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ORIGINAL ARTICLE

One-year effectiveness and safety of open-label losartan/hydrochlorothiazide combination therapy in Japanese patients with hypertension uncontrolled with ARBs or ACE inhibitors

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The long-term antihypertensive efficacy and safety of losartan/hydrochlorothiazide (HCTZ) combinations have not been appropriately evaluated in Japan. In this study, treated hypertensive patients taking angiotensin-receptor blocker (ARB) or angiotensin-converting enzyme inhibitor (ACEI) regimens not at blood pressure (BP) goals proposed by the Japanese Society of Hypertension (JSH) were switched to losartan/HCTZ combinations and followed for 1 year. Data analysis included 244 patients aged 64.5 ± 10.7 years, 56% male, 27% with diabetes mellitus and 36% with dyslipidemia. Pre-switching BP 157 ± 16/88 ± 10 mm Hg promptly decreased and maintained a steady state, reaching 132 ± 15/77 ± 9 mm Hg ($P<0.001$) 1 year later. After 1 year of treatment, 50% of patients cleared the goals of the JSH guideline for systolic BP and 79% for diastolic BP. Patients with maximal doses of ARBs tended to show larger decreases in BP (159 ± 11/90 ± 10 to 128 ± 10/75 ± 8 mm Hg, $P<0.001$, $n=32$). Clinical and laboratory adverse events were reported for 29 patients (11%), but serious abnormalities were not observed. In particular, plasma levels of uric acid (UA) were well-maintained for 1 year, and significant decreases in UA were observed in patients with higher levels of UA ($\geqslant 7.0$ mg dl^{-1}). Losartan/HCTZ combinations showed strong and steady hypotensive abilities and acceptable safety and tolerability in patients currently not at BP goals with regimens including ARBs or ACEIs in Japan.

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Keywords: angiotensin-receptor blocker (ARB); Japanese; losartan/hydrochlorothiazide; uric acid

INTRODUCTION

Guidelines for hypertension treatment, including those of the Japanese Society of Hypertension (JSH), have recommended strict blood pressure (BP) control, with the aim of improving protection against cardiovascular and renal accidents.[1,2] However, considerable numbers of hypertensive patients have not achieved the recommended goals of BP in Japan.[3] The JSH guideline recommends angiotensin-receptor blockers (ARBs), angiotensin-converting enzyme inhibitors (ACEIs), Ca^{2+} channel blockers (CCB), β-blockers and diuretics as first-line drugs for hypertensive treatment.[1] The guideline also recommends appropriate combinations of the drugs, in particular low-dose (quarter to half dose) diuretics are recommended as an important candidate for satisfactory BP control.[1] However, the prescribing rate of diuretics was quite low (under 10%) in cases of monotherapy or combination therapy for hypertension in Japan.[4] The principal reason for reluctance to prescribe thiazide diuretics is the metabolic side effects of the drugs. However, low-dose thiazide diuretics retain their hypotensive abilities with minimal side effects.[5] Therefore, proper application of low-dose diuretics, particularly in combination therapies, is desirable in Japan to improve BP control.

A fixed dose combination of losartan (50 mg)/hydrochlorothiazide (HCTZ, 12.5 mg) (Preminent; Banyu/Merck, Tokyo, Japan) is the first combination of an ARB and a diuretic for hypertensive treatment in Japan, and is expected to be effective and safe from the pharmacological properties of both drugs. However, limited data were available on the combination drug in Japan, especially with regard to long-term treatment, large numbers of patients and its use in a clinical setting.[6–8] We organized a study group mainly consisting of clinical physicians in Miyazaki Prefecture in Japan (Preminent Assigned League in Miyazaki by Primary care physicians: PALM-1 study group), and evaluated the

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efficacy and safety of the fixed combination of losartan/HCTZ for patients with essential hypertension for 1 year.

METHODS

Study subjects

This study was conducted at 43 centers for the PALM-1 study group (Appendix). Patients with essential hypertension (20–79 years old) were considered for screening and potential recruitment into the trial. They had visited the attending clinics from February 2007 to March 2008 and had not reached BP goals with antihypertensive therapy regimens, including ARBs or ACEIs, but not diuretics, over 1 month. Patients were excluded from the study if there was any evidence of secondary hypertension, renal failure (serum creatinine $\geqslant 2.0\,mg\,dl^{-1}$), severe liver dysfunction and symptomatic heart failure (New York Heart Association functional class-III or IV for dyspnea at exertion). Patients with concomitant use of two or more ARBs and/or ACEIs and any type of diuretics were also excluded.

Study protocol

The study was conducted in accordance with the principles of the declaration of Helsinki. The investigational protocol was approved by the ethics committee for human studies at the University of Miyazaki. Informed consent was obtained from all patients prior to recruitment.

This was an open-label, multicenter study consisting of a 3-month screening/baseline period and 1-year treatment period. Under antihypertensive treatment with regimens including ARBs or ACEIs, at least two BP measurements were conducted within 3 months of the baseline period to confirm baseline BP measurements were over the recommended BP goals of the JSH. The BP goals were 130/85 mm Hg for patients aged less than 65 years, 140/90 mm Hg for those aged 65 years or more, 130/80 mm Hg for patients with diabetes and/or chronic kidney disease and/or history of myocardial infarction, and 140/90 mm Hg for patients with a history of stroke.[1] After screening 311 patients, 266 entered the trial. Then only ARBs or ACEIs were switched to the fixed dose combination of losartan/HCTZ and patients were followed for 1 year. Changed prescriptions were kept for the initial 3 months and then, if needed, adjustments of antihypertensive drugs were allowed except for ARBs, ACEIs and diuretics. Symptoms, sitting BP, pulse rate and blood tests, including potassium, uric acid (UA), lipid profile, creatinine, glucose, hemoglobin-A1c (HbA1c, diabetic patients only), were evaluated every 3 months. Major complications were also evaluated. The criteria for diabetes and dyslipidemia were as follows: diabetes, using antiglycemic drugs or fasting blood glucose $\geqslant 126\,mg\,dl^{-1}$; dyslipidemia, using lipid-lowering drugs or total cholesterol $\geqslant 220\,mg\,dl^{-1}$ and/or high-density lipoprotein-cholesterol $<40\,mg\,dl^{-1}$, and/or triglyceride $\geqslant 150\,mg\,dl^{-1}$.

Statistical analysis

All data are expressed as mean ± s.d. The significance of differences was evaluated by one-factor analysis of variance with repeated measures on the time course of variables followed by Bonferroni/Dunn *post hoc* comparison tests. Comparisons of parameters among subgroups were made by unpaired Dunnet's C-test or analysis of variance followed by Scheffe's *post hoc* comparison test. *P*-value <0.05 was the criterion for statistical significance.

RESULTS

As indicated in Figure 1, 22 of the 266 enrolled patients dropped out within the first 3 months. The remaining 244 patients were considered as full analytical objects. Finally, 222 patients completed the entire trial and were used for evaluation of efficacy.

The baseline characteristics of the study population are summarized in Table 1. Patients' age was 64.5 ± 10.7 years, 56% were male and major complications included 27% of patients with diabetes, 36% with dyslipidemia and 18% with mild heart failure. Pre-prescribed ARBs or ACEIs were well distributed from among drugs on the market and, noteworthy, the average doses per day of the drugs were very close to the usual dosage of each drug (Table 1). ARBs or ACEIs were used as monotherapy for 93 patients (38%) and as combined therapy, mainly with CCB, for 151 patients (62%). Other pre-prescribed drugs were as follows and these drugs were not altered after introduction of the losartan/HCTZ combination: antiglycemic drugs for 38 of 266 patients (37 of 222), lipid-lowering drugs for 58 of 266 (53 of 222) and UA-lowering drugs for 14 of 266 (14 of 222).

The time course of BP in all patients is illustrated in Figure 2. Baseline BP 157 ± 16/88 ± 10 mm Hg significantly decreased to 134 ± 14/77 ± 9 mm Hg at 3 months ($P<0.001$) (fixed prescription period), and then steady levels were maintained throughout the remaining treatment period. The respective goals of BP were cleared by 50% of the patients for systolic BP and 79% of the patients for diastolic BP in the final assessment 1 year later. Interestingly, 32 of 222 patients who were switched from the maximum dose of ARBs showed a similar to larger decrease in BP

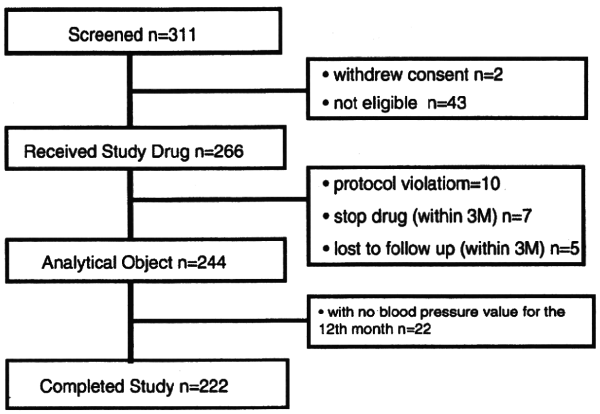


Figure 1 Patient disposition and reasons for exclusion.

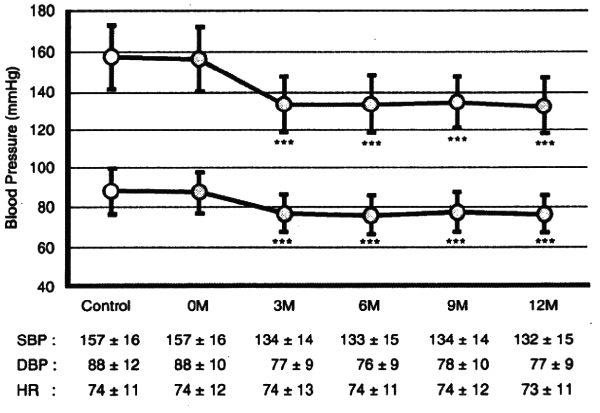
Table 1 Baseline characteristics (*n*=244)

Variable	*Value*	*Average doses (mg day^{-1})*
Age (years)	64.5 ± 10.7	
Male (*n*)	136 (56%)	
Body mass index (kg m^{-2})	25.2 ± 5.6	
Waist circumference (cm)	85.9 ± 8.6	
Obesity (*n*)	110 (48%)	
Diabetes (*n*)	66 (27%)	
Dyslipidemia (*n*)	88 (36%)	
Heart diseases (*n*)	43 (18%)	
Renal insufficiency (*n*)	8 (3%)	
Antihypertensives (n)		
One drug	93 (38%)	
Over two drugs	151 (62%)	
Pre-prescribed drugs (n)		
Valsartan	68 (28%)	88.8 ± 40.1
Candesartan	54 (22%)	8.4 ± 2.2
Losartan	34 (14%)	51.5 ± 8.6
Telmisartan	32 (13%)	40.3 ± 10.0
Olmesartan	31 (13%)	22.6 ± 8.6
ACE inhibitors	25 (10%)	

Abbreviation: ACE, angiotensin-converting enzyme.

