Original Article

A Newly Developed Angiotensin II Type 1 Receptor Antagonist, CS866, Promotes Regression of Cardiac Hypertrophy by Reducing Integrin β_1 Expression

Nan JIA, Hiroshi OKAMOTO, Toshihiro SHIMIZU, Satoru CHIBA, Yutaka MATSUI, Takeshi SUGAWARA, Masatoshi AKINO, and Akira KITABATAKE

Previous studies have demonstrated that integrins link the extracellular matrix to the hypertrophic response pathway of cardiac myocytes *in vitro*. To examine the direct relation between integrin β_1 and cardiac hypertrophy *in vivo*, we studied the effects of a newly developed angiotensin II type 1 (AT₁) blocker, CS866 (ARB; 10 mg/kg/day), an angiotensin-converting enzyme inhibitor, temocapril (ACEI, 10 mg/kg/day), or both on modulation of integrin β_1 in the hypertrophied hearts of stroke-prone spontaneously hypertensive rats (SHRSP) 6 to 12 weeks of age. Treatments with ARB, ACEI, and combination therapy significantly reduced systolic blood pressure. However, the reduction in cardiac hypertrophy was greater in SHRSP treated with ARB or combination therapy than in those treated with ACEI. Multiplex reverse transcription-polymerase chain reaction revealed significantly higher mRNA expression of atrial natriuretic factor, AT₁ receptor, and integrin β_1 in untreated SHRSP than in normotensive Wistar-Kyoto rats (WKY). The mRNA levels of ANP, AT₁ receptor, and integrin β_1 in SHRSP were significantly decreased by treatment with ARB, ACEI, or combination therapy. Decreased mRNA expression of ANP, AT₁ receptor, and integrin β_1 in the treated SHRSP was associated with reductions in blood pressure; ARB and combination therapy produced greater decreases in expression than did ACEI. These observations suggest that CS866 has a beneficial effect on myocyte hypertrophy and that down-regulation of AT₁ receptor and suppression of integrin β_1 participate in the regression of pressure-induced cardiac hypertrophy *in vivo*. The correlation between the expression of integrin β_1 and AT₁ receptor was significant. Our results also suggest that integrin expression by myocytes might be modulated by angiotensin II *via* AT₁ receptor. (*Hypertens Res* 2003; 26: 737–742)

Key Words: integrin, angiotensin II, angiotensin II type 1 receptor, stroke-prone spontaneously hypertensive rats

Introduction

Hypertrophy is an adaptive response of the heart to hemodynamic overload caused by conditions such as hypertension (1, 2). The initial compensatory responses can progress to decompensated heart failure. The molecular mechanisms and specific cellular signaling pathways coordinating these re-

sponses are poorly understood. Considerable evidence demonstrates that the cardiac renin-angiotensin system is linked to the development of pressure-overload hypertrophy (3) and that angiotensin II (Ang II), *via* Ang II type 1 (AT₁) receptor, plays a pivotal role in cardiac hypertrophy and remodeling (4, 5).

Integrins are heterodimeric cell-surface receptors composed of α and β subunits. These molecules not only function

From the Department of Cardiovascular Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan.

This study was funded in part by Research Grants on Cardiomyopathy from the Ministry of Health and Welfare of Japan and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Address for Reprints: Hiroshi Okamoto, M.D., Ph.D., Department of Cardiovascular Medicine, Hokkaido University Graduate School of Medicine, Kita 15, Nishi 7, Kita-ku, Sapporo 060–8638, Japan. E-mail: okamotoh@hucc.hokudai.ac.jp

Received December 27, 2002; Accepted in revised form May 1, 2003.

as simple mediators of cell adhesion but also can transduce biochemical signals across the cell membrane to activate cell-signaling pathways (6–8). Integrin β_1 is a dominant integrin β subunit expressed in the heart (9). Studies have directly linked β_1 integrin to hypertrophy of neonatal ventricular myocytes and the development of cardiomyopathies (10, 11).

