be useful to find initial atherosclerotic inflammation on calcified aortic valves. However, little information exists regarding the biomarkers in patients with NR-AS.

Neopterin is produced by monocytes and macrophages upon activation by pro-inflammatory stimuli like interferon-γ [3] and serves as a soluble maker of monocytes and macrophages activation. However, to our knowledge, no previous studies have investigated serum neopterin levels in patients with NR-AS. Therefore, in this study we assessed serum neopterin levels in patients with NR-AS to find the useful biomaker of inflammation on NR-AS.

2. Methods

Serum samples were collected from 51 patients undergoing valve replacement surgery [37 NR-AS and 14 aortic regurgitation (AR)] and from 6 age matched controls (age 72 ± 7, range 41–83 years old). All of our protocols were approved by the Ethics Committee of our institution. Patients with significant diseases such as infections, renal failure, cancer, or carotid and peripheral artery atherosclerosis were excluded. Fasting blood samples were collected at the time of surgical valve replacement. Blood was kept into nonheparinized tubes on ice, and then centrifuged at 3000 rpm for 15 min at 4 °C. After immediate centrifugation, serum samples were stored at − 80 °C until they were assayed. Serum neopterin levels were measured by enzyme-linked immunosorbent assay kit (IBL, Hamburg, Germany) according to the manufacturer's instruction. In addition, serum high-sensitivity C-reactive protein (hs-CRP) levels were assessed by high-sensitivity CRP assay (N-Latex CRP II, Dade Behring, DE, USA).

All patients underwent a comprehensive examination including 2D, M-mode, pulsed-wave Doppler, continuous-wave Doppler and color Doppler echocardiography. Standard Doppler measurement of the left ventricular outflow tract and the aortic valve were recorded to obtain the maximum velocity and the peak velocity. We utilized a simplified Bernoulli equation to determine the transaortic pressure gradient.

Data are expressed as mean ± SEM. Analysis of variance (Kruskal–Wallis test, followed by Mann–Whitney *U* test) was used for statistical comparisons. Spearman's rank correlation test was used for correlations. A value of $p<0.05$ was considered to indicate statistical significance.

3. Results

The clinical characteristics of patients are shown in Table 1. Left ventricular end-diastolic and end-systolic dimension were larger in patients with AR than in those with NR-AS, whereas mean ejection fraction was lower in patients with AR than in those with NR-AS. Serum neopterin levels were higher in patients with AS than in those with AR and controls (controls, AR, and NR-AS: 7.4 ± 0.7, 6.7 ± 0.7, and 10.0 ± 0.9 ng/mL, respectively, $p<0.05$ versus AR and controls; Fig. 1A), while serum hs-CRP levels were not different

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doi:10.1016/j.ijcard.2010.02.035

Table 1
Patient characteristics.

	NR-AS	AR
n	37	14
Age (years)	75 ± 1	64 ± 2
Male/female	16/21	9/5
Left ventricular end-diastolic dimension (mm)	47 ± 1	68 ± 2*
Left ventricular end-systolic dimension (mm)	29 ± 1	47 ± 3*
Left ventricular ejection fraction (%)	67 ± 2	53 ± 4*
Bicuspid valve	6 (16%)	2 (14%)
Peak jet velocity (m/s)	4.0 ± 0.2	–
Mean pressure gradient (mmHg)	40 ± 4	–
Aortic valve area (cm^2)	0.75 ± 0.04	–
Medications		
ACEIs/ARBs	18 (49%)	8 (57%)
Diuretics	14 (39%)	6 (43%)
β-blockers	10 (27%)	2 (14%)

Values are expressed as means ± SEM and as numbers of patients and percentages for categorical variables. ACEIs; angiotensin converting emzyme inhibitors, ARBs; angiotensin II type1 receptor blockers. *$p < 0.05$ compared with patients with NR-AS.

among three groups (controls, AR, and NR-AS: 0.6 ± 0.2, 0.5 ± 0.2 and 0.7 ± 0.1 mg/L, respectively; Fig. 1B). Serum neopterin levels did not differ between NR-AS patients with and without coronary artery disease (CAD) (Fig. 2A). In addition, there was no difference in serum neopterin levels between bicuspid and tricuspid valves in patients with NR-AS (Fig. 2B). Serum neopterin levels did not correlate with echocardiographic hemodynamic parameters such as peak jet velocity, mean pressure gradient, and aortic valve area in patients with NR-AS.

4. Discussion

Inflammation, i.e. activated monocytes and macrophages, occurs in the earliest lesions on both atherosclerosis and calcified aortic valves. Neopterin, a pteridine derivative, is produced by activated monocytes and macrophages and serves as a maker for the active status of monocytes and macrophages [3]. In fact, elevated neopterin levels have been reported in patients with CAD compared with controls [4,5]. In this study, serum neopterin levels were higher in patients with NR-AS than those with AR and controls, while there was no difference between NR-AS patients with and without CAD. Therefore, increased serum neopterin levels in patients with NR-AS may reflect local monocytes and macrophages activation in aortic valves.

Neopterin is produced by monocytes and macrophages in response to interferon-γ [3]. We have previously reported that interferon-γ inducible factor, also called interleukin-18 (IL-18), express in aortic valves and IL-18 expression is increased in patients with NR-AS [6]. Thus, increased IL-18 in aortic valves may be associated with neopterin production via interferon-γ in patients with NR-AS.

Little information exists regarding the biomarkers in patients with NR-AS. Serum neopterin levels may be a useful maker in patients with

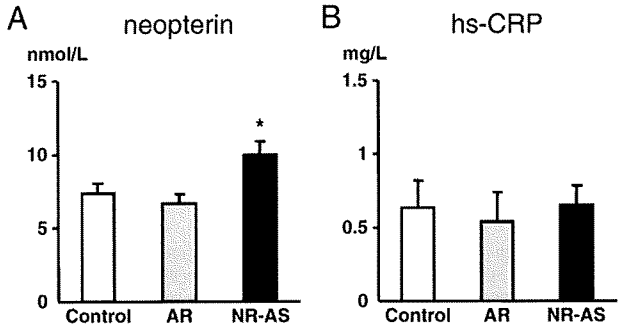

Fig. 1. Increased serum neopterin levels in patients with nonrheumatic aortic valve stenosis. Bar graph shows comparison of (A) serum neopterin and (B) serum hs-CRP levels among control ($n = 6$), patients with AR ($n = 14$), and those with NR-AS ($n = 37$). *$p < 0.05$ compared with other groups.

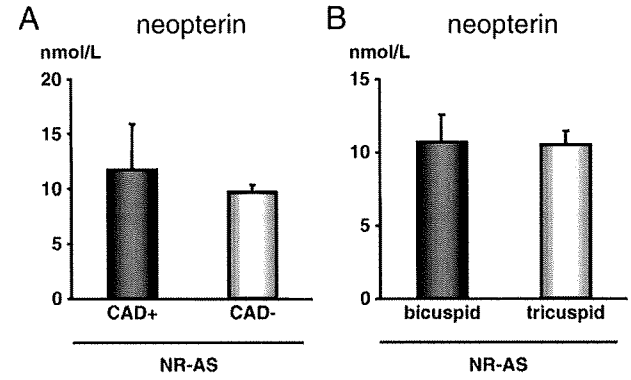

Fig. 2. Comparison of serum neopterin levels in patients with nonrheumatic aortic valve stenosis. (A) Bar graph shows comparison of serum neopterin levels between NR-AS patients with (CAD+; $n = 6$) and without coronary artery disease (CAD-; $n = 31$). (B) Bar graph shows comparison of serum neopterin levels between NR-AS patients with bicuspid ($n = 6$) and with tricuspid valves ($n = 31$).

the early development of inflammation on calcified NR-AS and in whom intensive pharmacotherapy may contribute to attenuate the progression of NR-AS.