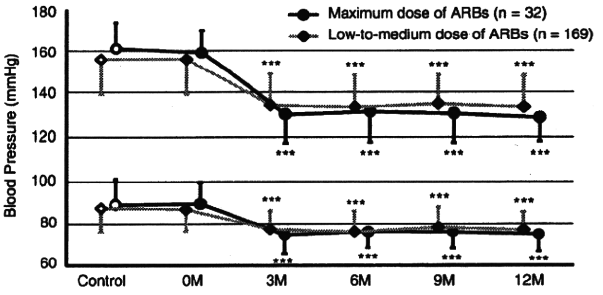
as compared with patients with low-to-medium dose of ARBs (Figure 3). There was a significant difference in the changes of BP from 3 months to 1 year between patients switched from low-to-medium dose of ARBs and maximum dose of ARBs (at 1 year: systolic BP, 23 ± 19 *vs.* 31 ± 13 mm Hg, P=0.005; diastolic BP, 10 ± 11 *vs.* 15 ± 10 mm Hg, P=0.027). As shown in Figure 4, similar and significant decreases in systolic and diastolic BP were achieved in all patients grouped based on pre-prescribed drugs at 1 year. Also there was no difference in BP changes among all ARBs and ACEI-receiving patients. The systolic and diastolic BPs at 0 and 12 month (changes of the BPs) for each drug were as follows: losartan, 154 ± 17 to 135 ± 10 mm Hg (−19 ± 17 mm Hg, P<0.001) and 87 ± 11 to 78 ± 8 mm Hg (−9 ± 10 mm Hg, P<0.001); candesartan, 156 ± 14 to 131 ± 14 mm Hg (−24 ± 17 mm Hg, P<0.001) and 87 ± 9 to 76 ± 9 mm Hg (−11 ± 10 mm Hg, P<0.001); valsartan, 160 ± 16 to 134 ± 13 mm Hg (−26 ± 18 mm Hg, P<0.001) and 89 ± 9 to 77 ± 8 mm Hg (−12 ± 10 mm Hg, P<0.001); telmisartan, 156 ± 17 to 132 ± 20 mm Hg (−24 ± 15 mm Hg, P<0.001) and 85 ± 12 to 75 ± 11 mm Hg (−10 ± 8 mm Hg, P<0.001); olmesartan, 153 ± 18 to 129 ± 14 mm Hg (−24 ± 24 mm Hg, P<0.001) and 88 ± 15 to 77 ± 10 mm Hg (−11 ± 15 mm Hg, P<0.001); and ACEIs, 159 ± 16 to 133 ± 19 mm Hg (−26 ± 20 mm Hg, P<0.001) and 87 ± 9 to 76 ± 12 mm Hg (−11 ± 12 mm Hg, P=0.001). There were very limited number of alterations in antihypertensive drugs after 3 months (8 of 222): two terminations of CCBs, one decrease of CCB, four introductions of low doses of CCBs for patients receiving low-to-medium dose of ARBs and one introduction of atenolol (12.5 mg) for a patient with maximum dose of ARBs.

To determine the difference in receptivity to losartan/HCTZ between specific backgrounds of the patients, we compared BP



	Control	0M	3M	6M	9M	12M
SBP :	157 ± 16	157 ± 16	134 ± 14	133 ± 15	134 ± 14	132 ± 15
DBP :	88 ± 12	88 ± 10	77 ± 9	76 ± 9	78 ± 10	77 ± 9
HR :	74 ± 11	74 ± 12	74 ± 13	74 ± 11	74 ± 12	73 ± 11

Figure 2 The time course of BP in all patients (n=222). ***P<0.001 compared with month 0. BP, blood pressure.



	Control	0M	3M	6M	9M	12M
Maximum dose of ARBs						
SBP :	161 ± 13	159 ± 11	130 ± 13	131 ± 13	130 ± 13	128 ± 10
DBP :	89 ± 12	90 ± 10	75 ± 9	76 ± 8	76 ± 8	75 ± 8
HR :	71 ± 11	70 ± 12	71 ± 12	72 ± 11	72 ± 11	72 ± 12
Low-to-medium dose of ARBs						
SBP :	156 ± 16	156 ± 17	134 ± 15	133 ± 15	135 ± 13	133 ± 15
DBP :	88 ± 11	87 ± 11	77 ± 9	76 ± 10	78 ± 10	77 ± 9
HR :	75 ± 11	74 ± 11	74 ± 13	74 ± 11	74 ± 12	74 ± 10

Figure 3 The time course of BP in patients switched from maximum dose (n=32) and low-to-medium dose (n=169) of ARBs. ***P<0.001 compared with month 0. ARB, angiotensin-receptor blocker; BP, blood pressure.

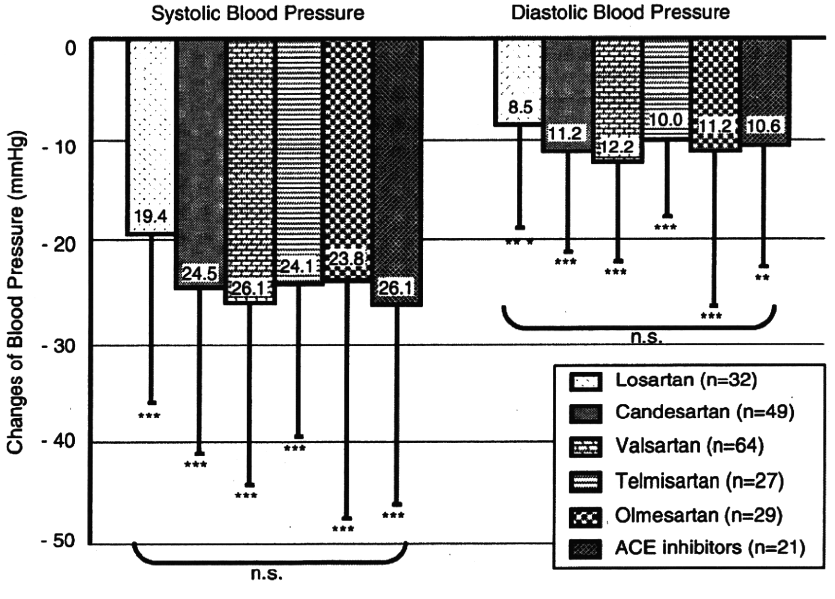


Figure 4 Decreases in BP after 12 months for each pre-prescribed drug. **P<0.01, ***P<0.001 compared with month 0. BP, blood pressure.

changes at 1 year for various subgroups. However, there was no difference among the subgroups and specific factors contributing to resistance against losartan/HCTZ were not detected. For example, if patients are grouped according presence (+) or absence (−) of diabetes (D) and obesity (O) (body mass index, $\geqslant 25\,kg\,m^{-2}$), decreases in systolic BP were 24 ± 18 mm Hg (D+/O+, n=35), 23 ± 17 mm Hg (D+/O−, n=27), 24 ± 15 mm Hg (D−/O+, n=63) and 25 ± 21 mm Hg (D−/O−, n=97). This indicates that the losartan/HCTZ combination is effective even for patients with diabetes and obesity.

Remarkable changes were not observed in metabolic parameters after 1 year of treatment with losartan/HCTZ. Figure 5 shows changes in UA levels in all patients (5.46 ± 1.43 to 5.62 ± 1.43 mg dl^{-1}) and subgroups with high levels of UA at baseline and others. UA level was slightly increased in patients with relatively low levels of UA (UA < 7.0 mg dl^{-1}, middle panel): 5.02 ± 1.11 to 5.37 ± 1.34 mg dl^{-1} (P<0.001). But, interestingly, UA level was significantly decreased in patients with high level of UA (UA ⩾ 7.0 mg dl^{-1}, right panel): 7.66 ± 0.57 to 6.88 ± 1.16 mg dl^{-1} (P=0.004). Other changes (month 0 to 12) concerning parameters in blood tests are summarized in Table 2.

Adverse events were observed in 29 of 266 patients (10.9%) who received the losartan/HCTZ combination, including accidental events, and 16 (5.4%) discontinued the losartan/HCTZ combination, while

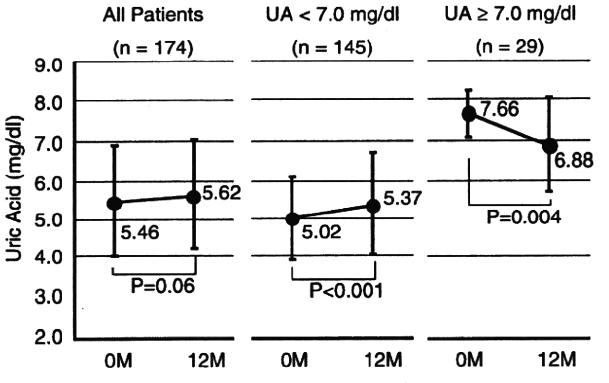


Figure 5 Changes in serum UA levels in all patients (left panel) and in those with high (middle panel) and low-to-medium levels (right panel) of UA. UA, uric acid.

Table 2 Changes of parameters in blood tests

	Month 0	*Month 12*	P-*value*
All patients			
Potassium (mEq l^{-1})	4.13 ± 0.48	4.15 ± 0.52	0.67
Total cholesterol (mg dl^{-1})	199 ± 34	191 ± 31	0.001
HDL-cholesterol (mg dl^{-1})	56.4 ± 14.6	55.1 ± 13.6	0.075
Triglyceride (mg dl^{-1})	147 ± 96	149 ± 96	0.74
Creatinine (mg dl^{-1})	0.83 ± 0.29	0.88 ± 0.30	<0.001
Glucose (mg dl^{-1})	118 ± 46	121 ± 52	0.24
Diabetic patients only (n=52)			
Glucose (mg dl^{-1})	154 ± 62	155 ± 73	0.83
HbA1c (%)	6.45 ± 1.22	6.46 ± 1.15	0.91

Abbreviations: ACE, angiotensin-converting enzyme; HDL, high-density lipoprotein.

the remaining 13 patients continued receiving the drug. Among the 16 patients who discontinued, 13 events (4.9%) were considered possibly, probably or definitely drug-related. Laboratory abnormalities were observed for 13 patients. The 13 drug-related adverse events included three cases of hypokalemia, two patients who complained of skin rash, one patient who suffered photosensitive dermatoses, worsening of diabetes in one patient and excessive BP depression in six patients. Four patients of 266 discontinued the losartan/HCTZ combination because of patient circumstances or requests, without adverse events. No death occurred during the study.