The Ang II proliferative effect is mediated indirectly *via* integrin binding and activation of specific integrin-signaling pathways (12). Ang II has been shown to modulate integrin expression (13, 14). *In vitro*, Ang II increases the protein levels of β_1 , β_3 , β_5 , and α_v integrins and α -actinin. Ang II-induced stimulation of α_v , β_3 , and β_5 integrins and α -actinin is inhibited by AT₁-receptor blockade. In the spontaneously hypertensive rat (SHR), treatment with losartan, an AT₁-receptor blocker, attenuates the increased expression of the extracellular matrix (ECM) proteins α_v , β_5 , and α -actinin (15). In a murine model of pressure-overload hypertrophy caused by transverse aortic constriction, expression of integrins β_1 , α_1 , and α_5 transcripts is increased (16). Abnormal cardiac function and cardiomyopathy occur in a murine model with cardiac-specific β_1 integrin deficiency (11). It is also possible that integrins act in concert with growth factor receptors at focal clusters to maximize cell-anchorage-dependent growth (17). However, the interaction between integrin and the Ang II-signaling pathway remains unclear.

The aim of this study was to investigate the modulation in integrin β_1 expression during cardiac hypertrophy and the correlation with AT₁ receptor expression in stroke-prone SHR (SHRSP). We addressed the hypotheses that down-regulation of AT₁ receptor and suppression of integrin β_1 are implicated in the regression of stress-induced cardiac hypertrophy *in vivo* and that integrin β_1 expression in myocytes might be modulated by Ang II *via* AT₁ receptor.

Methods

Experimental Protocol

All procedures were in accordance with institutional guidelines for animal research. Male SHRSP 5 weeks of age ($n = 65$) and age-matched normal Wistar-Kyoto rats (WKY) ($n = 13$) were purchased from Japan SLC (Hamamatsu, Japan) and were given standard laboratory chow (CE-2) and tap water *ad libitum*. Blood pressure and heart rate were measured once a week by the tail-cuff method (TK-370A; Unicom Inc., San Diego, USA). CS866, temocapril, or combination therapy were administered daily to the SHRSP from 6 to 12 weeks of age. The dose of each drug was as follows: CS866, 10 mg/kg/day, $n = 13$; temocapril, 10 mg/kg/day, $n = 13$; and CS866 + temocapril, 3 mg/kg/day + 3 mg/kg/day, $n = 13$. In preliminary experiments, these doses of CS866, temocapril, and combination therapy were shown to produce similar decrements in blood pressure. As a control, another group of SHRSP ($n = 13$) was given vehicle from 6 to 12 weeks of age. At the end of the treatment, body weights were mea-

sured. The SHRSP and age-matched normal WKY were killed by decapitation, the whole heart was rapidly excised and rinsed with cold saline, and the left ventricle (LV) was separated from the atria in precooled phosphate buffered saline (PBS) (pH 7.4). The LV was weighed and rapidly frozen in liquid nitrogen and stored at -80°C until RNA isolation. For histological examination, one-third of the LV tissues were fixed in 10% formaldehyde in saline and embedded in paraffin.

Histomorphological Study and Myocyte Breadth

For histomorphological analysis, coronal sections ($4\ \mu\text{m}$ thick) of the left ventricle were obtained from the paraformaldehyde-fixed, paraffin-embedded specimens ($n = 5$). The equator was considered representative of the entire ventricle. Short diameters of myocytes were measured by using an eyepiece micrometer with a $10\text{-}\mu\text{m}$ scale at a magnification of $\times 400$. Lines that ran transversely on the LV wall were selected from the anterior, lateral, posterior, and septal walls. The mean value for each specimen was used for analysis.

Immunohistochemical Analysis to Detect Integrin β_1

The primary antibody used was rabbit anti-rat integrin β_1 (Santa Cruz Biotechnology, Santa Cruz, USA). An anti-rabbit immunoglobulin G (IgG) biotinylated secondary antibody was also used. Slides were counterstained with hematoxylin.

