國 立 交 通 大 學

生 物 科 技 學 院
生 物 科 技 學 系
碩 士 論 文

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中 華 民 國 九 十 八 年 七 月

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中 文 摘 要

腎素-血管收縮素系統 (renin-angiotensin system, RAS) 中的組成要素已經被廣泛使用在作為治療多種不同疾病的藥物標靶。這些治療方式常藉由抑制特定的受器 (receptor) 或其合成酵素來減低血管收縮素 II (angiotensin II, Ang II) 的胜肽含量並達到抑制高血壓的效應。在西元兩千年，一個嶄新的酵素被發現，並被命名為血管收縮素轉化酶 II (angiotensin-converting enzyme II, ACE2)。ACE2 與其耳熟能詳的類似物血管收縮素轉化酶 I (ACE) 同樣成為眾所矚目的焦點。與 ACE 相同的是，ACE2 同樣是一種第一型跨膜金屬肽酶 (type I transmembrane metallopeptidase)，並可作為一羧基胜肽水解酶 (carboxypeptidase)，切除特定受質 (substrate) C 端的殘基 (residue)，但兩者所切除的 residue 數目不同。ACE2 之所以可以作為一個調節心血管疾病的潛力標靶，是由於其可扮演將 Ang II 代謝成具有保護血管效用的另一胜肽血管收縮素 1-7 (angiotensin 1-7, Ang 1-7)。Ang II 和 Ang 1-7 同為 RAS 中的重要調控胜肽。

Ang II 已經被證實在心臟重塑 (remodeling) 過程中扮演要角，在許多疾病例如心肌梗塞 (myocardial infarction, MI)、心臟衰竭 (heart failure, HF) 或是心房顫動 (atrial fibrillation, AF) 的病理狀態下，都可以同時測得 Ang II 以及 ACE2 的高量表現。有研究顯示，心臟內高量表現的 ACE2 可能參與防止 Ang II 異常表現所引起的高血壓或心臟纖維化，這些現象指出 ACE2 直接參與心臟保護的角色，並提供在醫療上可能的新契機。更進一步的證據指出，在心臟衰竭病患的心臟組織中可同時測得高量表現的 ACE2 以及 Ang 1-7，這也顯示了 ACE2 在心血管疾病中可能是藉由調節 Ang II 的含量來維持體內的自我平衡。因此，我們提出一個假設，ACE2 表現量的提升可能是心臟為抵抗異常高

量表現的 Ang II 所產生的自我保護機制。

在目前的研究中，我們使用人類心臟纖維母細胞 (human cardiofibroblast, HCF) 作為探討 Ang II 以及 Ang 1-7 對於 ACE2 在轉錄及轉譯調節上的重要模型。而當前的實驗結果也證實 Ang II 可以提高 ACE2 在人類心臟纖維母細胞的表現量，而此活化機制是經由血管收縮素 II 第一型受器 (angiotensin II type 1 receptor, AT1R) 進行調控。Ang II 所引起的 ACE2 高量表現更可以被 AT1R 及其下游諸如菸醯胺腺嘌呤二核酸磷酸氧化酵素 (Nicotinamide adenine dinucleotide phosphate oxidase, NADPH oxidase)、Extracellular signal-regulated kinase - Mitogen-activated protein kinase, ERK–MAPK 的拮抗劑所阻斷，這樣的結果更確立 Ang II 對於 ACE2 調控可能經由的訊號傳遞路徑。此外，promoter assay 的結果顯示在 Ang II 的刺激下，ACE2 promoter 活性顯著提升，而此提升的效應也可在加入 AT1R 的阻斷劑纈沙坦 (Valsartan) 後被阻斷，顯示出 Ang II 參與調控 ACE2 啟動子 (promoter) 的活性。

除此之外，我們的研究結果更發現 Ang 1-7 也可以提升 ACE2 在心臟纖維母細胞的表現量，而 ACE2 的向上調控則可在加入 Mas receptor 的抑制劑 A779 後被阻斷。我們的結果推論，Ang 1-7 對於 ACE2 的調控是經由 Mas receptor 並可以透過其下游的 NADPH oxidase 以及 ERK–MAPK 的訊息路徑調控 ACE2 的表現。共軛焦螢光顯微鏡的影像結果也更進一步提供 Ang II 和 Ang 1-7 對 ACE2 調控的證據，並呈現出 ACE2 及 AT1R 在心臟纖維母細胞的實際分布，而此影像的結果也與先前的發現一致。

簡而言之，據我們的實驗結果證實了 Ang II 所誘導產生的 ACE2 可以增加 Ang II 代謝成 Ang 1-7 的量，而增量的 Ang 1-7 又更進一步增強 ACE2 的表現。根據這樣的結果，我們提出 ACE2 在心臟調控中具備一正回饋機制 (positive feedback loop)，以維持人體內 RAS 的穩定平衡。我們的結果也提供產學界一個可發展治療因 RAS 失常所引起心血管疾病的潛力新標靶。

【關鍵詞】血管收縮素 II、血管收縮素 1-7、血管收縮素轉化酶 II、人類心臟纖維母細胞、腎素-血管收縮素系統

Interplay of Angiotensin II and Angiotensin 1-7 in the Modulation of Cardiac Angiotensin-Converting Enzyme II of Human Cardiofibroblasts

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Abstract

Components of renin–angiotensin system (RAS) are well established targets for pharmacological intervention in a variety of disorders. Many such therapies abrogate the effects of the hypertensive and mitogenic peptide, angiotensin II (Ang II), by antagonising its interaction with its receptor, or by inhibiting its formative enzyme, angiotensin-converting enzyme (ACE). At the turn of the millennium, a novel homologous enzyme, termed ACE2, was identified which increasingly shares the limelight with its better-known homologue. In common with ACE, ACE2 is a type I transmembrane metallopeptidase; however, unlike ACE, ACE2 functions as a carboxypeptidase, cleaving a single C-terminal residue from a distinct range of substrates. ACE2 is a potential therapeutic target for the control of cardiovascular disease owing to its key role in the formation of vasoprotective peptides angiotensin 1-7 (Ang 1-7) from Ang II [cleavage from angiotensin I by angiotensin-converting enzyme (ACE)]. Ang II and Ang 1-7 are both critical regulatory peptides in RAS.

Ang II has been documented to play important role in the progression of cardiac remodeling. Elevated Ang II paralleled to cardiac ACE2 upregulation was reported in some pathophysiological conditions, such as myocardial infarction, heart failure and atrial fibrillation. Intracardiac overexpression of ACE2 prevents Ang II induced hypertension and cardiac fibrosis, implicating a direct *in vivo* cardioprotective role for ACE2, in addition to suggesting possible therapeutic utility. Further evidence for a role of ACE2 in maintaining cardiovascular homeostasis is *via* Ang II regulation which detected increased ACE2 and Ang

1-7 forming activity in failing human hearts. Hence, we tested the hypothesis that upregulation of ACE2 may provide cardio-protection effects to counteract the elevated Ang II.

In the present study, human cardiofibroblast (HCF) cells were used to test the regulatory effects of Ang II and Ang 1-7 on the ACE2 expression at transcriptional and translational level. The results show that Ang II could upregulate ACE2 expression and this action may modulate through the activation of Ang II type I receptor (AT1R). Ang II-mediated ACE2 upregulation could be blocked by the antagonists of downstream targets of AT1R, NADPH oxidase and ERK–MAPK cascades. To test the Ang II mediated ACE2 promoter activity, our result showed that human cardiac ACE2 promoter activity was significantly upregulation with Ang II stimulation. Additionally, Ang II-induced ACE2 promoter activity could be abolished when the HCF cells pretreated with Valsartan.

Furthermore, Ang 1-7 also could up-regulate ACE2 expression in the HCF cells and this upregulation could be inhibited by Mas receptor blocker, A779. Our result shows that the Ang 1-7–depedent ACE2 upregulation is *via* Mas receptor signaling pathway and even go through the NADPH oxidase and ERK-MAPK cascades. The confocal fluorescence imaging results provide further validation for Ang II– and Ang 1-7–mediated ACE2 expression and an actual presentation of AT1R and ACE2 localization in HCF cells. Additionally, the image data also show the consistent results with our previous data.

In conclusion, our observation implicate that Ang II-induced ACE2 may increase Ang 1-7 formation from Ang II and then the ACE2 expression is further enhanced by the Ang 1-7. According to the results, we proposed a positive feedback-like loop on the cardiac ACE2 regulation for heart to maintain a steady state of RAS. Our results may point out new targets and possibilities for developing novel therapeutic strategies in cardiovascular diseases induced by the dysfunction of RAS.

Keywords: angiotensin II, angiotensin 1-7, angiotensin-converting enzyme II, human cardiofibroblasts, renin-angiotensin system

Abbreviation

ACE	Angiotensin-converting enzyme
ACE2	Angiotensin-converting enzyme II
ACEIs	Angiotensin-converting enzyme inhibitors
AF	Atrial fibrillation
Ang 1-7	Angiotensin 1-7 (Asp-Arg-Val-Tyr-Ile-His-Pro)
Ang 1-9	Angiotensin 1-9 (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His)
Ang II	Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)
ARBs	Angiotensin receptor blockers
AT1R	Angiotensin II type I receptor
AT2R	Angiotensin II type II receptor
CR	Cardiac remodeling
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
HCF	Human cardiac fibroblasts
HCM	Human cardiac myocytes
HF	Heart failure
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated/ERK kinase
MI	Myocardial infarction
NADPH	Nicotinamide adenine dinucleotide phosphate
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
IDC	Idiopathic dilated cardiomyopathy
ICM	Ischemic cardiomyopathy

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I. Literature review

1-1. Cardiac remodeling

1-1-1. Concepts of cardiac remodeling

Cardiovascular disease will be the greatest health care burden of the twenty-first century [Crackower *et al.*, 2002]. The term "remodeling" implies changes that result in the rearrangement of normally existing structures [Swynghedauw, 1999]. Cardiac remodeling (CR) is defined as genome expression resulting in molecular, cellular and interstitial changes and manifested clinically as changes in size, shape and function of the heart resulting from cardiac load or injury, cardiac remodeling is influenced by hemodynamic load, neurohormonal activation and other factors still under investigation [Cohn *et al.*, 2000]. The concept of myocardial remodeling excludes concomitant changes in the cardiac atria, valves, blood vessels, and pericardium [Swynghedauw, 1999].

Cardiac remodeling is generally accepted as a determinant of the clinical course of heart failure (HF). Heart failure is an all-too-frequent outcome of hypertension and arterial vascular disease, making it a major concern in public heath and preventive medicine. It is a common cause of morbidity and mortality, and the incidence is increasing [Tyagi *et al.*, 1995; Kannel, 2000; Rodeheffer, 2003; Izzo and Gradman, 2004; Mathew *et al.*, 2004; Franklin and Aurigemma, 2005; Hunt *et al.*, 2005; Weir *et al.*, 2006]. Following a specific cardiovascular stress, a cascade of compensatory structural events occurs within the myocardium and contributes to eventual left ventricular (LV) dysfunction and the manifestation of the heart failure syndrome.

The time course of events is influenced, however, by the severity of the underlying disease, secondary events (such as recurrent MI), other factors (such as ischemia or neuroendocrine activation), genotype and treatment [Hutchins and Bulkley, 1978; Weisman *et al.*, 1985]. Animal studies also show that infarct expansion, regional dilation and thinning of the infarct zone can occur within one day of an MI [Weisman *et al.*, 1985]. Severe impairment of global ventricular function, a functional and clinical phenomenon that can be differentiated clearly from LV remodeling, can be observed within two days of an insult [Anversa *et al.*, 1991]. The changes that occur after an insult are summarized in **Table 1-1**.

There is little doubt that remodeling and its role in disease progression are

multi-mechanistic and complex. Few clinical trials have specifically addressed the role of remodeling in disease progression. The key next steps will be the determination of how the information generated from cellular and molecular models can be used, together with data from clinical trials, to ensure that patients receive optimal therapy at an appropriate time to slow disease progression [Cohn *et al.*, 2000].

Table 1-1. Processes occurring in ventricular remodeling

Processes occurring	Description	References
Cardiomyocyte lengthening	Cardiomyocyte lengthening due to series addition of new sarcomeres and consequent fall in the short/long axis ratio.	Weisman *et al.*, 1985; Anversa *et al.*, 1991
Ventricular wall thins	Ventricular compliance depends upon the thickness of the ventricular wall and on factors, such as fibrosis, that alter the stiffness of the ventricle.	Weisman *et al.*, 1985; McKay *et al.*, 1986; Anversa *et al.*, 1991
Infarct expansion rather than extension occurs	Infarct expansion and infarct extension are events early in the course of myocardial infarction with serious short- and long-term consequences. Expansion has an adverse effect on infarct structure and functional infarct size is increased because of infarct segment lengthening, and expansion results in over-all ventricular dilatation. Infarct extension is defined clinically as early in-hospital reinfarction after a myocardial infarction.	Hutchins and Bulkley, 1978; Weisman *et al.*, 1985
Inflammation and resorption of necrotic tissue	If early thinning and dilatation did not occur after myocardial infarction, the process of remodeling with resorption of necrotic tissue, laying down of granulation tissue and scar formation would probably result in a healed area that was somewhat thinned but generally preserved normal LV contour.	Hochman and Bulkley, 1982; Weisman *et al.*, 1985
Scar formation	Scar formation is a natural part of the healing process. A scar forms from excess amounts of collagen in the wound as the body attempts a repair.	Zdrojewski *et al.*, 2002
Continued expansion of infarct zone	Infarct expansion is a progressive thinning and dilation of the infarcted zone. A progressive increase in infarct expansion is associated with increased left ventricular volume and predisposes to remodeling of the non infarcted segment.	Hutchins and Bulkley, 1978

Table 1-1. Continued

Processes occurring	Description	References
Dilation and reshaping of the left ventricle	Surgically reshaping the adversely remodeled dilated left ventricle is a concept that holds promise in the management of patients with dilated cardiomyopathy.	Weisman *et al.*, 1985; McKay *et al.*, 1986; Olivetti *et al.*, 1990; Gaudron *et al.*, 1993
Myocyte hypertrophy	Hypertrophy of the surviving myocytes is an important adaptive response to loss of contractile fuction. A decrease in cardiac fuction leads to increased levels of norepinephrine and activation of the renin-angiotensin system, leading to release of angiotensn II. Angiotensin II and mechanical stress induce a number of cellular signaling pathways important in the development of cellular hypertrophy.	Olivetti *et al.*, 1992; Kajstura *et al.*, 1994
Ongoing myocyte loss	Recent studies in experimental animals have shown that cardiac myocyte loss through apoptosis, or programmed cell death, occurs following myocardial infarction, in the presence of cardiac hypertrophy, in the aging heart, and in the setting of chronic heart failure.	Weisman *et al.*, 1985; McKay *et al.*, 1986; Olivetti *et al.*, 1990; Anversa *et al.*, 1991
Excessive accumulation of collagen in the cardiac interstitium	The accumulation of excess collagen is believed to be an important pathophysiological process that contributes to diastolic heart failure. Diastolic heart failure accounts for 30% to 50% of heart failure in clinical practice, and hypertensive disease is the major cause of this type of heart failure.	Weber and Brilla, 1991; Dostal, 2001

1-1-2. The effect of cardiac remodeling

Cardiac remodeling can be described as a physiologic and pathologic condition that may occur after myocardial infarction (MI), pressure overload (aortic stenosis, hypertension), inflammatory heart muscle disease (myocarditis), idiopathic dilated cardiomyopathy or volume overload (valvular regurgitation) [Fedak *et al.*, 2005].

With an increased workload during hypertension, the heart eventually undergoes hypertrophic (enlargement) and fibrotic responses. Myocyte hypertrophy, when accompanied by fibrosis can lead to a decrease in cardiac function. This cardiac hypertrophy and inappropriate interstitial collagen formation can contribute to increased wall stiffness and diastolic dysfunction. Thus the remodeling process, which could accompany hypertension, would consist of changes in the architecture of the heart, including myocardial fibrosis, and medial thickening of intramyocardial coronary arteries, in addition to the myocyte hypertrophy. Therefore, ventricular remodeling after myocardial infarction is a risk factor for development of heart failure and sudden cardiac death [Cohn *et al.*, 2000; Fedak *et al.*, 2005; Grobe *et al.*, 2007]. The prevention of ventricular remodeling after myocardial infarction is an important strategy in reducing mortality from myocardial infarction.

1-1-3. Critical factors involved in cardiac remodeling

The renin-angiotensin system (RAS) has previously been established to play an important role in the progression of cardiac remodeling, and inhibition of a hyperactive RAS provides a protection from cardiac remodeling and subsequent heart failure [Dzau, 1993; Cockcroft *et al.*, 1995; Parmley, 1998; Bader *et al.*, 2001; Ruiz-Ortega *et al.*, 2001a; Grobe *et al.*, 2007].

1-2. Renin-angiotensin system (RAS)

1-2-1. Physiological and patho-physiological roles of local RAS

RAS is a coordinated hormonal cascade in the control of cardiovascular, renal, and adrenal function that governs body fluid and electrolyte balance, as well as arterial pressure [Peach, 1977]. RAS is well known for its effects on the cardiovascular system and fluid homeostasis. Classically, these effects were thought to result primarily from the systemic production of angiotensin II (Ang II) [Paul *et al.*, 2006]. Circulating Ang II stimulates Ang II type 1 receptors (AT1R) present in the kidney and the vasculature to produce vasoconstriction but also water and salt reabsorption [Davisson, 2003; Lavoie and Sigmund, 2003].

It has become clear that a local RAS is present in several tissues, for example, the heart, adipose, vasculature, and bone marrow, with similar effects to the endocrine RAS but also more specific functions depending on the individual system [Paul *et al.*, 2006]. One of these local systems, the brain RAS, has long been considered pivotal in cardiovascular regulation and important in the pathogenesis of hypertension and heart failure [Davisson, 2003]. Yet the brain RAS remains poorly understood, because of the difficulty in experimentally dissecting the brain RAS at the cellular, regional, and whole organism levels.

1-2-2. RAS in regulation of cardiovascular homeostasis

Numerous clinical and laboratory data are now available supporting the hypothesis that the renin-angiotensin system (RAS) is relevant in the pathogenesis of cardiovascular diseases [Ruiz-Ortega *et al.*, 2001a; Boos and Lip, 2004; Levy, 2004; Healey *et al.*, 2005]. RAS plays a major role in regulating the cardiovascular system, and disorders of the RAS contribute largely to the pathophysiology of hypertension, renal diseases, myocardial infarction [Hanatani *et al.*, 1995; Sutton and Sharpe, 2000], atrial fibrillation [Freestone *et al.*, 2004; Savelieva and John Camm, 2004], and chronic heart failure [Weber *et al.*, 1993; Shi *et al.*, 2002]. This is to say that the emergence of cardiovascular diseases is largely related to the regulation of RAS.

1-2-3. Heart failure and RAS

Most cardiovascular diseases are multifactorial quantitative traits controlled by both genetic and environmental factors [Jacob, 1999]. One major factor for cardiovascular disease is the RAS. Due to this continuing morbidity and mortality, significant efforts have

been made to identify new drug targets in the RAS. We note that in human patients, inhibition of angiotensin-converting enzyme (ACE) or Ang II receptors can improve the outcome of heart failure [Garg and Yusuf, 1995; Boos and Lip, 2004; Madrid *et al.*, 2004; Healey *et al.*, 2005]. To solve the severe issue in the increasingly cardiovascular diseases such as heart failure, the endless stage of heart diseases, a novel efficient approach may be considered.

