Original Article

Increased Expression of Angiotensin Converting Enzyme 2 in Conjunction with Reduction of Neointima by Angiotensin II Type 1 Receptor Blockade

Michiya IGASE[1)], Katsuhiko KOHARA[1)], Tokihisa NAGAI[1)], Tetsuro MIKI[1)], and Carlos M. FERRARIO[2)]

Angiotensin converting enzyme 2 (ACE2), a newly recognized homolog of ACE that converts angiotensin II (Ang II) to angiotensin-1–7 (Ang-(1–7)), is found in vascular smooth muscle cells. Expression of ACE2 may be a local determinant of vascular Ang-(1–7) production and, when increased, may augment the increasingly recognized protective effects of this peptide within injured tissues. We previously showed that treatment with the angiotensin II type 1 (AT1) receptor blocker (ARB) olmesartan increased aortic ACE2 and Ang-(1–7) in conjunction with improved vascular remodeling in spontaneously hypertensive rats (SHR). In the present study, we investigated balloon injury–related ACE2 in the vasculature by determining the effect of sustained AT1 blockade on ACE2 protein expression in the carotid arteries of 12-week-old male SHR treated with either vehicle (n=5) or 10 mg/kg olmesartan (n=5) in drinking water for 14 days. Olmesartan treatment caused a 61% reduction in the cross-sectional area of the neointima, from 0.27±0.01 mm^2 in vehicle-treated rats to 0.11±0.01 mm^2 in olmesartan-treated rats. In contrast, olmesartan treatment had no effect on the medial area of injured or uninjured carotid arteries compared to that in vehicle-treated rats. Quantitative analysis of ACE2 immunostaining intensity in the carotid artery of SHR was significantly greater (p<0.05) in the neointima of olmesartan-treated SHR compared to that in vehicle-treated animals. In contrast, ACE2 immunostaining intensity was not quantitatively different in uninjured carotid arteries of olmesartan and vehicle-treated animals. These studies suggest that changes in ACE2 within the vascular system of SHR are regulated by a factor other than arterial pressure. (*Hypertens Res* 2008; 31: 553–559)

***Key Words*: angiotensin converting enzyme 2, angiotensin-1–7, angiotensin type 1 receptor antagonist, spontaneously hypertensive rat, vascular remodeling**

Introduction

Percutaneous coronary intervention (PCI), a balloon catheter–based interventional procedure, is a useful treatment strategy for coronary artery stenosis. However, the recurrence of restenosis in 30–50% patients within 6 months following angioplasty is a major shortcoming (*1*). One of the causes of arterial reocclusion after PCI has been thought to be the proliferation of smooth muscle cells (*2*).

Accumulating evidence indicates that the renin-angiotensin system (RAS) plays an important role in the pathophysiology of vascular remodeling. An angiotensin II (Ang II) type 1 (AT1) receptor blocker (ARB) significantly prevented neointimal hyperplasia in balloon-injured rat arteries independent of its hypotensive effect (*3*). However, to date, clinical trials

From the [1)]Department of Geriatric Medicine, Ehime University School of Medicine, Toon, Japan; and [2)]Hypertension and Vascular Disease Center, Wake Forest University School of Medicine, Winston-Salem, USA.

Address for Reprints: Michiya Igase, M.D., Ph.D., Department of Geriatric Medicine, Ehime University Graduate School of Medicine, Toon 791–0295, Japan. E-mail: migase@m.ehime-u.ac.jp

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of pharmacologic treatment have failed to demonstrate a clinically significant impact on restenosis. Effective pharmacologic therapy to prevent restenosis, therefore, remains desirable.

Angiotensin converting enzyme (ACE) 2 (ACE2) is a newly recognized homolog of ACE, having about 42% nucleotide sequence homology with conservation of active-site residues (*4*). Similar to ACE, ACE2 is present in a wide variety of cells and tissues, including heart, kidney, testis and large conduit arteries (*5*–*7*). Crackower *et al*. (*8*) found that targeted disruption of ACE2 in mice resulted in a severe cardiac contractility defect, increased Ang II level and upregulation of hypoxia-induced genes in the heart. These results strongly indicate that ACE2 plays an important role in cardiac function.

