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Figure S1

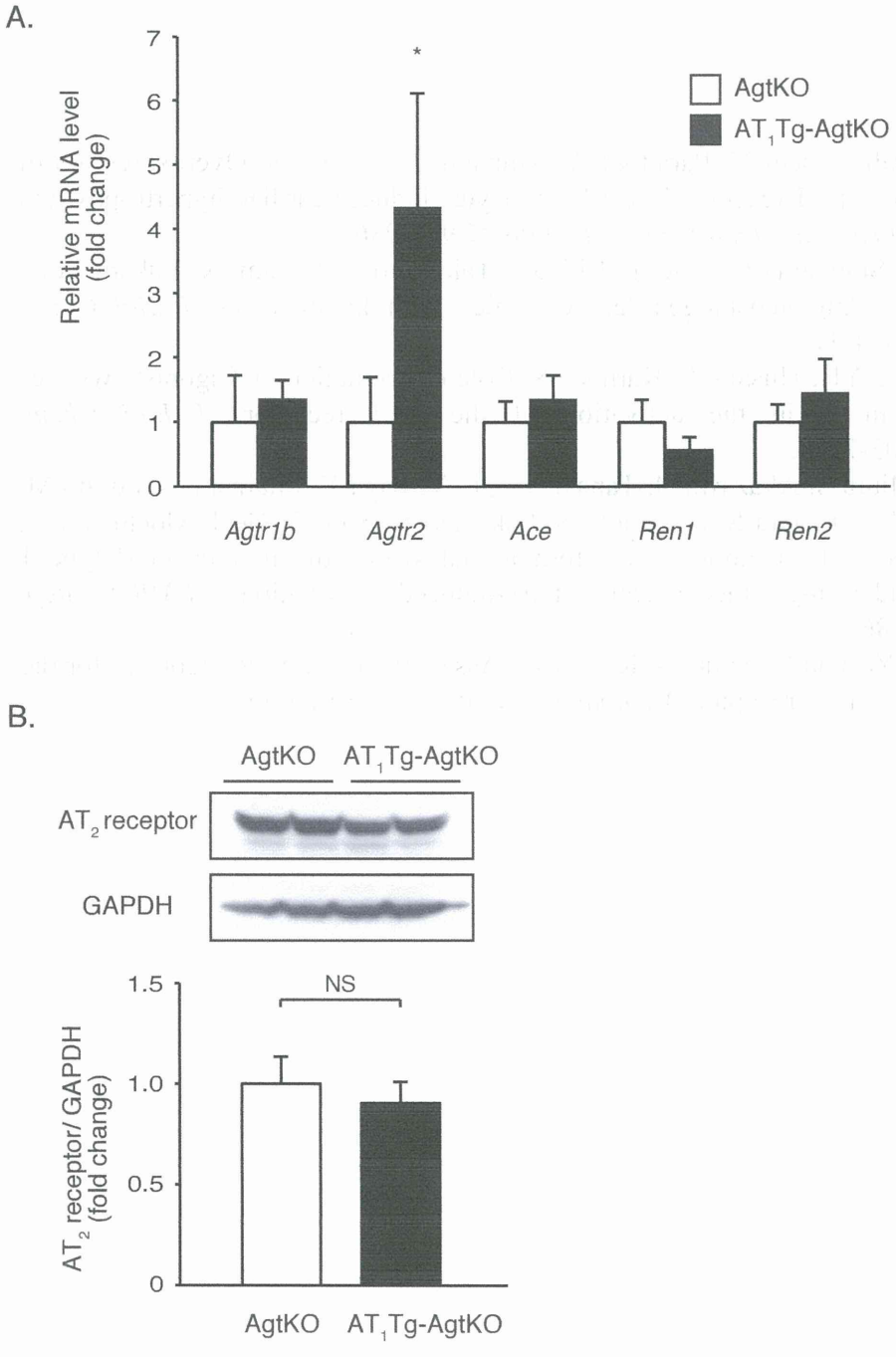

Figure S1. Expression levels of the renin-angiotensin system components in AT_1Tg-Agt KO and AgtKO hearts. (A) The mRNA expressions of the renin-angiotensin system components in AT_1Tg-Agt KO ($n = 6$) and AgtKO hearts ($n = 6$) at 20 weeks of age. Data are presented as mean ± SEM. * $P < 0.05$ versus AgtKO mice. (B) Immunoblot analysis of AT_2 receptor in AgtKO ($n = 4$) and AT_1Tg-AgtKO ($n = 4$) hearts at 20 weeks of age. GAPDH was used as an internal control for loading. The quantitation of the AT_2 receptor /GAPDH is shown as a bar graph. Data are presented as mean ± SEM. NS, not significant ($P > 0.05$).