1-2-4. The components of peptide converting involved in RAS

The protease renin is synthesized and released from the kidney and acts on a circulating inactive peptide, angiotensinogen [angiotensinogen (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile)], produced by the liver, giving rise to angiotensin I [angiotensin I; Ang I (Asp-Arg-Val-Tyr- Ile-His-Pro-Phe-His-Leu)]. Ang I is then transformed into the biologically active octapeptide, Ang II [Ang II; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe], through enzymatic cleavage by ACE [Skeggs *et al.*, 1980; Carey and Siragy, 2003a; Lambert *et al.*, 2008], which is a critical regulator of the RAS and the target of a number of highly effective therapeutic agents used to treat cardiovascular and renal diseases. ACE is also a metalloproteinase which converts the inactive decapeptide Ang I into the potent vasoconstrictor and mitogen Ang II [Skeggs *et al.*, 1980; Lambert *et al.*, 2008], which can contribute to hypertension by promoting vascular smooth muscle vasoconstriction and renal tubule sodium reabsorption [Skeggs *et al.*, 1980].

In common with ACE, ACE2 is a type I transmembrane metallopeptidase; however, unlike ACE, ACE2 functions as a carboxypeptidase, cleaving a single C-terminal residue from a distinct range of substrates. One such substrate is angiotensin II, which is hydrolysed by ACE2 to the vasodilatory/anti-hypertrophic peptide angiotensin 1-7 [Ang 1-7; Asp-Arg-Val-Tyr-Ile-His-Pro] [Lambert *et al.*, 2008]. The schematic conversion of angiotensin peptides and balance between ACE/ACE2 was shown in **Figure 1-1**.

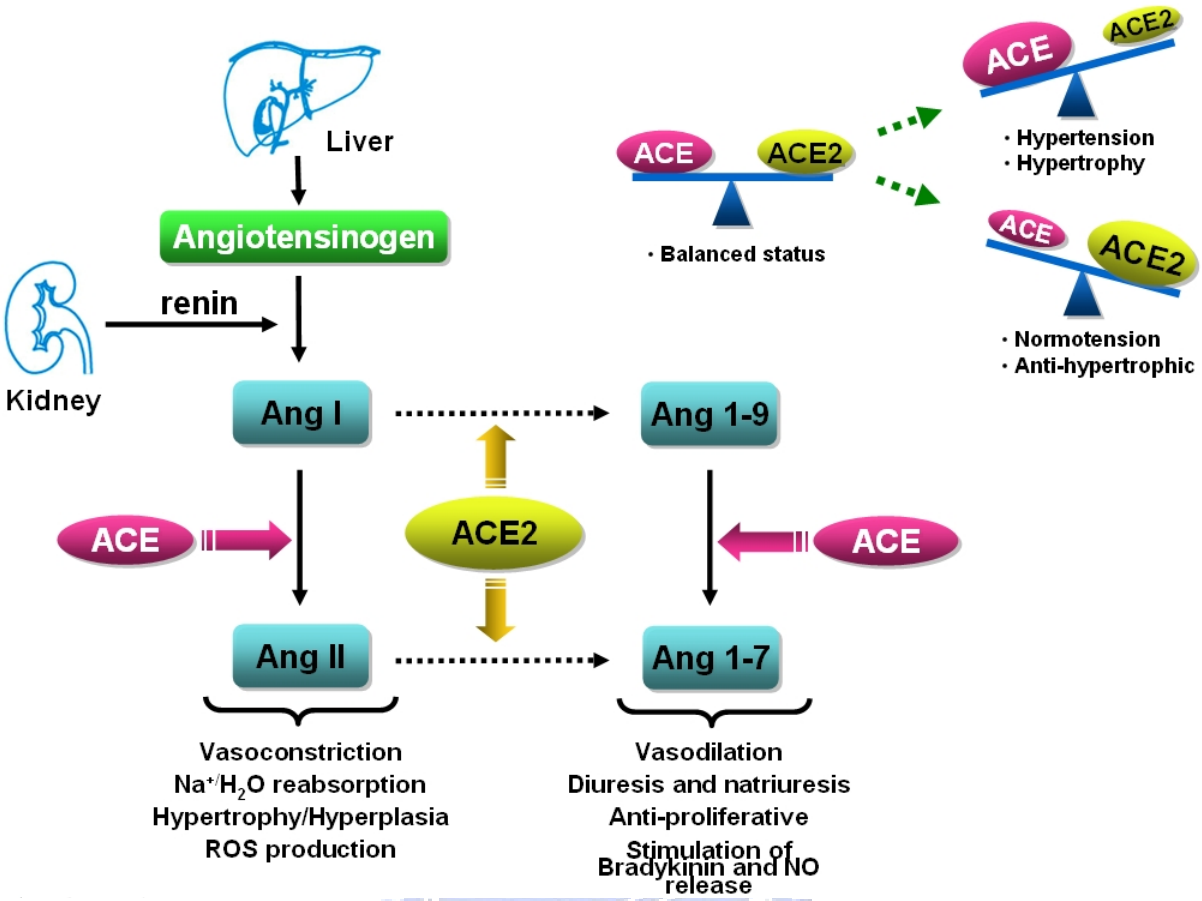


Figure 1-1. Schematic represents conversion of angiotensin peptides and balance between ACE/ACE2 in RAS.

1-3. Angiotensin peptides and core enzyme

1-3-1. Angiotensin II

Ang II is the main effector peptide of the RAS, acting in an endocrine, autocrine/paracrine, and intracrine hormone involved in the regulation of blood pressure, vascular tone, waster as well as electrolyte balance [Skeggs *et al.*, 1980; Parfrey, 2008]. Historically, Ang II was only seen as a regulatory hormone that regulates blood pressure, aldosterone release, and sodium reabsorption. Now it is generally accepted that locally formed Ang II could activate the cells regulating the expression of many substances, including growth factors, cytokines, chemokines, and adhesion molecules, which are involved in cell growth/apoptosis, procoagulation, fibrosis, and inflammation (**Figure 1-2**) [Matsubara, 1998; Sadoshima, 2000; Bader *et al.*, 2001; Ruiz-Ortega *et al.*, 2001b].

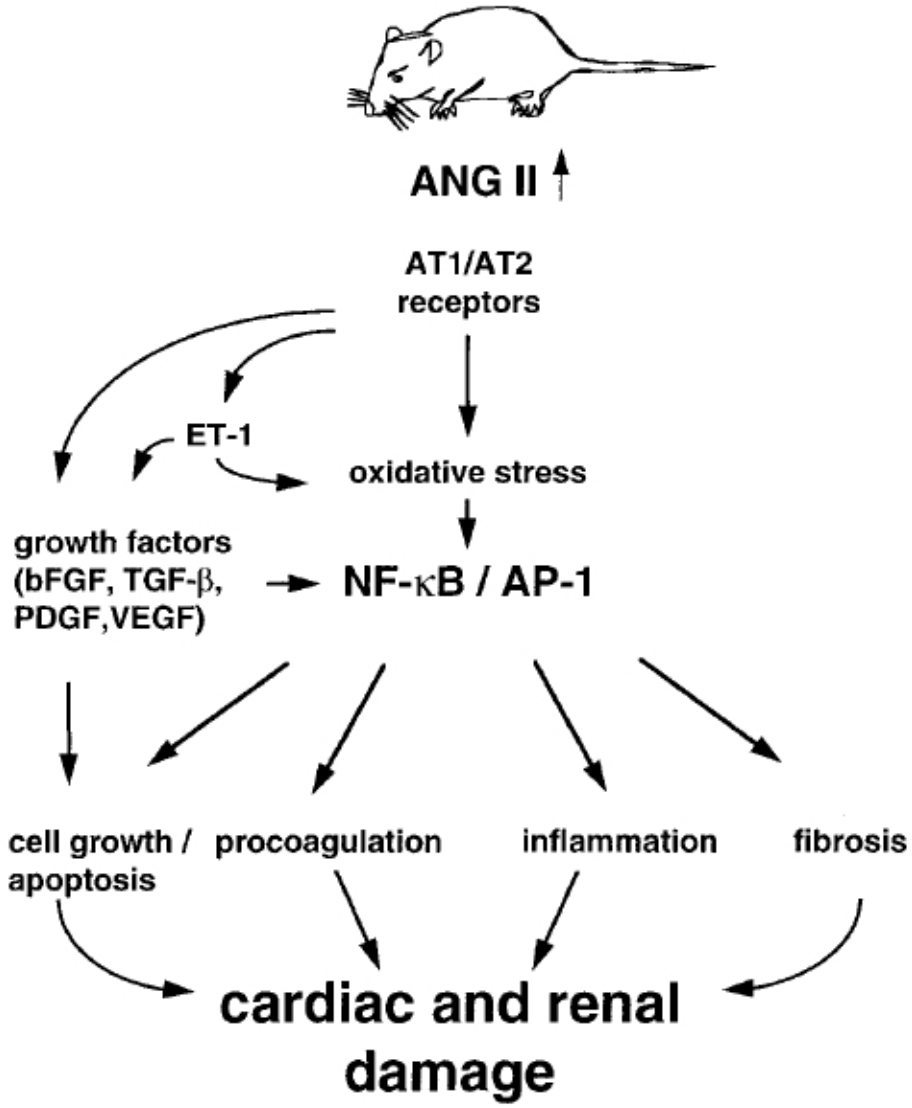


Figure 1-2. Abnormal Ang II generation results in cardiac and renal damage. Ang II activates the transcription factors nuclear factor κB (*NF-κB*) and activator protein 1 (*AP-1*) as well as proinflammatory and profibrotic genes. Ang II signaling also leads to impaired balance of cell growth and apoptosis and pro- and anti- coagulative systems. *bFGF* Basic fibroblast growth factor; *TGF*-β transforming growth factor β; *PDGF* platelet-derived growth factor; *VEGF* vascular endothelial growth factor [Bader *et al.*, 2001].

Ang II binds and activates G protein–coupled receptors, the AT1R and angiotensin II type 2 receptor (AT2R), to mediate its actions [Carey *et al.*, 2000; Shi *et al.*, 2002]. Activation of AT1R mediates most of the cardiovascular responses attributed to Ang II (ie, vasoconstriction, mitogenic and hypertrophic effects, fibrosis, inflammation, and fluid retention) [Booz and Baker, 1995; Booz and Baker, 1996; Unger, 2002]. In contrast, AT2R activation may cause opposing physiological responses that are increased in several disease processes [Ohkubo *et al.*, 1997; van Kesteren *et al.*, 1997]. Multiple lines of evidence indicate that stimulation of Ang II plays a key role in the development of pathological ventricular remodeling [Pfeffer and Braunwald, 1990]. Role of Ang II in the inflammatory response and cardiac damage was shown in **Figure 1-3**.

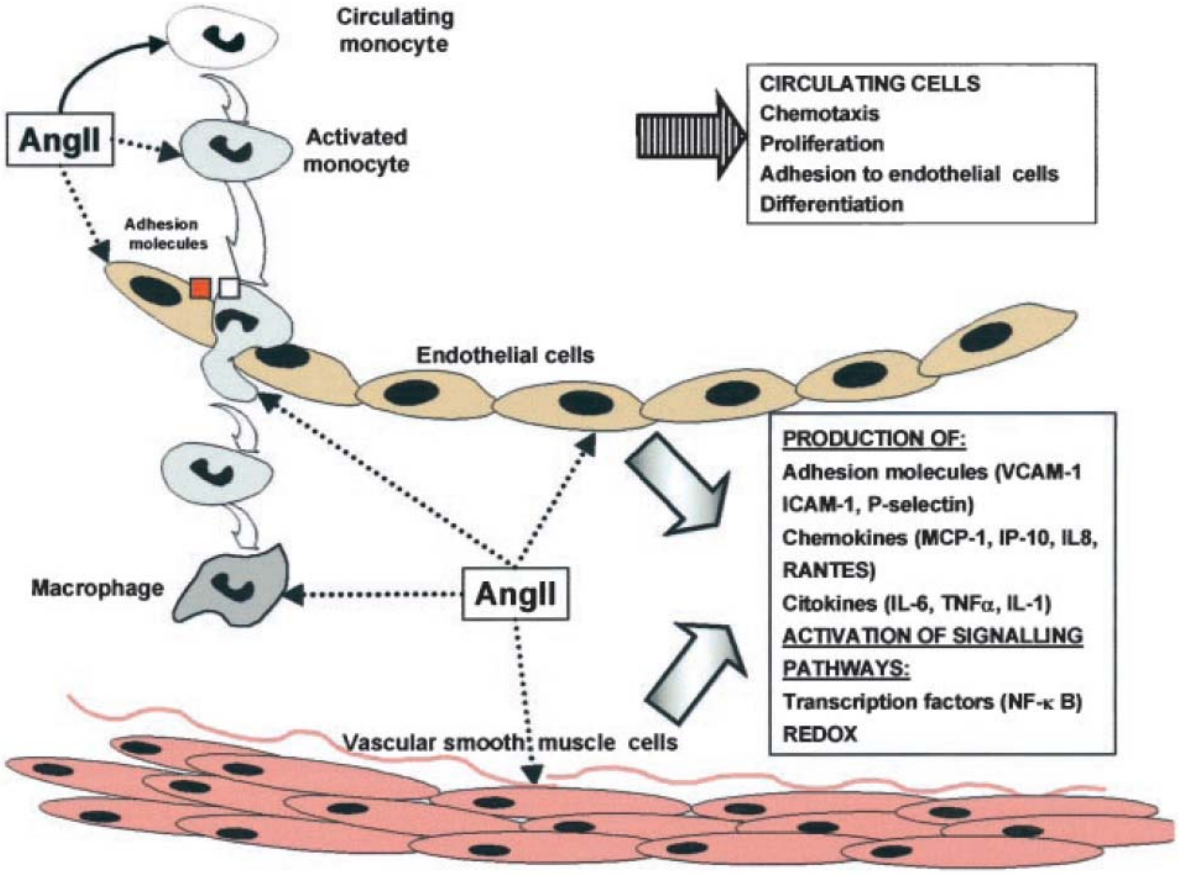


Figure 1-3. Role of Ang II in the inflammatory response in vascular injury. Ang II activates mononuclear cells, causing direct chemotaxis and proliferation. In both resident and infiltrating cells, Ang II, by NF-κB pathway and redox mechanisms, upregulates proinflammatory mediators, such as adhesion molecules, chemokines, and cytokines.

1-3-2. Angiotensin 1-7

Ang 1-7 is a biologically active peptide of the RAS that is known to potentiate the vasodilatory effects of bradykinin [Greco *et al.*, 2006], stimulate NO and prostaglandin release [Rajendran *et al.*, 2005], and antagonize the actions of Ang II [Grobe *et al.*, 2007]. Ang 1-7 has been reported to act as an antagonist to the AT1R and may also work by antagonizing ACE, which is involved in both the production of Ang II and the degradation of Ang 1-7 [Ferrario, 1998; Castro *et al.*, 2005; Igase *et al.*, 2005].

In the current study, Ang 1-7 infusion prevented cardiac hypertrophy and fibrosis without having any effect on the elevated blood pressure induced by chronic Ang II treatment. It has been well documented that Ang 1-7 levels are elevated during pharmacological ACE inhibition and blockade of AT1R [Ferrario *et al.*, 2005a; Ferrario *et al.*, 2005c; Igase *et al.*, 2005], and it has been proposed that these cardioprotective inhibitors may actually work through the actions of increased Ang 1-7 [Ferrario, 1998]. Correlative studies have shown that ACE2 and Ang 1-7 levels are increased by cardiac myocytes in hearts following myocardial infarction in both rats and human [Averill *et al.*, 2003; Burrell *et al.*, 2005].

Iwata *et al.* [2005] recently demonstrated that Ang 1-7 attenuates profibrotic signaling within the myocardium, through direct actions on cardiac fibroblasts. In the current study, chronic *in vivo* administration of Ang 1-7 also appears to have effects on hypertrophic actions on the cardiomyocytes that are induced by Ang II. This observation has also been observed *in vitro*, as Tallant *et al.* [2005a] showed that Ang 1-7 acts on cultured cardiac myocytes to inhibit hypertrophic responses through the Mas receptor.

Collectively, these findings suggest that elevated Ang 1-7 may also protect against cardiac hypertrophy in some forms of hypertension. Ang 1-7 delivery has been shown to delay development of cardiac hypertrophy [Santos *et al.*, 2004], inhibit vascular growth [Tallant and Clark, 2003], attenuate development of heart failure [Loot *et al.*, 2002], reduce cardiac Ang II levels [Mendes *et al.*, 2005], and reduce Ang II receptor populations [Clark *et al.*, 2003]. Evidence presented here would support the hypothesis that Ang 1-7 is a cardioprotective peptide.

1-3-3. Angiotensin-converting enzyme II (ACE2)

At the turn of the millennium, a homologous enzyme, termed ACE2, was identified which increasingly shares the limelight with its better-known homologue, ACE [Donoghue *et*

al., 2000b; Tipnis *et al.*, 2000; Lazartigues *et al.*, 2007]. *In vivo*, ACE2 is predominantly expressed in the heart, kidneys and testes. In human study, confirming that ACE2 is expressed in human heart, kidney and testis, consistent with a possible role in cardio-renal function [Harmer *et al.*, 2002]. The quantitative expression map for ACE 2 across 72 human tissues is shown in **Figure 1-4**.

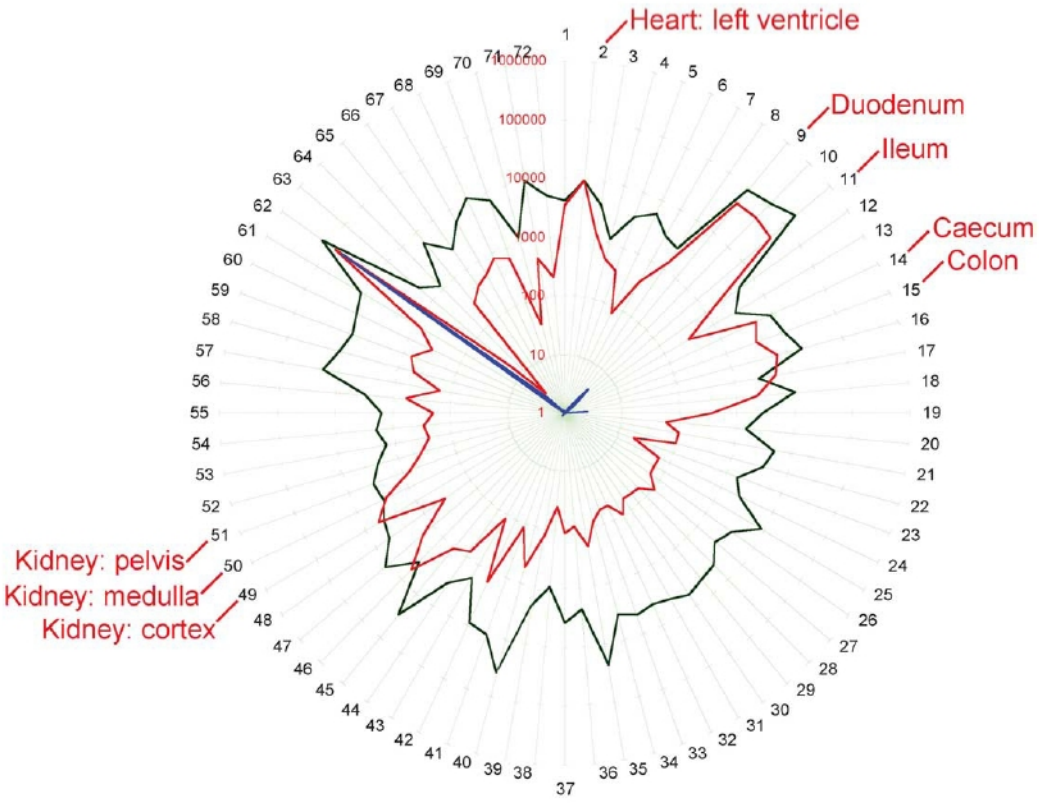


Figure 1-4. Stellar plot illustrating the mRNA copy number in logarithmic form in 72 human tissues. For ACE (black), ACE 2 (red) and ACE testicular (blue). Each point represents the geometric mean copy number from determinations in three donors. Gene copy number increases logarithmically moving from the centre to the periphery of the circle. The tissues used are: 1. heart: left atrium; 2. heart: left ventricle; 3. blood vessel: coronary artery; 9. duodenum; 11. ileum; 14. caecum; 15. colon; 49. kidney: cortex; 50. kidney: medulla; 51. kidney: pelvis [Harmer *et al.*, 2002].