Although ACE2 may act on several substrates, it exhibits high catalytic efficiency specifically for the hydrolysis of Ang II to the vasodilator and growth-inhibitor heptapeptide, Ang-(1–7) (*9*). In previous experiments, we showed that Ang-(1–7) mediates the vasodilator effects of combined ACE inhibition and AT1 receptor blockade (*10*). Furthermore, continuous intravenous infusion of Ang-(1–7) reduced neointimal growth in carotid arteries subjected to endothelial denudation (*11*). More recently we examined ACE2 gene expression, Ang-(1–7) protein level and vascular morphometry in the aorta and carotid artery of spontaneously hypertensive rat (SHR) with or without ARB treatment (*7*). In the aorta, the ACE2 gene expression and protein level, Ang-(1–7) level and lumen area were increased after ARB treatment. Our data demonstrated ACE2 and Ang-(1–7) expression in the aorta in response to ARB treatment and provided evidence that this pathway is regulated by AT1 receptors and may be important in mediating the pressure-independent vascular remodeling effects of Ang peptides. Since Ang-(1–7) opposes the actions of Ang II, regulation of Ang-(1–7) production by ACE2 may be an effective strategy against restenosis after PCI. Therefore, in this study, we examined the effect of an ARB in association with ACE2 on neointimal hyperplasia in balloon-injured rat arteries.

Methods

Animals

Experiments were performed in 12-week-old male SHR (body weight, 301±6 g) that were allowed to acclimatize to environmental conditions for 1 week. Rats were housed in individual cages in a room in which lighting was controlled (12-h light/dark cycle) and given access to food and water ad libitum. All experimental procedures complied with the policies implemented by our institutional Animal Care and Use Committee.

Table 1. Hemodynamic Parameters in SHR Treated with Olmesartan or Vehicle

	Vehicle (n=5)	Olmesartan (n=5)
BW (g)	320±5	317±4
HW (g)	1.30±0.01	1.12±0.08*
HW/BW	4.12±0.10	3.50±0.20*
MBP (mmHg)	142±2	107±10*
HR (bpm)	269±11	259±12

Values are mean±SEM. *$p<0.05$ *vs*. vehicle. SHR, spontaneously hypertensive rat; BW, body weight; HW, heart weight; MBP, mean blood pressure; HR, heart rate.

Experimental Protocol

Ten SHR (13 weeks of age) were randomly assigned to drink either tap water (vehicle, n=5) or tap water to which the angiotensin AT1 receptor blocker olmesartan medoximil (olmesartan, 10 mg/kg/day, n=5; Sankyo Co. Ltd., Tokyo, Japan) was added from 1 day before balloon injury until the end of the experiments. Balloon injury of the left common carotid artery was performed on day 0 under halothane anesthesia using a 2F Fogarty balloon catheter (Baxter Healthcare, Irvine, USA) to induce neointimal formation, as described by Igase *et al*. (*12*). Briefly, under a microscope, the left common, external and internal carotid arteries were exposed by a longitudinal midline cervical incision and blood flow was interrupted temporarily by ligation of the common and internal carotid arteries using vessel clips. The external carotid artery was ligated permanently. The balloon catheter was introduced into the common carotid artery and passed to the aorta. To produce carotid artery injury, the balloon was inflated with 0.1 mL 0.9% NaCl and pulled back through the common carotid artery. This process was repeated 3 times before removal of the catheter. After this, the balloon was deflated and the catheter was withdrawn. After withdrawal of the catheter, the proximal end of the external carotid artery was closed and the rats were allowed to recover from anesthesia. Fourteen days after balloon injury, the rats were weighed and anesthetized as described above. Mean arterial blood pressure and heart rate were measured with a computer-based data acquisition system (Biopac Instruments; BIOPAC Systems, Goleta, USA) by insertion of a 20-gauge angiocatheter (Baxter Healthcare) into the abdominal aorta and attachment of the catheter to a transducer. Arterial blood (7 mL) was withdrawn and processed for determination of ACE activity and plasma Ang II and Ang-(1–7) concentrations as described previously (*13*). A 5% solution of Evans blue dye (0.3 mL) was administered *via* the catheter to identify denuded and injured arteries, followed by a bolus injection of pentobarbital sodium (25 mg/kg). Exsanguination was followed by whole body perfusion with 1× phosphate buffered saline (PBS). The carotid arteries were perfusion-fixed *in situ* after flushing with PBS with 4% paraformaldehyde at a pressure of 100