Figure S2

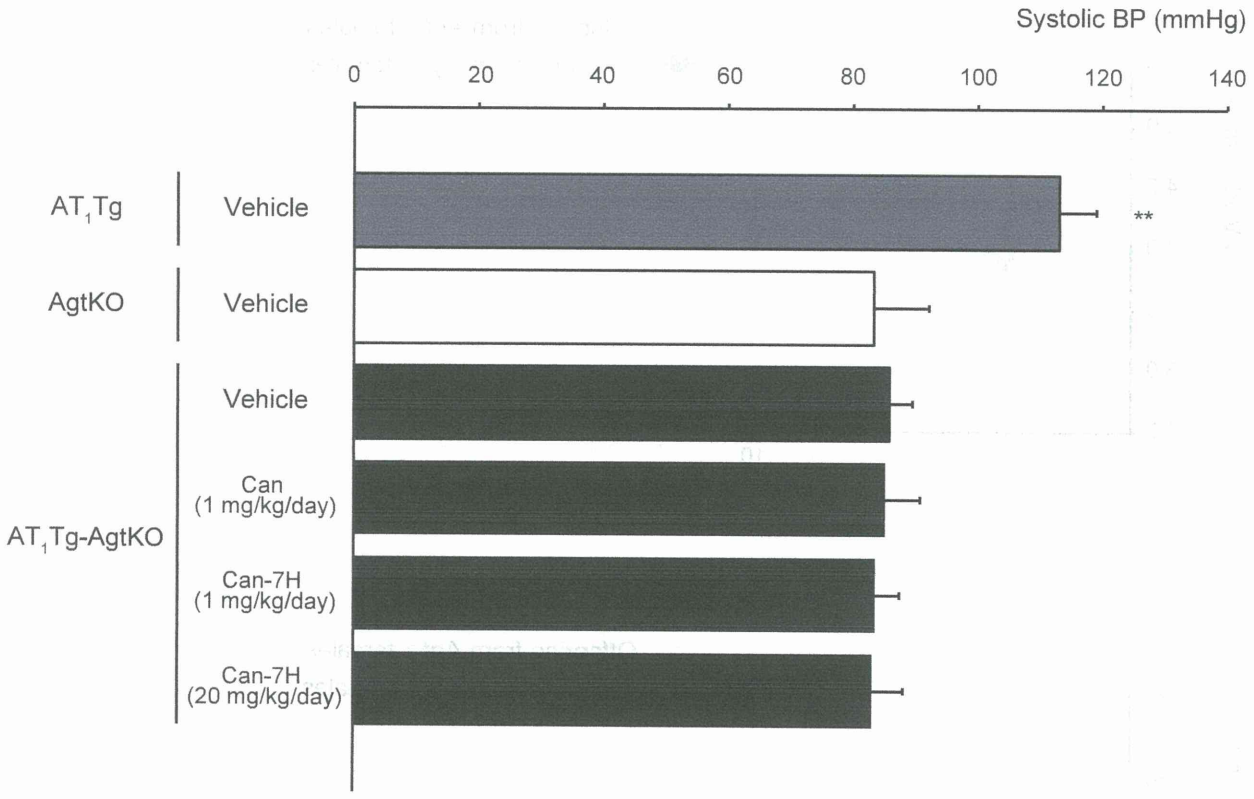

Figure S2. Systolic BP in AT_1Tg mice treated with vehicle ($n = 9$), AgtKO mice treated with vehicle ($n = 6$), AT_1Tg-AgtKO mice treated with vehicle ($n = 6$), candesartan cilexetil (Can) (1 mg/kg/day, $n = 8$) or candesartan-7H (Can-7H) (1 mg/kg/day, $n = 5$ or 20 mg/kg/day, $n = 5$). BP was measured in 20-week-old mice after the treatment for 14 weeks. Data are presented as mean ± SEM. $^{**}P < 0.01$ versus AgtKO mice.