RNA Extraction and Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) Analysis

Multiplex RT-PCR was used to determine atrial natriuretic factor (ANF), AT₁ and integrin β_1 mRNA levels in SHRSP ventricular tissue. Semi-quantitative RT-PCR (multiplex RT-PCR) and quantitative real-time RT-PCR are currently used widely for the quantitative evaluation of gene expression. Because multiplex RT-PCR has been proven accurate and sensitive for relative quantification of target mRNAs (18–20), quantitative methods such as real-time RT-PCR were not used in present study. Tissue was homogenized and RNA was isolated according to the TRIZOL protocol (Gibco Life Technologies, Gaithersburg, USA). RNA was dissolved in DEPC-treated water, quantified spectrophotometrically at 260 nm, and stored at -80°C . First-strand cDNA was generated by adding RNA ($10\ \mu\text{g}$) to a mixture containing 0.5 mM dNTP, 1 U/ μl RNase inhibitor, 2.5 U/ μl Moloney murine leukemia virus reverse transcriptase, 2.5 $\mu\text{mol/l}$ random primer, and $5\times$ 1st strand buffer in a final volume of 20 μl . RT was carried out at 42°C for 50 min followed by heat inactivation of the reverse transcriptase at 70°C for 15 min. For semi-quantitative analysis of ANF, AT₁, and integrin β_1 , the rat GAPDH gene served as an internal control for calculation of the densitometric results. The GAPDH and

Table 1. Anatomical Parameters and Myocyte Breadth after 6-Week Treatment

	<i>n</i>	WKY	SHRSP	ARB	ACEI	ARB + ACEI
SBP (mmHg)	13	127 $\pm$ 2*	219 $\pm$ 7	134 $\pm$ 3*	135 $\pm$ 3*	131 $\pm$ 2*
HW/BW (mg/g)	13	2.83 $\pm$ 0.08*	4.45 $\pm$ 0.16	2.94 $\pm$ 0.09* [†]	3.20 $\pm$ 0.07*	2.94 $\pm$ 0.07* [†]
LVW/BW (mg/g)	13	2.17 $\pm$ 0.07*	3.84 $\pm$ 0.45	2.45 $\pm$ 0.07* [†]	2.70 $\pm$ 0.07*	2.46 $\pm$ 0.08* [†]
Myocyte breadth (μ m)	5	14.89 $\pm$ 0.16*	20.24 $\pm$ 0.42	16.56 $\pm$ 0.26* [‡]	17.85 $\pm$ 0.43*	16.51 $\pm$ 0.40* [‡]

WKY, Wistar-Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats; ARB, angiotensin II type 1 blocker; ACEI, angiotensin-converting enzyme inhibitor; SBP, systolic blood pressure; BW, body weight; HW, heart weight; LVW, left ventricle weight. Values are the mean $\pm$ SEM. * $p < 0.01$ vs. untreated SHRSP. [†] $p < 0.01$ vs. ACEI-treated SHRSP. [‡] $p < 0.05$ vs. ACEI-treated SHRSP.

Fig. 1. Distribution of integrin β_1 in myocardium. Representative photomicrographs of myocardium stained with integrin β_1 antibody. The immunolocalization of integrin β_1 is indicated by arrows.

aimed mRNA were co-amplified by multiplex PCR. The PCR solution contained 1 μ l of first-strand cDNA, 5 μ l 10 $\times$ PCR buffer, 4 μ l 2.5 mmol/l dNTP mix, 2 U Taq polymerase (Takara, Kyoto, Japan), 0.05 μ mol/l sense (5'-TATGATGAC ATCAAGAAGGTGG-3') and antisense (5'-CACCACCCT GTTGCTGTA-3') GAPDH primer, and 0.5 μ mol/l of one of the following primers: sense (5'-GTAGCCAAAGTCACCT GCAT-3') and antisense (5'-TCAGGCAATTGTTAAATA AGCTAT-3') AT₁ primers; sense (5'-ACCTGCATGAGTGT TGATAGG-3') and antisense (5'-CAGGTCAATGACTGCT ATAACCTCA-3') Ang II type 2 (AT₂) primers; sense (5'-ATATTGGAGCAAATCCCGTA-3') and antisense (5'-AGG GTATTCACCACCTCTCA-3') ANF primers; or sense (5'-A AAGTTGCGATCAGGAGAAC-3') and antisense (5'-ATCT CCAGCAAAGTGAAACC-3') integrin β_1 primers. Water was added to a final volume of 50 μ l. The amplification profile involved denaturation at 95°C for 60 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s for 20 cycles (ANF, integrin β_1) or 30 cycles (AT₁). PCR products were run on 2% agarose gel electrophoresis and exposed to ultraviolet light. The band density (AT₁, ANF, integrin β_1) was measured with the public domain NIH Image program, and the ratios of AT₁, ANF, and integrin β_1 to GAPDH were determined.