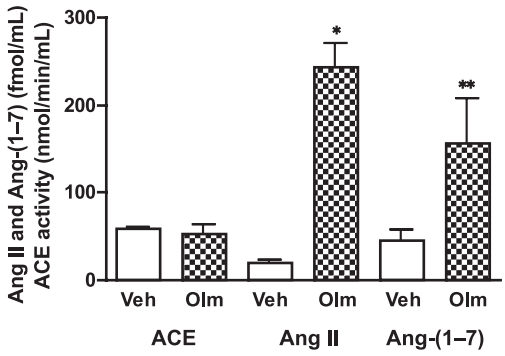

Fig. 1. *Changes in serum levels of Ang II, Ang-(1–7) and plasma ACE activity. *$p < 0.01$ vs. vehicle (Veh), **$p < 0.05$ vs. vehicle. Olm, olmesartan.*

mmHg for 10 min, and processed for immunostaining, as described previously (*7*). After perfusion fixation, the balloon-injured artery and corresponding region of the right carotid artery were removed rapidly, cut into 5-mm pieces and embedded in paraffin for histological analysis. Thin sections (5 μm thick) were cut and mounted on glass slides and stained with hematoxylin-eosin for morphometric analysis. Tissue sections were examined using light microscopy, photographed with a MicroPublisher 3.3 RTV camera (QImaging, Burnaby, Canada) and analyzed using QCapture Pro software (QImaging). Cross-sectional areas (3 per vessel) were measured morphometrically using Image J 1.32 software. The area of the media was determined by measurement of the lengths of the internal and external elastic laminae traced manually on digitized images using Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, USA) The medial cross-sectional area was obtained by subtracting the area encompassed by the internal elastic lamina from the area encompassed by the external elastic lamina. The neointima was defined as the area delimited by the internal elastic lamina and the surface of the artery lumen. The neointimal cross-sectional area was obtained by subtracting the area encompassed by the surface of the artery lumen from the area encompassed by the internal elastic lamina. The pixel values were divided by mm^2 to convert them to the laminae area. For each arterial cross section, the neointima and medial areas were measured, and the intima/media area ratio was calculated. ACE2 immunohistochemical staining was performed as described elsewhere (*14*). Briefly, cross-sections adherent to glass slides were washed in PBS and incubated overnight at 4°C with affinity-purified rabbit polyclonal antibodies to ACE2 produced in our laboratory at a dilution of 1:1,200 in 1% bovine serum albumin (BSA). The specificity and applicability of the antibodies for immunohistochemical staining were previously reported (*14*). After washing, sections were incubated for 3 h at 4°C with biotinylated goat anti-rabbit antibody diluted 1:400 in 1% BSA. Sections were rinsed, and a peroxidase-conjugated avidin-biotin method (VectaStain, Vector Laboratories, Burlingame, USA) in combination with 3,3′-diaminobenzidine (DAB, Sigma, St. Louis, USA) in Tris-buffered saline (0.05 mol/L, pH 7.65) was used according to the manufacturer's instructions to visualize primary antibody location. Sections were counterstained with hematoxylin (Sigma). To validate the staining procedure, some sections were incubated with secondary antibody alone without the primary antibody. ACE2 immunostaining was quantified as previously described (*7*).

Statistical Analysis

All data are presented as the mean±SEM. Statistical analyses of differences between rats treated with either vehicle or olmesartan were performed using Student's *t*-test (GraphPad Software, San Diego, USA). The criterion for statistical significance was set at $p < 0.05$. Mean values of density and morphometric values were calculated from numerical data obtained in four sequential tissue sections from each rat.

Results

Effect of Olmesartan Treatment on Blood Pressure, Heart Rate, and Angiotensin Peptides

At 14 days after the initiation of drug treatment, body weight, heart rate, blood pressure and angiotensin peptides were measured. Body weight was increased after 2 weeks in both olmesartan- and vehicle-treated rats (317±4 and 320±5 g, respectively). Heart weight was also not different between the groups (1.12±0.08 and 1.30±0.01 g, respectively). Olmesartan and vehicle groups had equivalent systolic blood pressure (210±3 and 201±6 mmHg, respectively) and heart rate (300±15 and 295±15 bpm, respectively) before starting treatment. Hemodynamic parameters and plasma Ang II and Ang-(1–7) concentrations in SHR treated with olmesartan or vehicle are shown in Table 1. After 14 days of treatment with olmesartan, systolic blood pressure was decreased ($p < 0.05$) compared to that before treatment, whereas vehicle treatment had no effect on blood pressure. In contrast, heart rate was not affected by either treatment. Olmesartan treatment caused a 12-fold increase in the circulating level of Ang II, from 19.6±4.5 fmol/mL in vehicle-treated rats ($n = 5$) to 243.5±29.6 fmol/mL in olmesartan-treated rats ($n = 5$; $p < 0.01$). Olmesartan treatment was associated with increased Ang-(1–7). Plasma ACE activity was not affected by olmesartan or vehicle (Fig. 1).