DISCUSSION

Only 42% of hypertensive patients reached the guideline BP goals in the J-HOME (Japan Home versus Office Blood Pressure Measurement Evaluation) study.[3] Mori *et al.*[4] reported that hypertensive patients attaining BP under 140/90 mm Hg by monotherapy were limited to 34.0% with ARBs and 40.3% with CCBs. Additionally, strict BP goals (130/80 mm Hg) are recommended for hypertensive patients with diabetes, chronic kidney disease and old myocardial infarction.[1] Addition of low-dose diuretics is recommended as a key combination therapy for better BP control in the JSH guideline.[1] However, the prescription rate of diuretics remains low in Japan, for example, 9.3% in the J-HOME study.[9] Additionally, combination therapy with diuretics seems to contribute to organ protection. Many large-scale clinical trials have shown organ-protective effects of losartan, and, importantly, the majority of patients in these trials concomitantly used diuretics, for example, 72% in the LIFE (Losartan Intervention For Endpoint) trial and 84% in the RENAAL (Reduction of Endpoints in NIDDM with the Angiotensin-II Antagonist Losartan) trial.[10,11] Therefore, an acceptable and safe way to introduce low doses of diuretics for hypertension therapy is desirable in Japan.

The losartan/HCTZ combination is composed of losartan, which displays superior activity under the activated renin–angiotensin system[12] and a thiazide-diuretic that activates renin–angiotensin system through a diuretic effect,[13] so this combination is expected to be efficient in BP lowering by the synergistic effect of both the drugs. In this study, BP was decreased by 23 ± 17/11 ± 10 mm Hg at 3 months and 24 ± 18/11 ± 11 mm Hg at 12 months after switching from ARBs or ACEIs alone to the losartan/HCTZ combination for patients who did not reach the BP goal with regimens including ARBs or ACEIs. Similar decreases in BP were observed with all types of pre-prescribed ARBs and ACEIs (Figure 4), and thus these strong and steady decreases in BP seem to depend on the HCTZ 'add-on' effect. Salt intake of the Japanese is relatively high,[14] and thus excess salt may suppress the renin–angiotensin system and disturb the ability of ARBs or ACEIs. In particular, this possibility seems high for patients whose BP was not satisfactorily suppressed by ARBs or ACEIs. Alternatively, HTCZ probably works well in that situation, and this possibility is indirectly supported by evidence that patients pre-using the maximum dose of ARBs showed larger decreases in BP than those using the low-to-medium dose of ARBs following introduction of the losartan/HCTZ combination (Figures 2 and 3). Also this synergistic effect is effective in a comprehensive range of patients; over 90% of patients showed meaningful reductions in diastolic BP (⩾10 mm Hg) and 79% of patients reached the BP goals of the JSH guideline, and thus specific cases of diabetes or obesity resistant against losartan/HCTZ combination were not detected.

Diuretics such as HCTZ have been avoided in Japan for fear of their negative effects on metabolic parameters.[4] In particular, hypokalemia and increase in UA are associated with HCTZ. In combination with losartan, hypokalemia may be canceled by the anti-aldosterone effect

of ARBs and UA elevation may be enfeebled by the UA-decreasing ability of losartan. Losartan has a unique effect of stimulating UA excretion in urine by suppressing UA transporters URAT1 and URATv1, with a resulting decrease in the serum levels of UA.[15,16] In this study, these expectations were well achieved and potassium and UA levels were kept within normal ranges. Additionally, a significant decrease in UA was observed for patients with high levels of UA (Figure 5). Except for losartan, clinical doses of ARBs do not have suppressive properties on the UA transporters.[17] This property of losartan should be profitable in combination with HCTZ.

Another concern with HCTZ is worsening of glucose metabolism. A recent cohort study in Taiwan showed that diuretic or β-blocker monotherapy increased the risk of new-onset diabetes, but combination therapies composed of diuretics or β-blocker with ACEI or ARB did not. Conversely, there was a decrease in the risk of new-onset diabetes.[18] In this study, blood glucose and HbA1c levels were stable in patients with diabetes (Table 2), as was glucose level in all patients, and so the losartan/HCTZ combination appears to be safe for glucose metabolism. However, the sensitivity of glucose metabolism under diuretics use could be changed by gene variation,[19] and thus there may be small numbers of susceptible patients. In fact, one patient dropped out because of worsening of diabetes in this study. Therefore, careful monitoring of glucose metabolism is required.

Fixed dose combination drugs decrease the number of pills taken and may contribute to better adherence. Patients on a fixed-combination regimen showed better persistence after 1 year of antihypertensive treatment, namely 58% for combination therapy with ACEI plus diuretics in two pills, and 70% for one-pill fixed combination.[20] In this study, a limited number of patients, 44 of 266 (16.5%), dropped out despite the clinical setting, so this fixed combination could be beneficial in clinical use.

In summary, a fixed dose combination of losartan/HCTZ for 1 year of treatment in a clinical setting resulted in sufficient and steady BP decrease in a majority of Japanese hypertensive patients who had not been controlled with a regimen including ARBs or ACEIs. Also this combination showed acceptable safety and tolerability. A fixed dose combination of losartan/HCTZ is an available tool to introduce low-dose diuretics for treatment of uncontrolled hypertension in Japan.

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APPENDIX

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Structure–function analysis of helix 8 of human calcitonin receptor-like receptor within the adrenomedullin 1 receptor

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ABSTRACT

Adrenomedullin 1 (AM_1) receptor is a heterodimer composed of calcitonin receptor-like receptor (CLR) – a family B G protein-coupled receptor (GPCR) – and receptor activity-modifying protein 2 (RAMP2). Both family A and family B GPCRs possess an eighth helix (helix 8) in the proximal portion of their C-terminal tails; however, little is known about the function of helix 8 in family B GPCRs. We therefore investigated the structure–function relationship of human (h)CLR helix 8, which extends from Glu430 to Trp439, by separately transfecting nine point mutants into HEK-293 cells stably expressing hRAMP2. Glu430, Val431, Arg437 and Trp439 are all conserved among family B GPCRs. Flow cytometric analysis revealed that Arg437Ala or Trp438Ala mutation significantly reduced cell surface expression of the receptor complex, leading to a ~20% reduction in specific ^{125}I-AM binding but little change in their IC_{50} values. Both mutants showed 6–8-fold higher EC_{50} values for AM-induced cAMP production and ~50% reductions in their maximum responses. Glu430Ala mutation also reduced AM signaling by ~45%, but surface expression and ^{125}I-AM binding were nearly the same as with wild-type CLR. Surprisingly, Glu430Ala and Val431Ala mutations significantly enhanced AM-induced internalization of the mutant receptor complexes. Taken together, these findings suggest that within hCLR helix 8, Glu430 is crucial for Gs coupling, and Arg437 and Trp439 are involved in both cell surface expression of the hAM_1 receptor and Gs coupling. Moreover, the Glu430–Val431 sequence may participate in the negative regulation of hAM_1 receptor internalization, which is not dependent on Gs coupling.

1. Introduction

Adrenomedullin (AM) receptors are heterodimers composed of calcitonin receptor-like receptor (CLR) – a family B G protein-coupled receptor (GPCR) – and receptor activity-modifying protein 2 (RAMP2) or RAMP3, both of which are single membrane-spanning domain accessory proteins [19]. CLR/RAMP2 (AM_1 receptor) has proven to be the most highly specific AM receptor and is particularly sensitive to the AM receptor antagonist AM-(22-52) in all species tested [20]. By contrast, CLR/RAMP3 (AM_2 receptor) binds calcitonin gene-related peptide (CGRP) at lower concentrations than it binds AM and is more sensitive to the CGRP receptor antagonist CGRP-(8-37) than is the AM_1 receptor, particularly in rodents [20]. Although intermedin (AM2), like AM, belongs to the calcitonin/CGRP family and is more closely related to AM than to CGRP [23,29], intermedin elicits cAMP production in cells expressing CLR/RAMP3, but not CLR/RAMP2 [23,29].

AM, the endogenous agonist, is a novel vasodilator also shown to be necessary for development of the fetal cardiovascular system and able to powerfully act against various vascular diseases including hypertension, atherosclerosis and secondary lymph edema [7,11,16,18,30]. All of these effects are mediated via the AM_1 receptor [7,11,18,30]; the in vivo function of the AM_2 receptor remains unclear.

When acting as chaperones, RAMP2 and -3 transport CLR molecules from the endoplasmic reticulum to the cell surface, where the CLR/RAMP complex mediates AM-induced intracellular cAMP production and Ca^{2+} mobilization [17]. Upon AM binding, both AM receptors undergo rapid internalization via a clathrin- and β-arrestin-dependent pathway without dissociation of the CLR and RAMP molecules [8,17].

At present the crystal structures of four family A GPCRs are available: rhodopsin, β_1-adrenergic receptor, β_2-adrenergic receptor and adenosine A_{2A} receptor [21,24]. The structural findings revealed the presence of an eighth helix (helix 8), oriented per-

Abbreviations: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; C-tail, cytoplasmic C-terminal tail; CLR, calcitonin receptor-like receptor; GPCR, G protein-coupled receptor; GRK, GPCR kinase; h, human; HEK, human embryonic kidney; Hyg, hygromycin; RAMP, receptor activity-modifying protein; RCP, CGRP-receptor component protein; WT, wild-type.

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pendicular to the seven transmembrane helixes, in the proximal portion of the C-terminal tail (C-tail). To date, helix 8 of family A GPCRs has been shown to play key roles in the cell surface expression of GPCRs, stabilization of GPCRs at the cell-surface, the conformational switch involved in GPCR activation, Gs coupling, Gq coupling and activation of GPCR kinases [4,25,28,31,34]. On the other hand, no crystal structures of family B GPCRs are currently available, and little is known about the function of helix 8 within the receptors. Recently, Conner et al. [2] used mutagenesis and various synthetic peptides to characterize helix 8 in human (h)CLR. They showed that putative hCLR helix 8 extends from Glu430 to Trp439 and contains four residues, Glu430, Val431, Arg437 and Trp439, which are strictly conserved among family B GPCRs. In addition, putative hCLR helix 8 possesses an Arg436–Arg437 sequence, which an earlier study suggests may be part of a consensus motif involved in Gs coupling [32]. But to our knowledge, there is still no detailed information concerning the structure–function relationship of putative hCLR helix 8 complexed within AM receptors. To address that issue, we examined the effects of nine hCLR helix 8 point mutants separately expressed in human embryonic kidney (HEK)-293 cells stably expressing hRAMP2 [13], which enables hCLR to function as a hAM_1 receptor.

2. Materials and methods

2.1. Reagents and antibodies

Human AM was kindly donated by Shionogi & Co. (Osaka, Japan). FITC-conjugated mouse anti-V5 monoclonal antibody (anti-V5-FITC antibody) was from Invitrogen. All other reagents were of analytical grade and obtained from various commercial suppliers.