In the heart, ACE2 is essentially confined to the endothelia [Donoghue *et al.*, 2000b; Tipnis *et al.*, 2000; Guy *et al.*, 2008; Pan *et al.*, 2008].

ACE2 is able to cleave both Ang I and Ang II, to Ang 1-9 and Ang 1-7, respectively. The high level of expression of ACE2 in the heart together with its ability to hydrolyse angiotensin peptides have suggested a role for ACE2 in maintaining cardiovascular physiology [Lambert *et al.*, 2008]. The potential role of Ang 1-7 as a cardioprotective peptide having vasodilator, anti-growth and antiproliferative actions has been recognized [Ferrario, 1992b; Ferrario, 1992a; Clark *et al.*, 2001; Carey and Siragy, 2003b; Burrell *et al.*, 2004; Lambert *et al.*, 2008]. It was shown that ACE2 provides a counter-regulatory system to Ang II [Carey and Siragy, 2003b; Burrell *et al.*, 2004]. Crackower *et al.* [2002] showed that deletion of ACE2 in mice resulted in elevated cardiac and plasma Ang II together with impaired cardiac contractility which increased with age. These changes were associated with an upregulation of hypoxia-induced genes, consistent with a role for Ang II in the ACE2 null phenotype.

In humans, single nucleotide polymorphisms associated with increased risk of cardiovascular disease have been identified within the ACE2 gene locus [Yang *et al.*, 2006]. Disturbance of the balance of expression of ACE2 and its homologue ACE could alter the levels of Ang II and contribute to the development of a range of pathologies.

1-3-4. The counterbalance between Ang II and Ang 1-7

Further evidence for a role of ACE2 in maintaining cardiovascular homeostasis via Ang II regulation is provided by studies conducted by Zisman *et al*. [2003] which detected increased ACE2 and Ang 1-7 forming activity in failing human hearts. Hence, the ability of ACE2 to degrade Ang II and simultaneously increase Ang 1-7 would effectively oppose the actions of ACE, suggesting the balance of the levels of the two enzymes would be critical in pathologies in the aetiologies of which Ang II is implicated. It is likely that ACE2 may play a protective role in the early stages of heart failure by elevating Ang 1-7 levels. In another study, Grobe *et al*. [2007] suggested that infusion of Ang II into adult Sprague-Dawley rats resulted in significantly increased blood pressure, myocyte hypertrophy, and midmyocardial interstitial fibrosis. Coinfusion of Ang 1-7 resulted in significant attenuations of myocyte hypertrophy and interstitial fibrosis, without significant effects on blood pressure. Another findings demonstrate that, in human endothelial cells, Ang 1-7 negatively modulates Ang II/AT1R–activated c-Src and its downstream targets ERK1/2 and NADPH oxidase. These

phenomena may represent a protective mechanism in the endothelium whereby potentially deleterious effects of Ang II are counterregulated by Ang1-7 [Sampaio *et al.*, 2007]. The cascade of the processing of angiotensin peptides and their interaction with AT1R and Ang 1-7 receptor systems was shown in **Figure 1-5**.

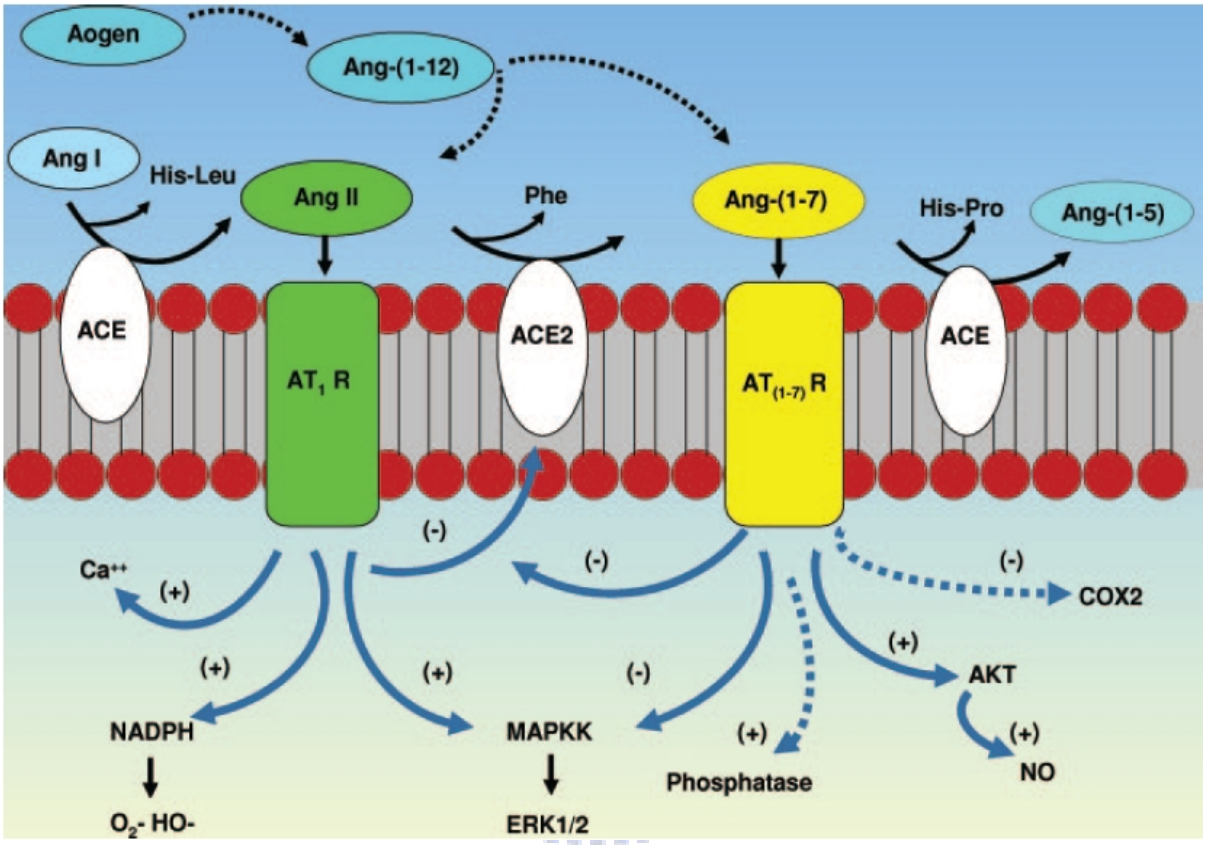


Figure 1-5. Cascade of the processing of angiotensin peptides and their interaction with AT1R and Ang 1-7 receptor systems. ACE cleaves Ang I, releasing the dipeptide His-Leu to form Ang II, and ACE2 subsequently hydrolyzes Ang II to Ang1-7. ACE also metabolizes Ang 1-7 to Ang 1-5 and the dipeptide His-Pro. Ang 1-12 may be cleaved from angiotensinogen (Aogen) and potentially processed (→) directly to Ang II or Ang 1-7. Ang1-7 may attenuate the inflammatory and fibrotic actions of the Ang II-AT1R pathway through inhibition (-) of the MAP kinase kinase (MAPKK) pathway, the potential stimulation (+) of cellular phosphatases, the inhibition of cyclooxygenase-2 (COX2) and other proinflammatory agents, as well as the stimulation of NO. Although not shown, the AT2R and bradykinin receptor systems may interact with these pathways as well [Chappell, 2007].

1-4. Angiotensin associated receptors

1-4-1. Angiotensin receptors and blockades

Angiotensin II has two major receptor subtypes, the angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R). Two subtypes of angiotensin II (Ang II) receptors have been defined on the basis of their differential pharmacological and biochemical properties. AT1R, which are involved in most of the well-known physiological effects of Ang II, and AT2R, which have a less well-defined role but appear capable of counterbalancing some of the effects of AT1R stimulation [Levy, 2004]. Importantly, AT1R antagonists are associated with a rise in plasma Ang II concentration due to the inhibition of the AT1R–mediated negative feedback on renin release [Campbell, 1996]. Drugs that block Ang II actions, such as ACE inhibitors or angiotensin receptor antagonists, are currently employed in the treatment of hypertension, heart failure, atherosclerosis, and other cardiovascular diseases [Matsubara, 1998; Sadoshima, 2000; Ruiz-Ortega *et al.*, 2001b].

1-4-2. Angiotensin II type 1 receptor (AT1R)

AT1R are widely distributed throughout the body, including vascular smooth muscle, kidney, heart, and brain. It is to say that AT1R are responsible for mediating most of the known actions of Ang II, including vasoconstriction and aldosterone release [Griendling *et al.*, 1996]. AT1R gene has been mapped to chromosome 3, and is highly expressed in smooth muscle cells, fibrolasts, as well as in atrial and ventricular myocytes [Allen *et al.*, 1999]. The amino terminal portion and the first and third loops of the transmembrane domain of this glycoproteic receptor are responsible for the interaction with Ang II [Hjorth *et al.*, 1994]. AT1R transactivates growth pathways and mediates major Ang II effects such as vasoconstriction, increased cardiac contractility, renal tubular sodium reabsorption, cell proliferation, vascular and cardiac hypertrophy, inflammatory responses, and oxidative stress [Levy, 2004].

Ang II, through its interactions with the AT1R, has been demonstrated to increase fibroblast gene expression (including collagen), fibroblast density and proliferation, and myocyte hypertrophy, all of which are hallmarks of myocardial fibrosis and remodeling [Sun *et al.*, 1997; Kawano *et al.*, 2000; Gonzalez *et al.*, 2002]. AT1R activation triggers a variety of intracellular systems, including tyrosine kinase-induced protein phosphorylation, production of arachidonic acid metabolites, alteration of reactive oxidant species activities,

and fluxes in intracellular Ca^{2+} concentrations [Berry *et al.*, 2001]. Interestingly, Ang II/AT1R activates the Jak-STAT pathway, which is also part of the signaling pathway of cytokine receptors, leading to activation of growth response genes which could contribute to cardiac tissue remodeling [Berk, 1999].

1-4-3. Angiotensin II type 2 receptor (AT2R)

Using *in situ* hybridization techniques, Shanmugam *et al.* [1995] reported that AT2R mRNA was detectable in the large arteries, in the mesenchymal tissues, such as the kidney and the urogenital tract, and variably in the cardiomyocytes of fetal rats. Vascular AT2R mRNA was most abundant in late gestation and in the early postnatal period, becoming undetectable in the cardiovascular system of adult rats.

The AT2R is thought to counteract the signals transmitted by the AT1R, eliciting vasodilatation [Brede *et al.*, 2001], inhibition of proliferation [Nakajima *et al.*, 1995; Stoll *et al.*, 1995; Mukawa *et al.*, 2003], NO production [Kurisu *et al.*, 2003] and apoptosis [Yamada *et al.*, 1996; Horiuchi *et al.*, 1997; Lehtonen *et al.*, 1999; Wang *et al.*, 2001; Suzuki *et al.*, 2002]. The AT2R may play a homeostatic role in the regulation of blood pressure in animal models of hypertension [Barber *et al.*, 1999]. In studies in rats with heart failure induced by coronary artery ligation, treatment with an AT1R antagonist was associated with improvements in left ventricular (LV) systolic function, LV end-diastolic diameter, and LV end-systolic volume. The beneficial effects on cardiac dimensions, but not function, were prevented by cotreatment with an AT2R antagonist [Liu *et al.*, 1997].

Recently, a number of studies have implicated the AT2R as having an opposing role to the AT1R in certain experimental settings, including endothelial cell proliferation and neointimal formation. In both situations, the AT1R causes stimulation, while the AT2R mediates inhibition of the response [Stoll *et al.*, 1995; Matsubara, 1998]. Therefore, it has been suggested that, at therapeutic doses of AT1R antagonists, endogenous Ang II may stimulate unopposed AT2R and thereby contribute to the decrease in blood pressure [de Gasparo and Levens, 1998].

1-4-4. Mas receptor

An anti-remodeling role for Ang 1-7 in cardiac tissue, which is not mediated through modulation of blood pressure or altered cardiac angiotensin receptor populations and may be at least partially mediated through an Ang 1-7 receptor [Grobe *et al.*, 2007]. Santos *et al.* [2003b] have reported that Ang 1-7 is an endogenous ligand for the G protein-coupled

receptor Mas. The genetic deletion of *Mas* abolished the binding of Ang 1-7 to mouse kidneys and abrogated the antidiuretic effect of Ang 1-7 in mice after acute water load. Santos *et al.* [2006] report impaired cardiac function in the Mas-deficient mouse that was associated with increased forms of collagen and fibronectin. Ang 1-7 also induced an increase of 3H-arachidonic acid release, endothelial NO synthase activation and NO release from Mas-transfected cells that were blocked by the specific Ang 1-7 receptor Mas. These findings further substantiate the role of the Mas receptor mediating the Ang 1-7 dependent activation of endothelial NO synthase.

Although, these studies clearly elevate the relevance of an Ang 1-7-Mas pathway by demonstrating a functional pathway in human cells, the acute vasodilatory effects of the peptide have not been demonstrated *in vivo* [Wilsdorf *et al.*, 2001]. Tallant *et al.* [2005a] have shown that Ang 1-7 reduces the growth of cardiomyocytes through activation of the Mas receptor. Ang 1-7-Mas axis may contribute to their beneficial effects on cardiac dysfunction and ventricular remodeling after myocardial infarction.

1-5. Oxidative stress in cardiovascular disease

1-5-1. Effect of oxidative stress in RAS

Several lines of evidence have been indicated that oxidative stress and reactive oxygen species (ROS) participate in the pathogenesis of cardiovascular diseases, including hypertension and atherosclerosis [Alexander, 1995; Griendling *et al.*, 2000b]. ROS is currently recognized as a modulator of intracellular redox state, which plays an important role as a second messenger in regulating signal transduction pathways and subsequent gene expression [Sundaresan *et al.*, 1995; Kunsch and Medford, 1999; Griendling *et al.*, 2000a].

Recent work has shown that NAD(P)H oxidases are major sources of superoxide in vascular cells and myocytes. It has recently been shown that many of these effects of Ang II are mediated by generation of ROS through the activation of vascular reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. For example, Ang II-induced VSMC hypertrophy is inhibited by blocking NADPH oxidase or scavenging intracellular ROS [Ushio-Fukai *et al.*, 1996], and Ang II-induced ERK1/2 activation and subsequent gene expression in VSMCs is also inhibited by pretreatment with antioxidants [Chen *et al.*, 1998; Frank *et al.*, 2001], suggesting its redox-sensitive signaling in VSMCs.

It has been well recognized that Ang II exerts its cardiovascular effects mainly via AT1-mediated generation of reactive oxygen species (ROS) [Yoshimoto *et al.*, 2004; Touyz, 2005; Yoshimoto *et al.*, 2005]. There is evidence that Ang II increases NADPH oxidase–mediated superoxide production through the activation of the AT1R, whereas inhibition of Ang II production ameliorates oxidative stress in the vasculature [Griendling *et al.*, 2000b; Keaney, 2005]. Ang II could significantly increase activation of NADPH oxidase in human endothelial cells [Sampaio *et al.*, 2007].

1-5-2. Downstream signaling of oxidative stress in RAS

One potential target for reactive oxygen species may be the mitogen-activated protein kinase (MAPK) family. MAPKs are serine/threonine kinases that transduce signals from the cell membrane to the nucleus in response to classical growth factors and G protein-coupled receptor agonists, as well as cellular stress [Davis, 1994; Cano and Mahadevan, 1995b; Cobb and Goldsmith, 1995b]. Four groups of MAPKs have been identified in mammalian cells: the extracellular signal-regulated kinases 1 and 2 (ERK1/2, also termed p42/44MAPK), the c-Jun NH2- terminal kinases (JNK, also termed stress-activated protein kinase, SAPK), p38MAPK (also termed CSBP) and Big MAPK 1 (BMK1, also termed ERK5) [Cano and Mahadevan, 1995a; Cobb and Goldsmith, 1995a; Abe *et al.*, 1996]. It is to say that the NADPH oxidase–mediated signaling pathway is associated with ERK–MAPK and p38-MAPK [Abe *et al.*, 1996; Ushio-Fukai *et al.*, 1998; Wenzel *et al.*, 2001].

1-6. ACE2-Ang 1-7 axis in regulation of cardiovascular disease

1-6-1. Role of ACE2 in recent studies of cardiovascular disease

Recently, the relationship between ACE2–Ang 1-7 axis and cardiovascular disease has been largely elucidated. It was shown that ACE2–Ang 1-7 axis play a critical role in heart disease development. The high level of expression of ACE2 in the heart together with its ability to hydrolyse angiotensin peptides have suggested a role for ACE2 in maintaining cardiovascular physiology from the outset, a hypothesis subsequently supported by experimental data. In the ACE2 studies, Huentelman *et al.* [2005] utilize transduction with lenti-mACE2 resulted in significant attenuation of the increased heart/body weight and myocardial fibrosis induced by Ang II infusion. In addition, ACE2 also plays an important role in dampening the hypertrophic response to pressure overload mediated by Ang II.

Disruption of this regulatory function may accelerate cardiac hypertrophy and shorten the transition period from compensated hypertrophy to cardiac failure. Increase of ACE2 mRNA and its protein expression in hypertrophic myocardium can attenuate cardiac hypertrophy due to pressure overload effectively [Yamamoto *et al.*, 2006; Qin *et al.*, 2008]. These observations demonstrate that ACE2 overexpression results in protective effects on Ang II-induced cardiac hypertrophy and fibrosis.