Effect of Olmesartan Treatment on Arterial Intimal and Medial Area at 14 Days after Balloon Injury

Balloon injury of the left carotid artery caused neointimal formation in both olmesartan- and vehicle-treated rats. The contralateral uninjured carotid artery showed no discernible

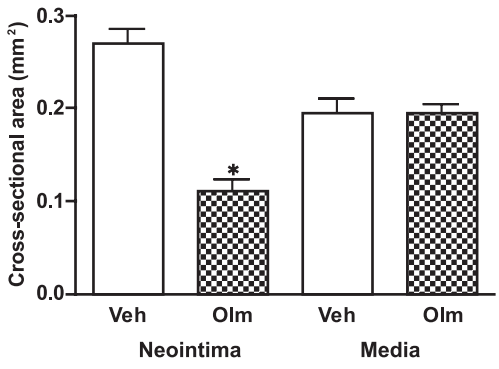

Fig. 2. *Changes in the cross-sectional area. *p < 0.01 vs. vehicle (Veh). Olm, olmesartan.*

neointima. Olmesartan treatment caused a 61% reduction in the cross-sectional area of the neointima, from 0.27±0.01 mm^2 in vehicle-treated rats to 0.11±0.01 mm^2 in olmesartan-treated rats ($n=5$; $p<0.01$). In contrast, olmesartan treatment had no effect on the medial area of injured or uninjured carotid arteries compared to that in vehicle-treated rats (from 0.20±0.02 mm^2 in vehicle-treated rats to 0.19±0.01 mm^2 in olmesartan-treated rats) (Fig. 2). Correspondingly, the neointima/media ratio was reduced in olmesartan-treated rats compared with that in vehicle-treated rats (from 1.39±0.03 mm^2 in vehicle-treated rats to 0.56±0.04 mm^2 in olmesartan-treated rats, $n=5$; $p<0.01$).

Effect of Olmesartan Treatment on ACE2 Staining Intensity

Representative cross-sections of the carotid artery stained for ACE2 are shown in Fig. 3. Figure 3A illustrates the weak ACE2 immunoreactivity found in the neointima and media of the carotid artery in vehicle-treated SHR. In contrast, Figure 3B shows the more intense ACE2 immunolabeling in the neointima and media of the carotid artery from olmesartan-treated SHR. Quantitative analysis of ACE2 immunostaining intensity in the carotid artery of SHR given olmesartan or vehicle is shown in Fig. 4. The ACE2 staining intensity was significantly greater ($p<0.05$) in the neointima of olmesartan-treated SHR compared to that in vehicle-treated animals. The increased ACE2 staining found in the neointima of SHR treated with olmesartan was associated with significantly increased intensity of ACE2 immunostaining in the media. In contrast, ACE2 immunostaining intensity was not quantitatively different in uninjured carotid arteries of olmesartan- and vehicle-treated animals.

Discussion

The important findings of the present study were as follows: 1) ACE2 protein was expressed not only in the media of the carotid artery but also in the neointima of the balloon-injured carotid artery in SHR. 2) The increase in ACE2 protein expression in the neointima following exposure of the rats to an ARB compared to vehicle was associated with a reduction in neointima. 3) These results lead to the hypothesis that there is a strong correlation between the remodeling effects seen and the observation of an elevation of ACE2 followed by Ang-(1–7).