2.2. Expression constructs

Double V5-tagged hCLR (V5-hCLR) [14] was cloned into pIRES1/Hyg, yielding pIRES1-V5-hCLR [13]. Nine point mutations within putative hCLR helix 8 were introduced using a QuikChange® kit (Stratagene) according to the manufacturer's instructions, with pIRES1-V5-hCLR serving as the template. For each mutation, two complementary 30- to 40-mer oligonucleotides (sense and antisense) were designed with the mutation in the middle. The resultant mutants were all sequenced using an Applied Biosystems 310 Genetic Analyzer.

2.3. Cell culture and DNA transfection

Several hygromycin (Hyg)-resistant HEK 293 cell clones stably expressing hRAMP2 were isolated previously [15]. Among them, the clone that most efficiently promoted AM-mediated V5-hCLR internalization [15] was selected and maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 100 μg/ml Hyg at 37 °C under a humidified atmosphere of 95% air/5% CO_2.

Transient transfection of HEK-293 cells stably expressing hRAMP2 was performed using LipofectAMINE as previously described [13]. Briefly, the cells were seeded into 12-well plates (for flow cytometric analysis) or 24-well plates (for binding and cAMP assays) and, upon reaching 70–80% confluence, were transfected with empty vector (pIRES1/Hyg) (*Mock*) or V5-tagged wild-type (WT) or mutant constructs; at all times, V5-hCLR was included in each transfection set. This was accomplished by incubating the cells for 4 h in OptiMEM 1 medium containing plasmid DNAs (0.2 μg/well for 24-well plates; 0.4 μg/well for 12-well plates), Plus regent (2 μl/well for 24-well plates; 2.5 μl/well for 12-well plates) and LipofectAMINE (2 μl/well for 24-well plates; 2.5 μl/well for 12-well plates), and all experiments were performed 48 h after transfection. Separate transfections were carried out with different passage number (1–5) hRAMP2-expressing cells; the intra- and inter-assay coefficients of variance were less than ~10%.

2.4. Flow cytometric analysis

Following transfection of the indicated V5-tagged cDNAs into hRAMP2-expressing HEK-293 cells in 12-well plates, the cells were exposed to selected concentrations of hAM in prewarmed serum-free DMEM containing 20 mM Hepes and 0.5% bovine serum albumin for 60 min at 37 °C. Receptor internalization was stopped by adding ice-cold PBS, after which the cells were harvested, resuspended in ice-cold buffer for flow cytometric analysis [17] and labeled with anti-V5 FITC antibody (1:1000 dilution) for 60 min at 4 °C in the dark. Following two successive washes, the cells were subjected to flow cytometry in an EPICS XL flow cytometer (Beckman Coulter), and cell surface expression of each V5-tagged receptor, before and after exposing cells to AM, was analyzed using EXPO 2 software (Beckman Coulter) [17].

2.5. Radioiodination and radioligand binding

^{125}I-hAM (specific activity 5 μCi/pmol) was produced in our laboratory, as previously described [12]. Briefly, hAM (10 μg) in 25 μl 0.4 M sodium acetate buffer (pH 5.6) was introduced into a tube followed by the addition of $Na^{125}I$ (0.5 mCi/5 μl, MP Biomedicals). Lactoperoxidase (Calbiochem: 600 ng/10 μl of 0.1 M sodium acetate, pH 5.6) and H_2O_2 (70 ng/5 μl of water) were then added. After letting the tube stand for 10 min at 33 °C, additional H_2O_2 (100 ng/5 μl of water) was added, and the tube was left for another 10 min at 33 °C. Immediately thereafter, the mixture was submitted to reverse-phase high-performance liquid chromatography, and mono-iodinated hAM was purified and used for the following binding assay.

To assess whole-cell radioligand binding, transfected HEK-293 cells in 24-well plates were washed twice with prewarmed PBS and then incubated with 20 pM ^{125}I-hAM for 5 h at 4 °C in the presence (for nonspecific binding) or absence (for total binding) of 1 μM unlabeled hAM in modified Krebs–Ringers–Hepes medium [17]. After washing the cells twice with ice-cold PBS, they were harvested in 0.5 M NaOH, and the associated cellular radioactivity was measured in a γ-counter. Specific binding was defined as the difference between the total binding (Bo, in the absence of hAM) and nonspecific binding (N, measured in the presence of 1 μM AM). The *Y*-axis for calculating IC_{50} values for hAM in competition with ^{125}I-hAM for binding to transfected HEK-293 cells is $(B - N)/(Bo - N) \times 100$ (%), where B is the ^{125}I-hAM bound.

2.6. Measurement of intracellular cAMP

Transfectants in 24-well plates were incubated for 15 min at 37 °C in Hanks' buffer containing 20 mM Hepes, 0.2% bovine serum albumin, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma) and the indicated concentrations of hAM. The reactions were terminated by addition of lysis buffer (GE Healthcare), after which the cAMP content was determined using a commercial enzyme immunoassay kit according to the manufacturer's instructions (GE Healthcare) for a non-acetylation protocol.

2.7. Data analysis and statistics

Competitive binding data (IC_{50} values) and cAMP concentration–response data (EC_{50} values and maximum responses) were analyzed using a four-parameter logistic equation with MasterPlex ReaderFit software (Hitachi Software Engineering America Ltd., USA). Results are expressed as means ± SEM of

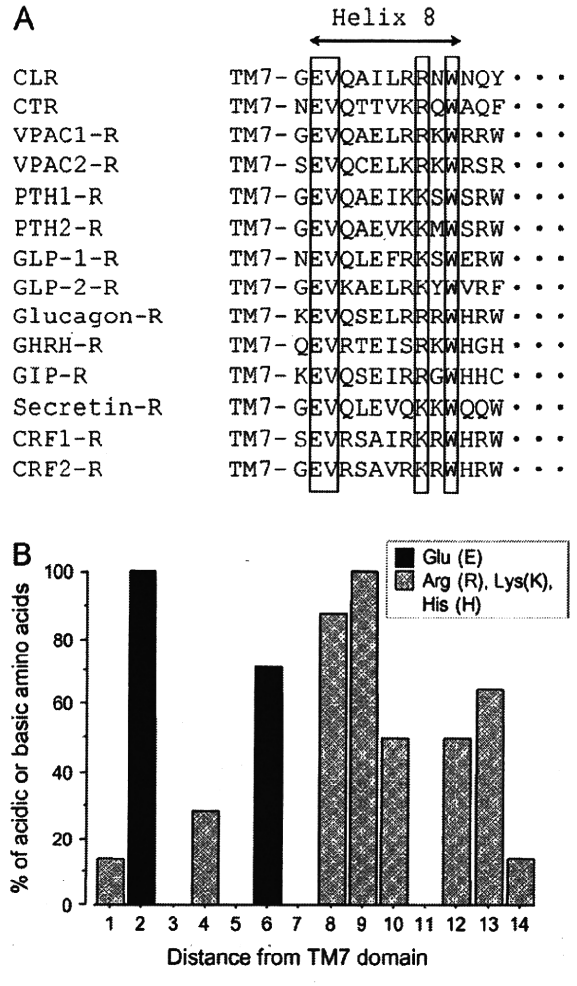

Fig. 1. (A) Alignment of the first 14 residues of the C-terminal tails of 14 family B hGPCRs. Four conserved residues, E (Glu), V (Val), R (Arg) and W (Trp), are *boxed*. CTR, calcitonin receptor; VPAC-R, vasoactive intestinal polypeptide, pituitary adenylyl cyclase activating peptide receptor; PTH, parathyroid hormone; GLP, glucagon-like peptide; GHRH, growth hormone releasing hormone. (B) Distribution of charged amino acid residues within the first residues of 14 family B hGPCR C-tails. Putative helix 8 of family B GPCRs is situated between positions 2 and 11. Acidic residues (E (Glu)) occur most frequently at positions 2 and 6, while dibasic residues (R (Arg) and K (Lys)) occur most frequently at positions 8 and 9.

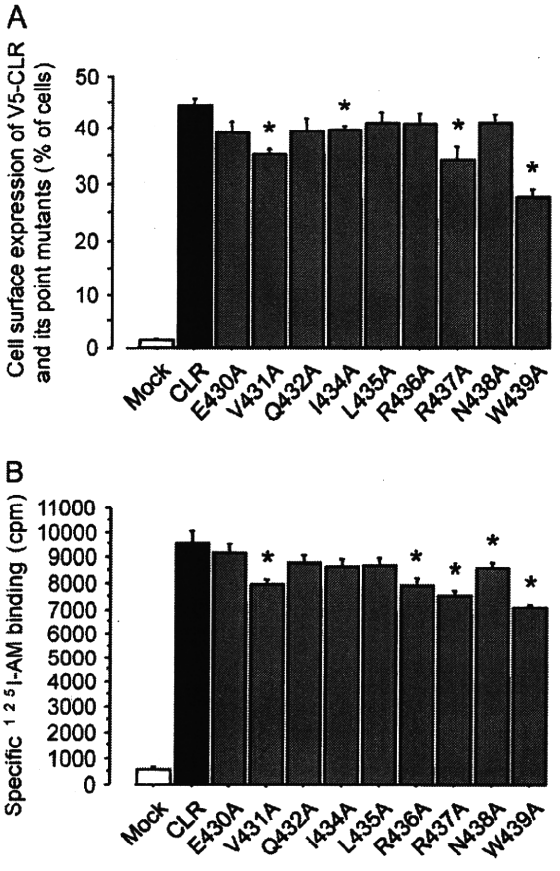

Fig. 2. Characterization of hCLR helix 8 point mutants in HEK-293 cells stably expressing hRAMP2. (A) Flow cytometric analysis of the cell surface expression of the indicated V5-tagged proteins. Transfected cells were incubated for 1 h at 4 °C with anti-V5-FITC antibody; mock incubation with the antibody served as the control. Surface V5-FITC-labeled proteins are expressed as % of cells. Cell surface expression of WT-hCLR and all hCLR helix 8 point mutants was significantly higher than that seen with mock ($p < 0.05$). Bars represent means ± SEM of six independent experiments. *$p < 0.05$ vs. hCLR. (B) Specific binding of ^{125}I-hAM. Transfected cells were incubated for 5 h at 4 °C with ^{125}I-AM (20 pM) in the presence or absence of 1 μM unlabeled hAM. Specific ^{125}I-AM binding to WT-hCLR and all hCLR helix 8 point mutants was significantly higher than that seen with mock ($p < 0.05$). Bars represent means ± SEM of six experiments. *$p < 0.05$ vs. hCLR.

at least five independent experiments; only bars in the cAMP dose–response curves (Fig. 3) are expressed as means ± SD due to limitations of the software. Differences between two groups were evaluated using Student's *t*-tests; differences among multiple groups were evaluated using one-way analysis of variance followed by Scheffe's tests. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Amino acid sequence alignment of putative helix 8 in family B GPCRs

Fig. 1A is based on results from Conner et al. [2] and shows the alignment of the first 14 residues of the C-terminal tails of 14 family B hGPCRs. Putative helix 8 is situated between positions 2–11 [2]. Among the 14 residues shown, the four boxed residues, E (Glu), V (Val), R (Arg) and W (Trp), all of which are within helix 8, are strictly conserved. It is also noteworthy that acidic residues (E (Glu)) occur most frequently at positions 2 and 6, while dibasic residues (R (Arg) and K (Lys)) occur most frequently at positions 8 and 9 (Fig. 1B).