Targeted disruption of ACE2 in mice causes enhanced susceptibility to Ang II–induced hypertension and results in a severe cardiac contractility defect [Crackower *et al.*, 2002; Gurley *et al.*, 2006]. Oudit *et al.* showed that the age-dependent cardiomyopathy in ACE2 null mice is related to increased Ang II-mediated oxidative stress, and defined a critical role of ACE2 in the suppression of Ang II-mediated heart failure [Oudit *et al*., 2007]. These researches showed that ACE2 is an essential regulator of heart function *in vivo*. Sluimer *et al.* [2008] suggested that RAS may play a role in the pathogenesis of atherosclerosis. During the progression of atherosclerosis, overexpression of ACE2 results in stabilized atherosclerotic plaques and the mechanism is probably the conversion of vasoconstrictive Ang II to vessel protective Ang 1-7 [Dong *et al.*, 2008]. The studies of ACE2 in regulation of cardiovascular disease in human studies and animal models were shown in **Table 1-2**.

Table 1-2. Studies of ACE2 in regulation of cardiovascular disease in human studies and animal models.

Authors	Year	Experimental model and study design	Key findings
Crackowe *et al.*	2002	* Generation of ACE2 mutant mice to test whether loss of ACE2 expression affects blood pressure homeostasis and/or kidney development or function. * Generation of ACE2 knockout mice.	Targeted disruption of ACE2 in mice results in a severe cardiac contractility defect, increased Ang II levels, and upregulation of hypoxia-induced genes in the heart. ACE2 is an essential regulator of heart function *in vivo*.
Donoghue *et al.*	2003	* The cDNA for human ACE2 was inserted into the mouse cardiac a-myosin heavy chain (αMHC) promoter expression vector and microinjected into FVB mouse pronuclei to generate transgenic mice.	Transgenic mice with increased cardiac ACE2 expression had a high incidence of sudden death. Spontaneous downregulation of the ACE2 transgene in surviving older animals correlated with restoration of nearly normal conduction, rhythm, and connexin expression.
Goulter *et al.*	2004	* 32 patients undergoing this study. * non-diseased hearts (n = 9), idiopathic dilated cardiomyopathy (IDC, n = 11) and ischemic cardiomyopathy (ICM, n = 12).	ACE2 is upregulated in human IDC and ICM and are consistent with the hypothesis that differential regulation of this enzyme may have important functional consequences in heart failure. ACE2 may be a relevant target for the treatment of heart failure.
Burrell *et al.*	2005	* Left ventricular (LV) free wall MI was induced in Sprague-Dawley rats (150-200 g) by ligation of the left coronary artery. * Rats were killed at days 1, 3, and 28 after MI, or treated for 4 weeks with the ACE inhibitor ramipril (1 mg/kg).	The increase in ACE2 after MI suggests that it plays an important role in the negative modulation of the renin angiotensin system in the generation and degradation of angiotensin peptides after cardiac injury.

Table 1-2. Continued

Authors	Year	Experimental model and study design	Key findings
Ferrario *et al.*	2005	* 36 male normotensive Sprague-Dawley rats (age range, 8 to 10 weeks) were used for these studies. * Rats were randomly assigned to drink either tap water (vehicle, n=12) or tap water to which lisinopril (10 mg $\cdot kg^{-1} \cdot d^{-1}$, n=8), losartan (10 mg $\cdot kg^{-1} \cdot d^{-1}$, n=8), or both drugs at the same doses (n=8) were added to their drinking water for 12 consecutive days.	ACE inhibition may result from the combined effect of reduced Ang II formation and Ang 1-7 metabolism, the antihypertensive action of AT1 antagonists may in part be due to increased Ang II metabolism by ACE2.
Huentelman *et al.*	2005	* Cloning of murine ACE2 in lentiviral vector and production of lenti-mACE2. * At 5 days of age, a single 40 μl bolus of 3×10^{8} particles of either lenti-GFP (n =6) or lenti-mACE2 (n =6) was injected into the left cardiac ventricular cavity as previously described.	ACE2 overexpression results in protective effects on angiotensin II-induced cardiac hypertrophy and fibrosis.
Igase *et al.*	2005	* Experiments were performed in 60 male 12-wk-old (287 ± 3 g body wt) SHR. * Olmesartan (10 mg$\times kg^{-1}\times day^{-1}$, n =13) compared with those that received atenolol (30 mg$\times kg^{-1}\times day^{-1}$, n=13), hydralazine (10 mg$\times kg^{-1}\times day^{-1}$, n= 13), or vehicle (n = 21).	AT1R regulate ACE2 and Ang 1-7 expression in the aorta of spontaneously hypertensive rats. Increased ACE2 and Ang 1-7 in association with altered dimensions of the thoracic aorta but not carotid arteries in response to olmesartan treatment provides evidence that this pathway is regulated by AT1R and may be important in mediating the pressure-independent vascular remodeling effects of angiotensin peptides.

Table 1-2. Continued

Authors	Year	Experimental model and study design	Key findings
Gurley *et al*.	2006	* Generation of ACE2-deficient mice. * Ang II peptide was administered by i.v. infusion in WT (n = 5) and ACE2-deficient mice (n = 6).	The absence of functional ACE2 causes enhanced susceptibility to Ang II–induced hypertension. No evidence for a role of ACE2 in the regulation of cardiac structure or function but the author suggest that ACE2 is a functional component of the renin-angiotensin system, metabolizing Ang II and thereby contributing to regulation of blood pressure.
Yamamoto *et al*.	2006	* 12 week-old ACE2 deletion mice and wild-type (WT) mice were divided into 3 groups: sham-operation, transverse aortic constriction (TAC), and TAC during candesartan treatment.	ACE2 plays an important role in dampening the hypertrophic response to pressure overload mediated by Ang II. Disruption of this regulatory function may accelerate cardiac hypertrophy and shorten the transition period from compensated hypertrophy to cardiac failure.
Yang *et al*.	2006	* 3 single nucleotide polymorphisms in the ACE2 gene (1075A/G, 8790A/G and 16854G/C) were genotyped by PCR-RFLP (restriction-fragment-length polymorphism) in 811 patients with CHD (of which 508 were patients with MI) and 905 normal controls in a Chinese population.	Common genetic variants in the ACE2 gene might impact on MI in females, and may possibly interact with alcohol consumption to affect the risk of CHD and MI in Chinese males.

Table 1-2. Continued

Authors	Year	Experimental model and study design	Key findings
Oudit *et al*.	2007	* ACE2 mutant mice develop a progressive age-dependent dilated cardiomyopathy with increased oxidative stress, neutrophilic infiltration, inflammatory cytokine and collagenase levels, mitogen-activated protein kinase (MAPK) activation and pathological hypertrophy. * The AT1R blocker, irbesartan, prevented the dilated cardiomyopathy.	The age-dependent cardiomyopathy in ACE2 null mice is related to increased Ang II-mediated oxidative stress and neutrophilic infiltration *via* AT1R. The combination of genetic and pharmacological approaches defines a critical role of ACE2 in the suppression of Ang II-mediated heart failure.
Takeda *et al*.	2007	* DS rats and Dahl salt-resistant (DR) rats fed high or low salt diets. * The rats were treated orally with or without eplerenone (100 mg/kg/d), candesartan (10 mg/kg/d), or both drugs combined for 8 weeks.	In DS rats, blockade of aldosterone or Ang II protects cardiac hypertrophy and fibrosis by inactivation of the local RAAS in the heart.
Dong *et al*.	2008	* Atherosclerotic plaques were induced in the abdominal aorta of 114 rabbits by endothelial injury and atherogenic diet. * Gene therapy was performed in 3 groups, a recombinant ACE2 expressing vector, a control vector AdEGFP and AdACE2+A779.	Overexpression of ACE2 results in stabilized atherosclerotic plaques and the mechanism is probably the conversion of vasoconstrictive Ang II to vessel protective Ang 1-7.

Table 1-2. Continued

Authors	Year	Experimental model and study design	Key findings
Koka *et al*.	2008	* 12 patients had been diagnosed with hypertensive nephropathy and 8 with hypertensive cardiomyopathy. * All hypertensive patients with unequivocal hypertension were treated with either ACE inhibitor or AT1R blockers.	The AT1R-mediated ERK/p38 MAP kinase signaling pathway may be a key mechanism by which Ang II down-regulates ACE2 expression, implicating an ACE/ACE2 imbalance in hypertensive cardiovascular and renal damage.
Qin *et al*.	2008	* Suprarenal abdominal aortic coarctation was performed to create the pressure overload induced left ventricular hypertrophy model in rats. * Rats were randomly divided into 5 groups: (1) normal control group (2) normal control group treated with atorvastatin (3) sham group (4) atorvastatin given orally by gastric gavage for 4 weeks (5) vehicle group.	ACE2 mRNA and its protein expression increase significantly in hypertrophic myocardium in rats; atorvastatin can attenuate cardiac hypertrophy due to pressure overload in rats effectively, and part of this anti-hypertrophy effect may be attributed to decrease ACE2 mRNA and protein expression.
Sluimer *et al*.	2008	* A total of 5 human veins, 5 non-diseased mammary arteries and 36 human atherosclerotic carotid arteries were collected from 46 donors undergoing vascular surgery.	Differential regulation of ACE2 activity during the progression of atherosclerosis and suggest that this novel molecule of the RAS may play a role in the pathogenesis of atherosclerosis.

Table 1-2. Continued

Authors	Year	Experimental model and study design	Key findings
Ye *et al*.	2008	* SHRs, 12 weeks old, were randomly divided into 4 groups: the model control group (A), the Verapamil group (B), and the two puerarin groups (C and D) treated by low dose and high dose of puerarin respectively.	High dose puerarin could increase the mRNA expressions of AT1R and ACE2 in kidney, while low dose puerarin could decrease them in heart; there might be a feed back correlation between AT1R and ACE2.

1-6-2. Role of Ang 1-7 in recent studies of cardiovascular disease

In the studies of Ang 1-7, Ferreira *et al.* demonstrated that the nonpeptide Ang 1-7 analogue, AVE, attenuates postischemic heart failure and has a cardioprotective effect on ISO-induced cardiac remodeling [Clark *et al.*, 2001; Heitsch *et al.*, 2001; Ferreira *et al.*, 2007a; Ferreira *et al.*, 2007b]. Furthermore, deletion or blockade of Mas receptor markedly induced the changes in contractile function in isolated hearts during ischemia, thus Mas receptor plays an important role in cardiac function and keeps with the cardiac and coronary effects previously described for Ang 1-7 [Castro *et al*., 2006]. Ang 1-7 can reduce hypertension-induced cardiac remodeling through a direct effect on the heart [Mercure *et al.*, 2008], and it also has beneficial effects on the failing heart by activating the sodium pump, hyperpolarizing the cell membrane and increasing the conduction velocity.

An optimal generation of Ang 1-7 must be achieved to permit a protective role of Ang 1-7 on cardiac arrhythmias [De Mello *et al.*, 2007]. These findings are in agreement with previous studies demonstrating that Ang 1-7 is a cardioprotective peptide. Moreover, Ang 1-7-forming activity from both Ang I and Ang II was increased in failing human heart ventricles [Zisman *et al.*, 2003]. The studies of Ang 1-7 in regulation of cardiovascular disease in human studies and animal models were shown in **Table 1-3**.

Table 1-3. Studies of Ang 1-7 in regulation of cardiovascular disease in human studies and animal models

Authors	Year	Experimental model and study design	Key findings
Davie and McMurray	1999	* 8 patients with chronic heart failure. * Patients were undergoing treatment with an ACE inhibitor. * 5 patients taking enalapril 10 mg BID, 1 patient lisinopril 10 mg QD, 1 patient captopril 25 mg TID, and 1 patient perindopril 4 mg BID.	Ang 1-7 is biologically inactive in the forearm circulation of patients with heart failure treated with an ACE inhibitor.
Zisman *et al.*	2003	* 35 patients undergoing open heart surgery. * 14 idiopathic dilated cardiomyopathy (IDC), 8 primary pulmonary hypertension (PPH), and 13 nonfailing human hearts.	Ang 1-7-forming activity from both Ang I and Ang II was increased in failing human heart ventricles.
Oudot *et al.*	2005	* 14 groups, each composed of six to nine hearts, were subjected to different perfusion protocols at 37°C. * Isolated perfused rat hearts underwent 45 min of non-ischemic perfusion, or 30 min of global ischemia followed by 30 min of reperfusion.	Ang 1-7 at pharmacological concentration activates NADPH oxidase, an enzyme thought to be involved in several angiotensin II effects.

Table 1-3. Continued

Authors	Year	Experimental model and study design	Key findings
Sakima *et al*.	2005	* Baroreflex sensitivity control of heart rate was tested in younger (3 to 5 months) and older (16 to 20 months) anesthetized male Sprague-Dawley rats before and after bilateral solitary tract injections of the AT1R antagonist candesartan (24 pmol) or Ang 1–7 antagonist A779 (144 fmol or 24 pmol).	The attenuated counterbalancing effect of Ang 1-7 on baroreflex function is lost in older rats, which may be attributable to diminished production of the peptide from neprilysin.
Castro *et al*.	2006	* Wild-type (WT, n=8) and Mas knockout (Mas KO, n=4) C57BL/6 mice (12-week-old).	Mas plays an important role in cardiac function during ischemia/reperfusion which is in keeping with the cardiac and coronary effects previously described for Ang 1-7.
Santos *et al*.	2006	* 7 male wild-type Mas and 4 Mas KO mice (3 months old) were used for isolated hearts experiments.	Ang 1-7-Mas axis plays a key role in the maintenance of the structure and function of the heart.

Table 1-3. Continued

Authors	Year	Experimental model and study design	Key findings
De Mello *et al*.	2007	* Small pieces of the right ventricle of four month-old cardiomyopathic hamsters were used.	Ang 1-7 has beneficial effects on the failing heart by activating the sodium pump, hyperpolarising the cell membrane and increasing the conduction velocity. An optimal generation of Ang 1-7 must be achieved to permit a protective role of Ang 1-7 on cardiac arrhythmias.
Ferreira *et al*.	2007	* Rats were treated either with AVE- 0991 (1 mg/kg, n = 9) or vehicle (0.9% NaCl, n =11) administered orally by gavage.	The compound AVE-0991 produces beneficial effects in isolated perfused rat hearts involving the Ang 1-7 receptor, Mas and the release of nitric oxide. In addition, our results indicate that AVE-0991 attenuates postischemic heart failure.
Ferreira *et al*.	2007	* Heart dysfunction was induced by daily injection of isoproterenol (2 mg/kg i.p. diluted in 0.9% NaCl) during 7 days. * The rats were divided into four groups: control (0.9% NaCl i.p. plus water by gavage), ISO (isoproterenol plus water by gavage), ISO+AVE (isoproterenol plus AVE 0991, 1 mg/kg by gavage), and AVE (0.9% NaCl i.p. plus AVE 0991).	The nonpeptide Ang 1-7 analogue, AVE, has a cardioprotective effect on ISO-induced cardiac remodeling.

Table 1-3. Continued

Authors	Year	Experimental model and study design	Key findings
Grobe *et al*.	2007	* 42 male Sprague-Dawley rats weighing 270–300 g were used for these studies. * Hypertension and cardiac remodeling were induced by chronic subcutaneous infusion of Ang II for 4 wk through the use of subcutaneous osmotic minipumps.	An anti-remodeling role for Ang 1-7 in cardiac tissue, which is not mediated through modulation of blood pressure or altered cardiac angiotensin receptor populations and may be at least partially mediated through an Ang 1-7 receptor.
Mercure *et al*.	2008	* Chronic overproduction of either Ang II or Ang 1-7 in the heart of transgenic mice and tested their effect on age-related contractility and on cardiac remodeling in response to a hypertensive challenge.	Ang 1-7 can reduce hypertension-induced cardiac remodeling through a direct effect on the heart and raise the possibility that pathologies associated with ACE2 inactivation are mediated in part by a decrease in production of Ang 1-7.

1-6-3. ACE2-Ang 1-7 axis play a key role in cardiac protection

The expression of ACE2 is upregulated in human Idiopathic dilated cardiomyopathy (IDC) and Ischemic cardiomyopathy (ICM), and this finding is consistent with the hypothesis that differential regulation of this enzyme may have important functional consequences in heart failure. For this reason, ACE2 may be a relevant target for the treatment of heart failure [Goulter *et al.*, 2004]. Burrell *et al.* [2005] also demonstrated that the increase in ACE2 after MI suggests that it plays an important role in the negative modulation of the renin angiotensin system in the generation and degradation of angiotensin peptides after cardiac injury. Briefly, ACE2-Ang 1-7 axis may play a key role in the maintenance of the structure and function of the heart [Santos *et al.*, 2006].

II. Research Purpose and Strategy

2-1. Developing the specific targets in cardiac regulation

Therapeutic approaches, such as ACE inhibition and beta-blockade, which reduce morbidity and mortality and, in some cases, improve a number of remodeling parameters, may offer such a therapeutic approach. Specific blockade of the AT1R with an ARB results in elevation of circulating angiotensin II and thus an overstimulation of the AT2R [Levy, 2004], indicating that the effect of an ARB might be partly mediated by its effect on the AT2R [Oishi *et al.*, 2006]. ARBs and ACE inhibitors may not be the best way to solve the urgent problems of heart diseases. The challenge is to develop new and more specific treatments that may be even more effective in reversing the structural abnormalities in the left ventricle. According to such reasons, revealing the importance of ACE2 as a regulator of the local RAS is becoming increasingly apparent.

2-2. ACE2 as a novel target in the regulation of Ang II-induced negative effect

Recent study has demonstrated that overexpression of ACE2 prevents cardiac remodeling and hypertrophy during chronic infusion of Ang II [Grobe *et al.*, 2007]. This, coupled with the knowledge that ACE2 is a key enzyme in the formation of Ang 1-7, led us to hypothesize that Ang 1-7 would prevent cardiac remodeling induced by chronic infusion of Ang II. Through its ability to metabolize Ang II to Ang 1-7 it is able to regulate local Ang II levels thereby modulating its effects.

2-3. The schematic representation of research strategy and experimental flowchart

In this study, we discuss the latest developments in the rapidly progressing study of the physiological and patho-physiological roles of ACE2-Ang 1-7 axis allied with an overview of the current understanding of its molecular and cell biology. Furthermore, we discuss the potential physiological cross talk that may be operative between the angiotensin receptor

subtypes in relation to health and cardiovascular disease. This may be clinically relevant, inasmuch as inhibitors of the RAS are increasingly used in treatment of hypertension and coronary heart disease, where activation of the RAS is recognized [Berry *et al.*, 2001]. Finally, we discuss recent advances in our understanding of the components and actions of the RAS, and discover the particular and detailed functional signaling pathway in the ACE2-Ang 1-7 axis. The schematic representation of research strategy and experimental flowchart is shown in (**Figure 2-1**).

