achieving partial restoration of the physiological nocturnal BP dipping pattern and exerting efficient cardiovascular and renal protection [36]. Although antihypertensive "chronotherapy" has not been formally demonstrated to affect CKD progression, that the anti-proteinuric efficacy of the ARB valsartan or candesartan was associated with an increased day:night BP-level ratio on ambulatory or home BP monitoring induced by evening dosing is noteworthy [37,38].

The proposed strategy of anti-hypertensive therapy in hypertensive patients with CKD is summarized as schema (**Figure 1**). In conclusion, employing anti-hypertensive therapy based on ambulatory BP profile in the management of hypertensive patients with CKD may be effective to slow the progression of renal impairment and suppress the development of cardiovascular disease in these patients. Further clinical studies to confirm of the prognostic value of ambulatory BP profile, particularly ambulatory short-term BP variability, would need to be provided by outcome studies focusing on whether a therapeutic intervention improving ambulatory BP profile such as reducing BP variability also carries additional prognostic benefit, by concomitantly reducing also the rate of renal deterioration and cardiovascular events (**Figure 2**).

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Please address correspondence to: Kouichi Tamura, MD, PhD, Department of Medical Science and Cardiorenal Medicine, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, JAPAN. Tel: +81-45-787-2635; Fax: +81-45-701-3738. E-mail: tamu-kou@med.yokohama-cu.ac.jp

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Prepubertal angiotensin blockade exerts long-term therapeutic effect through sustained ATRAP activation in salt-sensitive hypertensive rats

Toru Dejima[a], Kouichi Tamura[a], Hiromichi Wakui[a], Akinobu Maeda[a], Masato Ohsawa[a], Tomohiko Kanaoka[a], Sona Haku[a], Azushima Kengo[a], Shin-ichiro Masuda[a], Atsu-ichiro Shigenaga[a], Koichi Azuma[a], Miyuki Matsuda[a], Machiko Yabana[a], Tomonori Hirose[b], Kazuaki Uchino[a], Kazuo Kimura[c], Yoji Nagashima[d] and Satoshi Umemura[a]

Objective **We previously showed that the molecule interacting with Ang II type 1 receptor (AT1R), ATRAP, promotes AT1R internalization and attenuates AT1R-mediated pathological responses. In this study we examined whether the regulation of renal ATRAP expression is related to the development of salt-induced hypertension and renal injury as well as to the beneficial effects of AT1R blockade.**

Methods and results **Dahl Iwai salt-sensitive hypertensive and Dahl Iwai salt-resistant rats were divided into six groups for the administration of vehicle or olmesartan either continuously from 3 to 16 weeks, or transiently from weaning to puberty (3–10 weeks), and fed high salt diet from 6 to 16 weeks. In Dahl Iwai salt-sensitive rats, not only continuous, but also prepubertal olmesartan treatment improved hypertension at 15 weeks. Renal ATRAP expression was suppressed in vehicle-treated Dahl Iwai salt-sensitive rats, concomitant with up-regulation of renal oxidative stress, inflammation and fibrosis-related markers such as p22phox, TGF-β, fibronectin, monocyte chemotactic protein-1 and type 1 collagen. However, prepubertal as well as continuous olmesartan treatment recovered the suppressed renal ATRAP expression and inhibited the renal activation of p22phox, TGF-β, fibronectin, MCP-1 and type 1 collagen. In Dahl Iwai salt-resistant rats, such suppression of renal ATRAP expression or induction of renal pathological responses by salt loading was not observed.**

Conclusions **These results indicate that prepubertal transient blockade of AT1R signaling exerts a long-term therapeutic effect on salt-induced hypertension and renal injury in Dahl Iwai salt-sensitive rats, partly through a sustained enhancement of renal ATRAP expression, thereby suggesting ATRAP a novel molecular target in salt-induced hypertension and renal injury.** *J Hypertens* **29:1919–1929**

Journal of Hypertension 2011, 29:1919–1929

Keywords: angiotensin receptors, basic science, gene expression/regulation, hypertension (kidney), oxidative stress (kidney), receptors, salt-sensitive

Abbreviations: AQP1, aquaporin 1; ARB, AT1R-specific blocker; AT1R, Ang II type 1 receptor; AT2R, Ang II type 2 receptor; ATRAP, AT1R-assciated protein; HS, high salt loading; MCP-1, monocyte chemotactic protein-1; NS, normal salt loading; SHR, spontaneously hypertensive rats

[a]Department of Medical Science and Cardiorenal Medicine, [b]Department of Molecular Biology, [d]Department of Molecular Pathology, Yokohama City University Graduate School of Medicine and [c]Division of Cardiology, Yokohama City University Medical Center, Yokohama, Japan

Correspondence to Kouichi Tamura, MD, PhD, FACP, FAHA, Department of Medical Science and Cardiorenal Medicine, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan
Tel: +81 45 787 2635; fax: +81 45 701 3738;
e-mail: tamukou@med.yokohama-cu.ac.jp

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Introduction

Previous epidemiological studies showed that dietary salt intake during the prepubertal period affects the blood pressure profile after adolescence [1,2], and animal studies using salt-sensitive hypertension models, including Dahl Iwai salt-sensitive rats, also showed that high salt intake at a young age promotes the development of hypertension and tissue injury later in life [3–5]. On the contrary, activation of the renal renin–angiotensin system plays a critical role in overall renal pathophysiology via the development of salt-sensitive hypertension and diabetic nephropathy. In Dahl Iwai salt-sensitive rats, high salt loading results in the development of severe hypertension as well as extensive cardiovascular and renal damage. In Dahl Iwai salt-sensitive rats, high salt loading decreases the activity of the circulating renin–angiotensin system, but previous studies have shown significant increases in components of the tissue renin–angiotensin system at local tissue sites such as the brain, heart, vessel wall and kidney [6–8].

The pathophysiological effects of the tissue renin–angiotensin system are mainly mediated by the activation of the Ang II type 1 receptor (AT1R). The carboxyl-terminal portion of AT1R is involved in the control of AT1R internalization independent of G-protein-coupling, and

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plays an important role in linking receptor-mediated signal transduction to specific pathophysiological responses through the promotion of oxidative stress and inflammatory responses at local tissue sites [9–12]. The AT1R-associated protein (ATRAP), which is a molecule specifically interacting with the carboxyl-terminal domain of AT1R, was cloned using a yeast-two hybrid screening system [13,14]. The results of previous in-vitro studies showed that ATRAP suppresses Ang II-mediated pathological responses in cardiovascular cells by promoting AT1R internalization and decreasing the cell surface AT1R number [15,16], thereby suggesting ATRAP is an endogenous inhibitor of AT1R signaling [17,18].

With respect to the tissue distribution and regulation of ATRAP expression *in vivo*, ATRAP is broadly expressed in many tissues [13]. We showed that there is a tissue-specific regulatory balancing of the expression of ATRAP and AT1R during the development of hypertension in spontaneously hypertensive rats (SHRs) [19]. The activation of ATRAP in transgenic-models in which ATRAP expression was increased beyond baseline promoted Ang II-mediated AT1R internalization and inhibited renal angiotensinogen production and cardiac hypertrophy in response to Ang II stimulation [20].

The development of interventional approaches to preventing the excessive activation of the renin–angiotensin system and attenuating oxidative stress, inflammation and fibrosis at local tissue sites is crucial for achieving the ultimate goal of preventing hypertension and renal injury. The feasibility of transient inhibition of the renin–angiotensin system to prevent the development of hypertension in humans was reported in the Trial of Preventing Hypertension (TROPHY) study [21], and the results of animal studies also showed that transient blockade of the renin–angiotensin system during a prepubertal critical period resulted in an attenuation of hypertension and renal injury in genetically hypertensive rats [22,23]. Thus, in this study, we examined renal ATRAP modulation in salt-induced hypertension as well as in the therapeutic effects of the AT1R-specific blocker (ARB) olmesartan on high blood pressure and renal damage, including oxidative stress, inflammation and fibrosis in Dahl Iwai salt-sensitive rats, a representative animal model of human salt-sensitive forms of hypertension and renal injury.

Methods

The study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All of the animal studies were reviewed and approved by the Animal Studies Committee of Yokohama City University.

Materials

Ang II was purchased from Sigma. The ARB olmesartan (RNH6270) was supplied by Daiichi-Sankyo Pharmaceuticals.

Animals and treatments

Male Dahl Iwai salt-sensitive rats (3 weeks of age) were purchased from SLC (Shizuoka), fed a normal salt diet containing 0.3% NaCl (Oriental Yeast Kogyo), and randomly divided into four groups (groups 1–4; $n=6$ per group) for the dietary high salt loading and oral administration of vehicle or olmesartan (RNH6270; Daiichi-Sankyo Pharmaceuticals). Male Dahl Iwai salt-resistant rats (3 weeks of age) were also divided into two groups (groups 5 and 6; $n=6$ per group) for the dietary high salt loading.

Whereas the Dahl Iwai salt-sensitive rats in group 1 and Dahl Iwai salt-resistant rats in group 5 were fed a normal salt diet (0.3% NaCl) and treated with vehicle throughout the experimental period from 3 to 16 weeks of age, Dahl Iwai salt-sensitive rats in groups 2, 3, and 4 and Dahl Iwai salt-resistant rats in group 6 were initially fed a normal salt diet and then fed a high salt diet (8% NaCl) from 6 to 16 weeks. The Dahl Iwai salt-sensitive rats in group 2 were treated with vehicle throughout the experimental period until 16 weeks. The Dahl Iwai salt-sensitive rats in group 3 were transiently treated with olmesartan (8 mg/kg per day) in drinking water during the prepubertal period from 3 to 10 weeks. The Dahl Iwai salt-sensitive rats in group 4 were continuously treated with olmesartan (8 mg/kg per day) in drinking water throughout the experimental period from 3 to 16 weeks. The olmesartan dosage was determined from previous studies [19].

SBP was measured by the tail cuff method (BP-monitor MK-2000; Muromachi Kikai Co) at the age of 3, 7, 11 and 15 weeks [19]. The rats were anesthetized and the tissues were placed into liquid nitrogen at the end of the experimental period (16 weeks of age). Plasma renin activity was measured by radioimmunoassay as described previously [24].