It is well known that several RAS components are involved in neointimal formation after vascular endothelial damage (*15*). In particular, Rakugi *et al*. (*16*) showed that vascular endothelial damage results in the induction of vascular ACE. Their results suggested that inhibition of vascular ACE might be critical in the prevention of restenosis after balloon injury as well. However, in human clinical studies, an ACE inhibitor (ACEI) has not led to prevention of restenosis after percutaneous transluminal coronary angioplasty (PTCA) (*17*, *18*). Urata *et al*. (*19*) demonstrated chymase-dependent Ang II formation in the human heart *in vitro*, which means that the conversion of Ang I to Ang II occurs not only *via* ACE, but also *via* chymase produced in humans. After the landmark discovery of a new serine proteinase that forms Ang II, it began to occur to us that blocking of local Ang II production by both ACE and chymase is quite important to clarify the detailed mechanisms of tissue Ang II formation in humans and their contribution to the pathophysiological changes in cardiovascular disease.

ARBs are used for preventing restenosis after angioplasty because they can inhibit the action of Ang II generated by both enzymes. The Val-PREST trial (*20*) showed that the ARB valsartan reduced the in-stent restenosis rate after stent implantation. The inhibition of RAS by ARBs may help to prevent restenosis after angioplasty. In addition, specific blockade of the AT1 receptor with an ARB results in elevation of circulating Ang II and thus overstimulation of the Ang II type 2 (AT2) receptor (*21–24*). This pathway could also have the beneficial effect of reducing atherosclerotic lesions. On the other hand, we previously showed that ACE2 contributed to cardiac remodeling post-myocardial infarction (MI) in normotensive rats. We demonstrated a significant three-fold rise in ACE2 occurring in rats receiving olmesartan for 28 days after occlusion of a coronary artery, accompanied by an increased plasma concentration of Ang II and downregulation of cardiac AT1 receptor expression (*25*). This phenomenon means that olmesartan reversed not only cardiac hypertrophy but also impaired ventricular contractility. In addition, olmesartan increased the heart ACE2 protein level three-fold. Because these effects were not reproduced by PD-123319, the effect of olmesartan cannot be attributed to an action on Ang II at the AT2 receptor. These results suggest that ARBs may upregulate ACE2 expression, which theoretically could contribute to the beneficial effects of ARBs by facilitating increased cardiac Ang-(1–7) formation post-myocardial infarction. This study indeed provides hope that selective stimulation of the ACE2/Ang-(1–7) axis of RAS may have

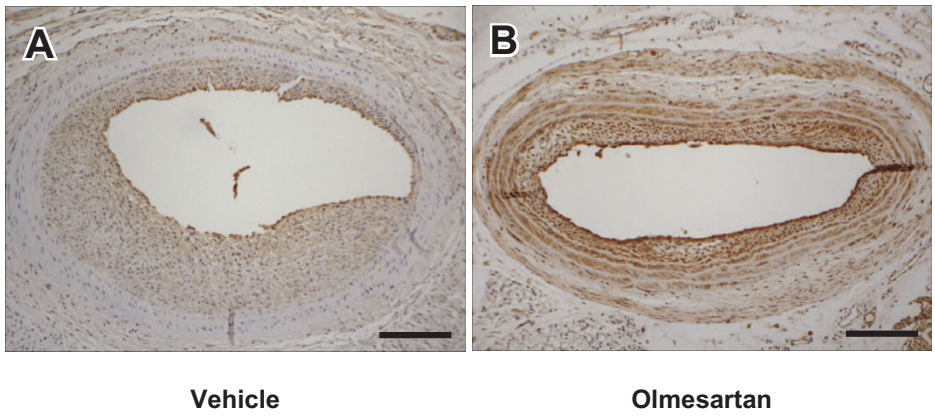

Fig. 3. *Representative cross-sections of the carotid artery stained for ACE2. Scale bar: 100 μm.*

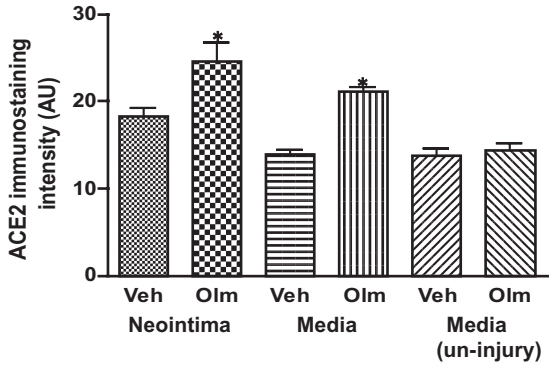

Fig. 4. *Quantitative analysis of ACE2 immunostaining intensity in the carotid artery of SHR given olmesartan (Olm) or vehicle (Veh).*

beneficial effects on post-infarction ventricular remodeling and left ventricular function in congestive heart failure.