In conclusion, we demonstrated for the first time that serum neopterin levels were elevated in patients with NR-AS. Early detection of NR-AS by echocardiography and serum neopterin levels may contribute to prevent the progression of NR-AS.

Acknowledgement

This work was supported by the Medical Research Grant of Osaka Heart Club and the Grant for Clinical Vascular Function (to Naito Y). The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [7].

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Please cite this article as: Naito Y, et al, Increased serum neopterin in patients with nonrheumatic aortic valve stenosis, Int J Cardiol (2010), doi:10.1016/j.ijcard.2010.02.035

IJCA-11749; No of Pages 4

ELSEVIER

International Journal of Cardiology xx (2009) xxx–xxx

International Journal of Cardiology

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Letter to the Editor

Increased interleukin-18 expression in nonrheumatic aortic valve stenosis

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Received 4 January 2009; accepted 10 January 2009

Abstract

Background: It is unknown whether interleukin-18 (IL-18) participates in the pathophysiology of nonrheumatic aortic stenosis (NR-AS).
Methods: We examined IL-18 expression in human NR-AS valves by immunohistochemistry and Western blot analysis.
Results: Immunohistochemistry revealed that NR-AS valves showed increased IL-18 expression compared with controls. IL-18 receptor was also expressed in NR-AS valves. Western blot analysis showed that IL-18 was expressed as an active form in NR-AS valves. Furthermore, increased IL-18 expression was correlated with the advanced clinical severity of NR-AS.
Conclusions: IL-18 was expressed in the aortic valves and up-regulated in NR-AS valves. IL-18 may contribute to the pathophysiology of NR-AS.

Keywords: Aortic stenosis; Cytokine; Inflammation; Interleukin-18

1. Introduction

The number of patients with nonrheumatic aortic stenosis (NR-AS) has been increasing recently [1]. Although clinical trials were carried out to prevent the progression of NR-AS, it is still controversial [2]; thus, it is important to elucidate the molecular pathogenesis of NR-AS to find targets for the prevention of NR-AS. Many recent observations demonstrated that the etiology of NR-AS is associated with the pathophysiology of atherosclerosis [1].

Interleukin-18 (IL-18) is a member of the interleukin-1 family of cytokines [3] and plays an important role in the pathogenesis of atherosclerosis [4]. However, to our knowledge, no previous studies have investigated IL-18 expression in human NR-AS valves. Therefore, in this study we examined IL-18 expression in NR-AS valves to better understand IL-18 actions in NR-AS.

2. Methods

NR-AS valves were obtained from 41 consecutive patients undergoing valve replacement surgery because of symptomatic aortic stenosis. Control valves ($n=10$; age 48±7, range 21–82 years old; 5 male, 5 female) were obtained from patients without aortic stenosis at autopsy. All of our protocols were approved by the Ethics Committee of our institution. Aortic valve tissues were collected at the time of surgical valve replacement. They were fixed with buffered 10% formalin, and pieces of them were snap-frozen in liquid nitrogen in O.C.T. embedding medium (Tissue Tek O.C.T., Sakura Finetek Japan

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doi:10.1016/j.ijcard.2009.01.022

Table 1
Patient characteristics

n	41
Age (years)	73±1
Male/Female	17/24
NYHA class, I/II/III/IV	2/19/18/2
Peak jet velocity (m/s)	4.3±0.2
Mean pressure gradient (mmHg)	46±4
Aortic valve area (cm^2)	0.73±0.04
Left ventricular ejection fraction (%)	64±2
Left ventricular mass index (g/m^2)	156±10
Prevalence of dyslipidemia	12 (29%)
Bicuspid/tricuspid valve	10/31
Medications	
ACEI	9 (22%)
ARB	14 (34%)
Diuretics	19 (46%)
β-blocker	8 (20%)
Statins	8 (20%)

Values are expressed as means±SEM and as numbers of patients and percentages for categorical variables. ACEI; angiotensin converting emzyme inhibitor, ARB; angiotensin II type1 receptor blocker.

Inc., Tokyo, Japan) and stored at −80 °C. For Western blot analysis, aortic valve tissues were frozen in liquid nitrogen and stored at −80 °C. 4 μm sections from paraffin-embedded samples were stained with hematoxylin-eosin and were incubated with either a primary rabbit anti-human IL-18 antibody (MBL, Nagoya, Japan), a primary mouse anti-human CD68 antibody (Dako, Kyoto, Japan) for macrophages, a primary mouse anti-human alpha smooth muscle actin (SMA) antibody (Dako, Kyoto, Japan) for smooth muscle cells or myofibroblasts, and a primary mouse anti-human CD31 (Dako, Kyoto, Japan) for endothelial cells. For IL-18 receptor (alpha-chain) (IL-18Ralpha), 6 μm cryostat sections were incubated with a primary goat anti-human IL-18Ralpha antibody (R&D Systems, Minneapolis, MN). Immunostains were visualized with the use of an avidin-biotin-peroxidase conjugate and 3,3′-diaminobenzidine substrate. Every section was counterstained with hematoxylin. Non-immune IgG of the same species was used as a negative control. Protein extracts from aortic valves were separated by SDS-PAGE and transferred onto PVDF membranes. The expression levels of molecules using antibodies against rabbit anti-human IL-18 (MBL, Nagoya, Japan) and mouse anti-human IL-18Ralpha (R&D Systems, Minneapolis, MN) were detected by an enhanced chemiluminescence kit (PIERCE, Rockford, IL). IL-18 levels in NR-AS valves were also measured by ELISA kit (MBL, Nagoya, Japan) and normalized to total protein concentration. Data are expressed as mean± SEM. Statistical analysis was performed using one way