Western blot analysis

The characterization and specificity of the anti-ATRAP antibody and the anti-AT1R antibody (sc-1173; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) were described previously [19,20,25,26]. The anti-p22phox antibody (sc-20781; Santa Cruz Biotechnology Inc.), anti-p47phox antibody (sc-14015; Santa Cruz Biotechnology, Inc.), anti-Rac1 antibody (DAM1522857; Millipore) and anti-Ang II type 2 receptor (AT2R) antibody (sc-9040; Santa Cruz Biotechnology Inc.) were also used in this study. Western blot analysis was performed essentially as described [19,20,25,26].

Immunohistochemical analysis

The 4-μm thick sections were blocked for endogenous biotin activity using peroxidase blocking reagent (DAKO, Tokyo, Japan) and were incubated with either anti-ATRAP antibody diluted at 1 : 100; anti-AT1R antibody diluted at 1 : 100; anti-p22phox antibody diluted at

1 : 100; or antiaquaporin 1 (AQP1) antibody (ab9566, Abcam) diluted at 1 : 100, essentially as described previously [25,27].

Real-time quantitative RT-PCR analysis

Real-time quantitative RT-PCR was performed by incubating the RT product with TaqMan Universal PCR Master Mix and a designed TaqMan probe (Applied Biosystems, Foster City, California, USA), essentially as described previously [20,26,28]. The RNA quantity was expressed relative to the 18S rRNA endogenous control.

Statistical analysis

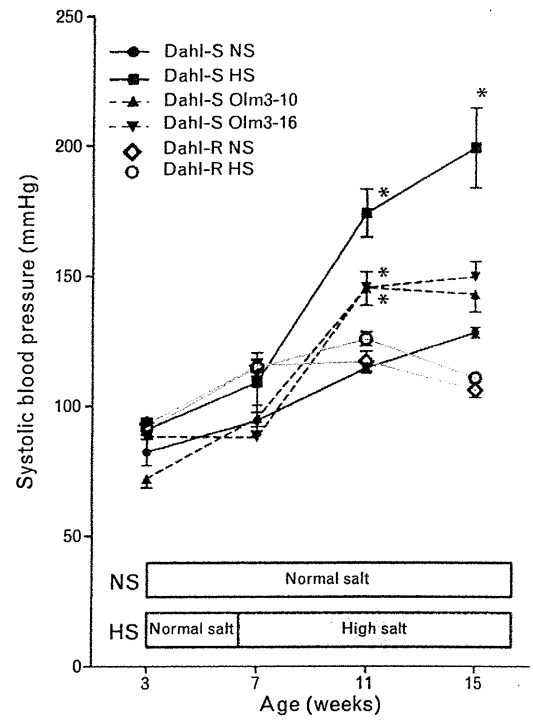
Data are expressed as mean ± SEM. Statistical significance was determined by unpaired Student's *t*-test, with *P* less than 0.05 being deemed statistically significant.

Results

Effects of prepubertal treatment with olmesartan on blood pressure in Dahl Iwai salt-sensitive rats

SBP did not differ among the six groups of Dahl Iwai salt-sensitive and Dahl Iwai salt-resistant rats at 3 weeks of age (Fig. 1). SBP of the Dahl Iwai salt-resistant rats in group 5 (normal salt loading) and group 6 (high salt loading) did not show any significant changes during the experimental period. Also, SBP of the Dahl Iwai salt-sensitive rats in group 1 (normal salt loading) remained normotensive at 15 weeks (128 ± 2 mmHg). In contrast, the group 2 Dahl Iwai salt-sensitive rats (high salt loading) showed a progressive increase in SBP at 11 weeks (174 ± 9 mmHg) and 15 weeks (199 ± 15 mmHg). On the contrary, the Dahl Iwai salt-sensitive rats in group 4 (Olm3-16) exhibited a significant suppression of the increase in SBP at 15 weeks (150 ± 6 mmHg). Furthermore, the group 3 Dahl Iwai salt-sensitive rats (Olm3-10) also exhibited a (comparably) significant suppression of the increase in SBP at 15 weeks (143 ± 7 mmHg).

Fig. 1



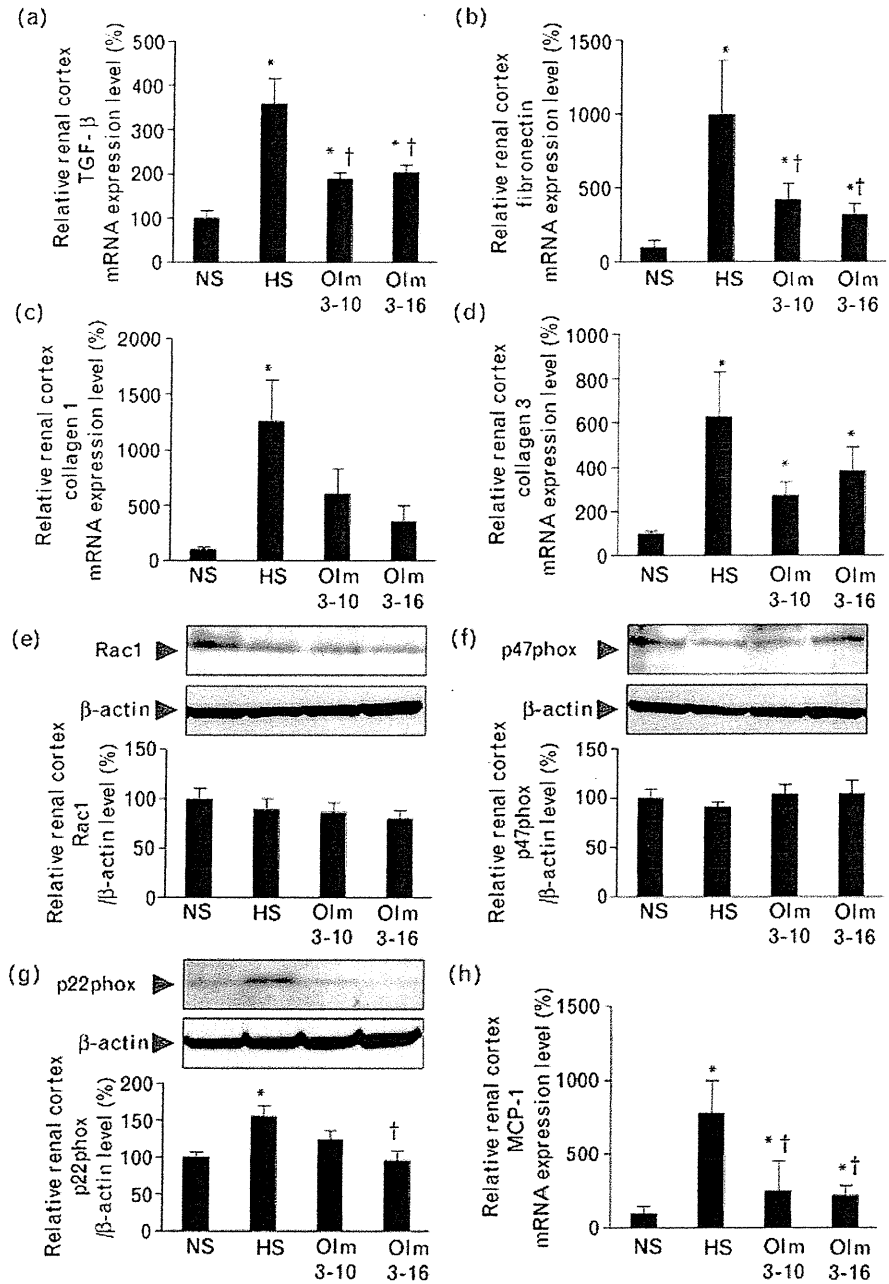
Effects of prepubertal transient olmesartan treatment on salt-induced hypertension in Dahl Iwai salt-sensitive rats. Male DS and DR rats (3 weeks of age) were randomly divided into six groups for dietary high salt loading and oral administration of vehicle or olmesartan. Normal salt loading (NS), DS and DR rats fed a normal salt diet (0.3% NaCl); high salt loading, DS and DR rats fed a high salt diet (8% NaCl) from 6 to 16 weeks and treated with vehicle throughout the experimental period until 16 weeks; Olm 3-10, DS rats fed a high salt diet (8% NaCl) from 6 to 16 weeks and transiently treated with olmesartan (8 mg/kg per day) during the prepubertal period from 3 to 10 weeks; Olm 3-16, DS rats fed a high salt diet (8% NaCl) from 6 to 16 weeks and continuously treated with olmesartan (8 mg/kg per day) throughout the experimental period from 3 to 16 weeks. The values of the SBP measured by the tail cuff method are expressed as the mean ± SE ($n = 6$ in each group). *$P < 0.05$ vs. NS. DR, Dahl Iwai salt-resistant; DS, Dahl Iwai salt-sensitive.

Effects of prepubertal treatment with olmesartan on development of renal oxidative stress, inflammation and fibrosis in Dahl Iwai salt-sensitive rats

Since the salt-induced hypertension was observed only in Dahl Iwai salt-sensitive rats, we next examined effects of high salt loading and olmesartan treatment on the renal pathological responses in Dahl Iwai salt-sensitive rats at 16 weeks. The results of real-time RT-PCR analysis showed that high salt loading increased the renal cortical TGF-β, fibronectin and type 1 and type 3 collagen mRNA, compared with normal salt loading (Fig. 2a–d). Among these upregulated mRNA levels in response to high salt loading, the mRNA expression of TGF-β, fibronectin and type 1 collagen was significantly suppressed by continuous treatment with olmesartan (Olm3-16). Furthermore, prepubertal transient treatment with olmesartan comparably inhibited the up-regulation of TGF-β, fibronectin and type 1 collagen mRNA levels induced by high salt loading (Olm3-10).