Statistical Analysis

All data are presented as the means $\pm$ SEM. The statistical

significance of differences between two groups was determined with unpaired Student's *t*-tests. For comparison among more than two groups, statistical significance was determined with one-way analysis of variance and the Duncan multiple range test. Pearson's correlation coefficient test was used to quantify the association. Differences were considered statistically significant at a value of $p < 0.05$.

Results

Effects of AT₁ Receptor Blocker (ARB), Angiotensin-Converting Enzyme Inhibitor (ACEI), and Combination Therapy on Blood Pressure and LV Weight

As shown in Table 1, after 6 weeks of treatment, ARB, ACEI, or combination therapy resulted in similar, significant decreases in the blood pressure of SHRSP. Despite their similar hypotensive effects, ARB and combination therapy reduced the increase in heart weight and LV weight to greater degrees than did ACEI in 12-week-old SHRSP.

Effects of ARB, ACEI, and Combination Therapy on Myocyte Breadth and the Distribution Pattern of Integrin β_1

The reductions in increased myocyte breadth with ARB and combination therapy were greater than that with ACEI in 12-week-old SHRSP (Table 1). Immunohistochemical analysis showed that the distribution pattern of integrin β_1 differed markedly between SHRSP and WKY. Integrin β_1 was distributed around myocytes in SHRSP and in intercalated disks in WKY (Fig. 1).

Effects of ARB, ACEI, and Combination Therapy on Expression of Integrin β_1 , AT₁, and ANF

Multiplex RT-PCR analysis demonstrated that the expression of integrin β_1 , AT₁, and ANF in vehicle-treated SHRSP was slightly higher than that in WKY and SHRSP treated with ARB, ACEI, or combination therapy (Fig. 2A–C). ARB, but not combination therapy, reduced the increase in ANF expression to a greater degree than did ACEI in

Fig. 2. A: Ratio of the mRNA expression level of ANF to GAPDH for 12-week-old WKY, untreated SHRSP, and SHRSP treated with ARB, ACEI, or both ARB and ACEI for 6 weeks (from 6 to 12 weeks of age). Each bar represents the mean $\pm$ SEM ($n = 5$). * $p < 0.01$ vs. untreated SHRSP. † $p < 0.05$. B: Ratio of the mRNA expression level of AT₁ to GAPDH for 12-week-old WKY, untreated SHRSP, and SHRSP treated with ARB, ACEI, or both ARB and ACEI for 6 weeks (from 6 to 12 weeks of age). Each bar represents the mean $\pm$ SEM ($n = 5$). * $p < 0.01$ vs. untreated SHRSP. † $p < 0.01$. C: Ratio of the mRNA expression level of integrin β_1 to GAPDH for 12-week-old WKY, untreated SHRSP, and SHRSP treated with ARB, ACEI, or both ARB and ACEI for 6 weeks (from 6 to 12 weeks of age). Each bar represents the mean $\pm$ SEM ($n = 5$). * $p < 0.01$ vs. untreated SHRSP. † $p < 0.01$. D: Scatter-plots showing an association between the expression of integrin β_1 ($n = 25$) and AT₁ receptor ($n = 25$). The association is expressed as a Pearson correlation coefficient.

SHRSP. ARB and combination therapy reduced the increase in the expression of integrin β_1 and AT₁ receptor to a greater degree than did ACEI in SHRSP. There was a significant correlation between the expression of integrin β_1 and AT₁ receptor (Fig. 2D).