Figure S3

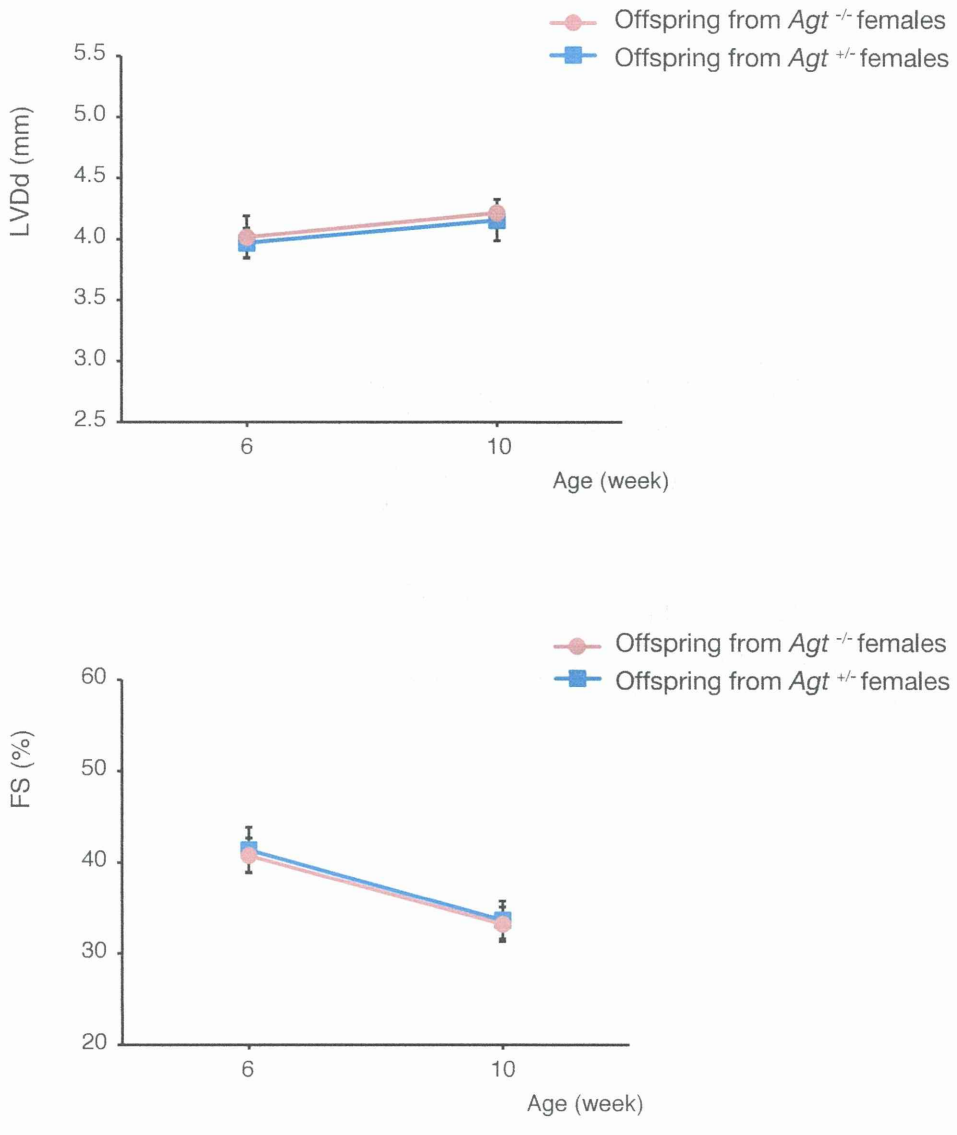

Figure S3. AT_1Tg-AgtKO mice developed cardiac remodeling independently of the effects of maternal or placental angiotensinogen during the fetal period. Left ventricular end-diastolic dimension (LVDd) and fractional shortening (FS) of AT_1Tg-AgtKO offspring of *Agt* $^{+/-}$ females ($n = 4$) or *Agt* $^{-/-}$ females ($n = 4$), measured by echocardiogram at 6 and 10 weeks of age. Data are presented as mean ± SEM.

Figure S4

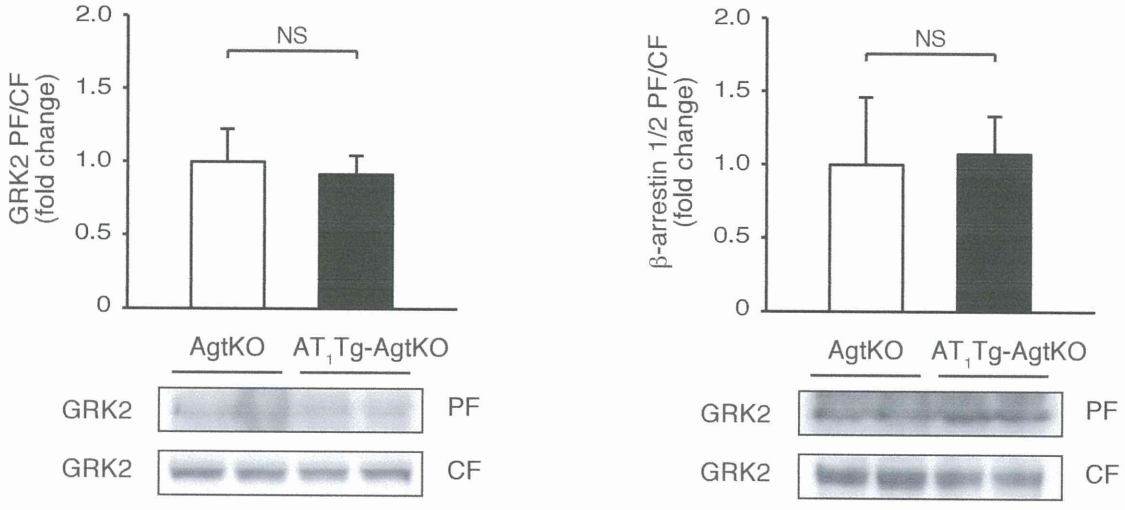

Figure S4. Immunoblot analysis of GRK2 and β-arrestin 1/2 in particulate fraction (PF) and cytosolic fraction (CF) extracted from AgtKO ($n = 4$) and AT_1Tg-Agt KO ($n = 4$) hearts. The quantitation of GRK2 in PF/CF and β-arrestin 1/2 in PF/CF is shown as bar graphs. Data are presented as mean ± SEM. NS, not significant ($P > 0.05$).

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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

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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

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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) $= 194 \times \text{serum creatinine}^{-1.094} \times \text{age}^{-0.287} \times 0.739$ (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

interval measurements are needed to evaluate sudden, quick, and short-lasting BP changes comparable with continuous intra-arterial monitoring (54). On the other hand, another previous study compared the different measurement intervals of ambulatory BP monitoring and showed that a minimum sampling frequency of two readings per hour (i.e., a 30-min interval) is required to obtain an accurate assessment of short-term BP variability (55). Therefore, we believe that the method of one measurement for every 30 min in the present study can ensure a good assessment of ambulatory short-term BP variability (53).

In conclusion, the results of the present study showed that the intensified multifactorial medical therapy, including ARB and statin, improved ambulatory mean BP and short-term BP variability concomitant with the reduction of albuminuria without a decrease in eGFR in hypertensive patients with type 2 diabetic nephropathy. Particularly, the results of multivariate regression analysis showed a significant negative association between eGFR and nighttime systolic BP variability, in addition to that between eGFR and nighttime diastolic BP during the intensified multitherapy in the present study. Also, a recent subanalysis of ASCOT-BPLA study suggested that the better inhibitory effects of calcium channel blockers on ambulatory BP variability than b blockers account for the superiority of calcium channel blockers over b blockers in the suppression of cardiovascular events, irrespective of no significant difference in mean BP levels (34). Accumulated evidence already indicated that it is critically important to achieve the target BP goal to efficiently inhibit organ damages including diabetic nephropathy. As a next step, more and better evidence on the intensified multifactorial medical therapy-mediated beneficial effects on renal function and ambulatory BP profile including short-term BP variability could help to lead to a more precise understanding of the pathogenesis, and hence potential treatment, of diabetic nephropathy and its cardiovascular complications (56–58).