In order to investigate the mechanisms involved in the prepubertal olmesartan treatment-mediated suppression of renal damage in response to high salt loading, we analyzed the renal cortical expression of oxidative stress-related proteins, including Rac1, p47phox and p22phox, at 16 weeks (Fig. 2e–g). Although neither high salt loading nor olmesartan treatment affected Rac1 or p47phox expression, high salt loading significantly increased the renal cortical p22phox expression compared with normal salt loading. On the contrary, continuous olmesartan treatment (Olm3-16) completely abolished the high salt loading-mediated increase in

Fig. 2



Effects of prepubertal transient olmesartan treatment on the expression of renal oxidative stress, inflammatory response, and fibrosis-related genes in Dahl Iwai salt-sensitive rats. The effects of high salt loading and continuous (Olm 3-16) or prepubertal (Olm 3-10) treatment with olmesartan on TGF-β (a), fibronectin (b), type 1 collagen (c), type 3 collagen (d) and MCP-1 (h) mRNA expression in the renal cortex of DS rats at 16 weeks are shown. Values are calculated relative to those achieved with extracts in DS rats with normal salt loading (NS) and are expressed as the mean ± SEM ($n = 6$ in each group). *$P < 0.05$, vs. NS, †$P < 0.05$, vs. HS. Representative western blot and quantitative analysis of the effects of high salt loading (HS) and continuous (Olm 3-16) or prepubertal (Olm 3-10) treatment with olmesartan on Rac1 (e), p47phox (f) and p22phox (g) protein expression in the renal cortex of DS rats at 16 weeks are also shown. Values are calculated relative to those achieved with extracts in DS rats fed a NS and are expressed as the mean ± SEM ($n = 6$ in each group). *$P < 0.05$, vs. NS, †$P < 0.05$, vs. HS. DS, Dahl Iwai salt-sensitive; MCP-1, monocyte chemotactic protein-1.

p22phox expression, and prepubertal transient olmesartan treatment (Olm3-10) had a moderate inhibitory effect on the renal cortical p22phox expression (Fig. 2 g). Furthermore, whereas high salt loading significantly increased the renal cortical monocyte chemotactic protein-1 (MCP-1) expression compared to normal salt loading, both continuous olmesartan treatment (Olm3-16) and prepubertal transient olmesartan treatment

(Olm3-10) significantly suppressed the high salt loading-mediated increase in the renal cortical MCP-1 expression (Fig. 2 h).

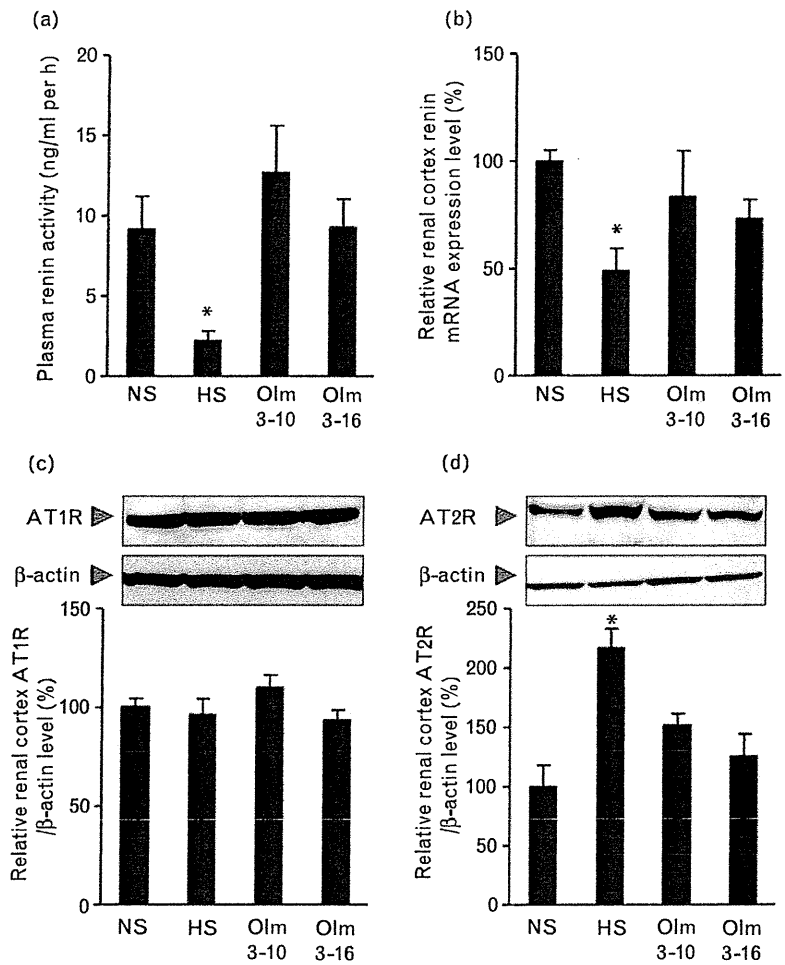
Effects of prepubertal olmesartan treatment on renal expression of renin, AT1R and AT2R in Dahl Iwai salt-sensitive rats

In Dahl Iwai salt-sensitive rats, the high salt loading significantly decreased plasma renin activity and renin mRNA expression at 16 weeks, which was reversed by either continuous (Olm 3-16) or prepubertal transient (Olm3-10) olmesartan treatment (Fig. 3a and b). The results of western blot analysis showed that high salt loading did not affect renal cortex AT1R protein levels, whereas AT2R protein levels were significantly increased by high salt loading and olmesartan treatment suppressed this increase (Fig. 3c and d).

Effects of prepubertal olmesartan treatment on renal expression of ATRAP in Dahl Iwai salt-sensitive rats

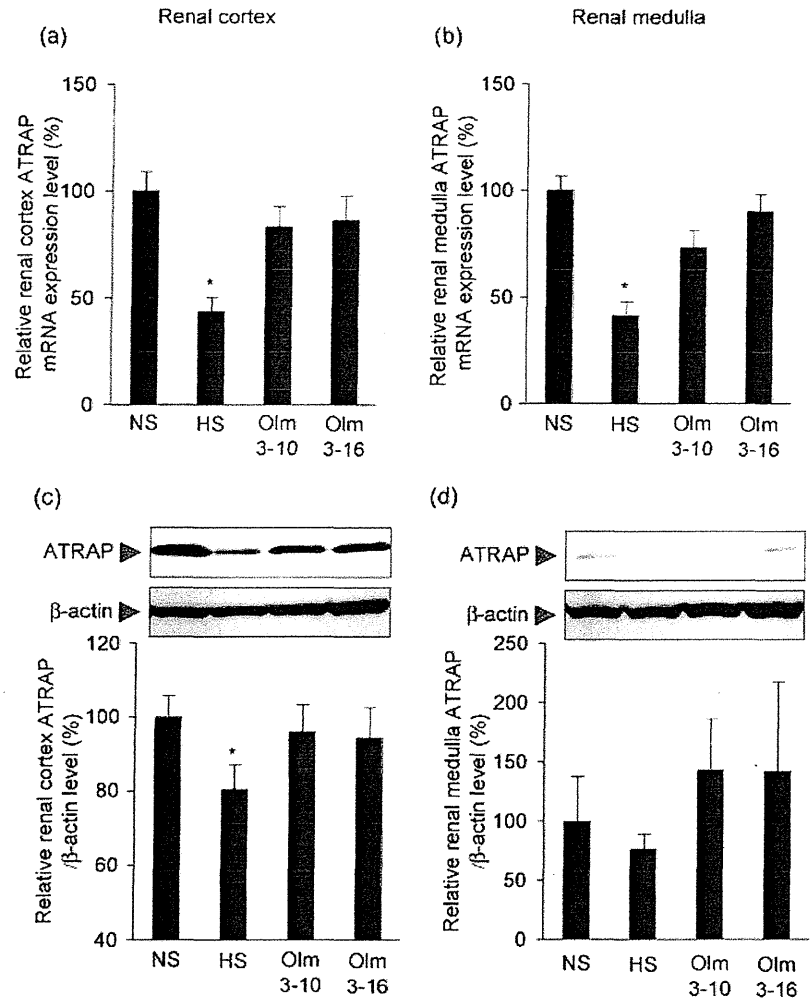
We also examined the effects of high salt loading and olmesartan treatment on endogenous ATRAP gene expression in the kidneys of Dahl Iwai salt-sensitive rats at 16 weeks. The results of real-time RT-PCR analysis showed that high salt loading significantly decreased ATRAP mRNA expression in the renal cortex and medulla, which was reversed by either continuous (Olm 3-16) or prepubertal transient (Olm3-10) olmesartan treatment (Fig. 4a and b). The results of western blot analysis also demonstrated a significant reduction of renal

Fig. 3



Effects of prepubertal transient olmesartan treatment on plasma renin activity and expression of renal renin, AT1R and AT2R expression in Dahl Iwai salt-sensitive rats. The effects of high salt loading (HS) and continuous (Olm3-16) or prepubertal (Olm 3-10) treatment with olmesartan on PRA (a), renal cortex renin mRNA (b) and AT1R (c) and AT2R (d) protein expression in DS rats at 16 weeks are shown. Values are calculated relative to those achieved with extracts in DS rats with normal salt loading (NS) and are expressed as the mean ± SEM ($n = 6$ in each group). *$P < 0.05$, vs. NS. AT1R, Ang II type 1 receptor; DS, Dahl Iwai salt-sensitive; PRA, plasma renin activity.

Fig. 4



Effects of prepubertal transient olmesartan treatment on expression of ATRAP and AT1R in the renal cortex and medulla of Dahl Iwai salt-sensitive rats. The effects of high salt loading (HS) and continuous (Olm 3-16) or prepubertal (Olm 3-10) treatment with olmesartan on ATRAP mRNA levels in the renal cortex (a) and medulla (b) and on ATRAP protein levels in the renal cortex (c) and medulla (d) in DS rats at 16 weeks are shown. Values are calculated relative to those achieved with extracts in DS rats with normal salt loading (NS) and are expressed as the mean ± SEM ($n = 6$ in each group). *$P < 0.05$, vs. NS. AT1R, Ang II type 1 receptor; DS, Dahl Iwai salt-sensitive.

cortical ATRAP protein level, which was comparably recovered to the baseline level under normal salt loading by either continuous (Olm 3-16) or prepubertal transient (Olm3-10) olmesartan treatment (Fig. 4c). On the contrary, a lower level of ATRAP protein expression was detected in the renal medulla and the inhibitory effect of high salt loading on renal medullary ATRAP protein levels did not reach the statistical significance (Fig. 4d).