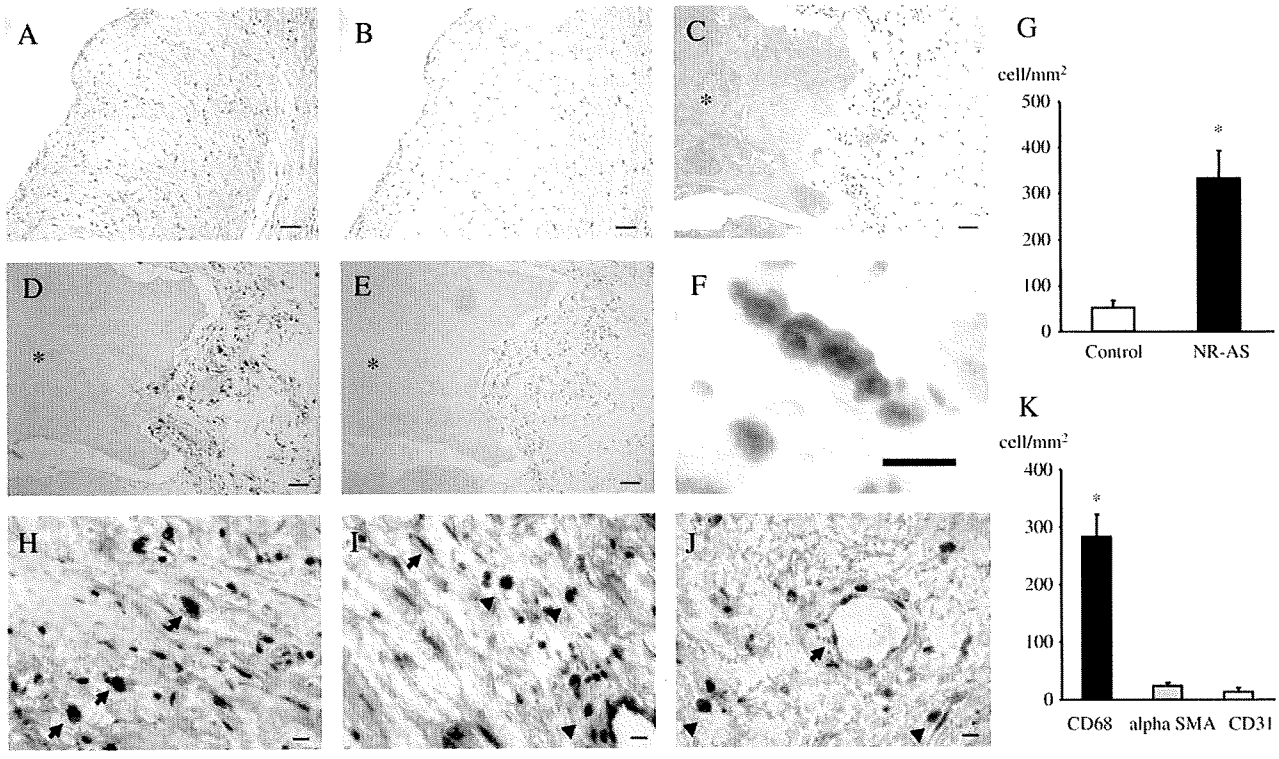

Fig. 1. Expression and cellular localization of IL-18 in human nonrheumatic aortic stenosis valves. Representative images of hematoxylin and eosin staining (A) and IL-18 staining (B) of human control valves. Representative images of hematoxylin and eosin staining (C), IL-18 staining (D), and non-immune IgG (negative) staining (E) of human NR-AS valves. *indicates massive calcified lesion. Scale bars: 50 μm. High magnification view shows that IL-18 appeared to be localized to cytoplasm (F). Scale bars: 10 μm. Bar graph shows the number of IL-18 positive cells in control and NR-AS valves ($n=10$ for each group).*:$p<0.05$ compared with control valves (G). Sections were anti-IL-18 (brown) and anti-CD68 (red) immunostaining (H), anti-IL-18 (brown) and anti-alpha SMA (red) immunostaining (I), anti-IL-18 (brown) and anti-CD31 (red) immunostaining in human NR-AS valves (J). Arrows indicate double positive cells and arrow heads indicate IL-18 positive cells. Scale bars: 10 μm. Bar graph shows the number of double positive cells (IL-18 and each staining) in NR-AS valves ($n=10$). *:$p<0.05$ compared with alpha SMA and CD31 positive cells (K).

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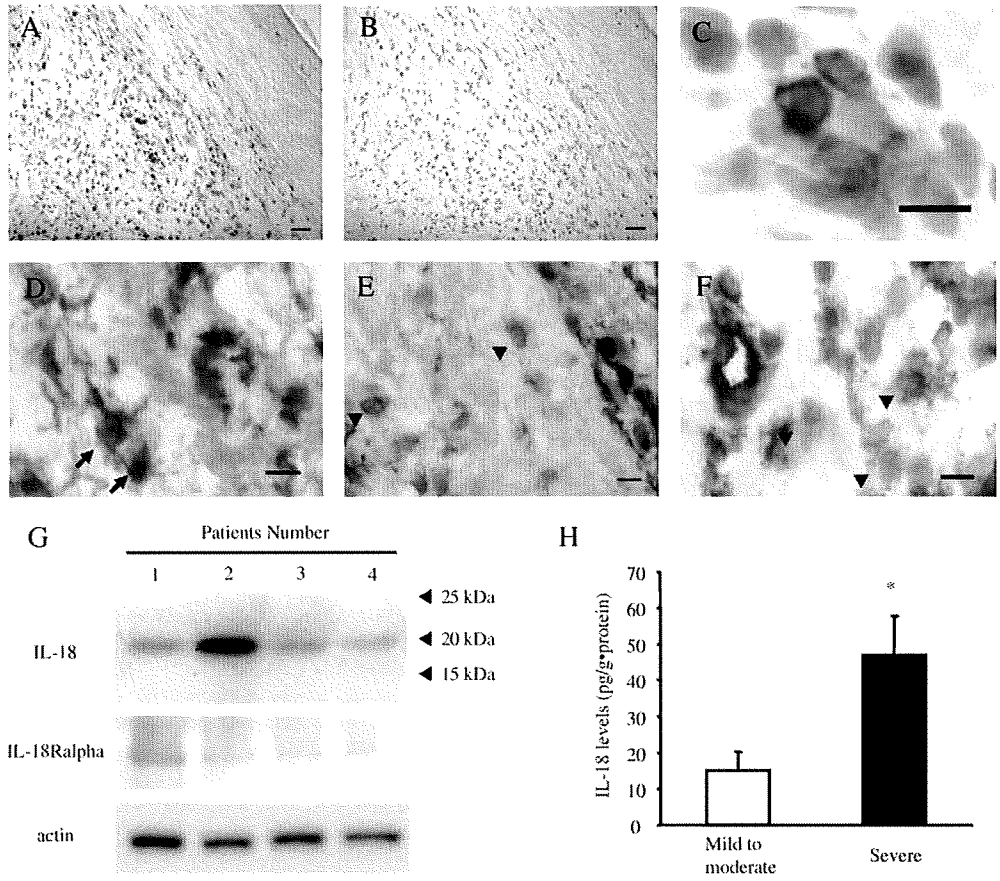

Fig. 2. Expression and cellular localization of IL-18Ralpha in human nonrheumatic aortic stenosis valves and relationship between IL-18 expression and severity in patients with nonrheumatic aortic stenosis. Representative images showing expression of IL-18Ralpha (A) and non-immune IgG (negative) staining (B) of human NR-AS valves. Scale bars: 50 μm. High magnification view shows that IL-18Ralpha appeared to be localized to the cell membranes (C). Sections were anti-IL-18Ralpha (red) and anti-CD68 (brown) immunostaining (D), anti-IL-18Ralpha (red) and anti-alpha SMA (brown) immunostaining (E), anti-IL-18Ralpha (red) and anti-CD31 (brown) immunostaining in human NR-AS valves (F). Arrows indicate double positive cells and arrow heads indicate IL-18Ralpha positive cells. Scale bars: 10 μm. Representative Western blot analysis showed expression of IL-18, IL-18Ralpha, and actin in human NR-AS valves (G). Bar graph shows comparison of IL-18 levels in NR-AS valves between patients with mild to moderate symptoms ($n=12$) and severe symptoms ($n=14$) by ELISA (H). Mild to moderate and Severe mean patients with mild to moderate symptoms and patients with severe symptoms, respectively. *$p<0.05$ versus patients with mild to moderate symptoms.

ANOVA or Student's t test. Differences were considered significant when the value was <0.05.