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Involvement of Runx3 in the basal transcriptional activation of the mouse angiotensin II type 1 receptor-associated protein gene

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Involvement of Runx3 in the basal transcriptional activation of the mouse angiotensin II type 1 receptor-associated protein gene

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Departments of [1]Medical Science and Cardiorenal Medicine, [3]Molecular Biology, and [4]Molecular Pathology, Yokohama City University Graduate School of Medicine, Yokohama; and [2]Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

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Matsuda M, Tamura K, Wakui H, Dejima T, Maeda A, Ohsawa M, Kanaoka T, Haku S, Azushima K, Yamasaki H, Saito D, Hirose T, Maeshima Y, Nagashima Y, Umemura S. Involvement of Runx3 in the basal transcriptional activation of the mouse angiotensin II type 1 receptor-associated protein gene. *Physiol Genomics* 43: 884–894, 2011. First published May 17, 2011; doi:10.1152/physiolgenomics.00005.2011.—We previously cloned a molecule that interacts with angiotensin II type 1 (AT1) receptor to exert an inhibitory function on AT1 receptor signaling that we named ATRAP/*Agtrap* (for AT1 receptor-associated protein). In the present study we examined the regulation of basal ATRAP gene expression using renal distal convoluted tubule cells. We found that serum starvation upregulated basal expression of ATRAP gene, a response that required de novo mRNA and protein synthesis. Luciferase assay revealed that the proximal promoter region directs transcription and that a putative binding site of runt-related transcription factors (RBE) is important for transcriptional activation. The results of RBE-decoy transfection and endogenous knockdown by small interference RNA showed that the runt-related transcription factor Runx3 is involved in ATRAP gene expression. Chromatin immunoprecipitation assay also supported the binding of Runx3 to the ATRAP promoter in renal distal convoluted tubule cells. Immunohistochemistry demonstrated the expression of Runx3 and ATRAP proteins in the distal convoluted and connecting tubules of the kidney in consecutive sections. Furthermore, the Runx3 immunostaining was decreased together with a concomitant suppression of ATRAP expression in the affected kidney after 7 days of unilateral ureteral obstruction. These findings indicate that Runx3 plays a role in ATRAP gene expression in renal distal tubular cells both in vitro and in vivo.

distal tubule; gene transcription; renin-angiotensin system; transcription regulation

ACTIVATION OF THE angiotensin II (ANG II) type 1 (AT1) receptor at local sites is involved in the pathogenesis of hypertension and the ensuing related target organ damages, as well as the development of renal inflammatory and fibrotic disease. We previously performed a yeast two-hybrid system screening of a murine kidney cDNA library and identified the molecule AT1 receptor-associated protein (ATRAP/*Agtrap*), which interacts specifically with the COOH-terminal cytoplasmic domain of the AT1 receptor (6, 17, 26). Previous in vitro studies using cardiovascular cells suggested that ATRAP promotes AT1 receptor internalization and modulates the signaling pathways of the AT1 receptor (1, 10, 19, 28).

With respect to the tissue distribution and regulation of ATRAP expression in vivo, the ATRAP mRNA and protein are abundantly and widely distributed along the renal tubules, including the distal and proximal tubules (18, 29). We also demonstrated that there is a tissue-specific regulatory balancing of the expression of ATRAP and the AT1 receptor during the development of hypertension in rats (25). The activation of tissue ATRAP in transgenic models in which ATRAP expression was increased beyond baseline promoted ANG II-mediated internalization of the AT1 receptor (33) and abolished cardiac hypertrophy in response to ANG II stimulation (34). A recent study showed that a genetic deficiency of ATRAP in mice caused an enhanced surface expression of AT1 receptor in the kidney and an elevation of blood pressure through volume expansion (21). Therefore, it is important to elucidate the molecular mechanism of the tissue-specific regulation of ATRAP gene expression to determine the regulatory machinery for the tissue ATRAP level and/or ATRAP activity under both physiological and pathological conditions. Thus, as a first step, in this study we examined the basal transcriptional regulation of the ATRAP gene using mouse distal convoluted tubule cells (mDCT cells). These cells have been shown to have the phenotype of a polarized tight junction epithelium with morphologic and functional features retained from the parental cells (8, 22). The mDCT cells also express the endogenous AT1 receptor and ATRAP genes, as shown by reverse transcriptase-polymerase chain reaction (RT-PCR) and immunoblot analysis (18).

In the present study, we found that serum starvation upregulates basal ATRAP gene expression in renal DCT cells and that Runx3, one of the Runt-related transcription factors, is involved in the transcriptional activation of ATRAP gene expression. The Runt-related transcription factors have a conserved 128-amino acid Runt domain, a name derived from its homology to the pair-rule related gene "runt," which plays a role in the segmented body patterning of Drosophila (5). The Runt-related transcription factors consist of Runx1, Runx2, and Runx3, and all three Runx proteins bind to the common DNA motif TGPyGGTPy (Py is a pyrimidine), and each of these heterodimerizes with CBFβ, which makes no direct contact with DNA but, rather, increases DNA binding to Runx proteins.

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MATERIALS AND METHODS

This 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.

Cell culture. The mDCT cells were kindly provided by Dr. Peter A. Friedman (University of Pittsburgh School of Medicine, Pittsburgh, PA). The cells had been previously isolated and functionally characterized as described (9, 22).

Animals and treatment. Adult C57BL/6 mice were purchased from Oriental Yeast Kogyo (Tokyo, Japan). The procedure of unilateral ureteral obstruction (UUO) was performed using C57BL/6 mice as described previously (23). Briefly, with the mice under anesthesia, the left ureter was ligated at two locations. Mice that were operated on were killed under anesthesia 7 days after UUO. Sham operation was also performed, in which the ureters were manipulated but not ligated.

Immunoblot analysis. Characterization and specificity of the anti-mouse ATRAP antibody have been described (23, 28, 29). The total membrane fraction was isolated using a Plasma Membrane Protein Extraction Kit (BioVision) according to the manufacturer's protocol. Immunoblot analysis was performed as described previously (23, 28, 29, 31, 34), and the images were analyzed quantitatively using a FUJI LAS3000 Image Analyzer (FUJI Film, Tokyo, Japan) for determination of the ATRAP protein levels.

Plasmid construction and transcriptional ATRAP promoter assay. The *Kpn*I/*Bam*HI fragment of the 374-bp mouse ATRAP promoter (−302 to +72 of the putative transcriptional start site) was amplified from C57BL/6J genomic DNA using the pair of primers in Table 1. Construction of mutations in the Runx-binding element (RBE) and SMAD-binding element (SBE) was performed by oligonucleotide (ODN)-directed mutagenesis (11, 13, 27). The sequences of the ODN used to create the mutated SBE (SBE-mt) and mutated RBE (RBE-mt) are shown in Table 1. To normalize transfection efficiency, we employed the Dual-Luciferase Assay System (Promega) as described previously (22).