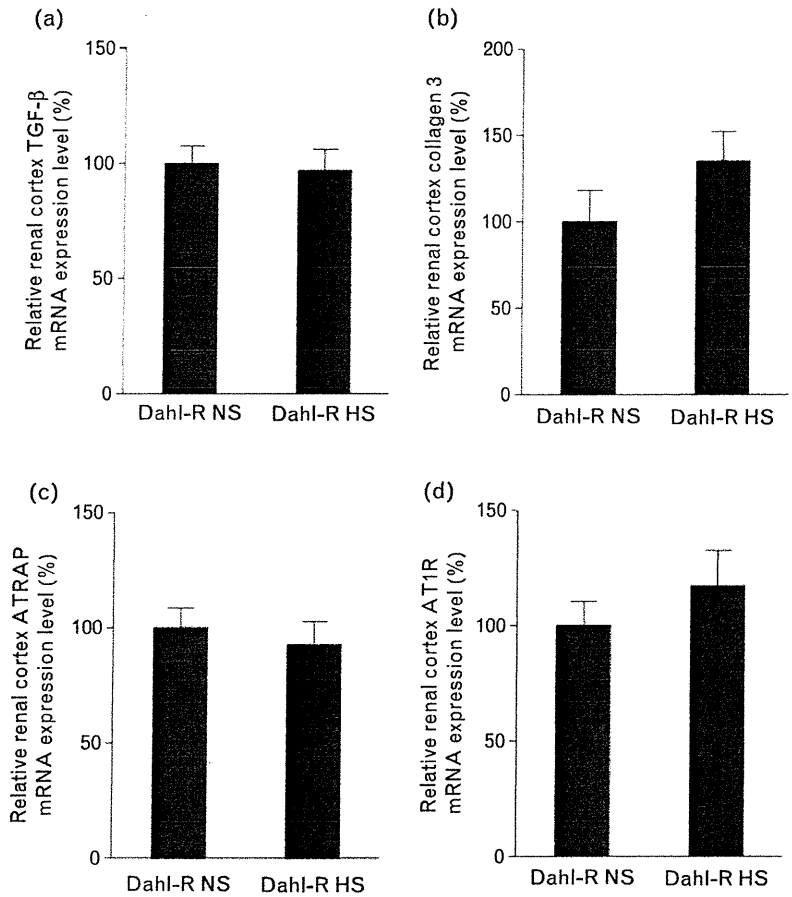
Effects of high salt loading on renal pathological responses and expression of ATRAP and AT1R in Dahl Iwai salt-resistant rats

In control Dahl Iwai salt-resistant rats, the high salt loading exhibited no apparent effects on the mRNA expression of TGF-β, type 3 collagen, ATRAP and AT1R in the renal cortex at 16 weeks (Fig. 5a–d).

Effects of prepubertal olmesartan treatment on intrarenal AT1R and p22phox immunostaining and interstitial fibrosis in Dahl Iwai salt-sensitive rats

The results of immunohistchemical analysis showed that neither high salt loading nor olmesartan treatment (Olm3-10, Olm3-16) affected the intensity or distribution of renal AT1R immunostaining in Dahl Iwai salt-sensitive rats at 16 weeks (Fig. 6). On the contrary, the results showed that high salt loading increased the renal cortical p22phox immunostaining, which was decreased to the same degree as in normal salt loading by either continuous (Olm3-16) or prepubertal transient (Olm 3-10) olmesartan treatment. With respect to the renal fibrotic response, the results of Masson's trichrome staining showed a prominent interstitial fibrosis in the high salt group compared to the normal salt group, which

Fig. 5



Effects of high salt loading on renal pathological responses and expression of ATRAP and AT1R in Dahl Iwai salt-resistant rats. The effects of high salt loading (HS) on TGF-β (a), type 3 collagen (b), ATRAP (c) and AT1R (d) mRNA expression in the renal cortex of DR rats at 16 weeks are shown. Values are calculated relative to those achieved with extracts in DR rats with normal salt loading (NS) and are expressed as the mean ± SEM ($n = 6$ in each group). AT1R, Ang II type 1 receptor; DR, Dahl Iwai salt-resistant.

was attenuated in both the prepubertal olmesartan treatment group (Olm3-10) and continuous olmesartan treatment group (Olm3-16).

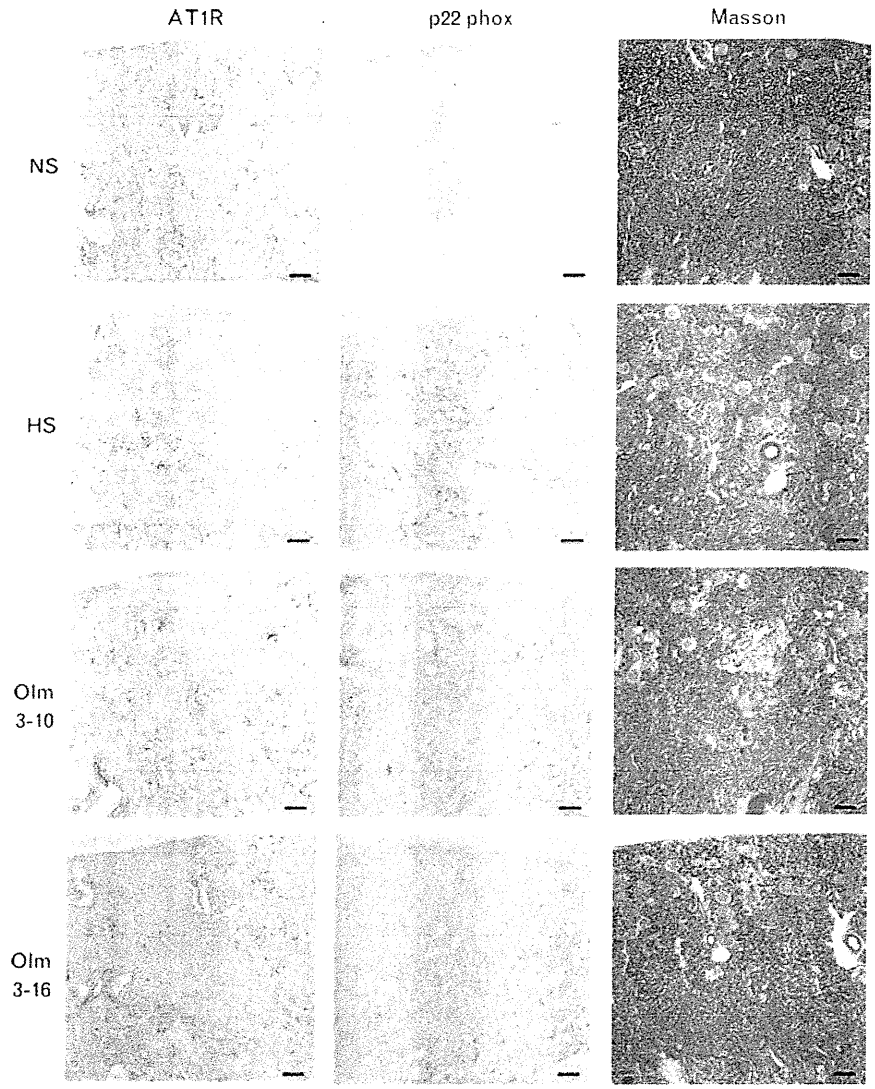
Effects of prepubertal olmesartan treatment on intrarenal distribution of ATRAP immunostaining in Dahl Iwai salt-sensitive rats

With respect to the intrarenal distribution of ATRAP, there was a relatively dominant ATRAP immunostaining in the inner cortex region of the kidney in Dahl Iwai salt-sensitive rats fed a normal salt diet, but ATRAP immunostaining in the inner cortex was decreased in Dahl Iwai salt-sensitive rats fed a high salt diet (Fig. 7a). However, either prepubertal (Olm3-10) or continuous (Olm3-16) olmesartan treatment recovered the suppressed ATRAP immunostaining to degree comparable with the Dahl Iwai salt-sensitive rats fed a normal salt diet. The results of further immunohistochemical analysis using consecutive sections stained for ATRAP and AQP1, which is specifically expressed in the proximal tubule [27], showed that the ATRAP immunostaining sites in the proximal tubules in the inner cortex were involved in the high salt loading-mediated decrease in the kidneys of Dahl Iwai salt-sensitive rats at 16 weeks (Fig. 7b).