3. Results

The clinical characteristics of NR-AS patients are shown in Table 1. Immunohistochemistry revealed that IL-18 was very weakly expressed in some interstitial cells in control valves (Fig. 1B). However, the expression of IL-18 in NR-AS valves was dramatically increased compared with controls (Fig. 1A–G). IL-18 was expressed mainly in macrophages, occasional alpha SMA positive myofibroblasts and endothelial cells (Fig. 1H–K). IL-18Ralpha was also expressed in human NR-AS valves and highly expressed in macrophages and very rarely in endothelial cells and alpha SMA positive myofibroblasts (Fig. 2A–F). Western blot analysis revealed that IL-18 was expressed in NR-AS valves as an active form, 18-kDa protein and its amount varied among the valves, while the amount of IL-18Ralpha was homogeneous (Fig. 2G). The amount of IL-18 expression was significantly greater in severe patients with the higher New York Heart Association functional class and those with symptom of angina pectoris compared with patients without

Table 2
Comparison of clinical characteristics in patients with mild to moderate symptoms and severe symptoms

	Mild to moderate	Severe
n	12	14
Age (yrs)	71±2	73±2
Male/Female	4/8	5/9
NYHA class, I/II/III/IV	0/12/0/0	0/0/13/1
Peak jet velocity (m/s)	4.3±0.3	4.2±0.3
Mean pressure gradient (mmHg)	44±7	43±7
Aortic valve area (cm^2)	0.76±0.08	0.72±0.07
Current symptoms of angina pectoris	0 (0%)	3 (21%)*
Prevalence of dyslipidemia	0 (0%)	5 (36%)*
Prevalence of bicuspid valve	3 (25%)	3 (21%)

Values are expressed as means±SEM and as numbers of patients and percentages for categorical variables. Mild to moderate; patients with mild to moderate symptoms, Severe; patients with severe symptoms. *$p<0.05$ vs patients with mild to moderate symptoms.

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those symptoms among 26 patients in whom frozen aortic valves were available using Western Blot Analysis and ELISA. (Fig. 2H). The clinical characteristics of these two subsets are summarized in Table 2.

4. Discussion

Inflammation plays a crucial role in the pathogenesis of not only atherosclerosis but also NR-AS [1]. IL-18 is a proinflammatory cytokine and takes part in the pathogenesis of atherosclerosis [4]. Indeed, atherosclerosis lesion size decreases in IL-18 deficient apolipoprotein E-knockout mice [5]. A strong IL-18 immunoreactivity was found mainly in macrophages in NR-AS valves. Thus, IL-18 appears likely to participate in the pathogenesis of NR-AS as well as atherosclerosis.

IL-18 is synthesized as an inactive 24 kDa precursor and cleaved by caspase-1 to an active 18 kDa mature form [3]. Interestingly, Western blot analysis showed that IL-18 was expressed as 18 kDa form in NR-AS valves. Since IL-18Ralpha represents the ligand-binding chain, active IL-18 and IL-18 receptor may signal in the progression of NR-AS valves. IL-18 induce some proinflammatory mediators, including adhesion molecules, matrix metalloproteinases, and IL-1beta. These mediators have been reported to increase in activated valvular cells [6]. Therefore, increased IL-18 may induce these mediators in NR-AS valves. In addition, IL-18 has been reported to express by osteoblasts and inhibit osteoclast formation [7]. IL-18 may be also associated with calcific deposits in NR-AS valves.

In conclusion, we demonstrated for the first time that IL-18 and IL-18Ralpha are expressed in human NR-AS valves. IL-18 was expressed as an active form in NR-AS valves. Moreover, increased IL-18 expression was correlated with the clinical severity of NR-AS. IL-18 may play some roles in the pathophysiology of NR-AS.

Acknowledgement

The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [8].

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Clinical and Experimental Hypertension, 31:625–638, 2009

ISSN: 1064-1963 print / 1525-6006 online
DOI: 10.3109/10641960903406993

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The Mechanism of Distinct Diurnal Variations of Renin-Angiotensin System in Aorta and Heart of Spontaneously Hypertensive Rats

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Diurnal variations in plasminogen activator inhibitor-1 mRNA expression are different between the spontaneously hypertensive rats (SHRs) and the Wistar-Kyoto (WKY) rats, and between the aorta and the heart. To elucidate the mechanisms, we examined diurnal changes in the circulating renin-angiotensin system in the SHR and WKY rats. Diurnal variations in plasma renin activity (PRA), plasma angiotensin I, and aldosterone concentrations were similar between the SHR and WKY rats. On the other hand, plasma angiotensin II (Ang II) concentration in the SHR was lower than that in the WKY rats at most time points, but increased to the level of the WKY rats in the late light phase. Treatment with AT1 receptor antagonist candesartan increased plasma Ang II concentration except at ZT 8 and lessened its diurnal variation in the SHR. At the peak in plasma Ang II in the SHR, Ang II regulated genes such as transforming growth factor-β1 and p22phox were upregulated in the aorta. On the other hand, these genes were upregulated throughout the day in the heart of SHR. Candesartan treatment increased AT1a receptor mRNA expression in the heart but not in the aorta of SHR. These findings suggest that an AT1 receptor-mediated mechanism might cause a surge in plasma Ang II concentration at the late light phase in the SHR. Homologous down-regulation of AT1a receptor by Ang II may dampen the effect of a surge in plasma Ang II concentration in the heart of SHR.

Keywords circadian rhythm, p22phox, renin-angiotensin system, spontaneously hypertensive rats, transforming growth factor-β1

Received August 20, 2008; revised December 4, 2008; accepted December 9, 2008.
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Introduction

Cardiovascular events, such as myocardial infarction and stroke, show well-defined patterns in their occurrence throughout the day (1). Peak activity of plasminogen activator inhibitor-1 (PAI-1) in the morning has been linked to increased onset of these events at that time (2). Recent works have shown that clock genes, essential components of a biological clock, directly regulate the PAI-1 promoter (3–5), while angiotensin II (Ang II) increases PAI-1 gene expression (6–9). We have recently shown that aortic PAI-1 mRNA exhibits circadian oscillation with a peak in the late light phase and its peak is strongly augmented in spontaneously hypertensive rats (SHRs) compared to their normotensive counterpart, Wistar-Kyoto (WKY) rats (10). On the other hand, cardiac PAI-1 mRNA exhibits circadian oscillation with a peak in the dark phase, and the expression level in the SHR was higher than that in the WKY rats throughout the day. A long-term blockade of angiotensin type 1a receptor (AT1a-R) suppressed the PAI-1 mRNA expression specifially in the late light phase and almost abolished the circadian changes in PAI-1 expression in the aorta of SHR. This finding shows that the major determinant of circadian rhythm in PAI-1 mRNA expression is circadian changes in the strength of renin-angiotensin system (RAS) but not clock genes in the aorta of SHR. On the other hand, AT1a-R blockade suppressed the PAI-1 mRNA expression throughout the day and did not change the circadian rhythm in PAI-1 mRNA expression in the heart of SHR. This finding suggested RAS is evenly activated throughout the day in the heart of SHR. However, it is unknown how distinct diurnal changes in the strength of RAS is generated in the aorta and heart of SHR. To elucidate it, in the present study, we investigated the diurnal variation in the circulating and tissue RAS in the aorta and the heart of the SHR and WKY rats. We found remarkably different diurnal variation in circulating and tissue RAS in these two strains of rats.

Methods

Materials

Angiotensin type 1 receptor (AT1 receptor) antagonist candesartan (CV-11974) was provided by Takeda Pharmaceutical (Osaka, Japan). Other materials and chemicals were obtained from commercial sources.

Animal Preparation

Experiments were carried out in 72 male SHR and 36 male WKY rats, descended from SHR and WKY rats provided by Kozo Okamoto (Kinki University, Osaka-Sayama, Japan) (11). Their genetic backgrounds have been reported previously (12). Rats were given a regular chow diet and *ad libitum* access to tap water and kept in a separate, environmentally controlled room (25 ± 1°C) in which a 12-h light/12-h dark cycle was maintained [lights on at 7 am; Zeitgeber time (ZT) 0]. All of our experimental procedures were approved by the Animal Research Committee of Hyogo College of Medicine.