In the present study, the lower neointima/media ratio in the carotid artery of olmesartan-treated SHR compared to vehicle-treated rats was the result of neointimal thinning and a lack of change in media area due to a lack of change in media cross-sectional area, suggesting a reversal of remodeling in the carotid artery. In keeping with these findings, the demonstration of increased ACE2 protein expression associated with reduced neointima in the carotid artery of olmesartan-treated SHR suggests that locally generated Ang-(1–7) through increased ACE2 activity may contribute to vascular remodeling in olmesartan-treated SHR. Given these results, there is no longer any doubt that ACE2 is at least partly responsible for the prevention of neointimal formation.

In relation to the cardiovascular system, both the ACE2/Ang-(1–7) axis and the ACE/Ang II axis within RAS appear to create an inner balance between proliferative and anti-proliferative effects, under normal conditions. It is only when an imbalance occurs, as a result of some pathological process, that abnormal smooth muscle cell proliferation occurs.

The possibility that the effect of olmesartan on neointimal ACE2 protein expression in the injured carotid artery was the result of reduced arterial pressure was ruled out because a similar effect was observed in the media of the non-injured carotid artery. There was no significant difference in ACE2 staining intensity in the media between the olmesartan treatment group and control group.

In summary, the observed association between an increase in ACE2 protein in the injured carotid artery of SHR and vascular remodeling during blockade of Ang II receptors poses the possibility that ACE2 plays a critical role in mediating the local effects of reversal of vascular hypertrophy in the carotid artery by a mechanism that is independent of arterial pressure.

The RAS plays a key role in the development of vascular structural changes that determine blood pressure, and extends the detrimental mechanical influence of elevated blood pressure on target organs. Since large arteries in patients with essential hypertension are thicker and stiffer than those in normotensive control subjects, normalization of wall dimensions is regarded as an important target of antihypertensive therapy. Our study suggests that ARBs may exert a beneficial vascular remodeling effect independent of blood pressure reduction in hypertensive patients by modifying local vascular RAS activity through increased ACE2 production.

Recent studies suggest that ACE2 plays a critical role in blood pressure regulation not only by modulating the balance of vasoconstrictor and vasodilator components of RAS, but also by reversing growth- and injury-related cardiovascular responses associated with increased Ang II (*26*, *27*).

Ang-(1–7) generated through ACE2 has a beneficial vascular protective effect. Grobe *et al*. (*28*) demonstrated that chronic Ang-(1–7) administration can prevent hypertension-induced cardiac myocyte hypertrophy and interstitial fibrosis. Recently Santos *et al*. (*29*) identified the orphan mas receptor as a functional binding site for Ang-(1–7). In addition, Tallant *et al*. (*30*) demonstrated that Ang-(1–7) reduces the growth of cardiomyocytes through activation of the mas receptor. ACE2

may thus emerge as a primary target for the prevention and treatment of cardiovascular disease. More recently, Agata *et al*. (*31*) demonstrated that the ARB olmesartan has dual actions as an Ang II receptor blocker and an ACE inhibitor through an increase in endogenous Ang-(1–7) *via* overexpression of ACE2. In their study, olmesartan increased plasma renin activity in the same manner as other ARBs, but it induced only a slight and nonsignificant increase in the plasma Ang II level. However, in our study, short-term (2 weeks) treatment with olmesartan caused a significant increase in the plasma Ang II level compared to the control group. This discrepancy between the studies may be related to the difference in treatment duration. In other words, the increase in plasma Ang II level may be attenuated only after long-term treatment with olmesartan. Although this effect may be unique to olmesartan, it can be said that at least one ARB has both an ACE2 antagonistic effect and an ACE inhibitory effect. This new concept will be tremendously valuable for investigating the pleiotropic mechanisms of ARBs in cardiovascular disease.

Perspectives

Taken altogether, the results of this and other related studies support the hypothesis that the ACE2/Ang-(1–7) axis of RAS may oppose the actions of the classical pathway in which ACE generates Ang II (ACE/Ang II axis). The AT2 receptor probably constitutes a separate beneficial counter-regulatory pathway of RAS. It would be interesting to determine whether overexpression of ACE2 in the heart would increase Ang-(1–7) and attenuate the detrimental structural and functional consequences of cardiac injury in future studies.

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