Discussion

The main findings of this study are as follows: prepubertal transient treatment with the AT1R-specific blocker olmesartan continued to exert a long-term blood pressure-lowering effect after the treatment, an effect which was comparable with that obtained by continuous olmesartan treatment in Dahl Iwai salt-sensitive rats under high salt loading; prepubertal transient treatment with olmesartan continued to maintain the inhibitory effects on renal oxidative stress and inflammatory and fibrotic responses after the treatment; the long-term therapeutic effects of the prepubertal transient treatment with olmesartan were accompanied by a sustained

Fig. 6

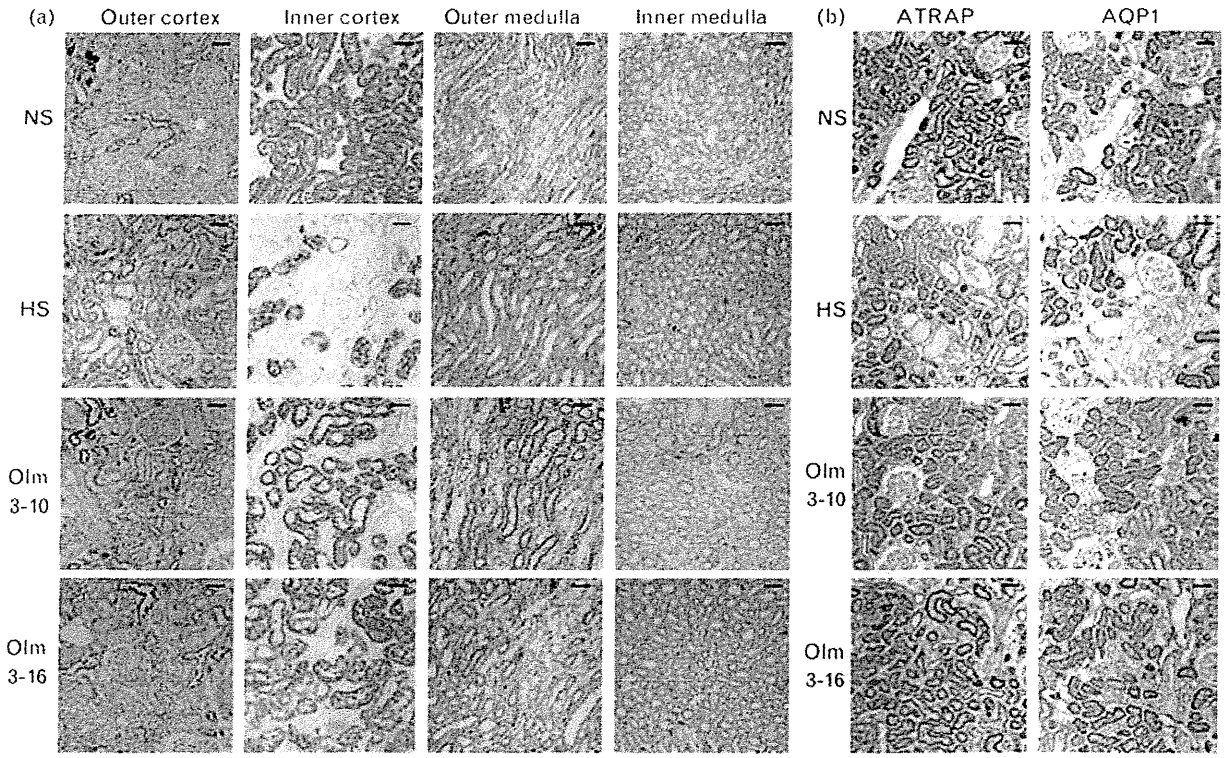


Representative kidney sections showing the effects of prepubertal transient olmesartan treatment on intrarenal AT1R and p22phox immunostaining and interstitial fibrosis in Dahl Iwai salt-sensitive rats. Representative immunohistochemical staining of the effects of high salt loading (HS) and continuous (Olm 3-16) or prepubertal (Olm 3-10) treatment with olmesartan on AT1R and p22phox immunostaining in the renal cortex of DS rats at 16 weeks are shown. The positive areas for AT1R and p22phox are evident as brown dots in the sections. Representative Masson's trichrome staining of the effects of high salt loading and continuous or prepubertal treatment with olmesartan on renal interstitial fibrosis in the renal cortex of DS rats at 16 weeks is also shown. Original magnification: ×40. Bars = 100 μm. AT1R, Ang II type 1 receptor; DS, Dahl Iwai salt-sensitive; NS, normal salt loading.

recovery of repressed renal ATRAP expression in Dahl Iwai salt-sensitive rats under high salt loading.

In the present study, the high salt diet-induced elevation of blood pressure was attenuated by transient administration of olmesartan only during the prepubertal period (from 3 to 10 weeks of age) in Dahl Iwai salt-sensitive rats (Fig. 1), thereby supporting the existence of the 'renin–angiotensin system block memory phenomenon' reported previously [29]. In several types of genetically hypertensive rats, including SHR and Dahl Iwai salt-sensitive rats fed a high salt diet, the blood pressure increases gradually from the age of 3–10 weeks, and subsequently the hypertensive state is established. Thus, the age of 3–10 weeks is called the critical period and corresponds to prehypertension in humans. The activity of the renin–angiotensin system during this critical period is suggested to have a major influence on the pathogenesis of hypertension [29]. Previous studies showed that transient pharmacological blockade of the renin–angiotensin system during the critical period significantly inhibits the vascular hypertrophy of renal arteries and arterioles as well as increases in blood pressure, and thus efficiently blocks this vicious cycle by

Fig. 7



Representative kidney sections showing the effects of prepubertal transient olmesartan treatment on intrarenal localization of ATRAP immunostaining in Dahl Iwai salt-sensitive rats. Representative high magnification of the kidney sections for the effects of high salt loading (HS) and continuous (Olm 3-16) or prepubertal (Olm 3-10) treatment with olmesartan on immunohistochemical localization of ATRAP expression in the outer cortex, inner cortex, outer medulla and inner medulla is shown (a). Consecutive sections also show the immunostaining of ATRAP and aquaporin 1 (AQP1), respectively (b). The positive areas for ATRAP or AQP1 are evident as brown dots in the sections. Original magnification: ×200. Bars = 50 μm. DS, Dahl Iwai salt-sensitive; NS, normal salt loading.

accelerating the 'reno-vascular amplifier' mechanism [29–31]. Furthermore, a persistent lowering of blood pressure and an attenuation of renal pathological changes were achieved by transient ARB treatment of Dahl Iwai salt-sensitive rats fed a high salt diet [23]. In this study, high salt loading caused glomerular sclerosis and vascular hypertrophy, and these pathological changes were attenuated, albeit not completely prevented, in the Dahl Iwai salt-sensitive rats treated either prepubertally or continuously with olmesartan (data not shown).

The increase in renal oxidative stress plays an important role in the pathogenesis of hypertension and the development of renal injury [12,32–35]. Ang II and high salt loading provoke organ injury in various tissues, including blood vessels, glomeruli and renal tubules, through an activation of NADPH oxidase and stimulation of oxidative stress at local tissue sites [9,12,33,36–39]. Previous studies showed that high salt loading-mediated paradoxical activation of the renal renin–angiotensin system, such as an enhancement of the renal angiotensinogen and (pro)renin receptors, in spite of the suppression of the circulating renin–angiotensin system, still contributed to hypertension and renal injury in Dahl Iwai salt-sensitive rats [7,8,40,41]. Because the biological actions of Ang II are influenced by the AT1R expression levels [42], and Ang II infusion in mice specifically lacking renal AT1R failed to develop hypertension [43], investigation of the renal activity of AT1R signaling in Dahl Iwai salt-sensitive rats fed a high salt diet is important in order to elucidate the mechanisms responsible for the salt-sensitive hypertension and renal injury observed in this hypertension model.

We previously cloned ATRAP as a molecule which interacts with AT1R, and showed that ATRAP suppressed the Ang II-induced hypertrophic and proliferative responses of cardiovascular cells by inducing AT1R internalization [17,18]. Thus, a tissue-specific regulatory balancing of ATRAP and AT1R expression may be involved in the modulation of AT1R signaling in each tissue. With respect to salt-induced hypertension, previous studies showed significant increases [8,44] or no evident changes [23,41] in AT1R mRNA and protein levels in response to high salt loading in Dahl Iwai salt-sensitive rats. In this study, whereas AT1R expression

did not appear to be modulated by high salt diet in the kidney of Dahl Iwai salt-sensitive rats, there was a significant decrease in renal ATRAP expression in the Dahl Iwai salt-sensitive rats fed a high salt diet along with the development of hypertension. This suppression of the renal ATRAP expression induced by salt-induced hypertension is AT1R-dependent, as it is prevented in Dahl Iwai salt-sensitive rats treated continuously with olmesartan (Fig. 3). Furthermore, because the high salt diet did not have any influence on renal ATRAP expression in normotensive Dahl Iwai salt-resistant rats in this study and the development of hypertension without high salt dietary regimen did not decrease the renal ATRAP expression in SHR [19], it is suggested that the high salt loading is not sufficient but the AT1R-dependent process during salt-induced hypertension is required for the renal suppression of ATRAP expression.

Interestingly, transient treatment with olmesartan during only the prepubertal period from 3 to 10 weeks of age was found to completely recover the repressed renal ATRAP expression in the Dahl Iwai salt-sensitive rats fed a high salt diet. This sustained effect of prepubertal olmesartan treatment on renal ATRAP expression was accompanied with significant suppression of the high salt-induced up-regulation of renal oxidative stress and inflammatory and fibrotic markers such as p22phox, TGF-β, fibronectin, MCP-1 and type 1 collagen (Fig. 2). Among the components of NADPH oxidase, p22phox is an essential subunit of NADPH oxidase by influencing the activity of this enzyme through the assembly of membrane-bound p22phox [12,45–47].

In the present study, there was a significant decrease in renal ATRAP expression, particularly in the AQP1-positive proximal tubules in the inner cortex, in the Dahl Iwai salt-sensitive rats fed a high salt diet along with the development of hypertension (Fig. 7), and the prepubertal transient AT1R blockade-mediated recovery of the repressed ATRAP expression in the inner cortex region of Dahl Iwai salt-sensitive rats on the high salt loading coincided with the sustained suppression of p22phox expression in the inner cortex region even after the discontinuation of AT1R blockade. Since our previous results showed that adenoviral overexpression of ATRAP suppressed pathological AT1R signaling such as Ang II-mediated TGF-β production in renal tubular cells [27], the results in this study suggest that the sustained recovery of renal tubular ATRAP exerts a long-term inhibitory effect on pathological AT1R signaling to maintain the suppression of p22phox expression in Dahl Iwai salt-sensitive rats on high salt loading.

A limitation of the present study is that this study is mainly an association study and no specific direct proofs are provided regarding the role of ATRAP on the consequence of the high salt loading-mediated process of hypertension in Dahl Iwai salt-sensitive rats and further studies are needed to elucidate the function of renal tubular ATRAP on AT1R signaling under pathological interventions *in vivo*, and these will be taken up in due course. Our previous in-vitro study showed that ATRAP associated specifically with AT1R but not with AT2R [13], and there was a significant increase in renal AT2R expression in the hypertensive Dahl Iwai salt-sensitive rats fed a high salt diet with an opposite direction of change from renal ATRAP expression (Fig. 3). However, previous studies demonstrated that AT2R signaling antagonizes AT1R signaling in the renal tubules and activation of renal tubular AT2R exerted natriuretic responses to reduce blood pressure *in vivo* [48,49]. Thus, although activation of the AT2R pathway is not likely to play a major role in the development of the salt-induced hypertension in Dahl Iwai salt-sensitive rats, it is still possible that ATRAP may function as a novel functional modulator of AT2R signaling *in vivo*.