For the control group, 14-week-old SHR and WKY rats (n = 36 for both groups) were sacrificed every 4 h from ZT0 to ZT20 under pentobarbital (for blood sampling) or ether (for ribonucleic acid (RNA) extraction) anesthesia. For the candesartan-treated group, we implanted subcutaneous osmotic minipumps (Alzet, Cupertino, CA) in a 12-week-old SHR (250 to 275 g; n = 36) under ether anesthesia to deliver candesartan (0.5 mg/kg per day for 14 days) at a constant rate. The systolic blood pressure (SBP) was measured using a noninvasive

tail-cuff method (Ueda Electronics, Tokyo, Japan) at ZT4, 1 week before euthanasia to avoid any effects of the stress induced by blood pressure measurement on circadian rhythm.

The heart, liver, lung, and thoracic aorta tissue were isolated, snap-frozen in liquid nitrogen, and stored at −80°C. Animals were sacrificed at each time point on 6 separate days to ensure reproducibility of the experiments.

Biochemical Assay

Under pentobarbital anesthesia, blood was withdrawn by aortic puncture and collected into prechilled tubes containing Na_2-EDTA at final concentrations of 5 mM. Plasma was separated after being spun in a refrigerated centrifuge and was stored at −80°C before analysis. Plasma renin activity (PRA) was measured using a radioimmunoassay (RIA) kit (GammaCoat Renin Kit, DiaSorin Inc., Stillwater, MN). Plasma immunoreactive Ang I and Ang II were measured using RIA (13). The Ang II antibody used in our experiment has been shown to have a very low cross-reactivity (0.037%) with Ang I. Aldosterone was measured using a direct RIA kit (Aldosterone-RIAKIT II Kit, Dainabot Co. Ltd., Tokyo, Japan).

RNA Extraction and Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the liver, aorta, and heart using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. We performed the real-time quantitative RT-PCR as previously described (14). Oligonucleotide probes and primers were designed according to cDNA sequences reported in the GenBank database, and shown in Table 1. The relative amount of each mRNA was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. We also examined the levels of GAPDH mRNA expression by Northern analysis as previously described (15). We confirmed that the levels of GAPDH mRNA expression in the liver, aorta, and heart were similar in the SHR and WKY rats and remained constant throughout the day (data not shown).

Angiotensin-Converting Enzyme Activity

Angiotensin-converting enzyme (ACE) activity in the lung and aorta was measured as previously described (16).

Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed using an unpaired student's *t*-test or analysis of variance (ANOVA) followed by Bonferroni's post-hoc test, as appropriate (Statview, Version 4, Abacus Concepts, Berkeley, CA). Differences were considered significant when the associated probability value was < 0.05. Because samples at each time point were from different animals, repeated-measures analysis was not applied.

Results

Characterization of Experimental Models

Baseline body weight and hemodynamic variables for the two groups are shown in Table 2. Systolic BP was significantly higher in the SHR than in the WKY rats at 13 weeks of age.

Table 1
Primer and probe sequences used in real-time quantitative RT-PCR analysis

Gene	Primer/Probe	Sequence
AT1aR	Forward	5'- TATCACAGTGTGCGCGTTTCA -3'
	Reverse	5'- TGGTAAGGCCCAGCCCTAT -3'
	Probe	5'- FAM- TGAGTCTCGGAATTCGACGCTCCC - TAMRA-3'
TGF-β1	Forward	5'-GGCACCATCCATGACATGAAC-3'
	Reverse	5'-CAGGTGTTGAGCCCTTTCCA-3'
	Probe	5'-FAM-CCTTCCTGCTCCTCATGGCCACC-TAMRA-3'
p22phox	Forward	5'-ACCGCTGTGGTGAAGCTGTT-3'
	Reverse	5'-AGCAGGTAGATCACACTGGCAAT-3'
	Probe	5'-FAM-CCACTTACTGCTGTCCGTGCCTGCA-TAMRA-3'
GAPDH	Forward	5'-TGACAACTCCCTCAAGATTGTCA-3'
	Reverse	5'-GGCATGGACTGTGGTCATGA-3'
	Probe	5'-FAM-TGCATCCTGCACCACCAACTGCT-TAG-TAMRA-3'

Abbreviations: RT-PCR-Reverse transcription-polymerase chain reaction; AT1aR-angiotensin type 1a receptor; TGF-β1-transforming growth factor-β1; GAPDH-glyceraldehyde-3-phosphate dehydrogenase.

Table 2
Body and heart weights and hemodynamic variables in SHR and WKY rats

Variable	SHR	WKY rats	SHR-candesartan
BW, g	259.6 ± 7.2	276.1 ± 8.1	258.8 ± 8.2
SBP, mm Hg	210.3 ± 8.6*	148.4 ± 7.7	153.2 ± 1.2†
Heart rate, bpm	334.8 ± 5.5	344.4 ± 12.1	348.0 ± 11.0
HW/BW ratio, mg/g	4.2 ± 0.1*	3.3 ± 0.2	3.4 ± 0.1†

*$p < 0.05$ vs. WKY rats.
†$p < 0.05$ vs. SHR.
Abbreviations: B = body weight; SBP = systolic blood pressure; HW = heart weight; SHR = candesartan-candesartan-treated SHR.

Candesartan (0.5 mg/kg/day) lowered the SBP and reduced the heart weight/body weight ratio (Table 2).

Circadian Variations in Circulating RAS of SHR and WKY Rats

All components of the circulating RAS exhibited rhythmic variations in their levels. Plasma renin activity peaked at ZT8, and was at a trough value at ZT16 in the SHR and at ZT20 in the WKY rats (Figure 1A). The PRA levels in both groups were almost the same throughout the day, except for a trend toward increased levels in the WKY rats.

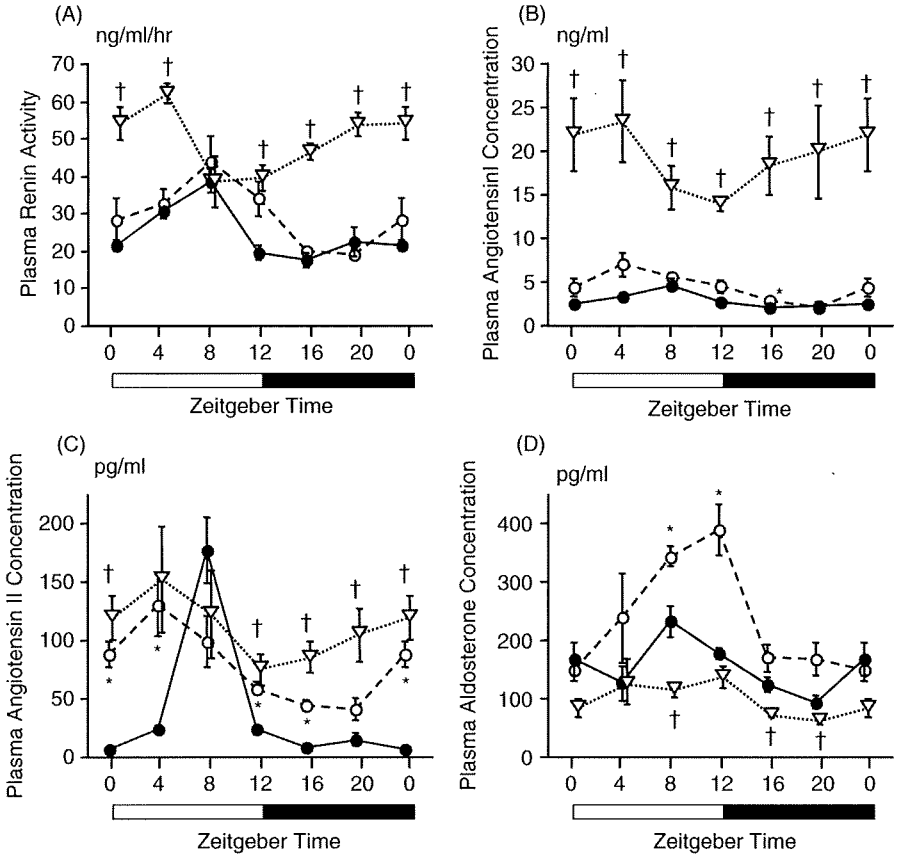

Figure 1. Diurnal changes in plasma renin activity (A), plasma angiotensin I (B), plasma angiotensin II (C), and plasma aldosterone (D) concentrations in the untreated SHR (•), candesartan-treated SHR (▽), and untreated WKY rats (○). Data represent the mean ± SEM for six independent experiments. *$p < 0.05$ (untreated SHR vs. untreated WKY rats); †$p < 0.05$ (untreated SHR vs. candesartan-treated SHR).