Discussion

Our results suggested that CS866, a new ARB, had a beneficial effect on myocyte hypertrophy and that down-regulation of AT₁ receptor and suppression of integrin β_1 led to the regression of pressure-induced cardiac hypertrophy *in vivo*. Our findings also provided evidence that integrin expression by myocytes was modulated by Ang II *via* AT₁ receptor. SHRSP is considered a useful animal model for investigating

the mechanisms of pathological cardiac hypertrophy and remodeling *in vivo*. In our experiments, expression of integrin β_1 , AT₁ receptor, and ANF was examined in the LVs of SHRSP treated with ARB, ACEI, or combination therapy. Despite similar lowering of blood pressure, CS866 and combination therapy reduced the increase in the expression of integrin β_1 and AT₁ receptor to greater degrees than did ACEI in SHRSP. The expressions of integrin β_1 and AT₁ receptor were positively correlated, suggesting the possibility that integrin expression is modulated by Ang II *via* AT₁ receptor.

The administration of ARB or ACEI attenuated the increased AT₁ receptor expression during cardiac hypertrophy. These observations were similar to the results described previously (21, 22). In the present study, treatment with ARB, ACEI, or combination therapy similarly attenuated the in-

crease in blood pressure. ARB or ARB + ACEI attenuated increased LV weight and myocyte breadth to greater degrees than did ACEI. Most of the known effects of Ang II in adults are attributable to AT₁ receptor, and signaling via AT₂ receptor has been shown to antagonize AT₁ receptor signaling. In the context of renin-angiotensin system (RAS) inhibition, ACEI and ARB might have the same effect when AT₁ receptor expression is abundant. However, ACEI not only blocks the RAS pathway but also inhibits the breakdown of bradykinin and then activates nitric oxide synthase. Previous studies have suggested that increased tissue bradykinin levels associated with ACE inhibition contribute to a blocking of the increase in left ventricular mass in SHR (23). The effect of ARB may not be entirely due to blockade of AT₁ receptors. During ARB therapy, plasma renin increases; hence Ang II increases and may act on AT₂ receptors. Increased kinin levels and amplified AT₂-receptor-mediated effects may contribute to the differences in treatment benefits between ARB and ACEI (24, 25). Thus, it is possible that the beneficial effects of ACEI in the present study resulted from effects other than RAS inhibition. Further experiments are necessary to elucidate the contribution of these alternative effects in cardiovascular diseases.

In vitro, Ang II increases the protein level of integrin β_1 (15). In SHR, treatment with losartan, an AT₁-receptor blocker, attenuated the increased expression of the ECM proteins α , β , and α -actinin (15). In the present study, the positive correlation between expression of AT₁ receptor and integrin β_1 suggested that integrin β_1 transcripts may be modulated by Ang II via AT₁ receptor. However, the mechanism linking AT₁ receptor to integrin β_1 remains unknown. The interleukin (IL)-6 family of cytokines have been shown to mediate Ang II-induced cardiac hypertrophy (26), but do not increase the expression of AT₁ receptor (27). Several mechanisms, such as activation of the small GTPase Rho, may play an intimate role in the cross-talk between the involved pathways (28–30).

An interesting finding of our study was that the distribution patterns of integrin β_1 in treated and untreated SHRSP differed markedly from that in WKY. Integrin β_1 was expressed only in the intercalated disks of WKY. In contrast, integrin β_1 was distributed around myocytes in treated and untreated SHRSP. Such differences in the distribution of integrin between SHRSP and WKY indicate that integrin β_1 is involved in the formation of pressure-induced cardiac hypertrophy *in vivo*. However, alterations of integrin β_1 distribution in WKY after transverse aortic constriction await characterization.

In conclusion, our study demonstrates that down-regulation of AT₁ receptor and suppression of integrin β_1 expression are involved in the regression of pressure-induced cardiac hypertrophy *in vivo*. Perhaps integrin expression in cardiac hypertrophy is modulated by Ang II via AT₁ receptor. Further studies are under way to evaluate the mechanisms and roles of the modulation of integrin expression by Ang II,

both *in vitro* as well as *in vivo*. Our results suggest that integrins may become a therapeutic target during cardiac remodeling.

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