3.2. Cell surface expression of point mutants of putative hCLR helix 8

We recently established a line of HEK-293 cells stably expressing hRAMP2 [13], which, prior to transfection with hCLR, lack AM receptors (Fig. 3). This enabled us to use these cells to examine the role of helix 8 in cellular trafficking and receptor signaling mediated via hCLR within the AM_1 receptor complex (hCLR/RAMP2). We accomplished this by testing the effects of nine helix 8 point mutations on receptor function.

Fig. 2A shows the cell surface expression of V5-tagged WT protein and the nine point mutants following their separate transfection into hRAMP2-expressing HEK-293 cells. When the cells were transfected with empty vector (*Mock*), surface binding of anti-V5-FITC antibody was within the 2% limit of resolution characteristic of flow cytometric analysis. Similar to WT-hCLR (44.2 ± 1.5%), the E430A, Q432A, I434A, L435A, R436A and N438A mutants appeared at the surface of 39–42% of cells. By contrast, cell

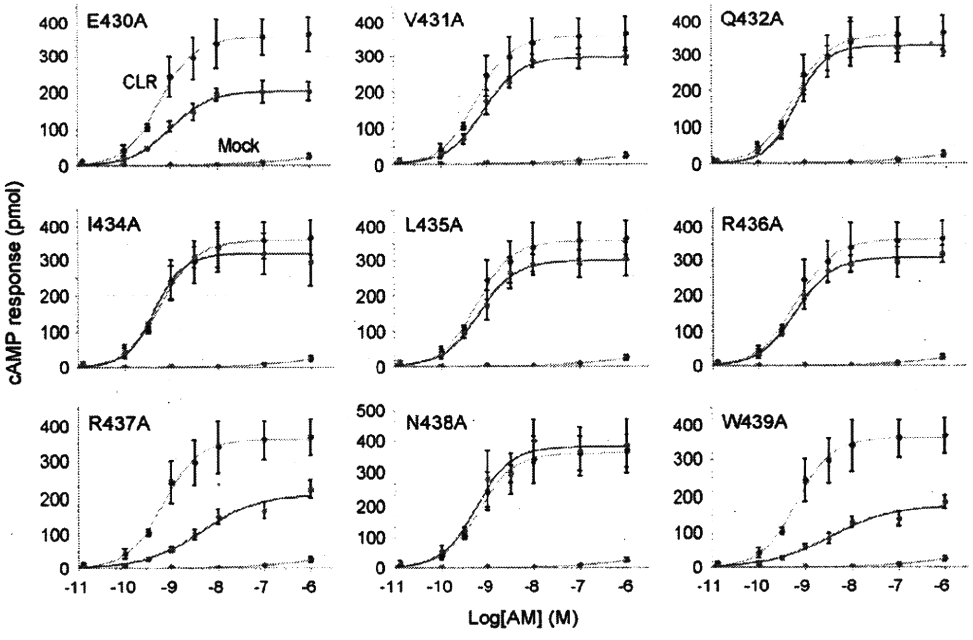

Fig. 3. AM-evoked cAMP production mediated via hCLR helix 8 point mutants in HEK-293 cells stably expressing hCLR. All cells transfected with wild-type or mutant hCLR were simultaneously exposed to the indicated concentrations of hAM for 15 min at 37 °C and then lysed. The resultant lysates were analyzed for cAMP content. Note that the same cAMP responses mediated via wild-type hCLR are shown in every mutant-mediated response. Bars are expressed as means ± SD of five experiments due to limitations of the MasterPlex ReaderFit software used.

surface expression of V431A (35.3 ± 1.0%), R437A (34.1 ± 2.4%) and W439A (27.4 ± 1.0%) was significantly lower than was seen with WT-hCLR.

3.3. Effect of helix 8 point mutation on ^{125}I-AM binding to hCLR

Fig. 2B shows that in cells expressing WT-hCLR (IC_{50} = 22.4 ± 3.0 nM), specific ^{125}I-AM binding was about 20-fold higher than in cells expressing empty vector (*Mock*), and similar levels of ^{125}I-AM binding were seen in cells expressing E430A, Q432A, I434A, L435A or N438A. On the other hand, the IC_{50} values for V431A (13.1 ± 2.0 nM), Q432A (15.0 ± 1.3 nM), R436A (14.4 ± 2.7 nM) and R437A (15.7 ± 1.4 nM) were somewhat lower, and the IC_{50} value for W439A (8.2 ± 1.2 nM) was significantly lower, than was seen with WT-hCLR ($p < 0.05$). The remaining mutants' IC_{50} values ranged from 18.3 to 19.4 nM.

3.4. Functionality of hCLR helix 8 point mutants

The functionality of the mutant receptors was assessed by measuring AM-induced intracellular cAMP production (Fig. 3 and Table 1). AM elicited little or no cAMP production in HEK-293 cells expressing hRAMP2 alone. Following transfection with WT-hCLR, however, AM elicited concentration-dependent increases in cAMP, and comparable responses were seen when cells were transfected with Q432A, I434A, L435A or N438A. The maximum cAMP levels obtained with the remaining five mutants were significantly lower than those obtained with WT-hCLR. In addition, the R437A and W439A mutants exhibited significant increases in their EC_{50} values.

3.5. Internalization of hCLR helix 8 point mutants

Our earlier flow cytometric analysis revealed that AM dose-dependently induced hRAMP2-mediated internalization of hCLR, with the peak of the receptor's internalization occurring after about 60 min [15]. In the same study, we also showed that there was no significant difference in the efficiency of receptor internalization induced by 0.1 μM and 1.0 μM AM [15]. We therefore evaluated the internalization of each point mutant after exposing hRAMP2-expressing cells to 0.1 μM AM for 60 min (Fig. 4). Surprisingly, we found that four mutants, E430A, V431A, Q432A and W439A, significantly enhanced AM-induced internalization of the mutant receptor complexes, despite the fact that E430A and W439A significantly reduced AM signaling (Fig. 3). The remaining mutants had little effect on receptor internalization.

4. Discussion

All family B GPCRs are preferentially coupled to Gs proteins responsible for stimulating cAMP production, and many undergo rapid internalization in response to an agonist. However, the roles of their C-tails in Gs coupling and in internalization have not been fully explored. We recently showed that AM elicits little or no cAMP production or internalization of receptor complexes composed of hRAMP2 and a hCLR truncation mutant totally lacking its

Table 1
AM-induced cAMP production in RAMP2-expressing HEK-293 cells co-transfected with wild-type CLR and its point mutants.

Construct	cAMP production	
	EC_{50} (nM)	Maximum response (pmol)
CLR	0.68 ± 0.10	372.4 ± 21.6
E430A	1.10 ± 0.11*	206.0 ± 9.5*
V431A	0.92 ± 0.05	300.4 ± 10.6*
Q432A	0.82 ± 0.11	328.2 ± 12.6
I434A	0.47 ± 0.04	319.3 ± 24.1
L435A	1.05 ± 0.44	306.8 ± 19.6
R436A	0.68 ± 0.09	305.6 ± 13.6*
R437A	5.15 ± 1.86*	215.7 ± 13.0*
N438A	0.59 ± 0.08	386.8 ± 29.4
W439A	3.99 ± 0.97*	182.8 ± 7.6*

The results represent the mean ± SEM of five independent experiments.
* $p < 0.05$ vs. CLR.

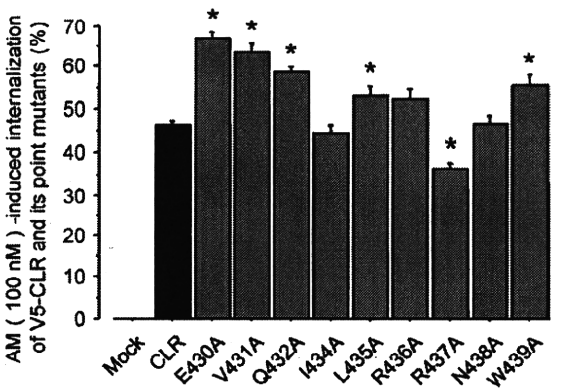

Fig. 4. Flow cytometric analysis of internalization of hCLR helix 8 point mutants in HEK-293 cells stably expressing hRAMP2. Surface expression of each construct was estimated by flow cytometry before and after exposing the cells to 0.1 μM hAM for 60 min. Internalization was estimated as the percentage of receptors lost from the cell surface after AM exposure. Internalization of WT-hCLR and all hCLR helix 8 point mutants was significantly higher than that seen with mock ($p < 0.05$). The results represent the means ± SEM of six independent experiments. *$p < 0.05$ vs. hCLR.

C-tail, despite their full cell surface expression and high-affinity ^{125}I-AM binding [13]. This means the hCLR C-tail is crucial for both AM-induced cAMP production and hAM_1 receptor internalization, which is in contrast to the earlier report that the hCLR C-tail is involved in agonist-mediated receptor internalization, but not cAMP production [2]. That finding was obtained using COS-7 cells transiently co-expressing hRAMP1 and an hCLR mutant in which the C-tail was completely removed. The discrepancies between that earlier study and our present one may reflect differences in the cell backgrounds, transfection methods, cell-surface expression levels of the target receptor complex, cAMP assays and/or the hRAMP isoform.

We observed that substituting Glu430 in putative hCLR helix 8 with Ala (E430A) reduced maximum cAMP levels by ~45% without altering the cell surface expression of the receptor or its affinity for ^{125}I-AM in HEK-293 cells stably expressing hRAMP2. This suggests Glu430 is required for the coupling of hCLR helix 8 to Gs. Notably, this negatively charged residue is strictly conserved among B GPCRs (Fig. 1), and a conserved Glu residue is also reportedly involved in Gs coupling to putative helix 8 in the hVPAC1 receptor in CHO cells [3]. In that case, substituting Glu394 with Ala (E394A) reduced cAMP responses by ~45%, although cell surface expression of the mutant receptor and agonist binding were identical to those in the wild-type receptor [3]. The C-tail of porcine calcitonin receptor is also required for evoked cAMP production [6], but the role of the conserved Glu residue in that receptor remains unknown. On the other hand, evidence suggests that the C-tails of the rat glucagon [33], parathyroid [10] and secretin receptors [9] may not be involved in coupling to Gs proteins. However, all of those studies made use of a truncation approach, which left some or all of helix 8 intact. It is therefore possible that the strictly conserved Glu residue in helix 8 is generally involved in coupling family B GPCRs to Gs.