Plasma Ang I peaked at ZT4 and was at a trough value at ZT20 in the WKY rats (Figure 1B). In the SHR, Ang I level peaked at ZT8 and was at a trough value at ZT20. Plasma Ang I level in the WKY rat group was greater than in the SHR, although a significant difference was observed only at ZT16.

The circadian pattern of the plasma Ang II levels was similar to that of Ang I in the WKY rats. The plasma Ang II level peaked at ZT4 and reached a trough value at ZT20 in the WKY rats (Figure 1C). In the SHR group, plasma Ang II peaked at ZT8, similar to Ang I, and was at a trough value at ZT0. Unlike plasma Ang I, diurnal variation in Ang II levels was markedly augmented in the SHR. The Ang II levels in the WKY rat group were several folds greater than in the SHR, except at ZT8 (Figure 1C). However, the Ang II level of the SHR increased to a level just above that of the WKY rats at ZT8, although the difference did not reach statistical significance.

Plasma aldosterone concentration (PAC) peaked at ZT12 and reached a trough value at ZT0 in the WKY rats (Figure 1D). Plasma aldosterone concentration peaked at ZT8 and reached a trough value at ZT20 in the SHR. The PAC was significantly greater in the WKY rats than in the SHR at ZT8 and ZT12.

The Effect of AT1 Receptor Antagonist on Circadian Variations in Circulating RAS of SHR and WKY Rats

Two-week treatment with candesartan increased PRA levels in the SHR at many time points (Figure 1A). However, candesartan treatment did not upregulate PRA at ZT8, when plasma Ang II was dramatically increased in the untreated SHR.

Candesartan treatment increased plasma Ang I in the SHR throughout the day (Figure 1B). Plasma Ang I levels of candesartan-treated SHR were several folds higher than untreated WKY. Candesartan treatment shifted the peak of plasma Ang I level in the SHR from ZT8 to ZT4, when plasma Ang I peaked in the untreated WKY rats.

Plasma Ang II levels of candesartan-treated SHR increased throughout the day except at ZT 8 (Figure 1C). Candesartan treatment lessened the amplitude of diurnal variation in the SHR.

Candesartan treatment decreased PAC in the SHR at ZT 8, ZT16, and ZT20 (Figure 1D).

Circadian mRNA Expression of Angiotensinogen in the Livers of SHR and WKY Rats

To examine the mechanisms of the altered circadian oscillatory pattern of circulating Ang II in the SHR, we examined angiotensinogen mRNA expression in the livers of SHR and WKY rats (Figure 2). Angiotensinogen mRNA showed little oscillation in both strains of the rats. Angiotensinogen mRNA expression in the liver was almost identical at most time points between the SHR and WKY rats.

ACE Activity in the Lungs and Aortas of SHR and WKY Rats

To investigate the mechanisms of marked fluctuation in plasma Ang II concentration in the SHR, we measured ACE activity in the lungs and aortas of SHR and WKY rats at ZT0 (time of plasma Ang II trough level in the SHR) and at ZT8 (time of plasma Ang II peak

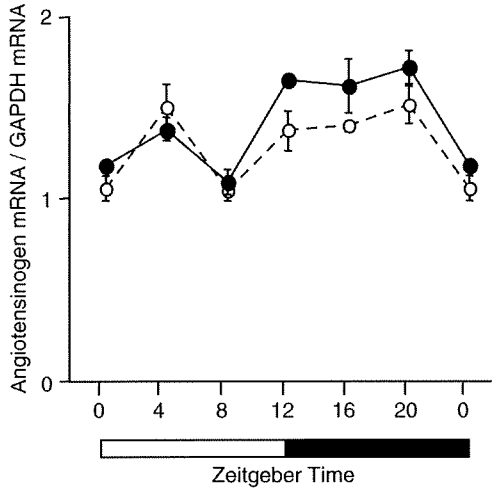

Figure 2. Diurnal expression of angiotensinogen in the SHR (•) and WKY rats (○). All expression values were normalized against the housekeeping gene product GAPDH. Trough values of WKY were defined as 1. Data represent the mean ± SEM for six independent experiments.

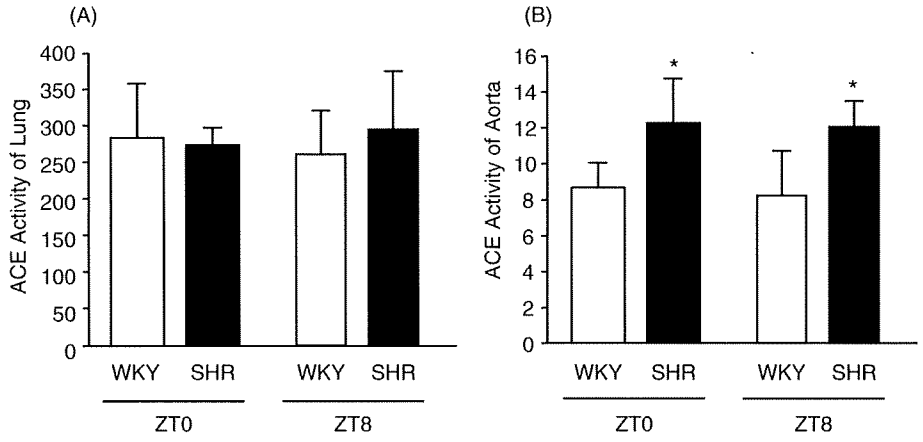

Figure 3. Angiotensin converting enzyme (ACE) activity of lung (A) and aorta (B) at ZT0 and ZT8 in the SHR (solid bar) and WKY rats (open bar). Values reported as nmol/mg wet weight/hour. Data represent the mean ± SEM for six independent experiments in the SHR and WKY rats. *$p < 0.05$ vs. WKY rats.

level in the SHR). Despite differences in plasma Ang II concentration, ACE activity was almost identical at ZT0 and ZT8 in both lungs and aortas in both strains of rats (Figure 3). However, ACE activity was greater in the SHR aortas than in the WKY aortas (Figure 3B).