Because the present study was performed only in Dahl Iwai salt-sensitive and Dahl Iwai salt-resistant rats, it is important to use caution in applying the findings to the pathophysiology of salt-sensitive hypertension and kidney damage in humans. Nevertheless, the findings of the present study may provide important information for further investigation *in vivo* of possible functional roles of ATRAP in the pathogenesis of salt-sensitive hypertension and kidney damage in humans, and suggest the potential benefit of an ATRAP activation strategy. Further studies to elucidate the molecular mechanisms of the antihypertensive and kidney-protecting properties of ATRAP may enable clinical applications of ATRAP in the near future, such as activating ligands for a more efficient inhibition of AT1R signaling in combination with inhibitors of the renin–angiotensin system. In conclusion, the present study shows that prepubertal temporary AT1R blockade attenuated salt-induced hypertension and renal injury, including oxidative stress overproduction, inflammation, and fibrosis, and this in turn was associated the sustained recovery of renal ATRAP expression.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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Effects of Multiple Factorial Intervention on Ambulatory BP Profile and Renal Function in Hypertensive Type 2 Diabetic Patients with Overt Nephropathy – A Pilot Study

Tomohiko Kanaoka,[1] Kouichi Tamura,[1] Tatsumi Moriya,[2] Keiji Tanaka,[2] Yusuke Konno,[3] Satoshi Kondoh,[3] Masao Toyoda,[4] Tomoya Umezono,[4] Tetsuya Fujikawa,[5] Masato Ohsawa,[1] Toru Dejima,[1] Akinobu Maeda,[1] Hiromichi Wakui,[1] Sona Haku,[1] Mai Yanagi,[1] Hiroshi Mitsuhashi,[1] Motoko Ozawa,[1] Yasuko Okano,[1] Nariaki Ogawa,[1] Tadashi Yamakawa,[6] Shunsaku Mizushima,[5] Daisuke Suzuki,[4] Satoshi Umemura[1]

[1]Department of Medical Science and Cardiorenal Medicine, Yokohama City University Graduate School of Medicine, Yokohama, Japan, [2]Department of Endocrinology, Diabetes and Metabolism, Kitasato University School of Medicine, Sagamihara, Japan, [3]Department of Nephrology and Hypertension, St. Marianna University School of Medicine, Kawasaki, Japan, [4]Division of Nephrology and Metabolism, Department of Internal Medicine, Tokai University School of Medicine, Isehara, Japan, [5]Department of Epidemiology and Public Health, Yokohama City University Graduate School of Medicine, Yokohama, Japan, [6]Department of Endocrinology and Diabetes, Yokohama City University Medical Center, Yokohama, Japan

Abstract

Accumulating evidence has shown that diabetic patients are increasing in number, and renal and cardiovascular complications are the most common cause of death in diabetic patients. Thus, it would be of considerable value to identify the mechanisms involved in the progression of renal impairment and cardiovascular injury associated with diabetes. Recent evidence also indicated that multifactorial intervention is able to reduce the risk of cardiovascular disease and death among patients with diabetes and microalbuninuria. In this pilot study, we examined the effects of intensified multifactorial intervention, with tight glucose regulation and the use of valsartan and fluvastatin on ambulatory blood pressure (BP) profile, estimated glomerular filtration rate (eGFR), and urinary albumin to creatinine ratio (UACR), in 20 hypertensive patients (16 male and 4 female) with type 2 diabetes mellitus and overt nephropathy. After 12 months of intensified treatment, office BP, fasting plasma glucose (FPG), and low-density lipoprotein cholesterol (LDLC) were significantly decreased compared to baseline (systolic blood pressure (SBP), 130 ± 2 vs. 150 ± 1 mmHg; diastolic blood pressure (DBP), 76 ± 1 vs. 86 ± 1 mmHg; FPG, 117 ± 5 vs. 153 ± 7 mg/dl; LDLC, 116 ± 8 vs. 162 ± 5 mg/dl, $P < 0.0001$). Also, compared to the baseline values, the daytime and nighttime ambulatory BP and short-term BP variability were significantly decreased after 12 months. Furthermore, while eGFR was not altered (44.3 ± 5.1 vs. 44.3 ± 6.5 ml/min/1.73 m^2, not significant (NS)), UACR showed a significant reduction after 12 months of intensified treatment (1228 ± 355 vs. 2340 ± 381 mg/g-cr, $P < 0.05$). These results suggest that the intensified multifactorial intervention is able to improve ambulatory BP profile, preserve renal function, and reduce urinary albumin excretion in type 2 diabetic hypertensive patients with overt nephropathy.

Keywords: ambulatory blood pressure monitoring, diabetic nephropathy, glomerular filtration rate, hypertension, proteinuria, valsartan, fluvastatin

INTRODUCTION

Cardiovascular complications are the main cause of death in diabetic patients with overt nephropathy (1), and major risk factors for cardiovascular disease in these patients include hypertension, dyslipidemia, albuminuria, and decreased glomerular filtration ratio (GFR) (2–7). A previous study showed that intensified multifactorial intervention, with tight glucose regulation and the use of renin-angiotensin system blockers, aspirin, and lipid-lowering agents, reduce the risk of cardiovascular disease and death among patients with type 2 diabetes mellitus and microalbuminuria (8,9), while the results of several recent studies questioned the beneficial

Address correspondence to Kouichi Tamura, MD, PhD, FACP, FAHA, Department of Medical Science and Cardiorenal Medicine, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004 Japan. E-mail: tamukou@med.yokohama-cu.ac.jp

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effects of intensive control of BP, lipid, or glucose alone on cardiovascular complications (10–13). Therefore, in this pilot study, we examined the possible effects of intensified multiple factorial intervention with tight control of BP, lipid, and glucose on the diurnal BP profile, including the ambulatory short-term BP and HR variability,and renal function, including urinary albumin excretion and estimated GFR (eGFR), in hypertensive type 2 diabetic patients with overt nephropathy.

SUBJECTS AND METHODS

Study Population and Design

The recruitment of the participants of this study was conducted from March 2007 to January 2008 in four specialized university hospitals in Kanagawa prefecture, Japan. This study was a pilot prospective trial consisting of a 4-week observation period and 12-month treatment period. After the observation period, along with the discontinuation of any previous ARB, eligible 20 hypertensive patients with type 2 diabetic nephropathy at Yokohama City University Hospital, Kitasato University Hospital, St. Marianna University Hospital, and Tokai University Hospital were subject to the intensified multifactorial medical therapy.

Inclusion criteria were an age $\geq$ 20 years, a history of type 2 diabetes with the presence of diabetic retinopathy, clinic systolic blood pressure (SBP) $\geq$ 125 mmHg and/or diastolic blood pressure (DBP) $\geq$ 75 mmHg, urinary protein creatinine ratio > 1 g per gram creatinine, and already under dietary nutritional guidance therapy. Urinalysis was performed to eliminate the possibility of other abnormalities, such as hematuria and so on. Exclusion criteria included patients who were receiving an angiotensin-converting enzyme inhibitor, women who were nursing or pregnant, non-diabetic renal disease, clinically significant heart disease, arrhythmia, stroke, renal artery stenosis, hepatic dysfunction, and known hypersensitivity to any component of the study medications.

The intensified multifactorial intervention for tight control of BP, lipid, and glucose was performed to control clinic BP to a level less than 125/75 mmHg, to control low-density lipoprotein (LDL) cholesterol to a level less than 100 mg/dl, and to control HbA1c to a level less than 6.5%. For BP control, the patients were initially given 40 mg of valsartan once daily and a dose of valsartan was titrated up to 160 mg daily as needed. We chose valsartan for a blockade of the renin-angiotensin system, since several previous studies reported that valsartan exerts renoprotective effects in hypertensive patients with type 2 diabetes mellitus and overt nephropathy (14,15). Other anti-hypertensive drugs prescribed in this study were calcium channel blockers, thiazide diuretics, loop diuretics, a blockers, and b blockers. For lipid control, the patients were initially given 10 mg of fluvastatin once daily and a dose of fluvastatin was titrated up to 60 mg daily as needed. We chose fluvastatin as the anti-dyslimidemia drug, since fluvastatin treatment significantly improved lipid parameters in patients with chronic renal disease with good tolerability and without any adverse effects on renal function in our previous study (16). Another anti-dyslipidemia drug was prescribed. For control of glucose, no specified treatment was recommended in this study. The participants were also strictly instructed on dietary control under stable sodium chloride intake (6 g/day) with dietary restrictions of protein (0.7 g/kg of body weight per day), phosphate (0.7 g/day), and potassium (1.5 g/day).

Ambulatory BP monitoring was performed before and 12 months after the start of treatment. Venous blood samples for the measurement of hematologic and biochemical parameters were drawn in the morning after an overnight fast on the same day the ambulatory BP monitoring and measurement of brachial-ankle pulse wave velocity (baPWV) were performed. We calculated eGFR with an application of a revised equation for the Japanese population: eGFR (mL/min/1.73 m^2) = 194 $\times$ serum creatinine$^{-1.094}$ $\times$ age$^{-0.287}$ $\times$ 0.739 (if female) (17). This pilot study was approved by the Ethics Committees of Yokohama City University Hospital, Kitasato University Hospital, St. Marianna University Hospital, and Tokai University Hospital, and written informed consent was obtained from every participant.