Transfection of decoy ODN and small interfering RNA. The sequences of the decoy ODN against RBE and control HA ODN are

Table 1. *Primer sequences used in the study*

Primers	Primer Sequences
Construction of wild-type and mutated ATRAP promoter-containing plasmids	
Wild-type ATRAP promoter	
forward	5′-ggggtacCTTGTGCAAGGGAAGTAAGA-3′
reverse	5′-cgggatccGAACTCGGGAACAAACTTCCT-3′
SBE-mt	
forward	5′-AGAGAGGATGTTCTGGGCCTCCACCAACTGTTACCACACCCGCAG-3′
reverse	5′-CTGCGGGTGTGGTAACAGTTGGTGGAGGCCCAGAACATCCTCTCT-3′
RBE-mt	
forward	5′-TGGGCAGACACCAACTGTTAGTGTACCCGCAGTTTCTGCCCGCTT-3′
reverse	5′-AAGCGGGCAGAAACTGCGGGTACACTAACAGTTGGTGTCTGCCCA-3′
Transfection of decoy ODN	
RBE-decoy ODN	5′-CTCGTCTACCACATGCACCGT-3′ and 5′-ACGGTGCATGTGGTAGACCAG-3′
HA-decoy ODN	5′-CCATACGATGTTCCAGATTAC-3′ and 5′-GGTATGCTACAAGGTCTAATG-3′
Transfection of siRNA	
siRunx1	
sense	5′-ccgucuuuacaaauccgccTT-3′
antisense	5′-ggcggauuuguaaagacggTG-3′
siRunx2	
sense	5′-cgaauggcagcacgcuauuAA-3′
antisense	5′-aauagcgugcugccauucgAG-3′
siRunx3	
sense	5′-uucaacgaccuucgauucgTG-3′
antisense	5′-cgaaucgaaggucguugaaCC-3′
siHA	
sense	5′-ccauacgauguuccagauuAC-3′
antisense	5′-aaucuggaacaucguauggGT-3′
Real-time RT-PCR analysis	
Runx1	
forward	5′-TAGCGAGATTCAACGACCTC-3′
reverse	5′-GTGGCGGATTTGTAAAGACG-3′
Runx2	
forward	5′-GTACTTCGTCAGCATCCTAT-3′
reverse	5′-AGCGTGCTGCCATTCGAGGT-3′
Runx3	
forward	5′-GGTTCAACGACCTTCGATTC-3′
reverse	5′-GGTTGGTGAACACGGTGATT-3′
Chromatin immunoprecipitation assay	
forward	5′-TCCTCTCCAACCCTCACTTTT-3′
reverse	5′-TAACAGTTGGTGTCTGCCCA-3′

ATRAP, AT1 receptor-associated protein; SBE, SMAD-binding element; mt, mutated; RBE, Runx-binding element; HA, hemagglutinin; ODN, oligonucleotide; siRNA, small interfering RNA.

shown in Table 1. To knockdown the endogenous expression of Runx proteins with small interfering RNA (siRNA), siRunx1, siRunx2, and siRunx3 primers were synthesized (Table 1). As a negative control, siHA primers were also synthesized. The annealed double-strand decoy ODN (100 nM) or siRNA (2.5–10 nM) was introduced into mDCT cells using Lipofectamine RNAiMAX (Invitrogen).

Real-time quantitative RT-PCR analysis. Total RNA was extracted and purified using the RNeasy Kit (QIAGEN), and real-time quantitative RT-PCR analysis was performed as described previously (34). The detection primer sequences for Runx1, Runx2, and Runx3 are shown in Table 1.

Co-immunoprecipitation. Kidneys were minced, homogenized in reaction buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1%