Circadian mRNA Expression of TGF-β1 and p22phox in the Aortas and Hearts of SHR and WKY Rats

To elucidate the physiological relevance of the dramatic increase in plasma Ang II at ZT8 in SHR, we compared two Ang II regulated genes, namely, TGF-β1 (17) and p22phox (18) mRNA expression in the aortas and hearts of SHR and WKY rats (Figure 4). In the aortas, TGF-β1 and p22phox mRNA showed little oscillation in the WKY rats (Figures 4A and 4B). In contrast, the expression of TGF-β1 and p22phox mRNA peaked at ZT8 and reached a trough value at ZT16 in the SHR (Figures 4A and 4B). Two-week treatment with candesartan decreased TGF-β1 mRNA at ZT8 and ZT12 and p22phox mRNA at ZT0, ZT8, and ZT12 in the SHR (Figures 4A and 4B). Meanwhile, in the hearts, TGF-β1 and p22phox mRNA also showed little oscillation in the WKY rats (Figures 4C and 4D). Unlike the aortas, cardiac TGF-β1 and p22phox expression in the SHR was higher throughout the day than in the WKY rats, except at ZT20 for TGF-β1 and ZT0 for p22phox (Figures 4C and 4D). Two-week candesartan treatment decreased TGF-β1 mRNA at ZT0, ZT12, and ZT16 and p22phox mRNA at ZT4, ZT8, ZT12, ZT16, and ZT20 (Figures 4C and 4D). Diurnal variations in TGF-β1 and p22phox mRNA of the heart and aorta in candesartan-treated SHR looked more similar to those in the WKY rats rather than those in the untreated SHR.

Effect of Candesartan Treatment on mRNA Expression of AT1a Receptor in the Aortas and Hearts of SHR and WKY Rats

To elucidate the mechanism of different diurnal variations in the strength of tissue RAS between the aorta and heart of SHR, we examined the circadian mRNA expression of

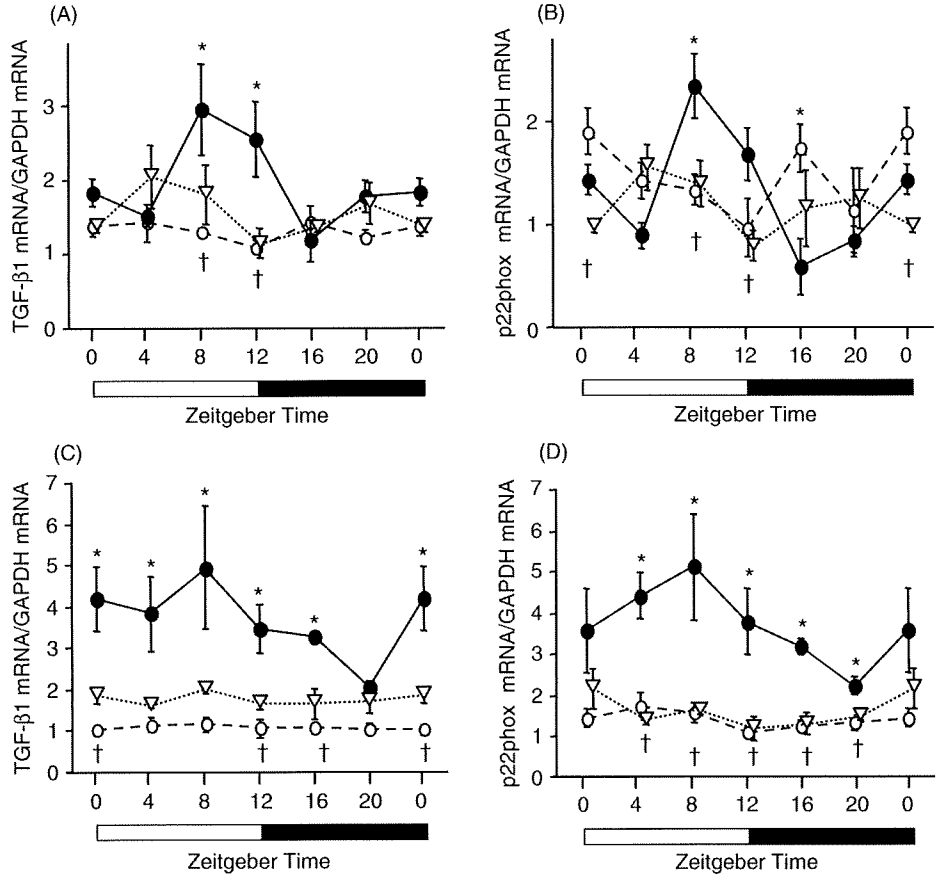

Figure 4. Diurnal expression of TGF-β1 and p22phox in the aortas (A, B) and hearts (C, D) of the untreated SHR (•), candesartan-treated SHR (▽), and untreated WKY rats (○). All expression values were normalized against the housekeeping gene product GAPDH. Trough values of untreated WKY were defined as 1. Data represent the mean ± SEM for six independent experiments. Abbreviation: TGF-β1-transforming growth factor-β1. *$p < 0.05$ (untreated SHR vs. untreated WKY rats). †$p < 0.05$ (untreated SHR vs. candesartan-treated SHR).

AT1a receptor in the aorta of SHR and WKY rats and compared that with the heart of SHR and WKY rats, which was previously published (14). In the aorta, the diurnal variations of mRNA expression of AT1a receptor were not different between the SHR and WKY rats (Figure 5). On the other hand, in the heart, the diurnal variations of mRNA expression of AT1a receptor were quite different between the SHR and WKY. Cardiac AT1a receptor mRNA expression in the SHR was significantly greater than in the WKY rats at many time points, but almost identical at ZT8 (14). To examine the effect of homologous downregulation by Ang II on the AT1a receptor mRNA levels, we examined the effect of chronic AT1 receptor antagonist treatment on the diurnal mRNA expression patterns of AT1a receptor in the SHR and WKY rats. Two-week treatment with candesartan had little effect on the diurnal mRNA expression patterns of the AT1a receptor in the aorta of SHRs (Figure 5). In contrast, cardiac AT1a receptor mRNA expression in candesartan-treated

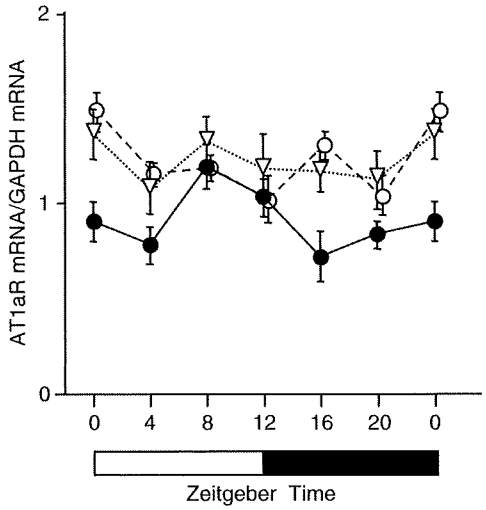

Figure 5. Diurnal expression of angiotensin type 1a receptor (AT1a R) in the aortas of the untreated SHR (•), candesartan-treated SHR (▽), and untreated WKY rats (○). All expression values were normalized against the housekeeping gene product GAPDH. Trough values of untreated WKY were defined as 1. Data represent the mean ± SEM for six independent experiments.

SHRs was significantly greater than in the untreated SHRs at ZT16, and a trend toward increased expression at ZT4, ZT8, ZT12, and ZT20 (14).

Discussion

All components of the circulating RAS exhibited rhythmic variations in their levels in both SHR and WKY rats. The most prominent finding of our study is the difference in the circadian variation in plasma Ang II levels. Plasma Ang II concentration was much lower in the SHR than in the WKY rats at most time points, but increased dramatically at ZT8 in the SHR and almost exceeded the level in the WKY rats. The expression of Ang II-regulated genes such as PAI-1 (10), TGF-β1, and p22phox (present study) were strongly upregulated at ZT8 in the aorta of SHR, and their peaks were abolished by candesartan treatment. These findings suggest that the surge in circulating Ang II at ZT8 in the SHR contribute to the peak expression of Ang II-regulated genes at that time in the SHR. On the other hand, mRNA expression of RAS components such as AT1a-R showed little diurnal changes in the aorta of SHR. These findings indicate that diurnal variation in the strength of RAS in the aorta is largely dependent on diurnal fluctuation in circulating Ang II in the SHR. In contrast to the aorta, candesartan treatment significantly decreased the mRNA expression of PAI-1 (10), TGF-β1, and p22phox almost throughout the day in the heart of SHR. Interestingly, cardiac AT1a-R mRNA expression level was downregulated at ZT8 (14), when circulating Ang II is markedly increased. Moreover, candesartan treatment increased AT1a receptor mRNA in the heart (14), but not in the aorta in the SHR. AT1a receptor in the heart might be more prone to receive homologous downregulation than that in the aorta, and the effect of a surge in the circulating Ang II concentration might be dampened in the heart. Otherwise, tissue RAS may exert a much stronger effect on cardiac tissue than on circulating RAS.