Determination of 24-h BP and Short-Term BP and HR Variability by Ambulatory BP Monitoring

Ambulatory BP monitoring was performed at the end of the observation period and each treatment period. The ambulatory BP and heart rate (HR) were monitored every 30 min with a fully automated device (TM-2425, A&D, Tokyo, Japan), essentially as described previously (18). The ambulatory BP monitoring was repeated in patients who had >20% missing values out of the expected number of readings, >30% error rate for the total readings, or missing values for more than two consecutive hours. The following readings were omitted because of technical artifacts: SBP > 250 mmHg or < 70 mmHg, DBP > 130 mmHg or < 30 mmHg, pulse pressure > 160 mmHg or <20 mmHg, systolic differences > 60 mmHg, or diastolic differences > 30 mmHg, compared to the immediately preceding or successive values (19). The patients were instructed to fill out a diary to record the time of sleeping, rising, and other daytime activities. Therefore, the term "day" and "night" hours in the present study reflect the average period during which the subjects were awake/upright and asleep/supine, respectively. Short-term BP variability, which is comprised of coefficients of variations of BP values obtained from ambulatory BP monitoring, is defined as the within-subject SD of all systolic and diastolic readings at 30-min intervals divided by the mean BP during the course of the measurement periods. Heart rate variability, which is comprised of the

coefficients of variation of HR values, is defined as the within-subject SD of all HR values at 30-min intervals divided by the mean HR (20–25).

Brachial-Ankle Pulse Wave Velocity (baPWV)

The baPWV values were determined with a PP analyzer (model: BP-203RPEII; Nihon Colin, Tokyo, Japan). Pulse volume waveforms were recorded with sensors placed over the right brachial artery and both tibial arteries. The baPWV values measured by this method are reported to significantly correlate with the aortic PWV measured by the catheter method (23,24,26).

Statistical Analysis

The quantitative data are expressed as the means ± SEM. For the statistical analysis of difference between groups, analysis of variance followed by Scheffe's *F*-test was used. Paired samples were compared by a paired comparison's *t*-test. A P value < 0.05 was considered statistically significant.

RESULTS

Effects of Multiple Factorial Intervention on Clinical Parameters Including Renal Function and On Ambulatory BP Profile

Baseline characteristics of the participants (male/female = 16/4, mean age = 57.6 ± 2.8 years, mean duration of diabetes = 9.3 ± 1.0 years, no smokers) and effects of multiple factorial therapy on clinical parameters are summarized in Table 1. The multifactorial medical therapy significantly improved the control status of clinic BP, glucose, and LDL-cho, concomitant with a significant reduction of urinary albumin to creatinine ratio (UACR) but without a decrease in eGFR. The multitherapy did not affect oxidative stress markers, high-molecular weight (HMW)-adiponectin and advanced glycation end-products (AGEs), and baPWV.

Table 2 shows the 24-h, daytime, and nighttime ambulatory BP and HR values, and their variability at baseline and after 12 months of multiple medical treatment. The multifactorial therapy significantly decreased all of the values of the 24-h, daytime, and nighttime ambulatory BP after the 12-month treatment. With respect to short-term BP variability, the multitherapy significantly decreased the values of the 24-h, daytime, and nighttime BP variability, other than nighttime DBP variability. On the other hand, HR variability was not affected as a whole.

Comparison of Effects of Multiple Factorial Intervention on Clinical Parameters Including Renal Function and On Ambulatory BP Profile Between Responders and Non-Responders

Subsequently, the patients were classified into two groups according to the changes in the eGFR at the 12 months after the start of treatment. The two groups were patients with improved eGFR (responders, n = 8; male/female = 5/3, mean age = 56.3 ± 3.9 years) and patients with worsened eGFR (non-responders, n = 12; (male/female =11/1, mean age = 58.4 ± 3.9 years). The baseline characteristics in each group are shown in Table 3. There was no significant difference between the two groups in clinic BP, HR, or parameters of glucose, lipid, oxidative stress, or renal and vascular functions. In the responders, the eGFR in the responders was significantly more preserved than that in the non-responders at the 12 months (59.0 ± 9.1 ml/min/1.73 m^2 vs. 32.4 ± 7.9 ml/min/1.73 m^2, $P = 0.043$). Also, in the responders, the UACR as well as the AGEs were significantly improved in the 12 months compared to baseline (from 2053 ± 552 mg/g-cr to 482 ± 157 mg/g-cr, $P = 0.016$; from 3.7 ± 0.5 mU/ml to 1.9 ± 0.3 mU/ml, $P = 0.010$) and tended to be lower than that in the non-responders (482 ± 157 mg/g-cr vs. 1726 ± 544 mg/g-cr, $P = 0.086$; 1.9 ± 0.3 mU/ml vs. 4.7 mU/ml, $P = 0.068$).

Table 1. Baseline patient characteristics and effects of multiple factorial medical therapy on clinical parameters

	Baseline ($N = 20$)	12 Months ($N = 20$)	P
BMI, kg/m^2	26.7 ± 1.1	26.1 ± 1.1	NS
Clinic BP:			
SBP, mmHg	150 ± 1	130 ± 2	$P < 0.0001$
DBP, mmHg	86 ± 1	76 ± 1	$P < 0.0001$
HR, beats/min	77 ± 1	75 ± 1	$P = 0.2631$
Glucose:			
FPG, mg/dl	153 ± 7	117 ± 5	$P < 0.0001$
HbA1c, %	6.7 ± 0.3	6.0 ± 0.2	$P = 0.0547$
Lipid:			
LDL cholesterol, mg/dl	162 ± 5	116 ± 8	$P < 0.0001$
Oxidative stress:			
HMW-adiponectin, μg/ml	7.7 ± 1.8	9.7 ± 2.1	NS
AGEs, mU/ml	3.3 ± 0.3	3.6 ± 0.8	NS
Renal function:			
UACR, mg/g-creatinine	2340 ± 381	1228 ± 355	$P = 0.0395$
eGFR, ml/min/1.73 m2	44.3 ± 5.1	43.1 ± 6.5	NS
Vascular function:			
baPWV, cm/sec	1818 ± 72	1710 ± 68	NS

Table 2. Effects of multiple factorial medical therapy on ambulatory BP profile

	Baseline ($N = 20$)	12 Months ($N = 20$)	P
24-h:			
SBP, mmHg	152 ± 2	138 ± 3	$P = 0.0003$
DBP, mmHg	86 ± 2	79 ± 2	$P = 0.0273$
HR, beats/min	72 ± 2	69 ± 2	NS
SBP variability, %	13.4 ± 0.6	11.4 ± 0.4	$P = 0.0051$
DBP variability, %	14.7 ± 0.8	12.0 ± 0.5	$P = 0.0065$
HR variability, %	14.4 ± 1.0	13.9 ± 1.2	NS
Daytime:			
SBP, mmHg	154 ± 3	141 ± 3	$P = 0.0013$
DBP, mmHg	88 ± 2	81 ± 2	$P = 0.0428$
HR, beats/min	74 ± 2	72 ± 3	NS
SBP variability, %	12.7 ± 0.6	10.9 ± 0.5	$P = 0.0205$
DBP variability, %	13.7 ± 0.7	10.7 ± 0.5	$P = 0.0014$
HR variability, %	13.7 ± 1.1	13.7 ± 1.3	NS
Nighttime:			
SBP, mmHg	146 ± 4	130 ± 3	$P = 0.0024$
DBP, mmHg	80 ± 2	72 ± 2	$P = 0.0371$
HR, beats/min	72 ± 2	69 ± 2	NS
SBP variability, %	11.1 ± 0.7	9.3 ± 0.5	$P = 0.0370$
DBP variability, %	11.5 ± 0.8	10.4 ± 0.6	NS
HR variability, %	9.8 ± 1.3	8.4 ± 0.7	NS

Table 3. Comparison of effects of multiple factorial intervention on clinic BP and parameters of glucose, lipid, oxidative stress, and renal and vascular functions

	Baseline		12 Months	
	Responder ($N = 8$)	Nonresponder ($N = 12$)	Responder ($N = 8$)	Nonresponder ($N = 12$)
BMI, kg/m^2	28.3 ± 2.0	25.6 ± 1.3	27.7 ± 1.9	25.0 ± 1.2
Clinic BP:				
SBP, mmHg	148 ± 2	151 ± 2	126 ± 1*	132 ± 4*
DBP, mmHg	85 ± 1	86 ± 1	76 ± 1*	76 ± 2*
HR, beats/min	76 ± 2	77 ± 1	73 ± 2	76 ± 2
Glucose:				
FPG, mg/dl	160 ± 8	149 ± 10	121 ± 9*	114 ± 6*
HbA1c, %	6.8 ± 0.4	6.7 ± 0.4	6.0 ± 0.3	6.1 ± 0.3
Lipid:				
LDL cholesterol, mg/dl	151 ± 9	168 ± 6	112 ± 8*	119 ± 12*
Oxidative stress:				
HMW-adiponectin, mg/ml	5.4 ± 1.1	9.2 ± 2.9	7.4 ± 1.4	11.3 ± 3.3
AGEs, mU/ml	3.7 ± 0.5	3.1 ± 0.2	1.9 ± 0.3*	4.7 ± 1.1
Renal function:				
UACR, mg/g-cr	2053 ± 552	2531 ± 530	482 ± 157*	1726 ± 544
eGFR, ml/min/1.73m^2	49.8 ± 7.3	40.6 ± 7.1	59.0 ± 9.1†	32.4 ± 7.9
Vascular function:				
baPWV, cm/sec	1824 ± 115	1814 ± 96	1617 ± 87	1773 ± 95*

*$P < 0.05$, 12 months vs. baseline; †$P < 0.05$, responder vs. nonresponder.

On the other hand, none of eGFRs, UACRs, or AGEs showed any statistically significant change during at the 12 months in the non-responders.

With respect to anti-hypertensive medication, the average dose of valsartan was 140.0 ± 13.1 mg daily in the responders and 146.7 ± 9.0 mg daily in the non-responders after 12 months of treatment, without statistical significance. Although more patients in the responders were prescribed a blocker than those in the non-responders at baseline and after 12 months of treatment (Table 4), there were no significant differences in other anti-hypertensive drugs between the groups (Table 4).