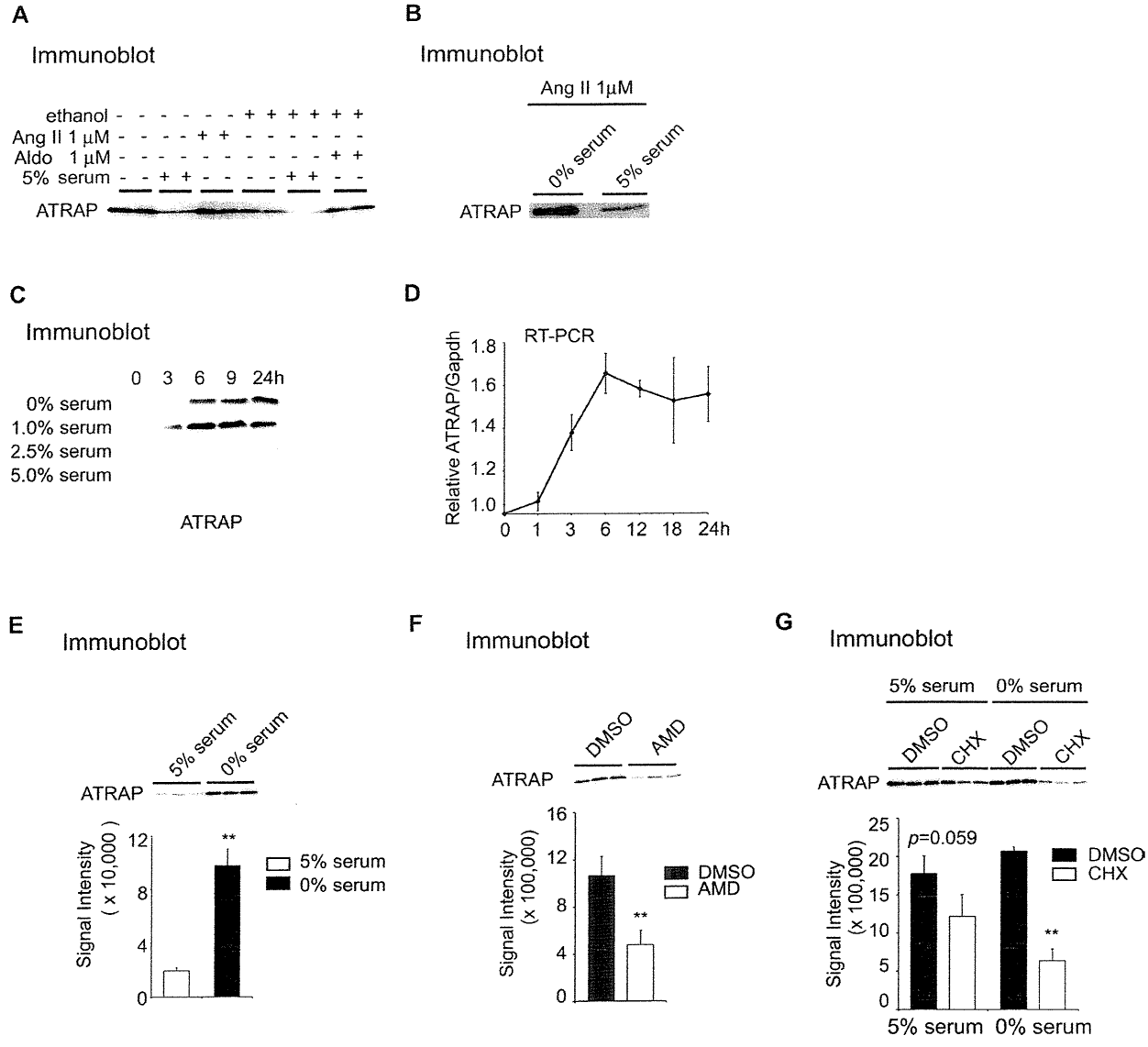

Fig. 1. Serum deprivation-mediated regulation of basal AT1 receptor-associated protein (ATRAP) expression involves the activation of transcription and translation in mouse distal convoluted tubule (mDCT) cells. *A*: representative immunoblot showing the effects of 24-h treatment with ANG II (1 μM), aldosterone (Aldo, 1 μM), or serum (5%) on ATRAP protein expression in whole mDCT extracts after serum starvation for 24 h. Ethanol (final concentration 0.1%) was used as a solvent for aldosterone. *B*: representative immunoblot showing the effects of 24-h treatment with ANG II (1 μM) with or without serum (5%) on ATRAP protein expression in whole mDCT extracts after serum starvation for 24 h. *C*: representative immunoblot showing the time- and concentration-dependent effects of serum starvation for the indicated times on relative ATRAP protein expression in whole mDCT extracts. *D*: quantitative real-time RT-PCR analysis showing time-dependent effects of serum starvation on the relative ATRAP mRNA levels. RNA quantity was normalized to the signal generated by constitutively expressed Gapdh and is expressed relative to those achieved with extracts at baseline (0 h) (n = 3). *E*: immunoblot showing the effects of 24-h serum deprivation (5% serum −) on ATRAP protein expression in the total membrane fraction of mDCT cells (n = 3). $^{**}P < 0.01$, vs. nondeprived cells (5% serum +). *F*: immunoblot showing the effects of actinomycin D (AMD, 1 μg/ml) on 6-h serum deprivation-mediated expression of ATRAP expression in the total membrane fraction of mDCT cells (n = 3). $^{**}P < 0.01$, vs. vehicle-treated cells (AMD-, DMSO). *G*: immunoblot showing the effects of 6-h treatment of cycloheximide (CHX, 10 μg/ml) on ATRAP protein expression in the total membrane fraction of the serum deprived (0% serum) or nondeprived (5% serum) mDCT cells (n = 3). $^{**}P < 0.01$, vs. vehicle-treated cells (CHX-, DMSO). Immunodetection of the membrane stained by Coomassie blue dye served as an internal control for the determination of equal protein loading.

NP-40, 1 mM DTT, and protease inhibitors), and centrifuged at 3,000 *g* for 30 min. Then, the solubilized proteins were subjected to absorption by protein G-beads to avoid a nonspecific reaction. For immunoprecipitation, anti-PY antibody (clone 6B4, MBL) cross-linked protein G-beads with Bis (sulfosuccinimidyl) suberate (#21580 Thermo Fisher Scientific) were used. Immunoblotting was performed using anti-Runx3 antibody (AV37263, Sigma-Aldrich).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay with anti-Runx3 antibody or normal rabbit IgG was performed using the detection primers in Table 1, essentially according to the manufacturer's protocol (Active Motif) (2, 15). Briefly, mDCT cells transiently transfected with the wild-type or mutated ATRAP promoter-containing plasmids were treated with paraformaldehyde to cross-link the protein-DNA complexes. Then, cell lysates were sonicated to reduce the DNA fragments to an average size of ~500 bp. Following immunoprecipitation with anti-Runx3 antibody or normal rabbit IgG as control, DNA was purified from the antibody-bound and unbound fractions, and enrichment of the ATRAP promoter sequences in the bound fraction was assayed by the overlap extension PCR method (13).

Immunohistochemistry. Immunohistochemistry was performed essentially as described previously (23, 29). The sections were incubated with anti-RUNX3 antibody (H-50, sc-30197; Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1:50. For the study of specific nephron markers, the sections were incubated with *1*) aquaporin 2 antibody (#178612, Calbiochem), *2*) calbindin D-28K antibody (C2724, Sigma-Aldrich), or *3*) Tamm-Horsfall glycoprotein antibody (sc-20631, Santa Cruz Biotechnology).