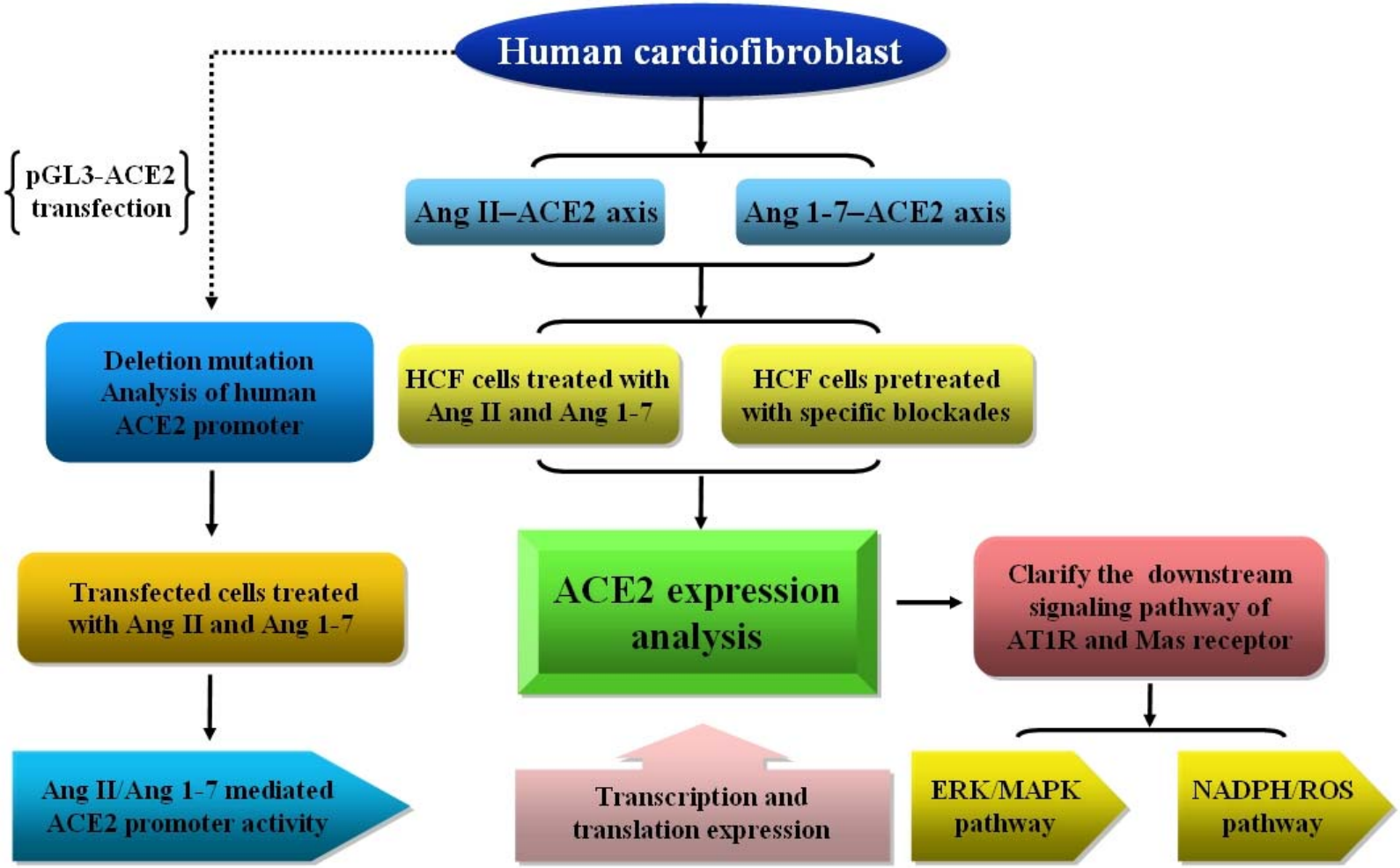


Figure 2-1. The schematic representation of research strategy and experimental flowchart.

III. Materials and Methods

3-1. Chemicals and reagents

Anti-GAPDH (#sc20357) antibody and HRP-labeled secondary antibodies against goat IgG (#sc2020), mouse IgG (#sc2005), and rabbit IgG (#sc2004) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti- phospho-MEK1/2 (#9121) and anti-phospho-ERK1/2 (#4370) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-ACE2 (#ab59351) antibody was purchased from Abcam (Cambridge, MA, USA). Ang II (#H1705), Ang 1-7 (#H1715) and (D-Ala^7)- Ang 1-7 (A779; #H2888) were purchased from Bachem (Merseyside, UK). Valsartan (#1708762) was from USP (Rockville, MD, USA). The promoterless luciferase reporter vectors, pGL3-Basic (#E1751), and luciferase assay system (#E1500) were purchased from Promega (Madison, WI, USA). All other reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK).

3-2. Cell culture

Primary human cardiac fibroblasts (HCF, #6300) were purchased from ScienCell Research Laboratories (San Diego, CA, USA). The cells were seeded in 100-mm Petri dishes (2×10^6 cells/dish) or 12-well plate (1×10^5 cells/well) coated with 0.01% poly-l-lysine (#P4832; Sigma-Aldrich) and cultured in commercial media (#2301; ScienCell Research Laboratories), according to the manufacturer's instructions. The cells were incubated in a humidified 5% CO_2 atmosphere at 37°C and culture media was changed every 2 days. The cells at passages 5–6 were used in all experiments. The grown cells were placed into serum-free medium for 24 h prior to experimental treatments. The cells were pretreated with/without the selective antagonists valsartan (1 μM) or A779 (1 μM) for 1 h, then stimulated with Ang II (1 μM) or Ang 1-7 (1 μM) for 24 h. The cells pretreated with specific receptor blockers, valsartan and A779, were used to confirm the receptor specific-effects of Ang II and Ang 1-7, respectively. Ang II, Ang 1-7, valsartan and A779 applied in the present studies were used at the concentration of 1 μM. For examine the regulation of ACE2 by Ang II, diphenyleneiodonium chloride (DPI) and PD98059 were used at concentration of 5 and 10 μM, respectively. Each experiment was performed independently three times.

3-3. Total RNA extraction

Total cellular RNA was extracted as recommended by the manufacturer of TRIzol™ (GIBCO BRL, Rockville, MD). Briefly, the TRIzol method consists of the addition of 1 mL of the TRIzol reagent to the cells (5×10^6 cells). The mixture was vigorously agitated for 30 sec and incubated at room temperature for 5 min. After this procedure, 200 μL chloroform was added to the tube, and mix well then centrifuged at 12,000 × *g* for 15 min. The aqueous phase was transferred to a clean tube, precipitated with 500 μL isopropyl alcohol, and centrifuged at 12,000 × *g* for 15 min. The resulting RNA pellet was then washed with 1 mL of 75% cold ethanol and centrifuged at 12,000 × *g* at 4°C for 10 min. The pellet was dried at room temperature in laminar flow, resuspended in 25 μL of diethylpyrocarbonate (DEPC)-treated water, and stored at −80°C. RNA was quantified by measuring absorbance at 260 nm and 280 nm and electrophoresed on a denaturing 1% agarose gel. The integrity and relative amounts of RNA were evaluated using ultraviolet visualization of ethidium bromide stained RNA.

3-4. Reverse transcription-polymerase chain reaction (RT-PCR)

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed as our previous report [Pan *et al.*, 2008]. For cDNA synthesis, 5 μg RNA was supplemented in a total reaction volume of 20 μL with 1 X reverse transcription buffer, 0.5 mM dNTPs, 2.5 μM oligo-dT (TOYOBO, Osaka, Japan), 1 U/μL RNase inhibitor (TOYOBO), and 5 U/μL ReverTra Ace™ reverse transcriptase (TOYOBO). After incubation for 60 min at 42°C, the mixture was incubated for 5 min at 99°C to denature the products. The mixture was then chilled on ice for further use.

PCR primer pairs used for RT-PCR were shown in **Table 3-1**. PCR reaction contains 2 μL cDNA, 2 μL of each primer (10 μM), 5 μL of 10 X PCR buffer, 2 μL of 10 mM dNTP, 1 μL of 5 U/μL Taq polymerase (Promega, Madison, WI) and 36 μL distilled water in a total volume of 50 μL. Thermal cycler (MiniCycler™; MJ Research, Waltham, MA) conditions were as follows: 1 cycle of 5 min at 94°C, 16 ~ 36 cycles of denaturation, annealing and elongation at 94°C for 30 sec, 55°C for 30 sec and 72°C for 45 sec, respectively. The resulting PCR products were visualized on 1.5% agarose gels stained with SYBR Safe DNA gel stain (Invitrogen, Eugene, OR, USA). The stained image was recorded by an image

analyzer (Kodak DC290 Digital camera System™; Eastman Kodak, Rochester, NY, USA), and the band intensity was quantified using densitometric analysis by Scion image™. The relative transcript expression of ACE2, AT1R and Mas were calculated as ratio to glyceraldehyde-3-phophate dehydrogenase (GAPDH) expression.

Table 3-1. The nucleotide sequences of the PCR primers used to assay gene expression by RT-PCR

Gene	Accession no.	Forward/Reverse primers sequence (5'→3')	PCR amplification protocol (cycle no.)	PCR product size (bp)
GAPDH	AF_261085	F-TGGCGCTGAGTACGTCGTG R-TTCAGCTCAGGGATGACCTT	94°C, 5 min → [94°C, 30 sec → 56°C, 30 sec → 72°C, 45 sec] (18) → 72°C, 3 min	413
ACE2	NM_021804	F-ACGACAATGAAATGTACCTGTTCCG R-TCCGATCTCTGATCCCAGTGAAG	94°C, 5 min → [94°C, 30 sec → 60°C, 30 sec → 72°C, 45 sec] (38) → 72°C, 3 min	399
AT1R	NM_004835	F-CCAAAAGCCAAATCCCACTCAAACC R-TCTGACATTGTTCTTCGAGCAGCC	94°C, 5 min → [94°C, 30 sec → 57°C, 30 sec → 72°C, 45 sec] (24) → 72°C, 3 min	362
Mas	NM_002377	F-ACAACACGGGCCTCTATCTG R-CTCATGGGCATAGCGAAGAT	94°C, 5 min → [94°C, 30 sec → 57°C, 30 sec → 72°C, 45 sec] (26) → 72°C, 3 min	388

ACE2, angiotensin-converting enzyme II; **AT1R**, angiotensin II type 1 receptor; **GAPDH**, glyceraldehyde-3-phophate dehydrogenase

3-5. Quantitative Real-time PCR

SYBR Green quantitative real-time reverse transcription-PCR (SYBR Green QPCR) was performed to detect the mRNA expression level of genes AT2R and GAPDH (as an internal control). SYBR Green QPCR was performed as recommended by the manufacturer of SYBR® Green Real-time PCR Master Mix (#QPK-201; TOYOBO). QCR primer pairs used for QPCR were shown in **Table 3-2**. QPCR amplification was performed on an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). According to the instructions of Applied Biosystems, the expression of each gene was quantified as $\triangle C_t$ (C_t of target gene - C_t of internal control gene) using GAPDH as the control and applying the formula $2^{-\triangle\triangle Ct}$ to calculate the relative fold changes [Winer *et al.*, 1999; Schmittgen and Zakrajsek, 2000].

Table 3-2. The nucleotide sequences of the PCR primers used to assay gene expression by Real-time PCR

Gene	Accession no.	Forward/Reverse primers sequence (5'→3')	PCR amplification protocol (cycle no.)	PCR product size (bp)
GAPDH	NM_008084	F- AGGTTGTCTCCTGCGACTTCA R- CCAGGAAATGAGCTTGACAAAGTTGTC	95°C, 1 min → [95°C, 15 sec → 60°C, 1 min] (40)	101
AT2R	NM_000686	F- CCTCGCTGTGGCTGATTTACTC R- CTTTGCACATCACAGGTCCAA	95°C, 1 min → [95°C, 15 sec → 60°C, 1 min] (40)	101

AT2R, angiotensin II type 2 receptor; **GAPDH**, glyceraldehyde-3-phophate dehydrogenase

3-6. Protein extraction and electrophoresis

The cultured cardiac cells (about 4×10^5 cells) were washed with 1 X PBS and lysed by adding 100 μL of PRO-PREP™ protein extraction solution (Intronbio, Gyeonggi-do, Korea), according to the manufacturer's instructions. The cell lysates were then centrifuged at 12,000 × *g* at 4°C for 10 min, and the supernatant was collected for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was measured by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard. Aliquots containing 30 μg protein were resolved on 10% slab SDS-PAGE gels.

3-7. Western blotting

The extracted proteins of the cardiac cells were electrophorized on SDS-PAGE and then transferred to PVDF membranes (POLYSCREEN™; PerkinElmer, Boston, MA). Briefly, nonspecific binding sites were blocked by incubating membranes in 5% non-fat milk. Primary antibodies against proteins were diluted as follows: 1:1,000 for ACE2, phosphorylated ERK1/2, phosphorylated MEK1/2 and GAPDH. The secondary antibodies were applied using a dilution of 1:2,000. Substrates were visualized by using ECL according to the manufacturer's instructions (Western Lightning Chemiluminescence Reagent Plus™; PerkinElmer, Boston, MA, USA) and by exposing the membranes to X-Ray film (Super Rx Medical X-Ray Film; Fujifilm, Kanagawa, Japan). The bands were detected at the expected size. The band intensity was quantified using densitometric analysis by imaging software (Scion image™; Scion, Frederick, MD). The amounts of ACE2, phosphorylated ERK1/2 and phosphorylated MEK1/2 are expressed relative to the amount of GAPDH in respective samples.

3-8. Human ACE2 promoter constructs

Human genomic DNA extracted by genomic DNA extraction kit (Geneaid, Taipei, Taiwan) was used as the template to obtain the promoter fragment of ACE2 genes by PCR-based approach. The upstream regulatory regions of the human ACE2 gene were constructed into a promotorless luciferase-based reporter vector, pGL3-Basic, to generate

pGL3-ACE2 (**Figure 3-1**). For amplify promoter fragment, the primers were introduced specific recognition sequence of restriction enzyme to facilitate cloning. The oligonucleotides used for amplify ACE2 promoters were shown in **Table 3-3**. All constructs used in this study were checked with restriction-mapped and sequenced to confirm their authenticity.

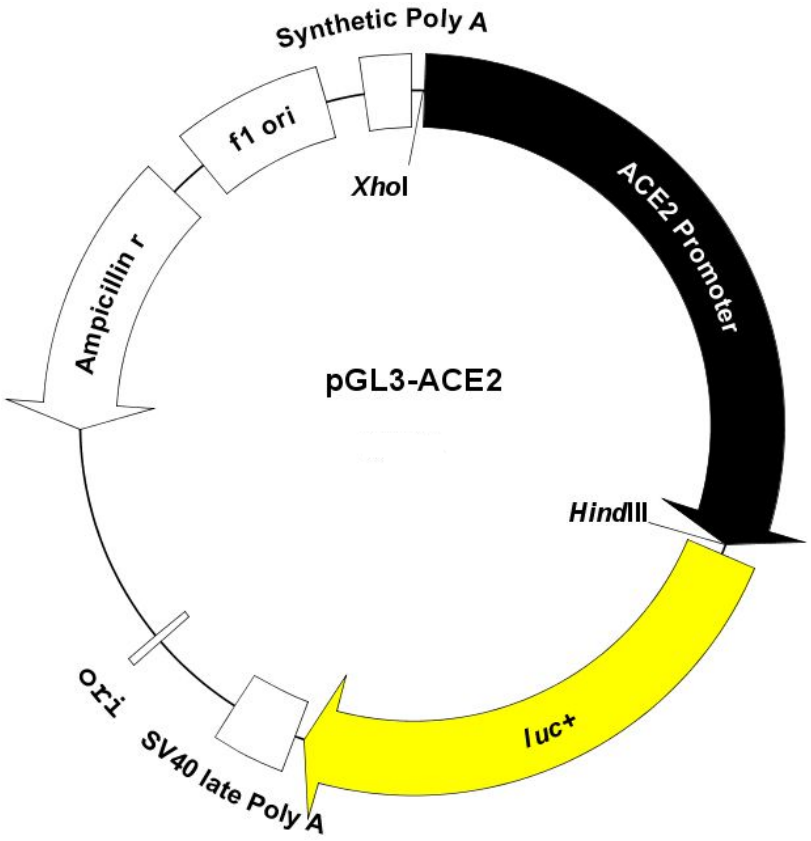


Figure 3-1. The vector map of the pGL3-ACE2 constructs. The cloned human ACE2 promoter region was inserted into the multi-cloning site (*Xho*I / *Hind*III) of a promotorless luciferase reporter vector, pGL3-Basic, to generate pGL3-ACE2. *Luc*$^{+}$, luciferase structural gene; Ampicillin r, ampicillin resistance gene; ori, origin of replication.

Table 3-3. Sequences of the primers used for construction of human ACE2 promoter plasmids, pGL3-ACE2

Constructs	Forward/Reverse sequence (5'→3')	Restriction Enzyme	Promoter region	Amplicon length (bp)
pGL3 (-2069/-49)	F- AACC**CTCGAG**TTTCATTTAGGA R- CTCAT**AAGCTT**TTCTCTCTTATCA	*Xho*I / *Hind*III	-2069~ -49	2021
pGL3 (-2069/+20)	F- AACC**CTCGAG**TTTCATTTAGGA R- GAGCT**AAGCTT**CGTCCCCTGTG	*Xho*I / *Hind*III	-2069 ~ +20	2089
pGL3 (-1493/+20)	F- GTTT**CTCGAG**ATGCTCAAATGA R- GAGCT**AAGCTT**CGTCCCCTGTG	*Xho*I / *Hind*III	-1493 ~ +20	1513
pGL3 (-1110/+20)	F- TGAC**CTCGAG**TGAGTTTTGAAT R- GAGCT**AAGCTT**CGTCCCCTGTG	*Xho*I / *Hind*III	-1110 ~ +20	1130
pGL3 (-516/+20)	F- TAAAGA**CTCGAG**CAAAGTCATG R- GAGCT**AAGCTT**CGTCCCCTGTG	*Xho*I / *Hind*III	-516 ~ +20	536

Restriction enzyme recognization sequences within primers are shown in red letters.
The promoter region is defined according to the position relative to the transcription start site (+1) in ACE2 mRNA sequence (GenBank No. AF_291820

3-9. Transient transfection and luciferase reporter assay

The cells were transfected with plasmid DNA using Turbofect™ *in vitro* transfection reagent (Fermentas, Glen Burnie, MD, USA) according to the manufacturer's instructions for the reverse transfection. Briefly, 2 μg DNA was diluted in 200 μL of cell growth medium. Add 4 μL of TurboFect to the diluted DNA and mix gently, and then the mixture was incubated for 15-20 min at room temperature. Gently layer 2 mL of fresh trypsinized cells (1×10^5 cells/well) on top of the TurboFect/DNA mixture in 12-well culture plate. Incubate cells at 37°C in CO_2 incubator for 24 h prior to testing for transgene expression.

Luciferase-based reporter gene assay was performed according to the manufacturer's instructions (Promega). Before harvesting, cell monolayers were rinsed twice with ice-cold 1 × PBS. Cells were, subsequently, scraped in 1 × luciferase cell culture lysis reagent (CCLR; Promega), then cell lysates were centrifuged at 4°C for 2 min. Total protein concentration of the supernatants was measured by the protein assay kit (Bio-Rad). To mix 20 μL of the supernatant containing equal amounts of total protein with 100 μL of luciferase assay reagent (Promega) prior to analysis. Luciferase enzymatic activities were measured with Lumat LB9507™ single tube luminometer (Brethold Techonologies, Bad Wildbad, Germany).