The mechanisms of different diurnal variations in plasma Ang II between SHR and WKY rats are currently unknown. To examine the role of AT1 receptor-mediated signaling for the different diurnal variations in plasma Ang II between SHR and WKY rats, we investigated the circadian variations of circulating RAS in candesartan-treated SHR. Candesartan treatment increased plasma Ang II concentration except at ZT 8 and lessened its amplitude of diurnal variation in the SHR. This finding indicates an AT1 receptor-mediated mechanism is at least partially responsible for the different circadian variation of plasma Ang II between the SHR and WKY rats. Candesartan treatment increased PRA at most time points. However, candesartan did not affect PRA in the SHR at ZT8. Ang II usually suppresses renin secretion via the so-called "short negative feedback." However, Ang II could also exert "positive feedback" on renin secretion by increasing reabsorption of sodium chloride in proximal tubule and decreasing sodium chloride that reaches to the macula densa (19). Our finding suggests that the effect of positive feedback of Ang II on the renin secretion is as strong as that of short negative feedback at ZT8, while the effect of positive feedback is weaker than that of short negative feedback at the time points except ZT8. Relatively strong positive feedback of Ang II on renin secretion may increase both Ang II and PRA at ZT8, resulting in a surge in plasma Ang II concentration. However, it is currently unknown what causes the imbalance between short negative feedback and positive feedback at ZT8 in the SHR. Further studies are necessary.

We investigated the mRNA expression of angiotensinogen in the liver and ACE activities in the lung and aorta of SHR and WKY rats. Angiotensinogen mRNA expression in the liver showed little circadian oscillation and did not differ between SHR and WKY rats. The difference in plasma Ang I between SHR and WKY rats was much smaller than that of Ang II. These findings suggest that the difference in the amount of Ang II precursor is not the major cause of difference in plasma Ang II concentration. The ACE activities in the lungs and aortas at ZT0 (time of plasma Ang II trough level in the SHR) were almost identical to those at ZT8 (time of plasma Ang II peak level in the SHR) in both SHR and WKY rats. We have not examined the circadian profile of serum ACE activity, but Stepien, Witte, and Lemmer reported that serum ACE activity in the SHR does not exhibit time-dependent differences (20). The diurnal variation in plasma Ang II concentration does not appear to be caused by the diurnal variation in ACE activity.

Rats are nocturnal and thus ZT8 corresponds to the time 4 hours before awakening in diurnal animals such as humans. A surge in plasma Ang II concentration may contribute to the increased onset of cardiovascular events early in the morning. However, care should be taken in extrapolating our results in the SHR to human hypertension. Circadian variation in PRA and PAC in human essential hypertension shows minor differences between that in normotensive subjects (21,22). However, diurnal variations in Ang II have been reported only in normotensive subjects (23). In the present study, diurnal variation of PRA was dissociated from that of Ang II concentration in the SHR. Circadian variation in plasma Ang II concentration in human essential hypertension should be examined to elucidate whether the same large fluctuation as seen in the SHR exists in humans.

The RAS plays pivotal roles not only in blood pressure regulation but also in target organ damage in hypertension (24–27). Many studies have been conducted to examine the differences in the circulating and tissue RAS in the SHR and WKY rats. However, discrepancies exist among these studies in the level of RAS components in the SHR and WKY rats (14,28–36) (Table 3). We have recently found that diurnal mRNA expression patterns of cardiac RAS components differ between SHR and WKY rats, and that most of the discrepancies can be explained by different diurnal expression (14). Our findings of different circadian patterns of plasma Ang II between the anesthetized SHR and WKY rats

Table 3
Previous studies on the different circulating renin-angiotensin system in SHR and WKY rats

	SHR < WKY*	SHR = WKY†	SHR > WKY#
PRA	Ref. 29 (8 weeks) Ref. 30 (6 weeks)	Ref. 32 (11 weeks) Ref. 28, 30, 31(14 week)	
Ang I		Ref. 32 (11 weeks) Ref. 28, 31 (14 weeks) Ref. 35 (12–16 weeks)	
Ang II	Ref. 30 (14 weeks) Ref. 32 (11 weeks)	Ref. 30 (6 weeks) Ref. 28, 30 (14 weeks) Ref. 35 (12–16 weeks)	Ref. 33, 34 (20 weeks)
PAC	Ref. 36 (34–35 weeks)		Ref. 29 (8 weeks) Ref. 32 (11 weeks)

*SHR < WKY means plasma concentration was greater in the WKY rats than in the SHR.
†SHR = WKY means plasma concentration was not statistically different between the SHR and the WKY rats.
#SHR > WKY means plasma concentration was greater in the SHR than in the WKY rats.
The age of rats at the time of examination is indicated in parentheses.
Abbreviations: PRA-plasma renin activity; Ang I-plasma angiotensin I concentration; Ang II-plasma angiotensin II concentration; PAC-plasma aldosterone concentration.

might explain in part the discrepancies between previously published observations. The timing of experiments should be carefully considered when investigating the RAS in *in vivo* experiments. However, contrary to some previous reports, PAC in the WKY rats was not lower than in the SHRs at any time point. Factors other than circadian variation (e.g., differences between substrains of SHR and/or WKY rats) may have caused some of these discrepancies.

Limitation of the Study

The major limitation of our study is that all the blood samples were obtained under pentobarbital anesthesia. Ether anesthesia raises the basal values of plasma renin activity and concentration, but it does not interfere with their circadian rhythms (37). Our data should be carefully interpreted to the condition without anesthesia. Second, we have no data about the effect of other anti-hypertensive agents on the diurnal variations of aortic TGF-beta and p22phox expression and circulating renin-angiotensin system. Thus, the effect of candesartan on the diurnal variations of these factors may be caused by blood pressure lowering rather than AT1 receptor blocking. However, the expression of another Ang II-regulated gene PAI-1 was strongly upregulated at ZT8 in the aorta of SHR like TGF-β1 and p22phox, and its peak was not affected by hydralazine treatment (10). This finding suggests that the surge in circulating Ang II at ZT8 in the SHR could contribute to the peak expression of Ang II-regulated genes at that time independently from the changes in blood pressure. The third limitation is the lack of data about degradation of Ang II. Recently, ACE2, an enzyme that degrades Ang II to Ang(1–7), has been shown to play a critical role in RAS (38). Alteration in Ang II degradation may be the cause of an increase in circulating Ang II concentrations at ZT8. Further studies are necessary.

Conclusion

Diurnal variations in the circulating RAS are present in the SHR and WKY rats. Circulating Ang II is dramatically upregulated during the late light phase in the SHR. In the aorta, expression of Ang II-regulated genes increases at the peak of circulating Ang II. An AT1 receptor-mediated mechanism might cause a surge in plasma Ang II concentration at the late light phase in the SHR. Homologous downregulation by Ang II of AT1a receptor may dampen the effect of surge in plasma Ang II concentrations in the heart of SHR.

Acknowledgement

We thank Noriko Kumon for excellent technical assistance.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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