Putative hCLR helix 8 also possesses two positively charged dibasic residues (Arg436–Arg437), both of which are highly conserved among family B GPCRs; in particular, Arg437 is strictly conserved (Fig. 1). In the present study, R437A mutation of hCLR reduced cell surface expression of the mutant receptor by ~25%, thereby reducing specific ^{125}I-AM binding by ~20%. Moreover, this mutant reduced maximum cAMP levels by ~40%, with an ~8-fold increase in the EC_{50} value. Thus, Arg437 also appears to participate in both the coupling to Gs proteins and the proper surface delivery of hCLR. Although Arg436 is next to Arg437, R436A mutation had little effect on the cell surface expression or the cAMP responses of the mutant receptor complex. By contrast, double mutation of Arg400–Arg401 to Ala400–Ala401 did not affect cell surface expression, radioligand binding or cAMP responses of the mutant hVPAC1 receptor [3]. It remains to be seen whether the highly conserved Arg residues in hCLR helix 8 are also important for other family B GPCR functions.

Unlike helix 8 in rhodopsin-like receptors (family A GPCRs), helix 8 in family B GPCRs, including hCLR, contains no cysteine residues, the palmitoylation of which has been shown to anchor the helix to the plasma membrane. Nonetheless, a recent analysis revealed that a synthetic hCLR helix 8 peptide readily anchors to liposomes in a membrane-parallel orientation via Trp439, which is strictly conserved among family B GPCRs [2]. In the intact receptor, therefore, a tethered Trp439 may partially fulfill the role of the lipid anchor seen at the equivalent position in many family A GPCRs. This function would be unaffected by the hRAMP isoform, as the hCLR C-tail does not interact with any of the three hRAMP isoforms [14]. In the present study, W439A mutation of hCLR reduced cell surface expression of the mutant receptor by ~40%, and there was a corresponding ~25% reduction in specific ^{125}I-AM binding, with a ~3-fold decline in the IC_{50} value, as compared to WT-hCLR. In addition, maximum cAMP levels were reduced by ~50%, with a ~6-fold increase in the EC_{50} value. Thus Trp439 appears to be important for both cell surface expression of the hAM_1 receptor and AM-mediated cAMP responses mediated via the receptor. Analysis of the crystal structure will be necessary to determine whether this hydrophobic residue serves as a lipid anchor in the intact form of hCLR.

It has been shown that CGRP-receptor component protein (RCP), an intracellular peripheral membrane protein, is specifically required for CLR/RAMP signal transduction [5,22]. In mouse NIH3T3 cells endogenously expressing RCP, CLR and RAMP1 or -2, RCP co-immunoprecipitates with CLR and appears to assist CGRP and AM receptor coupling to Gs [5,22]. However, nothing is known about the CLR domains responsible for the interaction with RCP, the mechanism by which RCP couples CLR to the cellular signal transduction pathway, or the role of RCP in vivo. In future experiments, it will be important to clarify whether RCP interacts with the three hCLR point mutants, E430A, R437A and W439A, all of which significantly reduced AM signaling when co-expressed with hRAMP2.

We also found that the E430A and V431A mutations significantly enhanced AM-induced internalization of the mutant receptor complexes, whereas the other mutations had little effect on internalization. As mentioned above, E430A mutation markedly reduced cAMP responses, and V431A also reduced the responses by ~20%. Taken together, these results suggest that internalization of hAM_1 receptors does not depend on Gs coupling, which is consistent with our earlier findings obtained using various C-tail deletion mutants [13]. Our present results are also supported by a recent report showing that introduction of a dileucine into helix 8 of the formyl peptide receptor (a family A GPCR) significantly enhances receptor internalization [27].

We previously showed that four GPCR kinases (GRK-2, -3, -4 and -5) bind to the Ser/Th-rich regions distal to putative hCLR helix 8, and that overexpression of these GRKs significantly enhanced internalization of the hAM_1 receptor in HEK-293 cells expressing endogenous GRKs 2–6 [13]. The helix 8 region of GPCRs moves significantly upon receptor activation [1,26,34], making it a candidate for recognition by GRKs. Perhaps the conformation of the E430A and V431A hCLR mutants favors interaction with intracellular binding partners such as GRKs.

In conclusion, we have shown that within putative hCLR helix 8, the strictly conserved Glu430 residue is crucial for Gs coupling, and that the strictly conserved Arg437 and Trp439 residues are involved in both cell surface expression of the hAM_1 receptor and Gs

coupling. We also suggest that the Glu430–Val431 sequence participates in the negative regulation of hAM_1 receptor internalization, which is not dependent on Gs coupling.

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ORIGINAL ARTICLE
Cardiovascular Intervention

Repeated Sirolimus-Eluting Stent Implantation to Treat Sirolimus-Eluting Stent and Bare-Metal Stent Restenosis

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Background: In-stent restenosis (ISR) remains a persistent, unresolved issue even in the era of percutaneous coronary intervention (PCI) using drug-eluting stents. The present study compares the clinical and angiographic outcomes of using sirolimus-eluting stents (SES) for re-intervention against ISR that was originally treated with sirolimus-eluting or bare-metal (BMS) stents.

Methods and Results: This prospective single-center registry investigated 179 ISR lesions in 158 consecutive patients (53 lesions in 49, and 126 in 109 patients originally treated with SES and BMS, respectively), who had undergone re-intervention with SES. The patients were clinically and angiographically followed up at 8 months after re-PCI. The incidence of re-restenosis (29 vs 12%, P<0.01), ischemia-driven target lesion revascularization (TLR; 21 vs 8%, P<0.05) and major adverse cardiac events (MACE; 21 vs 9%, P<0.05) were significantly greater in ISR lesions originally treated with SES than in those originally treated with BMS at 8 months after re-PCI. Moreover, late luminal loss was significantly greater in the group with post-SES restenosis (P<0.05). Even after adjustment, post-SES restenosis was the only independent predictor of re-restenosis and MACE (P<0.05, each).

Conclusions: Although the re-restenosis rate is acceptable, the incidence rates of late restenosis, ischemia-driven TLR and MACE are higher after repeated SES implantation to treat SES, than BMS restenosis. These results might affect the mid-term clinical outcomes of re-intervention with SES. (*Circ J* 2010; **74:** 2329–2333)

Key Words: Cardiovascular diseases; Coronary re-intervention; Ischemia; Revascularization

Although drug-eluting stents (DES) have significantly decreased the incidence of in-stent restenosis (ISR) and the need for repeated revascularization compared with bare-metal stents (BMS),[1–5] DES restenosis still develops and ISR remains an important clinical issue especially for patients with highly complex lesions.[2–7] The increasing use of DES in complex settings coupled with the worldwide implantation of >10 million DES[6] implies that DES restenosis will become a significant global problem. However, an optimal treatment for DES restenosis remains unknown, and some experts propose repeated DES implantation. In contrast, several studies have shown the effectiveness of DES in patients with BMS ISR.[4,5] A recent study suggested different pathological features between intra-DES and intra-BMS restenotic tissue.[8] These 2 types of ISR lesion might have different biological responses and clinical outcomes after DES implantation. We therefore compared the clinical and angiographic outcomes of re-intervention with sirolimus-eluting stents (SES; Cypher, Cordis/Johnson & Johnson, Warren, NJ, USA) for ISR lesions that were originally treated with SES or BMS.

Methods

Study Patients

The study population of this prospective single-center registry comprised 158 consecutive patients (179 lesions) who underwent re-intervention with SES for ISR between August 2004 and June 2007. Among them, restenosis developed in 49 patients with SES (53 lesions) and in 109 with BMS (126

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Table 1. Baseline Clinical Characteristics of Patients

Patients (n)	SES (n=49)	BMS (n=109)	P value
Age (years)	67±8.3	66.6±10.8	0.88
Male gender, n (%)	41 (84)	92 (84)	>0.99
Previous MI, n (%)	20 (41)	65 (60)	0.04
Previous CABG, n (%)	1 (2)	5 (5)	0.75
Risk factor, n (%)			
Hypertension	36 (73)	80 (73)	>0.99
Hyperlipidemia	28 (57)	42 (41)	0.09
Current smoking	6 (12)	19 (17)	0.55
Diabetes mellitus	28 (57)	36 (33)	<0.01

Data are expressed as mean±SD when appropriate.
SES, sirolimus-eluting stent; BMS, bare-metal stent; MI, myocardial infarction; CABG, coronary artery bypass graft.

Table 2. Baseline Angiographic and Procedural Characteristics

Target lesion (n)	SES (n=53)	BMS (n=126)	P value
Target vessel, n (%)			0.32
Left anterior descending	19 (36)	57 (45)	
Left circumflex	13 (24)	20 (16)	
Right coronary artery	21 (40)	49 (39)	
In-stent restenosis type, n (%)*			<0.01
Focal			
I	36 (68)	25 (20)	
Non-focal			
II	11 (21)	65 (52)	
III	5 (9)	29 (23)	
IV	1 (2)	7 (5)	
Pre-procedure			
Reference diameter (mm)	2.66±0.54	2.66±0.61	0.97
MLD (mm)	0.58±0.37	0.74±0.41	0.02
Diameter stenosis (%)	77.9±13.9	71±17.6	0.01
Lesion length (mm)	17±6.2	21±8.1	<0.01
Post-procedure MLD (mm)	2.68±0.48	2.79±0.51	0.18

Data are expressed as mean±SD when appropriate.
MLD, minimal lumen diameter. Other abbreviations see in Table 1.
*According to the classification by Mehran et al.[9]

lesions). Patients were eligible for the study if they had initial ISR in a native coronary artery with objective evidence of ischemia and without clinical contraindication against prolonged double antiplatelet therapy. All patients provided written, informed consent to participate in the study, and our institutional ethics committee approved the study protocol. We defined ISR using quantitative coronary angiography (QCA) as luminal stenosis of >50% within the stent or within 5 mm of the stent edges. The type of restenosis was categorized as focal (length <10 mm) and non-focal (diffuse, proliferative and occlusive) according to the classification of Mehran et al.[9]

Procedural anticoagulation therapy included heparin targeted to an activated clotting time of 200 to 300 s. All patients underwent repeated percutaneous coronary intervention (re-PCI) with SES according to current guidelines, and the choice of the implanted PCI devices was left to the operators' discretion. Then, if possible, intravascular ultrasound (IVUS)-guided re-PCI was performed to rule out a possible mechanism of stent failure such as underexpansion and to confirm neointimal growth in ISR lesions. All patients received aspirin (100 or 200 mg/day) before, and indefinitely after the procedure. Patients were also concomitantly treated with ticlopidine (200 mg/day) or clopidogrel (50 mg/day or 75 mg/day)[10] for at least 8 months. The patients were followed up angiographically at 8 months post re-PCI or earlier if non-invasive evaluation or clinical presentation suggested ischemia.