3-10. Immunocytochemistry

Human cardiac fibroblasts (HCF, #6300) were grown overnight on coverslips (1.2 mm, 0.01% Poly-L-Lysine treated 1 h) in commercial media (#2301; ScienCell Research Laboratories) and incubated in a humidified 5% CO_2 atmosphere at 37°C. The coverslips were washed in PBS, fixed with 4% formaldehyde for 15-20 min, treated with 0.5% Saponin (Sigma-Aldrich) for 10-15 min and nonspecific sites were blocked with 1% BSA for 30 min. Coverslips were then treated with anti-ACE2 and anti-AT1R antibody (1:100 dilution) at 37°C for 1 h followed by Alexa Fluor 488 and 594 second antibody (#A11032, #A11034; Invitrogen, Eugene, OR) (1:200 dilution) at 37°C for 1 h in a humidified chamber. Coverslips were also counterstained with DAPI (1:10000 dilution) at 37°C for 5 min in a humidified chamber. After washing in PBS, cover slips were mounted by DakoCytomation Fluorescent Mounting Medium **(**DakoCytomation, Denmark A/S**)** on glass slides and observed under FluoView^{TM} FV500 Confocal Microscope (Fluoview FV500, Olympus,

Tokyo, Japan).

3-11. Statistics

All values are expressed as mean ± standard deviation (SD). Data were compared with one-way analysis of variance (ANOVA) test to evaluate differences among multiple groups. A value of $P < 0.05$ was considered statistically significant.

IV. Results

4-1. Ang II-mediated cardiac ACE2 upregulation in human cardiofibroblast

The transcript expression of human ACE2 was examined in the Ang II–treated HCF cells. The expression of ACE2 mRNA was markedly elevated ($P < 0.01$) in a concentration-dependent manner in the HCF cells treated with Ang II (**Figure 4-1A**). Moreover, the HCF cells stimulated with 1 μM of Ang II resulted in time-dependent increase in ACE2 mRNA over 24 h (**Figure 4-1B**). The HCF cells were pretreated with an AT1R inhibitor, valsartan, and then stimulated with Ang II to check the role of AT1R in the ACE2 upregulation. Ang II–induced effect on the upregulation of cardiac ACE2 expression was abolished when the HCF cells were pretreated with valsartan. The result confirms that AT1R signaling transduction pathway is involved in the cardiac ACE2 upregulation by Ang II stimulation (**Figure 4-1C**).

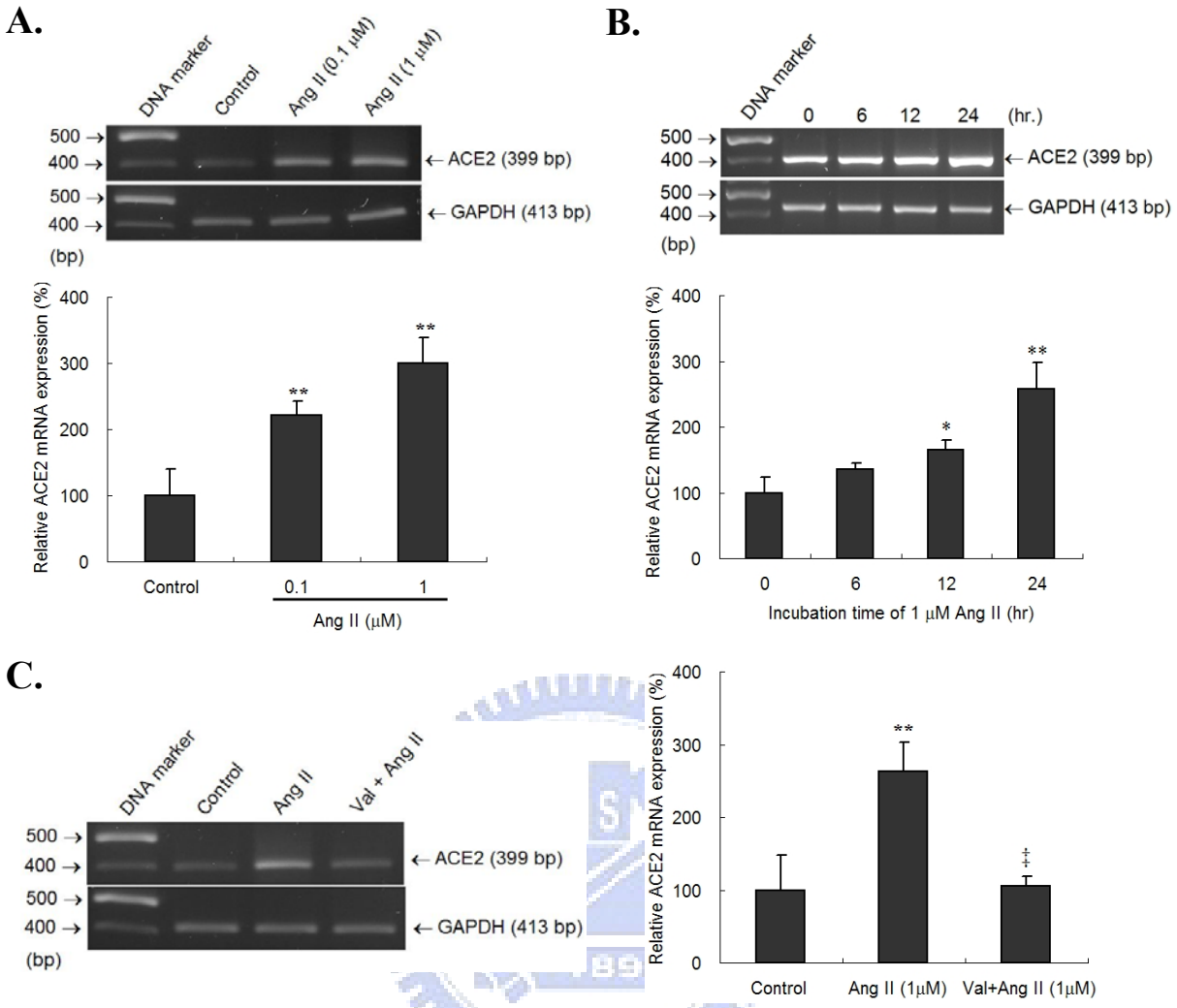


Figure 4-1. The mRNA expression of human ACE2 in the HCF cells treated with Ang II. The effect of Ang II in the regulation of ACE2 expression was determined in both dose-dependent (**A**) and time-dependent manner (**B**). (**C**) Ang II-mediated ACE2 expression was abolished by an AT1R blocker, valsartan (Val). The results for each experiment were normalized to the density of GAPDH PCR product. The relative transcript expression of ACE2 was calculated according to the values of control group as 100%. Histograms of all values are expressed as the mean ± S.D. * and ** indicate $P < 0.05$ and $P < 0.01$ as compared with the control group, respectively. ‡ indicates $P < 0.01$ as compared with the Ang II treated only.

4-2. Human AT1R and AT2R could be markedly increased after Ang II stimulation

The effect of Ang II in the regulation of human AT1R and AT2R were determined in time-dependent manner. The transcript expression of AT1R was markedly increased in time-dependent over 72 h. This result shows that the Ang II–mediated ACE2 mRNA expression could be strengthened by even more AT1R (**Figure 4-2A**). It is to say that AT2R is thought to counteract the signals transmitted by the AT1R, eliciting vasodilatation, inhibition of proliferation, NO production and apoptosis. The AT2R may play a homeostatic role in the regulation of blood pressure in animal models of hypertension. The transcript expression of AT2R was examined by Ang II treatment in time-dependent manner. The AT2R mRNA expression was upregulated over 48 h. This data suggest that AT2R expression was also increased by Ang II stimulation. According to the parallel effect of Ang II–mediated AT1R and AT2R expression, we suggest that the ratio of AT1R/AT2R could be considerably regard as the critical indicator of Ang II-induced heart dysfunction (**Figure 4-2B**).

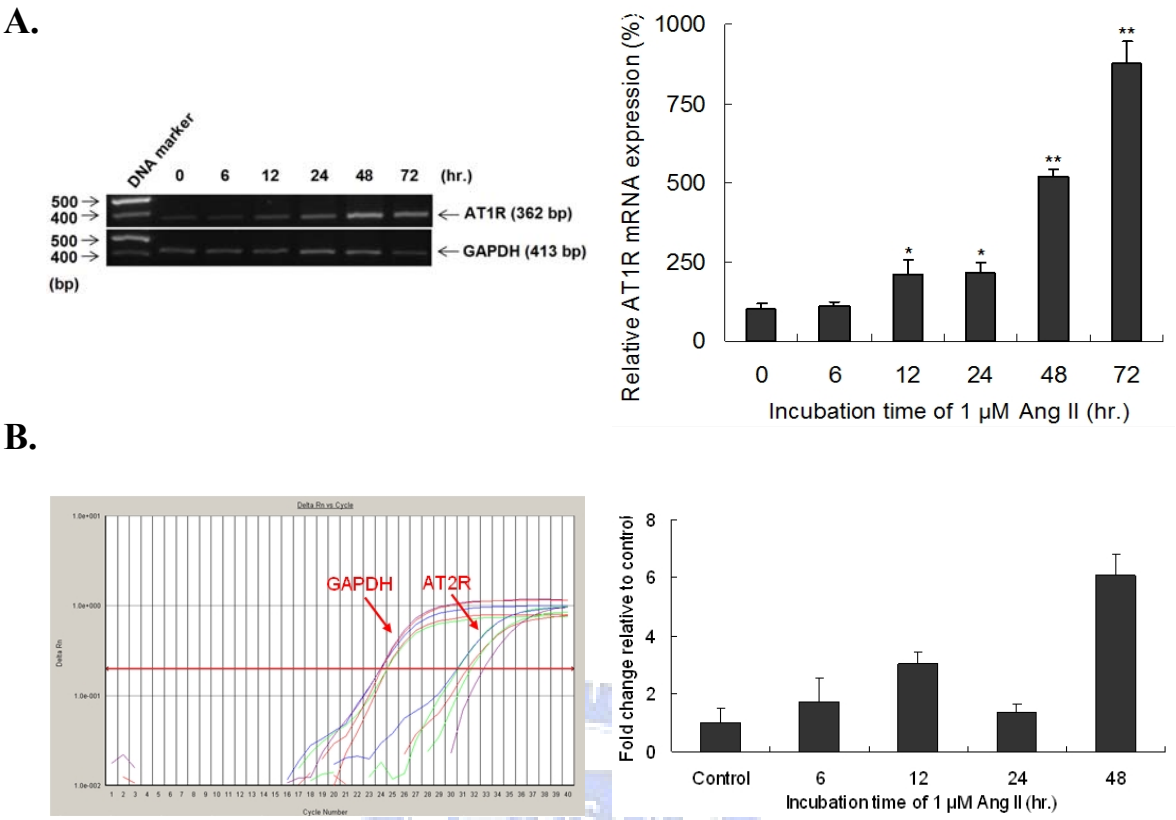


Figure 4-2. The mRNA expression of human AT1R and AT2R in the HCF cells treated with Ang II. The effect of Ang II in the regulation of AT1R (**A**) and AT2R (**B**) expression was determined in time-dependent manner. The results for each experiment were normalized to the density of GAPDH control. The relative transcript expression of AT1R was calculated according to the values of control group as 100%. The relative transcript expression of AT2R was calculated according to the fold change relative to control group as 1.0 fold. Histograms of all values are expressed as the mean ± S.D. * and ** indicate $P < 0.05$ and $P < 0.01$ as compared with the control group, respectively.

4-3. ERK–MAPK cascade is involved in Ang II-mediated upregulation of cardiac ACE2

The effect of Ang II-induced ACE2 expression in the HCF cells was further confirmed by the experiments of blocking the downstream signaling of AT1R, ERK–MAPK cascade. The HCF cells were pretreated with a MEK inhibitor, PD98059, and then treated with Ang II. The result shows that the mRNA expression of ACE2 increased by AT1R-dependent effect could be abolished by the PD98059 pretreatment (**Figure 4-3A**). Hence, the signal molecules within ERK-MAPK pathway were explored by Western blot to analyze whether ERK-MAPK cascade is involved in Ang II–mediated upregulation of cardiac ACE2. The results indicate that an upregulated ACE2 combined with increased phosphorylated MEK1/2 and activated ERK1/2 was found in the HCF cells after Ang II treatment, and all of upregulation of ACE2, phosphorylated MEK1/2 and activated ERK1/2 were attenuated in the Ang II-treated HCF cells as the cells pretreated with PD98059 (**Figure 4-3B**).

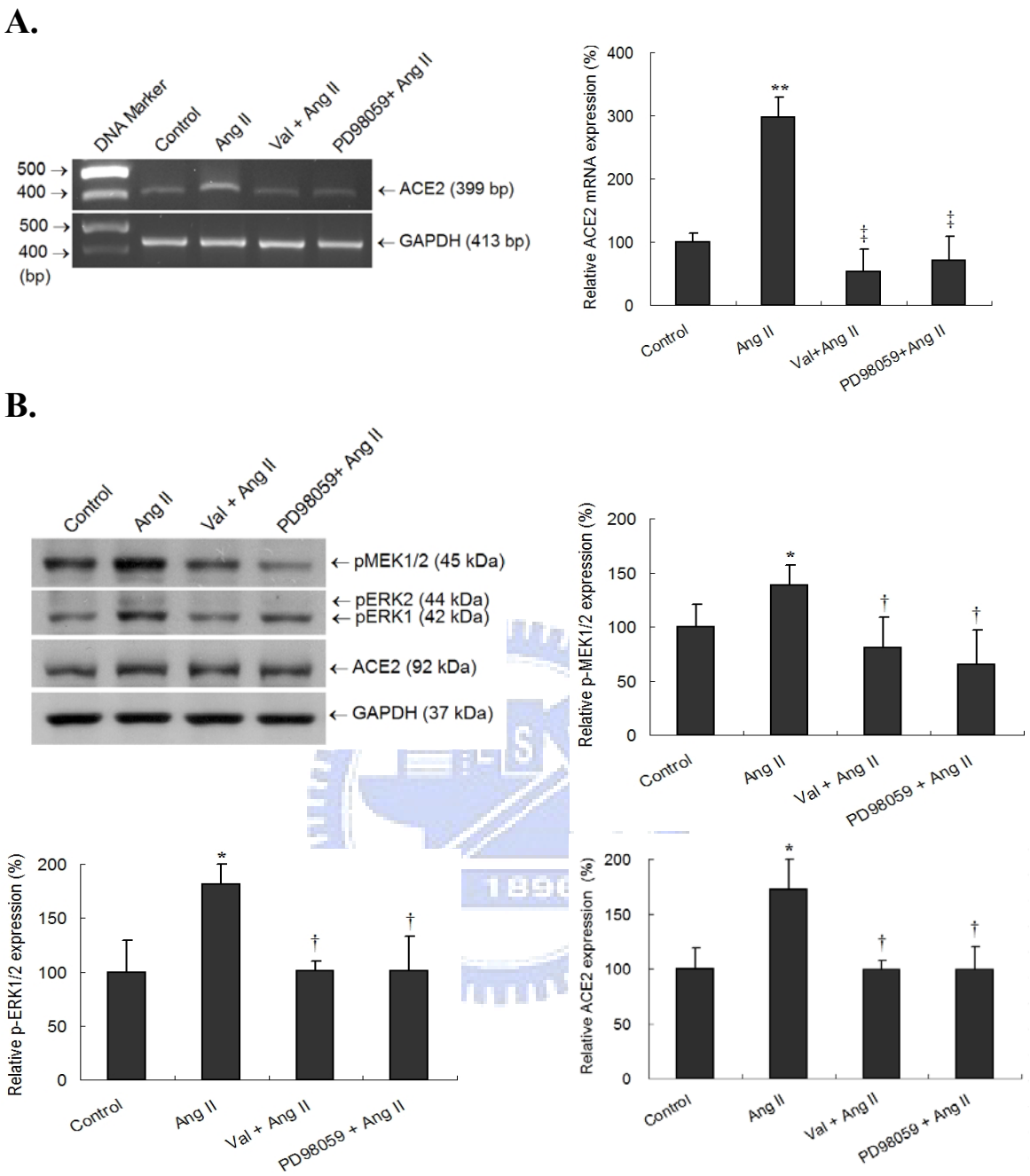


Figure 4-3. Role of ERK-MAPK signaling of AT1R in the ACE2 regulation by Ang II. **(A)** The effect of Ang II on ACE2 gene expression was determined in the HCF cells pretreated with AT1R blocker (valsartan, Val) and MEK1/2 inhibitor (PD98059) to confirm the AT1R-dependent effect. **(B)** ERK-MAPK signaling, including phosphorylated MEK1/2 and activated ERK1/2, was examined by Western blot to check the Ang II-mediated ACE2 expression. The results for each experiment were normalized to the density of GAPDH. The relative expression of ACE2, phosphorylated MEK1/2 and activated ERK1/2 were calculated according to the values of control group as 100%. Histograms of all values are expressed as the mean ± S.D. * and ** indicate $P < 0.05$ and $P < 0.01$ as compared with the control group, respectively. † indicates $P < 0.05$ as compared with the Ang II treated only.

4-4. NADPH oxidase signaling pathway is concerned with Ang II-stimulated ACE2 upregulation

NADPH oxidase signaling pathway in Ang II–AT1R axis was also analyzed to verify the role in Ang II-stimulated ACE2 upregulation in the HCF cells. The experimental result shows that the HCF cells pretreated with DPI, a NADPH oxidase inhibitor, could abolish the increase of ACE2 mRNA stimulated by Ang II (**Figure 4-4A**). Furthermore, hydrogen peroxide was used as a source of reactive oxygen species (ROS) to test the role of ROS in the ACE2 regulation in HCF cells. The transcript expression of cardiac ACE2 was markedly increased in the HCF cells after hydrogen peroxide treatment (**Figure 4-4B**).

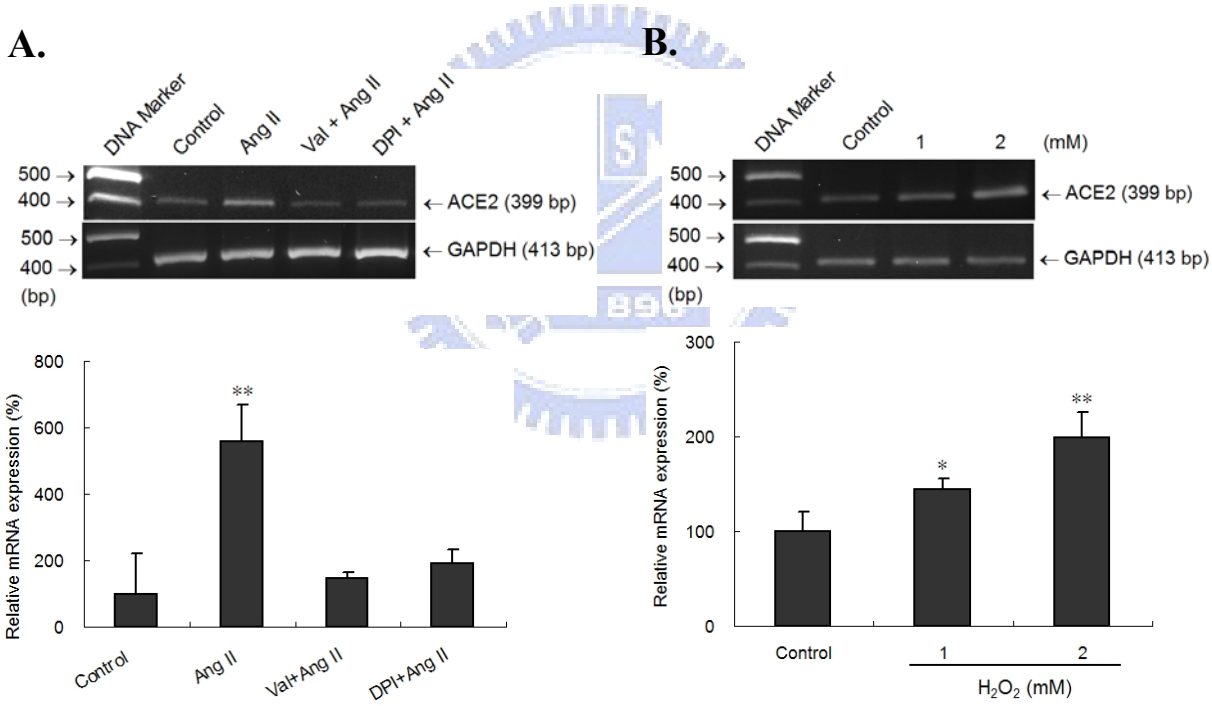


Figure 4-4. Role of NADPH oxidase in the regulation of ACE2 by Ang II. **(A)** The effect of Ang II in regulation of ACE2 expression was determined in HCF cells pretreated with NADPH oxidase inhibitor (DPI) to confirm AT1R-dependent effect by RT-PCR. **(B)** The effect of reactive oxygen species (ROS) in regulation of ACE2 was examined in HCF cells treated with hydrogen peroxide. The results for each experiment were normalized to the density of the GAPDH. The relative transcript expression of ACE2 was calculated according to the values of control group as 100%. Histograms of all values are expressed as the mean ± S.D. * and ** indicate $P < 0.05$ and $P < 0.01$ as compared with the control group, respectively. † and ‡ indicate $P < 0.05$ and $P < 0.01$ as compared with the Ang II treated only, respectively.