Statistical analysis. Data are expressed as the means ± SD. Statistical significance was determined using unpaired Student's *t*-test or analysis of variance followed by Bonferroni test, with $P < 0.05$ considered statistically significant.

RESULTS

Serum deprivation-mediated regulation of ATRAP expression involves the activation of transcription and translation in mDCT cells. To examine whether external stimuli exert an influence on the expression of the ATRAP gene in mDCT cells, we first assessed the effects of various vasoactive substances by immunoblot analysis, such as ANG II and aldosterone, on ATRAP protein expression. After mDCT cells were incubated with serum-free medium for 24 h, mDCT cells were incubated with the indicated medium for an additional 24 h. Although treatment with ANG II (1 μM) or aldosterone (1 μM) did not significantly affect ATRAP protein expression, the expression was markedly decreased after exposure of the cells to 5% serum (Fig. 1*A*). This inhibitory effect of serum on basal ATRAP expression was not affected by costimulation with ANG II (Fig. 1*B*). These results show that serum is a dominant inhibitor of ATRAP gene expression in mDCT cells.

To determine whether serum starvation increased ATRAP gene expression, we examined the time course of ATRAP protein expression in mDCT cells treated with various concentrations of serum. Expression of the ATRAP protein started to increase 3 h after treatment with 0 or 1.0% serum and peaked at 6 h, and at 24 h the level was still elevated compared with baseline, while treatment with 2.5 or 5% serum did not affect ATRAP protein expression (Fig. 1*C*). The results of real-time quantitative RT-PCR analysis showed that the serum starvation-induced increase in ATRAP protein expression was accompanied by an activation of ATRAP mRNA expression (Fig. 1*D*), indicating transcription plays a role in the basal expression of the ATRAP gene.

ATRAP is predicted to have three transmembrane domains (6, 17), so we also examined the effect of serum starvation on basal ATRAP protein expression using the total cellular membrane fraction. The results showed that serum starvation increased the basal ATRAP protein expression in the membrane

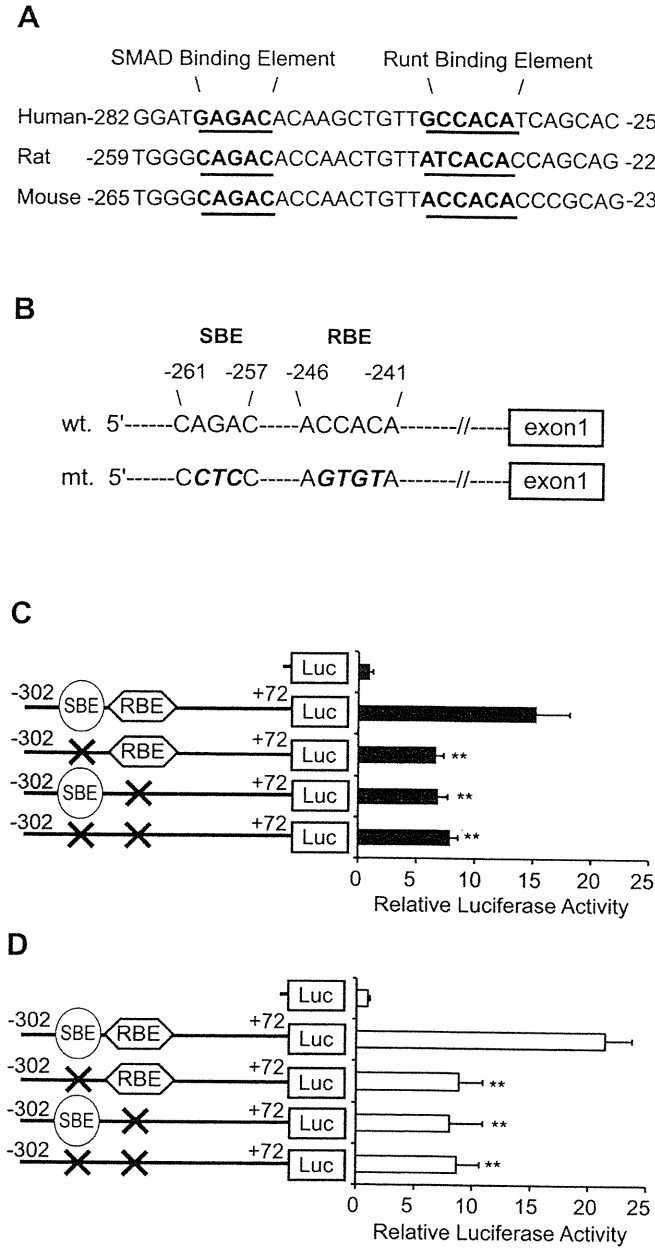

Fig. 2. The Runx binding element (RBE) and SMAD binding element (SBE) are important for transcriptional activation of the ATRAP promoter in mDCT cells. *A*: sequences of the SBE and RBE in the proximal promoter regions of the human, rat, and mouse ATRAP genes. *B*: construction of site-directed mutations in the SBE and RBE in the mouse ATRAP promoter sequence. Wild-type sequences (wt.) and mutated sequences (mt.) are shown. Effects of mutations in the SBE and RBE on the transcriptional activity of the mouse ATRAP promoter (−302 to +72 of the transcriptional start site)-luciferase hybrid gene in serum containing (*C*, 5% serum) or deprived (*D*, 0% serum) mDCT cells. The relative luciferase activities were calculated relative to those achieved with the promoterless control plasmid ($n = 3$). $^{**}P < 0.01$, vs. wild-type sequences.

fraction as well (Fig. 1*E*), and membrane fraction extracts were used for the analysis of ATRAP protein expression thereafter. Next, to determine whether de novo RNA or protein synthesis was required for the starvation-induced increase in basal ATRAP expression, mDCT cells were treated with actinomycin D or cycloheximide and incubated for 6 h in the presence or absence of 5% serum. The RNA synthesis inhibitor actinomycin D (Fig. 1*F*), as well as the protein synthesis inhibitor cycloheximide (Fig. 1*G*), significantly suppressed the starvation-mediated increase in basal ATRAP protein expression. These results demonstrate that both de novo mRNA transcription and de novo protein synthesis are required for the upregulation of basal ATRAP expression by serum starvation.