Quantitative Coronary Angiography

Coronary angiograms were analyzed using a validated edge detection system (CMS, MEDIS, Leiden, The Netherlands) by 2 experienced cardiologists (K.N. and T.N.) who were blinded to the clinical classification of the patients. Minimal lumen diameter (MLD), reference vessel diameter, and %diameter stenosis at baseline, post procedure and at follow up were measured. Angiographic re-restenosis was defined by QCA as stenosis of >50% diameter within a previously stented segment (within the stent or 5 mm of the stent edges) on 8-month follow-up angiograms. Late luminal loss was defined as the difference between MLD at the time of the post stenting procedure and that at follow up.

Clinical Follow up

Patients were followed up to assess the incidence of major

Table 3. Quantitative Angiographic Data and Clinical Outcomes at 8 Months of Follow up

Target lesion (n)	SES group (n=53)	BMS group (n=125)	P value
Follow up			
Reference diameter (mm)	2.89±0.45	2.93±0.54	0.66
MLD (mm)	2.01±1.08	2.41±0.91	0.01
Diameter stenosis (%)	30.3±33.6	17.9±26.6	0.02
Angiographic re-restenosis, n (%)	16 (29)	15 (12)	<0.01
TLR, n (%)	11 (21)	10 (8)	0.02
TVR, n (%)	11 (21)	11 (9)	0.04
MACE, n (%)	11 (21)	11 (9)	0.04
Stent thrombosis, n (%)	2 (4)	1 (1)	0.16

Data are expressed as mean±SD when appropriate.
TLR, target lesion revascularization; TVR, target vessel revascularization; MACE, major adverse cardiac event. Other abbreviations see in Tables 1 and 2.

adverse cardiac events (MACE) including all-cause death, myocardial infarction (MI), target lesion revascularization (TLR) and target vessel revascularization (TVR). We defined MI as the presence of a new Q wave in ≥2 contiguous ECG leads and/or a creatine kinase-MB concentration elevated to >3-fold the upper normal limit. We defined TLR as repeated re-intervention to treat an ISR accompanied by symptoms or objective signs of ischemia (ischemia-driven TLR). We then defined TVR as any repeated revascularization procedure involving a target vessel with an ischemic sign (ischemic-driven TVR). Stent thrombosis was defined according to the designation established by the Academic Research Consortium.[11]

Statistical Analysis

Data are expressed as mean±SD. The differences between the 2 groups were analyzed using an unpaired Student's t-test or the Mann-Whitney U test when the variance was skewed. Categorical variables were compared by Fisher's exact probability test. We also assessed independent predictors of angiographic re-restenosis and MACE using multivariate logistic regression analysis with independent variables that were considered significant according to univariate analyses. All tests were 2-sided and a P value of <0.05 was considered statistically significant.

Results

We studied a total of 179 ISR lesions in 158 consecutive patients (53 SES restenotic lesions in 49 patients, and 126 BMS restenotic lesions in 109 patients) between August 2004 and June 2007. All patents were successfully implanted with SES and 178 patients (99%) were followed up by angiography, for which 1 patient refused to undergo.

Tables 1 and **2** show baseline demographic, clinical, and angiographic characteristics and procedural variables. Patients with post-SES restenosis had a higher prevalence of diabetes mellitus and a lower prevalence of MI history than those with post-BMS restenosis (**Table 1**). Patients with SES restenosis also had more frequent focal restenosis, lesions with a significantly smaller MLD, a higher rate of %diameter stenosis and shorter lesions than those with BMS restenosis (**Table 2**). No periprocedural MI, TLR or stent thrombosis or in-hospital deaths occurred.

Table 3 and **Figure** show the QCA data and clinical outcomes at 8 months of follow up. The MLD was significantly smaller and the rate of %diameter stenosis was more increased in ISR lesions originally treated with SES than with BMS. Interestingly, the rates of angiographic re-restenosis (29% vs 12%, P<0.01), TLR (21% vs 8%, P=0.02), TVR (21% vs 9%, P=0.04) and MACE (21% vs 9%, P=0.04) were significantly higher in the group with post-SES than post-BMS restenosis (**Table 3**). Moreover, late luminal loss was significantly greater in the SES than in the BMS group (0.68±1.01 vs 0.36±0.77 mm, P=0.04; **Figure**). The type of re-restenosis was generally focal in both groups (data not shown). No cardiac death and Q-wave MI occurred during follow up. Stent thrombosis resulting in unstable or stable angina pectoris developed in 1 (1%) and 2 (4%) patients in the BMS and SES groups, respectively, but the difference was not statistically significant.

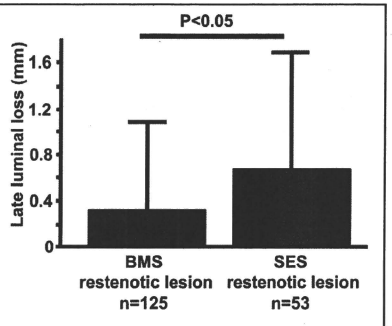

Figure. Late luminal loss in groups with post-BMS and post-SES restenosis. Data are expressed as means±SD. BMS, bare-metal stent; SES, sirolimus-eluting stent.

Multivariate logistic regression analysis also showed that repeated SES implantation to treat restenosis of SES is an independent predictor of angiographic re-restenosis and MACE (odds ratio (OR) 3.4, 95% confidence interval (CI) [1.28–9.02], P=0.01 and OR 3.37, 95%CI [1.08–10.49], P=0.04, respectively) compared with BMS, after including all variables that were significant in the univariate analyses listed in **Tables 1** and **2** (**Tables 4,5**).

Also, the incidence of re-restenosis and MACE did not differ between patients with and without diabetes mellitus (19% vs 16%, P=0.7 and 14% vs 11%, P=0.78, respectively).

Table 4. Univariate and Multivariate Predictors of Re-Restenosis

	Univariate			Multivariate		
	OR	95%CI	P value	OR	95%CI	P value
SES group	3.2	1.44–7.1	<0.01	3.4	1.28–9.02	0.01
Diabetes mellitus	1.24	0.57–2.71	0.59	0.92	0.4–2.12	0.85
Previous MI	0.68	0.31–1.47	0.68	0.81	0.36–1.85	0.62
Pre-procedure						
MLD	0.52	0.19–1.45	0.21	0.48	0.08–2.91	0.42
Lesion length	0.96	0.91–1.02	0.18	0.97	0.92–1.03	0.36
% Diameter stenosis	1.01	0.99–1.04	0.34	0.99	0.95–1.04	0.72
Lesion type: Focal	1.28	0.57–2.84	0.55	0.59	0.22–1.56	0.29

Variables were P<0.05 in univariate analyses listed in Tables 1 and 2.
OR, odds ratio; CI, confidence interval. Other abbreviations see in Tables 1 and 2.

Table 5. Univariate and Multivariate Predictors of MACE

	Univariate			Multivariate		
	OR	95%CI	P value	OR	95%CI	P value
SES group	3.04	1.2–7.67	0.02	3.37	1.08–10.49	0.04
Diabetes mellitus	1.37	0.55–3.42	0.5	1.05	0.4–2.74	0.92
Previous MI	0.54	0.21–1.35	0.19	0.61	0.23–1.61	0.32
Pre-procedure						
MLD	0.83	0.26–2.59	0.74	0.82	0.1–6.79	0.86
Lesion length	0.97	0.91–1.04	0.43	0.99	0.92–1.05	0.65
% Diameter stenosis	1	0.98–1.03	0.8	0.99	0.94–1.04	0.75
Lesion type: Focal	1.22	0.48–3.12	0.68	0.61	0.2–1.88	0.39

Variables were P<0.05 in univariate analyses listed in Tables 1 and 2.
Abbreviations see in Tables 1–4.

Discussion

The main finding of the present study was that repeated SES implantation for post-SES restenosis although safe, was associated with relatively higher rates of recurrent restenosis and MACE than that for post-BMS restenosis at 8 months of follow up. We also discovered significantly greater late luminal loss in the SES group.

The implantation of SES has significantly decreased the incidence of restenosis and the need for TLR.[1–3] In the clinical environment, SES are increasingly being implanted into patients with various higher risk indications such as ISR, small vessels, chronic total occlusions coronary artery calcification, and diabetes.[3–7,12,13] These applications have led to an observed rate of DES restenosis of >5%.[3,6,7,12,13] More uses of SES in complex settings will probably increase the incidence of restenosis. Thus, SES restenosis will become an important issue even in the DES era. However, because of the relatively low incidence of SES restenosis, few studies have evaluated the effectiveness of repeated SES implantation for SES restenosis, and the clinical and angiographic outcomes of re-intervention with SES to treat restenosis of SES and BMS have not been determined. The present study addressed these issues. Several retrospective studies[6,10,14–17] have suggested higher restenosis and TLR rates after treating restenosis of various DES, including SES, than after treating de novo lesions. Furthermore, current therapies for DES ISR or DES thrombosis are associated with a high long-term rate of MACE (42.9%).[18] The present study prospectively discovered that repeated SES implantation to treat SES restenosis is associated with a higher incidence of late restenosis, ischemia-driven TLR and MACE than SES implantation to treat BMS restenosis. The rates of re-restenosis, TLR or MACE in the present study were relatively similar to the findings of several retrospective studies.[6,10,14–17] Our results support previous findings and indicate that although SES is an excellent approach to treating patients with BMS restenosis, the same cannot be said for treating those with SES restenosis.

Local features, angiographic findings of restenosis type and diabetes mellitus might play important roles in post-SES restenosis.[5–7,12] Lemos et al[16] reported that recurrent SES restenosis is particularly high among patients with hypercholesterolemia, previous angioplasty, failed brachytherapy and post-SES restenosis treated with balloon dilation. Several investigators then also suggested that repeated SES implantation to treat non-focal SES restenosis and smaller-diameter vessels is an independent predictor of re-restenosis.[10,17] However, multivariate logistic regression analysis showed that diabetes mellitus, type of restenosis on angiograms, lesion length and procedural MLD did not significantly influence the outcomes after repeated intervention in our series.