4-5. Mas receptor is involved in the effect of Ang 1-7-mediated upregulation of ACE2

The effect of Ang 1-7 on cardiac ACE2 regulation was studied in the HCF cells. Ang 1-7 treatment could significantly increase the transcript and protein expression of cardiac ACE2 (**Figure 4-5**). For evaluate whether Mas receptor is involved in the effect of Ang 1-7–mediated upregulation of ACE2, the HCF cells were pretreated with a Mas receptor blocker, A779, and then treated with Ang 1-7. Both the mRNA and protein level of ACE2 upregulated by Ang 1-7 were depressed by the A779 pretreatment, which indicated that Mas receptor is associated with Ang 1-7-induced ACE2 upregulation.

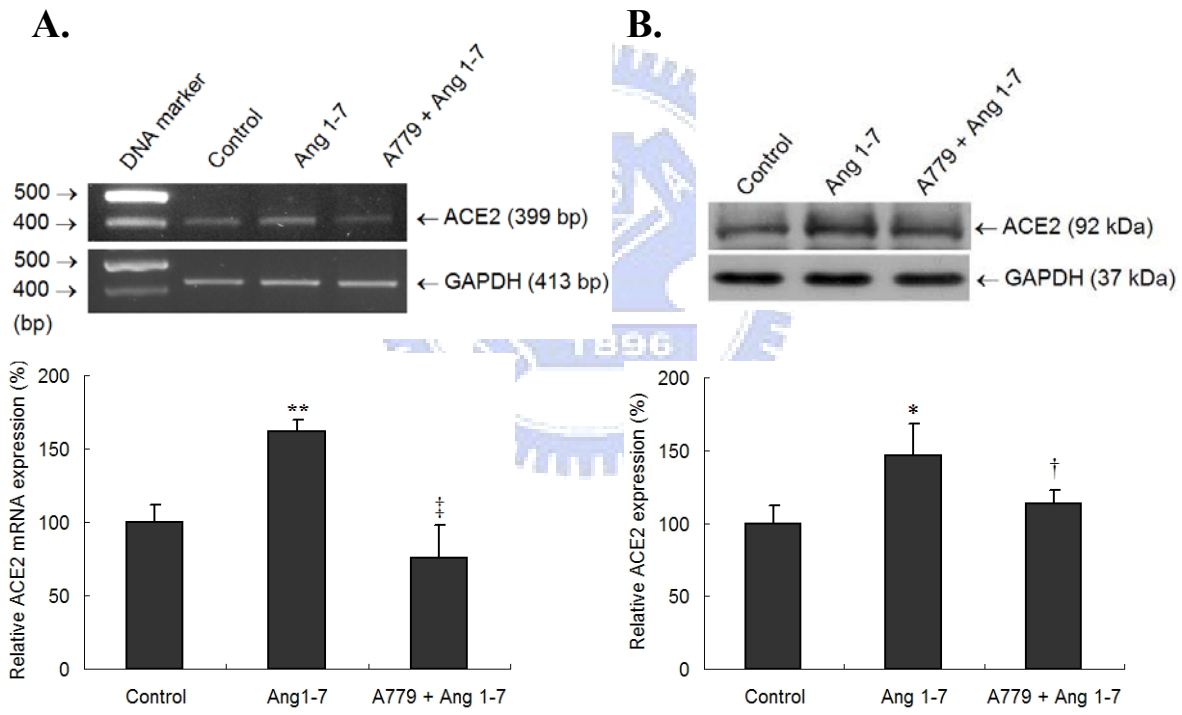


Figure 4-5. The regulation of ACE2 in HCF cells after Ang 1-7 treatment. The effect of Ang 1-7 on the regulation of ACE2 expression was examined by RT-PCR **(A)** and Western blot **(B)**. Ang 1-7 induced ACE2 was further confirmed by the cardiac cells pretreated with Mas receptor blocker, A779. The results for each experiment were normalized to the density of the GAPDH. The relative transcript and protein expression of ACE2 was calculated according to the values of control group as 100%. Histograms of all values are expressed as the mean ± S.D. * and ** indicate $P < 0.05$ and $P < 0.01$ as compared with the control group, respectively. † and ‡ indicate $P < 0.05$ and $P < 0.01$ as compared with the Ang II treated only, respectively.

4-6. ERK-MAPK cascade and NADPH oxidase signaling pathway were involved in the Ang 1-7 – ACE2 axis

The effect of Ang 1-7-induced ACE2 expression in the HCF cells was further confirmed by the experiments of blocking the downstream signaling of ERK–MAPK cascade and NADPH oxidase signaling pathway. The experimental result shows that the HCF cells pretreated with PD98059 and DPI could abolish the increase of ACE2 protein stimulated by Ang 1-7, respectively (**Figure 4-6A**). The results also indicate that an upregulated ACE2 combined with increased activated ERK1/2 was found in the HCF cells after Ang 1-7 treatment, and all of upregulation of ACE2 and activated ERK1/2 were attenuated in the Ang 1-7 treated HCF cells as the cells pretreated with PD98059 and DPI, respectively (**Figure 4-6B**).

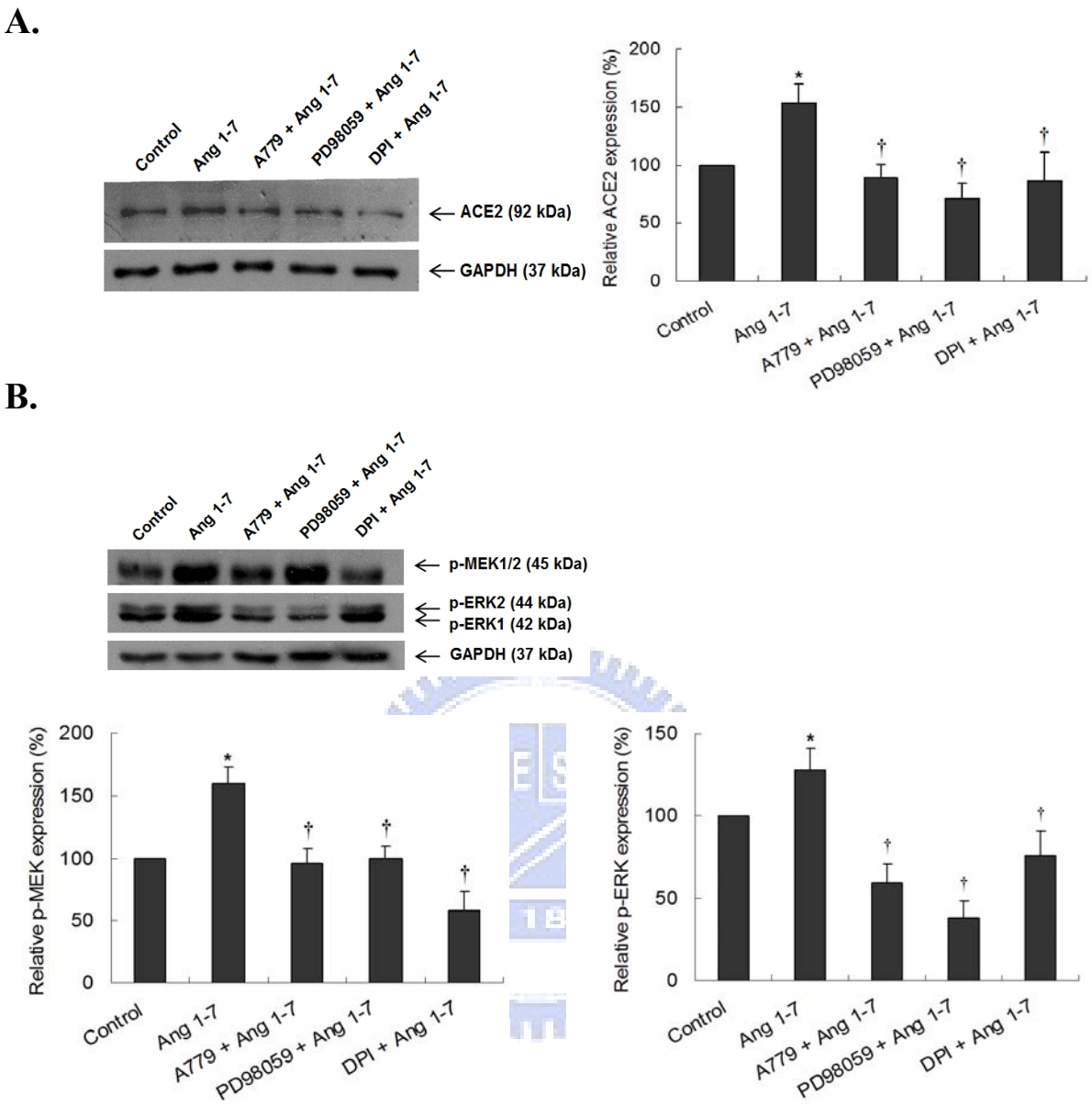


Figure 4-6. Role of ERK-MAPK signaling of Mas receptor and NADPH oxidase in the ACE2 regulation by Ang 1-7. **(A)** The effect of Ang 1-7 on ACE2 protein expression was determined by Western blot in the HCF cells pretreated with Mas receptor blocker (A779), MEK1/2 inhibitor (PD98059) and NADPH oxidase inhibitor (DPI) to confirm the Mas-dependent effect. **(B)** ERK-MAPK signaling, including phosphorylated MEK1/2 and activated ERK1/2, was examined by Western blot to check the Ang 1-7–mediated ACE2 expression. The results for each experiment were normalized to the density of GAPDH. The relative expression of ACE2 was calculated according to the values of control group as 100%. Histograms of all values are expressed as the mean ± S.D. * and ** indicate $P < 0.05$ and $P < 0.01$ as compared with the control group, respectively. † indicates $P < 0.05$ as compared with the Ang 1-7 treated only.

4-7. ACE2 upregulation stimulated by Ang 1-7 might be independent to Ang II–AT1R pathway

The effect of Ang 1-7 on the regulation of AT1R, Ang II receptor, in the HCF cells was examined. The result shows that Ang 1-7 treatment was insignificantly influence the AT1R expression in HCF cells at both of the transcriptional and translational level (**Figure 4-7**). To test the existence of Ang 1-7–AT1R pathway, HCF cells were pretreated with AT1R blocker (Valsartan) to confirm the AT1R-dependent ACE2 protein regulation. The result shows that HCF cells pretreating with Valsartan has no marked effect on Ang 1-7 induced ACE2 upregulation. The experimental results suggest the ACE2 upregulation stimulated by Ang 1-7 might be independent to Ang II–AT1R pathway (**Figure 4-8**).

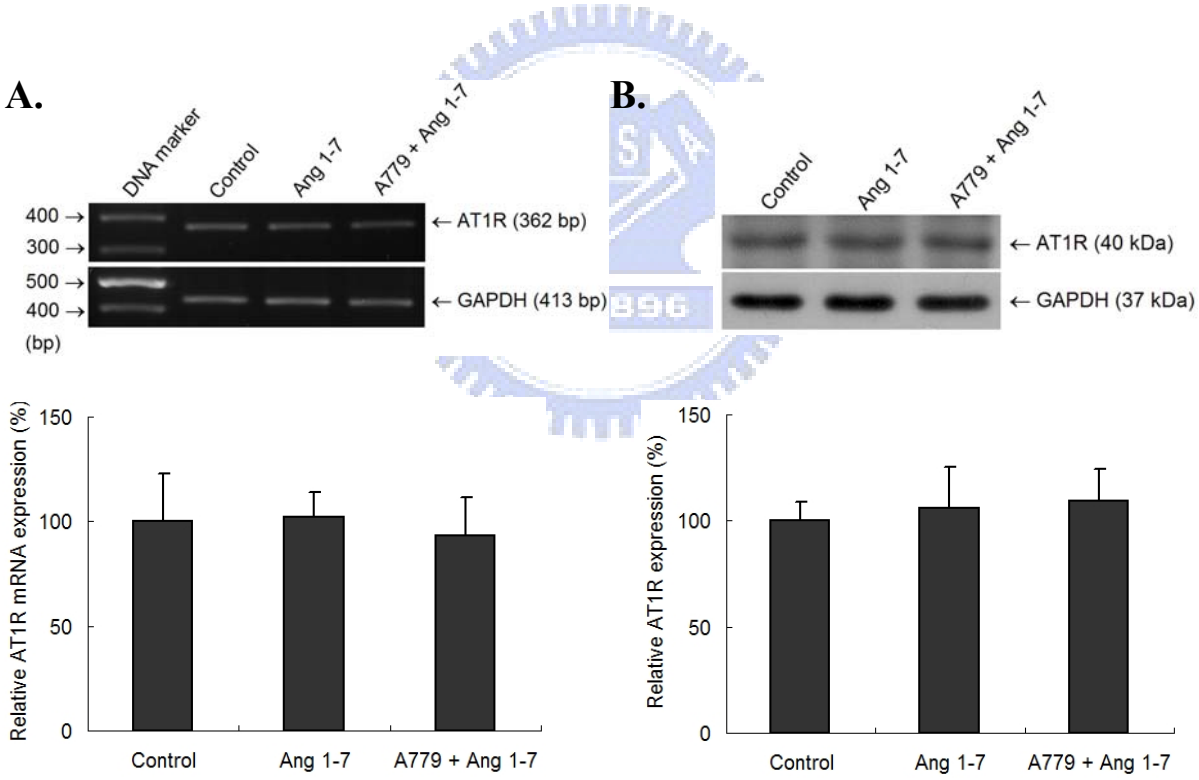


Figure 4-7. The regulation of angiotensin II type I receptors in the HCF cells treated with Ang 1-7. The effect of Ang 1-7 on the regulation of AT1R gene **(A)** and protein **(B)** expression were determined in the HCF cells pretreated with A779 to confirm Mas receptor-dependent effect. The results for each experiment were normalized to the density of the GAPDH. The relative expression of AT1R was calculated according to the values of control group as 100%. Histograms of all values are expressed as the mean ± S.D.

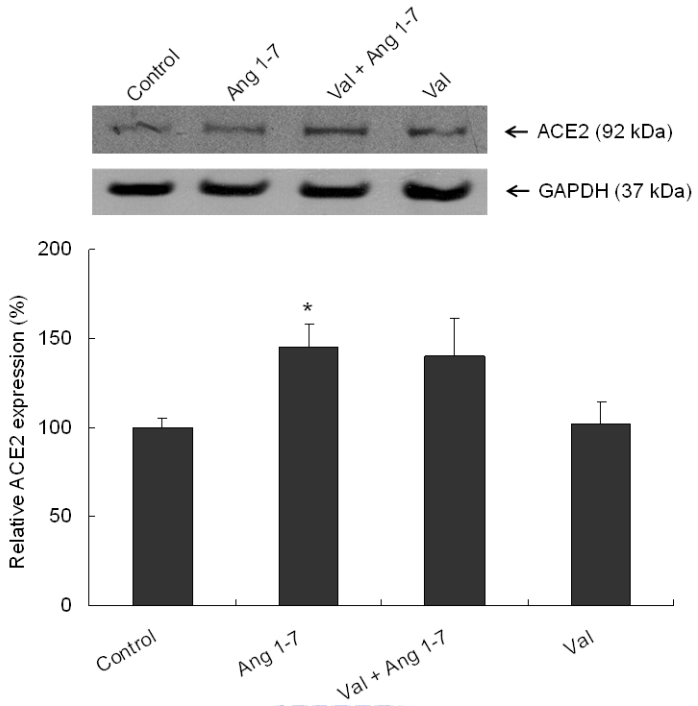


Figure 4-8. Role of the possibility of AT1R-dependent effect in the ACE2 regulation by Ang 1-7. The effect of Ang 1-7 on ACE2 protein expression was determined by Western blot in the HCF cells pretreated with AT1R blocker (Val) to confirm the AT1R-dependent effect. The results for each experiment were normalized to the density of GAPDH. The relative expression of ACE2 was calculated according to the values of control group as 100%. Histograms of all values are expressed as the mean ± S.D. * indicate $P < 0.05$ as compared with the control group.

4-8. The interference of each specific inhibitor or blocker was ruled out

To confirm the specific regulated mechanisms involved in the modulation of ACE2 and to exclude the effect of inhibitor treatment alone, the influence of each inhibitor on ACE2 regulation was determined by Western blot in the HCF cells pretreated with each signaling specific inhibitor (Valsartan, A779, PD98059 and DPI) to check the effect of each inhibitor on ACE2 regulation (**Figure 4-9**). The experimental results suggest that there is no markedly effect by inhibitor treatment alone. Thus, we could exclude the possibility of the interference of each specific inhibitor.

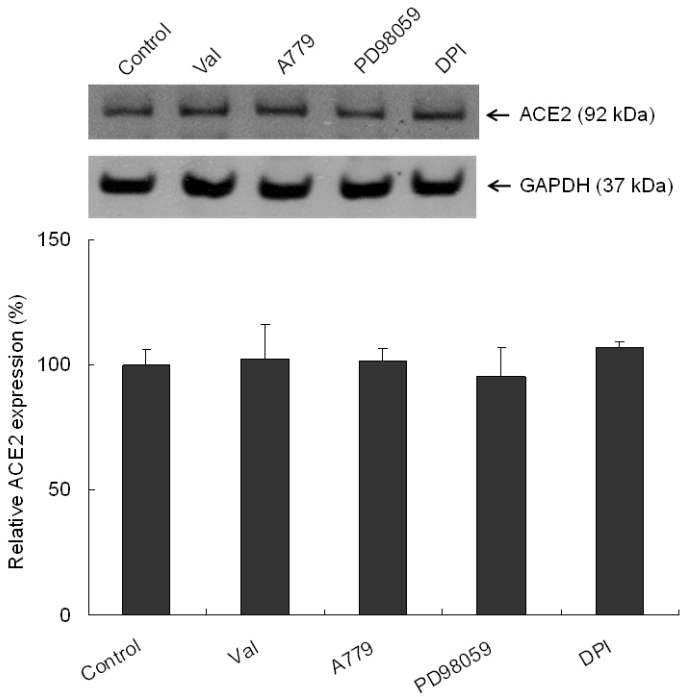


Figure 4-9. The influence of each signaling specific inhibitor on ACE2 regulation. The effect of specific inhibitor on ACE2 protein expression was determined by Western blot in the HCF cells pretreated with each signaling specific inhibitor (Val, A779, PD98059 and DPI) to check the effect of inhibitors on ACE2 regulation. The results for each experiment were normalized to the density of GAPDH. The relative expression of ACE2 was calculated according to the values of control group as 100%. Histograms of all values are expressed as the mean ± S.D.