The SMAD and Runx binding elements are important for the transcriptional activation of the ATRAP promoter in mDCT cells. The above results suggest that activation of transcription may play an important role in the serum starvation-induced upregulation of basal ATRAP expression in mDCT cells. The entire mouse ATRAP locus spanning 11002 nt on chromosome 4 consists of 5 exons (32). The first exon is 148 bp in length and contains 121 bp of untranslated nucleotides upstream of the initiating ATG. Sequence analysis of the 5′-flanking region of the ATRAP gene using the TF Search program and Signal Scan algorithm revealed the absence of a TATA box and the presence of a GC-rich region extending >200 nt from the initiating methionine. The percentage of GC dinucleotides in this region ranged between 65 and 80%, with a CG:GC ratio of 0.94, thus fulfilling the length and base composition criteria for a canonical CpG island (32).

Analysis of the proximal promoter region made up of approximately −300 bp of the putative transcriptional start site of the ATRAP gene, with the MOTIF Search program (http://motif.genome.jp/), showed the presence of a putative SBE and Runx binding site (RBE), which are highly conserved among the mouse, rat, and human forms (Fig. 2*A*). To examine the functional role of these conserved elements in the regulation of ATRAP gene transcription, we mutated the core binding sequences of SBE and RBE in the endogenous mouse ATRAP promoter by PCR-directed mutagenesis (Fig. 2*B*). Although the proximal promoter region from −302 to +72 of the putative transcriptional start site of the ATRAP gene exhibited substantial transcriptional activity, site-directed mutations of either SBE or

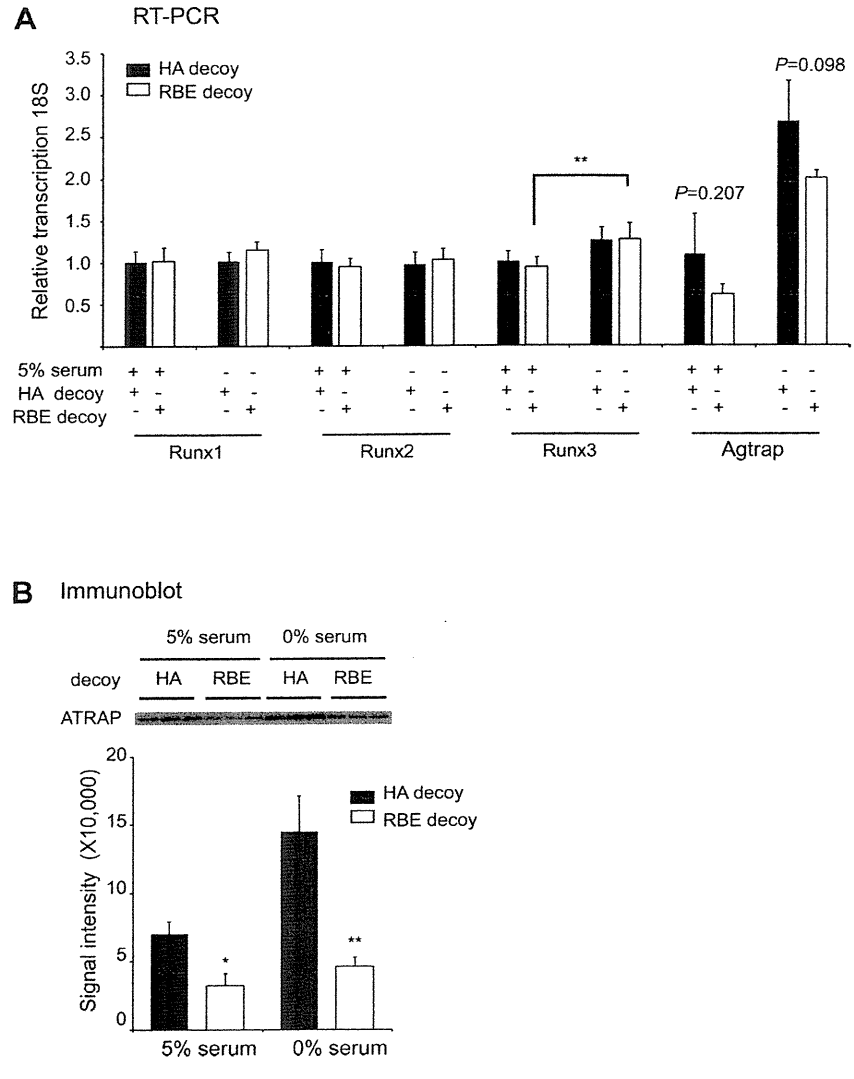

Fig. 3. RBE decoy decreases endogenous ATRAP gene expression in mDCT cells. *A*: quantitative real-time RT-PCR analysis showing the effects of RBE-decoy transfection on the relative mRNA levels of Runx1, Runx2, Runx3, and ATRAP. RNA quantity was normalized to the signal generated by the constitutively expressed 18S ribosomal RNA and expressed relative to extracts derived from nondeprived mDCT cells transfected with control hemagglutinin (HA) decoy (5% serum +, HA-decoy +) (n = 3). *P < 0.05, vs. HA, hemagglutinin; decoy; **P < 0.01, vs. HA decoy. *B*: immunoblot showing the effects of RBE-decoy transfection on ATRAP protein expression in the total membrane fraction of the serum deprived (0% serum) or nondeprived (5% serum) mDCT cells (n = 3). HA decoy was used as a control. Immunodetection membrane stained by Coomassie blue dye served as an internal control for the determination of equal protein loading. **P < 0.01, vs. HA decoy.