Table 5 shows the 24-h, daytime, and nighttime ambulatory BP and HR values, and their variability at baseline and after 12 months of treatment with the intensified multifactorial medical therapy. At baseline, the values of 24-h DBP variability and 24-h and daytime HR variability in the responders were significantly greater than those in non-responders. With respect to the effects of multifactorial medical therapy on ambulatory BP values, there were comparable decreases in the values of 24-h, daytime, and nighttime SBP after the 12-month treatment in both responders and non-responders. However, regarding short-term BP variability, the multifactorial therapy significantly

Table 4. Comparison of anti-hypertensive medication at baseline and after 12 months treatment with multiple factorial intervention between responder and nonresponder

	Baseline		12 months	
	Responder ($N = 8$)	Nonresponder ($N = 12$)	Responder ($N = 8$)	Nonresponder ($N = 12$)
Calcium channel blockers	4/8	9/12	4/8	10/12
Thiazide diuretics	4/8	4/12	4/8	4/12
Loop diuretics	0/8	2/12	0/8	2/12
α blockers	3/8*	0/12	3/8 *	0/12
β blockers	2/8	1/12	2/8	1/12

*$P < 0.05$, responder vs. nonresponder.

Table 5. Comparison of effects of multifactorial therapy on ambulatory BP profile between responders and nonresponders

	Baseline		12 Months	
	Responder ($N = 8$)	Nonresponder ($N = 12$)	Responder ($N = 8$)	Nonresponder ($N = 12$)
24-h:				
SBP, mmHg	149 ± 3	154 ± 4	135 ± 3*	140 ± 4*
DBP, mmHg	83 ± 3	87 ± 3	76 ± 3	80 ± 3
HR, beats/min	70 ± 4	73 ± 3	64 ± 3	73 ± 3
SBP variability, %	14.5 ± 0.8	12.8 ± 0.8	10.8 ± 0.5*	11.7 ± 0.6
DBP variability, %	16.8 ± 1.4†	13.3 ± 0.7	12.4 ± 0.7*	11.8 ± 0.6
HR variability, %	17.3 ± 0.9†	12.3 ± 1.2	15.4 ± 2.1	12.9 ± 1.6
Daytime:				
SBP, mmHg	152 ± 3	155 ± 4	139 ± 4*	142 ± 4*
DBP, mmHg	86 ± 3	89 ± 3	79 ± 4	83 ± 3
HR, beats/min	73 ± 4	75 ± 3	66 ± 3	75 ± 3
SBP variability, %	13.6 ± 0.5	12.1 ± 0.9	10.2 ± 0.6*	11.3 ± 0.6
DBP variability, %	15.1 ± 1.0	12.7 ± 0.9	10.8 ± 0.6*	10.6 ± 0.7
HR variability, %	16.4 ± 1.3†	11.9 ± 1.4	15.7 ± 1.9	12.5 ± 1.6
Nighttime:				
SBP, mmHg	141 ± 4	149 ± 6	127 ± 3*	132 ± 5*
DBP, mmHg	76 ± 5	82 ± 3	70 ± 4	74 ± 3
HR, beats/min	63 ± 4	68 ± 3	59 ± 3	68 ± 4
SBP variability, %	12.4 ± 1.3	10.2 ± 0.6	7.5 ± 0.6*†	10.4 ± 0.4
DBP variability, %	12.9 ± 1.0	10.6 ± 1.1	9.5 ± 0.9*	11.0 ± 0.8
HR variability, %	12.8 ± 2.3	7.8 ± 1.3	9.0 ± 1.6	8.0 ± 0.7

*$P < 0.05$, 12 months vs. baseline; †$P < 0.05$, responder vs. nonresponder.

decreased the values of 24-h, daytime, and nighttime SBP/DBP variability and the nighttime SBP variability in the responders was significantly lower than that in the non-responders after the 12-month treatment. On the other hand, the values of short-term BP variability were not affected in the non-responders after the multifactorial therapy for 12 months. Overall, the multifactorial therapy caused significantly greater improvement in short-term BP variability in the responders than in the non-responders on ambulatory BP monitoring.

Multiple Regression Analysis for Determination of the Factors Contributing to the eGFR and UACR

Finally, to determine the factors contributing to the eGFR and UACR, we performed the multivariate stepwise linear regression analysis. As shown in Table 6, the results of multivariate stepwise linear regression analysis indicated significant associations between eGFR and BMI, nighttime SBP variability, nighttime DBP, and daytime HR variability. The results also indicated

Table 6. Multiple stepwise linear regression analysis of eGFR and UACR

Variables	Coefficient	*P*-value
eGFR, ml/min/1.73m^2:		
BMI, kg/m^2	0.334	0.0030
Nighttime SBP variability, %	−0.388	0.0016
Nighttime DBP, mmHg	−0.327	0.0254
Daytime HR variability, %	0.331	0.0288
($R^2 = 0.616$, $P < 0.0001$)		
UACR, mg/g-creatinine:		
Daytime SBP, mmHg	0.331	0.0003
AGEs, mU/ml	0.354	0.0013
HMW-adiponectin, μg/ml	0.419	0.0106
LDL cholesterol, mg/dl	0.276	0.0315
($R^2 = 0.675$, $P < 0.0001$)		

The independent variables entered in the model for multivariate stepwise regression analysis are as follows; age, BMI, FPG, HbA1c, LDL cholesterol, HMW-adiponectin, AGEs, baPWV, and parameters of ambulatory BP monitoring including systolic/diastolic BP, BP variability, HR, and HR variability during the daytime and nighttime period. The variables which showed P-value of 0.05 or more in the multivariate relations were excluded from the table.

significant relationships between UACR and daytime SBP, AGEs, HMW-adiponectin, and LDL cholesterol.

DISCUSSION

Ambulatory BP monitoring allows the acquisition of valuable information on not only the average 24-h BP, but also the variations in the BP values that happen during the course of daily life. Among the information obtained by ambulatory BP monitoring, previous studies have shown that BP variability is a complex phenomenon that involves both short- and long-lasting changes (27). Thus, the 24-h BP varies not only because of a reduction in BP during nighttime sleep and an increase in the morning, but also because of sudden, quick, and short-lasting changes that occur both during the daytime and, to a lesser extent, at nighttime. This phenomenon, short-term BP variability, has been shown to depend on sympathetic vascular modulation and on atherosclerotic vascular changes (18,21,28,29). Several previous animal studies showed that exaggerated short-term BP variability without significant changes in mean BP induced chronic cardiovascular inflammation and remodeling (30,31). Short-term BP variability is also suggested to be clinically relevant by the fact that hypertensive patients with similar 24-h mean BP values exhibit more severe organ damage when the short-term BP variability is greater (18,21,23–25,29, 32–35).

The circadian pattern of BP in patients with diabetes has been found to exhibit a blunted nocturnal decrease in BP, which is associated with autonomic neuropathy and nephropathy (36,37). The loss of nocturnal BP dipping has been considered to be a risk factor for the progression of nephropathy and to be of prognostic value with respect to target organ damage and cardiovascular morbidity in both diabetic and hypertensive patients (38–42). However, the nighttime SBP/DBP values were similar in the responders and non-responders in this study, thereby indicating that nocturnal BP level was not critically involved in the response of eGFR to the intensified multifactorial medical treatment.

In the present study, the multifactorial therapy caused significantly greater improvement in short-term BP variability in the responders than in the non-responders, concomitant with a preferential reduction of plasma AGEs concentration and urinary albumin excretion in the responders. With respect to the pathologic significance of short-term BP variability in diabetics, a previous study showed nighttime short-term BP variability to be related to cardiovascular events in type 2 diabetic patients (43), and thus short-term BP variability may take part in the progression of diabetic nephropathy and associated cardiovascular complications.

Accumulated evidence has also shown that diabetes and renal dysfunction are associated with persistent oxidative and carbonyl stress as well as inflammation (44,45). AGEs are made up of a protein carbonyl compound, which is produced by protein-reactive oxygen species interaction, and the elevation of oxidative/carbonyl stress end products, including AGE, is likely to be at least partly responsible for the increased cardiovascular disease in diabetic patients (46,47). Furthermore, the recent results of ACCOMPLISH study and meta-analysis of several large-scale cohort studies showed that preservation of eGFR concomitant with reduction of albuminuria is important for the suppression of cardiovascular complications in patients with chronic kidney disease (48–50). The baseline comparison of ambulatory BP profile revealed that the multitherapy responders had higher HR variability than non-responders. The results of multivariate regression analysis also showed a significant positive association between eGFR and daytime HR variability during the intensified multitherapy in the present study. Lower HR variability occurs commonly in diabetic patients due to cardiac autonomic neuropathy and is associated with increased cardiovascular complications and mortality in diabetic patients (51).

There are several limitations of the present study. Firstly, this study was performed as a pilot study and a limitation of the study is the lack of control group. As another limitation of this study, the results of ambulatory BP monitoring may be affected by seasonal changes. Ambulatory BP levels and short-term BP variability are reported to be affected by the seasonal changes in environmental temperature (52). Since the recordings of ambulatory BP monitoring at baseline were performed from March 2007 to January 2008, this seasonal variation should be considered. However, with respect to the effects of the intensified multifactorial medical therapy on ambulatory BP profile, the seasonal effect is not likely to play a major role in each participant, because the recordings of ambulatory BP monitoring were performed at baseline and after the 12-month treatment in the same season.

Also, it is possible that diurnal BP profile including BP variability obtained by ambulatory BP monitoring may vary every measurement and we did not show the inter- and intra-variability of the methods of ambulatory BP monitoring employed in this study. The ambulatory BP and HR were monitored every 30 min with the fully automated device (TM-2425) and a recent study examined the reproducibility of ambulatory BP levels and BP variability monitored every 30 min with the same device (TM-2425) in hypertensive patients (53). The results showed good reproducibility of ambulatory BP levels and BP variability, thereby suggesting that each single ambulatory BP monitoring, before and after the treatment, is acceptable for the assessment of drug efficacy (53). Finally, although the ambulatory BP and HR were monitored every 30 min but not every 15 min in this study, a previous study showed that 15-min