The mechanisms of the high rates of recurrent restenosis after repeated SES implantation for SES restenosis are poorly understood. Possible explanations include enhanced allergic or inflammatory responses to the polymer or the drug as well as a late catch-up phenomenon, excessive intimal hyperplastic responses that are inadequately blocked by the drug or the amount of drug delivered, stent fracture and stent underexpansion.[6,19–22] In the present study, IVUS-guided PCI was performed to exclude underexpansion at the second intervention. In contrast, although the drug-resistance (sirolimus-resistance) hypothesis seems attractive, no clinical studies have yet demonstrated a clear clinical benefit of implanting a DES that is different from the original.[6,15,17] The small

sample size of these retrospective non-randomized studies might have limited the statistical power to demonstrate differences or detect potential adverse effects. Further study is required to evaluate this issue.

The limitations of this study are as follows. First, although this is a prospective study with a high rate of angiographic follow up, the small sample size might not have been sufficient to reach a definitive conclusion. However, to recruit a large number of patients is complicated by low SES restenosis rates. Further investigations are required to confirm our observations. Second, this is a non-randomized analysis at a single center. Third, in this study, we did not compare the effect of repeated SES implantation with that of other possible therapies, including balloon angioplasty or implantation with a different DES. Recently, some investigators have reported the efficacy of repeated DES implantation for DES restenosis compared with conventional balloon angioplasty.[10,23] In the preliminary study, we also assessed the rate of re-restenosis of balloon angioplasty for SES restenosis. The re-restenosis rate of balloon angioplasty for SES restenosis tended to be higher than in that of repeated SES implantation, but the difference did not reach significance (data not shown).

In conclusion, the incidence of late restenosis and MACE is higher after repeated SES implantation to treat restenosis of SES, than of BMS. These results might affect the mid-term clinical outcomes of re-intervention with SES.

Disclosure

Conflict of interest: None declared.

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NEPHROLOGY

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Original Article

Effects of mineralocorticoid and angiotensin II receptor blockers on proteinuria and glomerular podocyte protein expression in a model of minimal change nephrotic syndrome

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KEY WORDS:
nephrotic syndrome, podocyte, proteinuria, rennin–angiotensin–aldos terone system, slit diaphragm.

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SUMMARY AT A GLANCE

This is an interesting paper describing the effects of mineralocorticoid and angiotensin II receptor blockers in a model of minimal change disease that shows preservation of podocytes with treatment.

ABSTRACT:

Aim: **Several proteins constituting the slit diaphragm are considered important for maintaining capillary wall permselectivity. Early intervention with blockers of angiotensin II receptors (AR) and mineralocorticoid receptors (MR) is effective against proteinuria in models of chronic hypertensive and protein-induced renal damage. However, the effects of AR and/or MR blockers in a model of acute nephrotic syndrome remain unknown. The effects of AR and MR blockers were examined in puromycin aminonucleoside (PAN)-treated rats.**

Methods: **Six week old male Sprague–Dawley (SD) rats were injected with PAN or vehicle and assigned to groups as follows: vehicle (group C); PAN (group P); PAN followed 3 days later by administration of the MR blocker, eplerenone (group MR), and by the AR blocker, losartan (group AR). Blood pressure and urinary protein excretion were measured and all rats were killed for immunohistochemical investigation on day 14 after PAN administration.**

Results: **Blood pressure did not change throughout the study period. Proteinuria was decreased in groups MR and AR compared with group P (on day 14 after PAN administration, respectively; group P *vs* AR, $P < 0.01$; group P *vs* MR, $P < 0.05$). Nephrin, podocin and podocalyxin staining was preserved in the glomeruli of groups MR and AR compared with group P.**

Conclusion: **The MR and AR blockers decreased proteinuria in the acute model of nephrotic syndrome with preserved expression of glomerular podocyte protein independently of blood pressure.**

The filtration barrier of the kidney glomerulus that prevents plasma proteins from leaking into primary urine comprises endothelial cells, a glomerular basement membrane and visceral epithelial cells (podocytes). That the slit diaphragm located between the adjacent foot processes of podocytes functions as the final barrier of the glomerular capillary wall is becoming accepted.[1] Several proteins that constitute the slit diaphragm, such as nephrin and podocin, are considered important for maintaining capillary wall permselectivity, because mutations in the genes of these proteins cause congenital nephrotic syndrome and focal segmental glomerulosclerosis (FSGS).[2]

A dysfunction of the slit diaphragm is involved in the development of proteinuria. Recent studies show that early intervention with blockers of the renin–angiotensin–aldosterone system (RAAS) is effective against proteinuria in models of chronic hypertensive and protein-induced renal damage.[3–5] This mechanism is not well understood, but angiotensin II receptors (AR) and mineralocorticoid receptors (MR) are located in podocytes, and angiotensin II or aldosterone infusion induces proteinuria.[6,7] Therefore, the inhibition of AR or MR signalling in podocytes would improve impaired capillary wall permselectivity. However, the effects of AR and/or MR blockers in models of acute nephrotic syndrome remain unknown. To clarify the role of the RAAS on proteinuria and the components of slit diaphragm in minimal change nephrotic syndrome, we examined the effects of AR and MR blockers in puromycin aminonucleoside (PAN) rats.

METHODS

Animals

Six week old male Sprague–Dawley (SD) rats (Charles River Japan, Atsugi, Japan) weighing 180–220 g were maintained in individual metabolic cages in a temperature-controlled room with free access to water and normal salt diet. All experiments proceeded according to the regulations of the Animal Research Committee of the University of Miyazaki. This investigation also conformed to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health.

Experimental protocols

Puromycin aminonucleoside (Sigma Chemical, St Louis, MO, USA) was induced by a single dorsal i.v. injection at a dose of 10 mg per 100 g bodyweight in each group. Animals injected with vehicle only served as controls. Rats were assigned to the following groups: vehicle (group C, $n = 5$); PAN (group P, $n = 6$); PAN followed 3 days later by the MR blocker, eplerenone, 100 mg/kg per day (provided by Pfizer, Tokyo, Japan) (group MR, $n = 6$), and by the AR blocker, losartan, 30 mg/kg per day (provided by Merck, Whitehouse Station, NJ, USA) (group AR, $n = 6$). MR/AR blockers were given p.o. by using sonde. Blood pressure (BP), urinary protein excretion and serum markers were measured and then all rats were killed for immunohistochemical investigation on day 14 after PAN administration. Urine was collected for 24 h using metabolic cages. Systolic BP in conscious rats was measured by tail-cuff plethysmography after the animals were maintained in an incubator at 37°C for 15 min to ensure vasodilatation.

Real-time quantitative polymerase chain reaction (PCR)

The kidneys were fully perfused *in situ* with ice-cold saline and removed, and the cortex was dissected into small pieces. Glomeruli were isolated by sequentially pressing the kidney tissue through 125, 105 and 53 μm sieves. Each sieve was thoroughly washed with ice-cold saline. Tissue samples containing glomeruli were finally collected from the 53 μm sieve. Total RNA (2 μg) extracted from glomeruli (Total RNA Isolation Reagent; Invitrogen, San Diego, CA, USA) was reverse-transcribed using Superscript reverse transcriptase (Invitrogen) to yield the respective cDNA. Nephrin mRNA expression was analyzed by real-time quantitative PCR (7300 Fast Real-Time PCR System; Applied Biosystems, Foster City, CA, USA). The PCR products were used as standards and the mRNA levels were compared after normalization relative to those of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA. The forward and reverse primers for nephrin were 5′-CAACAATCCAAGCTGC CAGT-3′ and 5′-AAACGGGTGTTGTGAAGCGT-3′, and GAPDH were 5′-TCCTGCACCACCAACTGCTTAG-3′ and 5′-CACAGCCTTGGCAG CACCAGT-3′, respectively. The probe specific to nephrin was FAM-5′-CCAGACCCCTCCCTCATCTGGTTTAAGG-TAMRA-3′, and GAPDH was FAM-5′-TGACCACAGTCCATGCCATCACTGCCACTC-TAMRA-3′ in which FAM is 6 carboxyfluorescein and TAMRA (quencher) is 6-carboxy-tetramethylrhodamine.

Immunofluorescence microscopy

Immunofluorescence studies proceeded essentially as described[8] using the primary antibodies, rabbit antirat nephrin antibody,[8] rabbit antihuman podocin antibody (29040; Immuno-Biological Laboratories, Gunma, Japan), rabbit antirat podocalyxin antibody (KR064; Trans Genic, Kobe, Japan) and the secondary antibody, fluorescein isothiocyanate-conjugated swine antirabbit immunoglobulin (F234; DAKO, Tokyo, Japan).

Scoring of podocyte markers

For the evaluation of nephrin, podocin and podocalyxin, a semi-quantitative grading system was used (0, no; 1, weak; 2, intermediate; and 3, strong staining) and the average staining score was calculated.

Statistical analyses

All data are presented as mean ± standard errors of the mean. Parametric data between two groups were compared using an unpaired Student's *t*-test and among three or more groups using one-way ANOVA. The significance of individual difference was evaluated using Scheffe's *F*-test if the ANOVA findings were significant. $P < 0.05$ was considered statistically significant for all calculations.

RESULTS

Animal data

Table 1 shows serum markers in the groups C, P, MR and AR on day 14 after PAN administration. The serum albumin level in group P was significantly decreased compared with group C ($P < 0.05$), but did not differ from those in groups MR and AR. The serum total cholesterol level in group P was significantly increased compared with groups C, MR and AR ($P < 0.01$). Level of blood urea nitrogen (BUN) and potassium in group P were significantly increased compared with group C (BUN, $P < 0.05$; potassium, $P < 0.01$), but did not differ from those in groups MR and AR. Serum creatinine

Table 1 Biological parameters in groups C, P, MR and AR at 14 days after puromycin aminonucleoside administration

Group	Group C ($n = 5$)	Group P ($n = 6$)	Group MR ($n = 6$)	Group AR ($n = 6$)
Alb, g/dL	2.12 ± 0.06	1.85 ± 0.07*	1.97 ± 0.05	2.03 ± 0.03
T-cho, mg/dL	49.8 ± 3.68	151.8 ± 18.7**	81.0 ± 10.5‡	70.3 ± 7.90‡
BUN, mg/dL	17.8 ± 0.65	24.5 ± 1.96*	21.0 ± 1.03	21.9 ± 1.19
Cre, mg/dL	0.25 ± 0.01	0.31 ± 0.02	0.26 ± 0.02	0.27 ± 0.02
K, mEq/L	3.84 ± 0.10	4.93 ± 0.18**	4.30 ± 0.13	4.57 ± 0.25
Aldosterone, pg/mL	31.4 ± 7.87	36.0 ± 6.46	29.1 ± 6.94	27.0 ± 11.5

Values are means ± standard error of the mean. *$P < 0.05$ *vs* group C; **$P < 0.01$ *vs* group C; ‡$P < 0.01$ *vs* group P (tested by one-way ANOVA, followed by Scheffe's *F*-test). Alb, serum albumin; T-cho, serum total cholesterol; BUN, blood urea nitrogen; Cre, serum creatinine; K, serum potassium.