4-9. ACE2 was major represented at the peripheral of cell membrane with both Ang II and Ang 1-7 treatment

Immunofluorescence localization and regulation of the ACE2 and AT1R in cardiovascular-related areas could provide still more clear knowledge for exploring the biological modulation of RAS. We evaluated the localization of AT1R and ACE2 using immunofluorescence in the HCF cells and further elucidating the modulation of Ang II and Ang 1-7 on these two molecules. The localization of ACE2 was major represented at the peripheral of cell membrane and partially distributed over the inner nucleus. The result shows that an abundant labeling of ACE2 was found in the HCF cells with both Ang II and Ang 1-7 treatment. The effect of Ang II–, Ang 1-7–mediated ACE2 expression were further abolished by specific antagonist, Valsartan and A779, respectively.

4-10. AT1R representing at the boundary of cell membrane was increased by Ang II treatment but not Ang 1-7

The localization of AT1R was approximately represented at the boundary of cell membrane. The experimental result shows that intense AT1R-positive immunofluorescence was found in the cells treated with Ang II but was no apparent effect by Ang 1-7 treatment. The images in **Figure 4-10, 4-11** provide further validation for Ang II– and Ang 1-7–mediated ACE2 expression and an actual presentation of AT1R and ACE2 localization in HCF cells. The quantification of the fluorescence expression of ACE2 and AT1R in HCF cells was calculated in **Figure 4-12**.

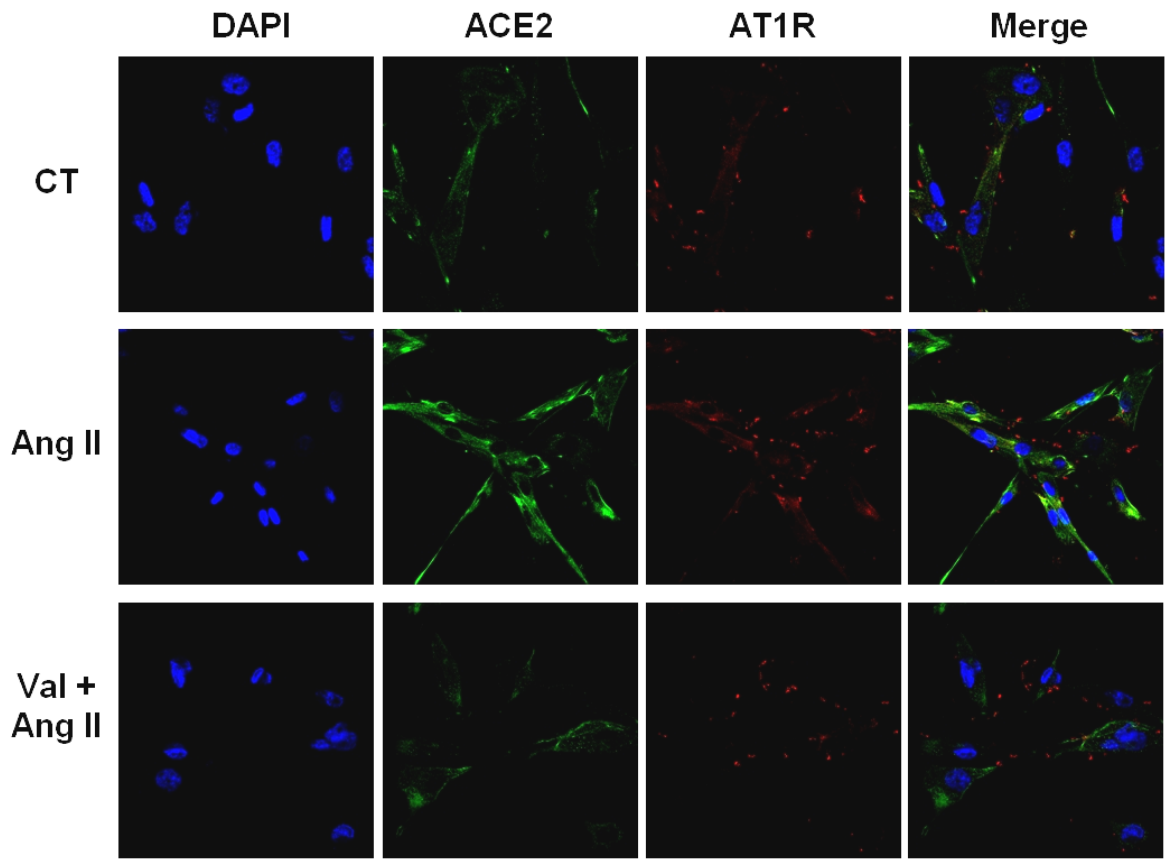


Figure 4-10. Localization and regulation of ACE2 and AT1R in HCF cells treated with Ang II. HCF cells grown on cover slips were treated with Ang II for 24 h. The cells after treatment were washed, then fixed and double-immunostained for ACE2 and AT1R. Colocalization appears as yellow after merging the images of Alexa 488-tagged ACE2 (green) and Alexa 594-tagged AT1R (red). The nuclear marker, DAPI, is shown in blue. Micrographs of cross sections were taken at a magnification of 600x.

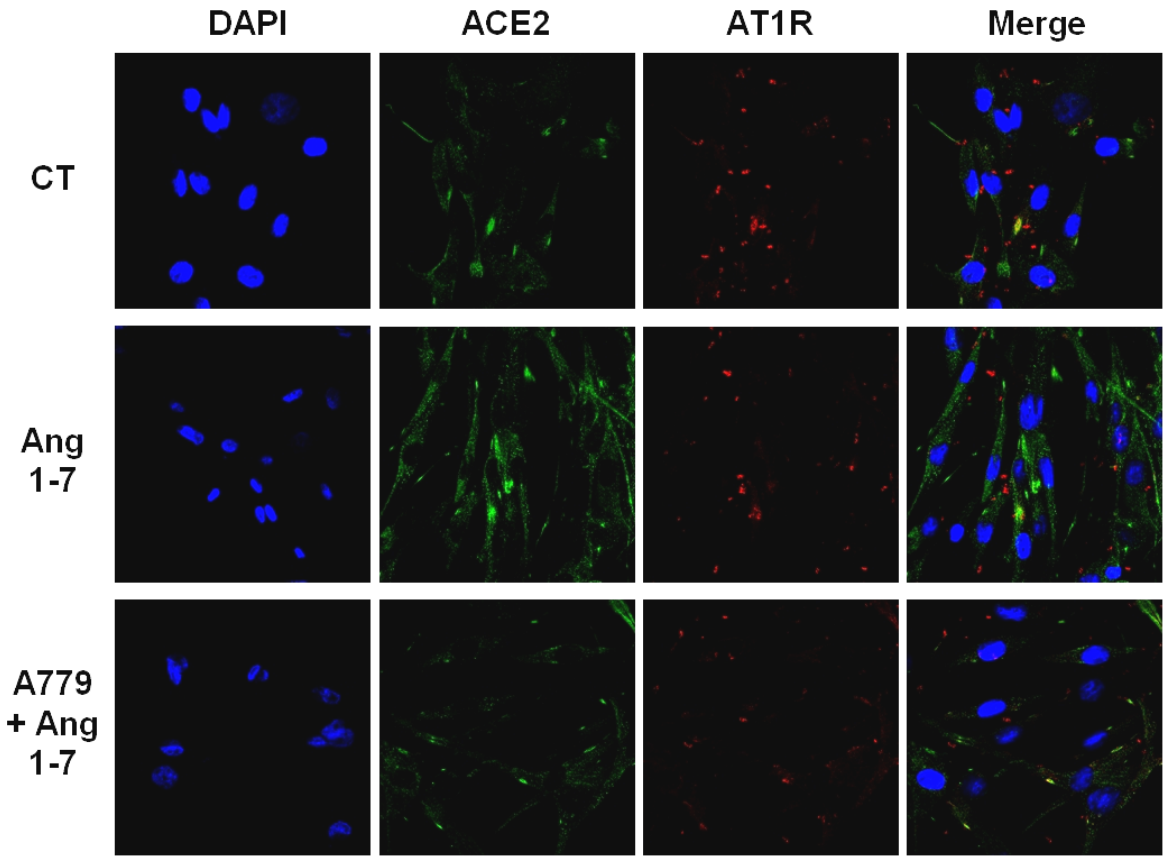
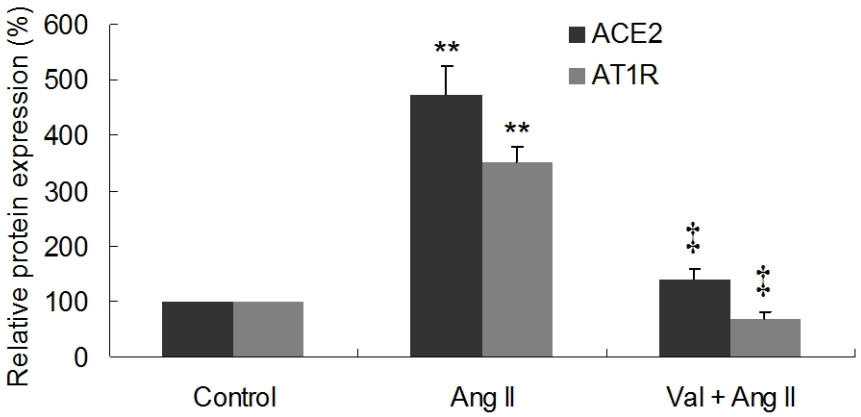


Figure 4-11. Localization and regulation of ACE2 and AT1R in HCF cells treated with Ang 1-7. HCF cells grown on cover slips were treated with Ang 1-7 for 24 h. The cells after treatment were washed, then fixed and double-immunostained for ACE2 and AT1R. Colocalization appears as yellow after merging the images of Alexa 488-tagged ACE2 (green) and Alexa 594-tagged AT1R (red). The nuclear marker, DAPI, is shown in blue. Micrographs of cross sections were taken at a magnification of 600x.

A.



B.

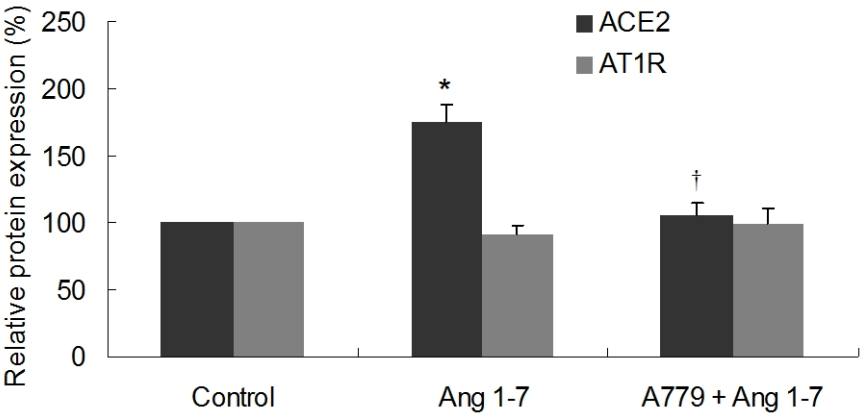


Figure 4-12. Quantification of the fluorescence expression of ACE2 and AT1R in HCF cells treated with Ang II and Ang 1-7. The fluorescence images were observed under Confocal Microscope and quantified by imaging software, Image J. **(A)** The effect of Ang II on the regulation of ACE2 and AT1R protein expression were determined in the HCF cells pretreated with Val. **(B)** The effect of Ang 1-7 on the regulation of ACE2 and AT1R protein expression were determined in the HCF cells pretreated with A779. The density of the fluorescence expression of ACE2 and AT1R were calculated according to the area of DIC view. The relative expression of ACE2 and AT1R were calculated according to the values of control group as 100%. Histograms of all values are expressed as the mean ± S.D. * and ** indicate $P < 0.05$ and $P < 0.01$ as compared with the control group, respectively. † and ‡ indicate $P < 0.05$ and $P < 0.01$ as compared with the Ang II or Ang 1-7 treated only

4-11. Deletion mutation of ACE2 promoter and figure out the intense promoter activity with Ang II and Ang 1-7 treatment

Serial truncated human ACE2 promoter (**Table 3-3**) was introduced into luciferase-based reporter vector, pGL3-basic, to rough out which region of constructed promoter might contain critical regulatory activity of ACE2 (**Figure 3-1**). The analyzed data showed that human cardiac ACE2 promoter activity was markedly upregulated with transfection of pGL3 (-516/+20) constructs (**Figure 4-13**). The pGL3 (-516/+20) constructs was further incubated with Ang II and Ang 1-7 for 24 h to verify the role in Ang II– and Ang 1-7–mediated ACE2 promoter activity. The result showed that human cardiac ACE2 promoter activity was significantly upregulated with Ang II stimulation. Ang II-induced ACE2 promoter activity could be abolished when the HCF cells pretreated with Valsartan. This result indicated that ACE2 promoter activity was parallel to the transcriptional and translational expression of ACE2. In order to confirm whether the Ang 1-7 regulated ACE2 promoter activity was the same as the data we have shown in transcript and protein expression of cardiac ACE2, HCF cells were treated with Ang 1-7 for 24 h. The analyzed data showed that Ang 1-7 could remarkably increase the human cardiac ACE2 promoter activity, but this upregulation has no noticeably effect by A779 pretreatment (**Figure 4-14**). The upstream region of the ACE2 gene and specific promoter binding site were shown in **Figure 4-15**.

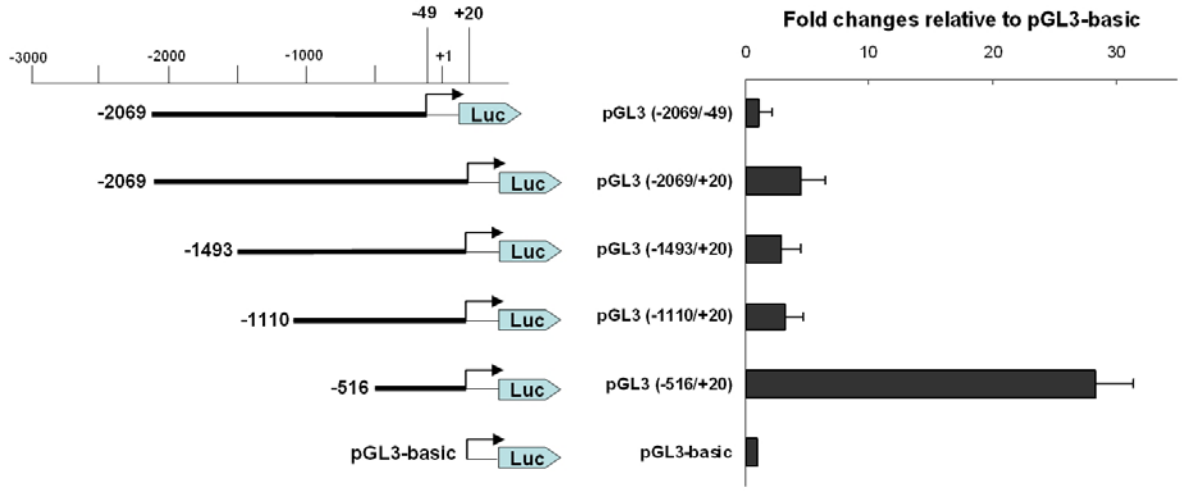


Figure 4-13. Deletion mutation analysis of the *ace2* promoter region in the HCF cells. Fragments of the 5'-flanking regions of the human ACE2 gene were fused to the firefly luciferase cDNA in the vector pGL3-basic. The position of the promoter fragments relative to transcription start site (+1) is indicated. HCF cells were transfected with the indicated reporter gene constructs. Cells were lysed 24 h after transfection, and luciferase activities were determined from duplicate wells. Five different constructs of pGL3-ACE2 plasmids are described in **Table 3-3**. Data are presented as relative fold changes of luciferase activity to the pGL3-basic construct. The diagram integrates results of three independent experiments. Histograms of all values are expressed as the mean ± S.D.

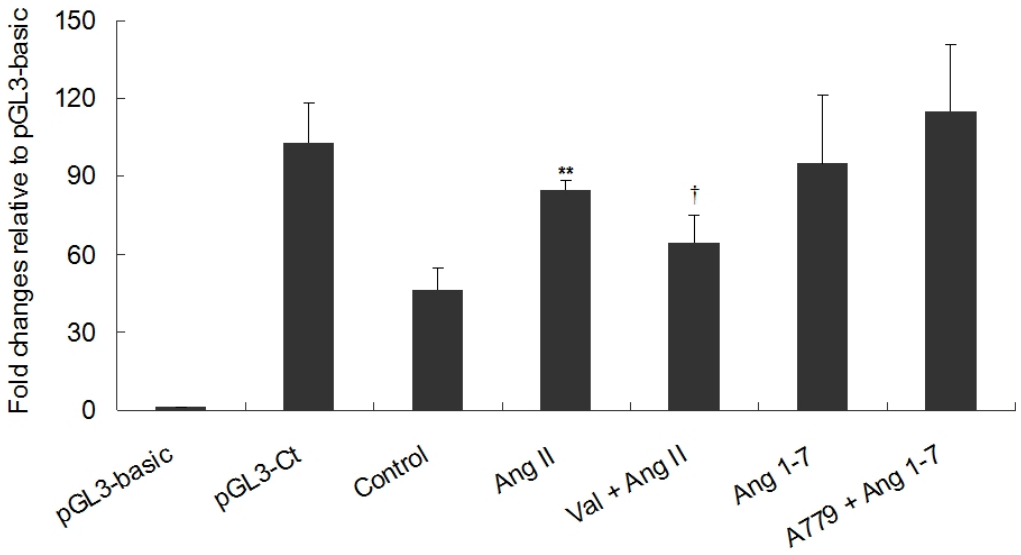


Figure 4-14. The regulation of ACE2 promoter activity in the HCF cells treated with Ang II and Ang 1-7. Fragments of the 5'-flanking regions of the human ACE2 gene were fused to the firefly luciferase cDNA in the vector pGL3-basic. HCF cells were transfected with the reporter gene constructs including the *ace2* promoter region of (-516/+20). The effect of Ang II and Ang 1-7 on the regulation of ACE2 promoter activity was determined in the HCF cells pretreated with Valsartan and A779 to confirm AT1R and Mas receptor-dependent effect, respectively. Cells were lysed 24 h after transfection, and luciferase activities were determined from duplicate wells. Data are presented as relative fold changes of luciferase activity to the pGL3-basic construct. The diagram integrates results of three independent experiments. Histograms of all values are expressed as the mean ± S.D.

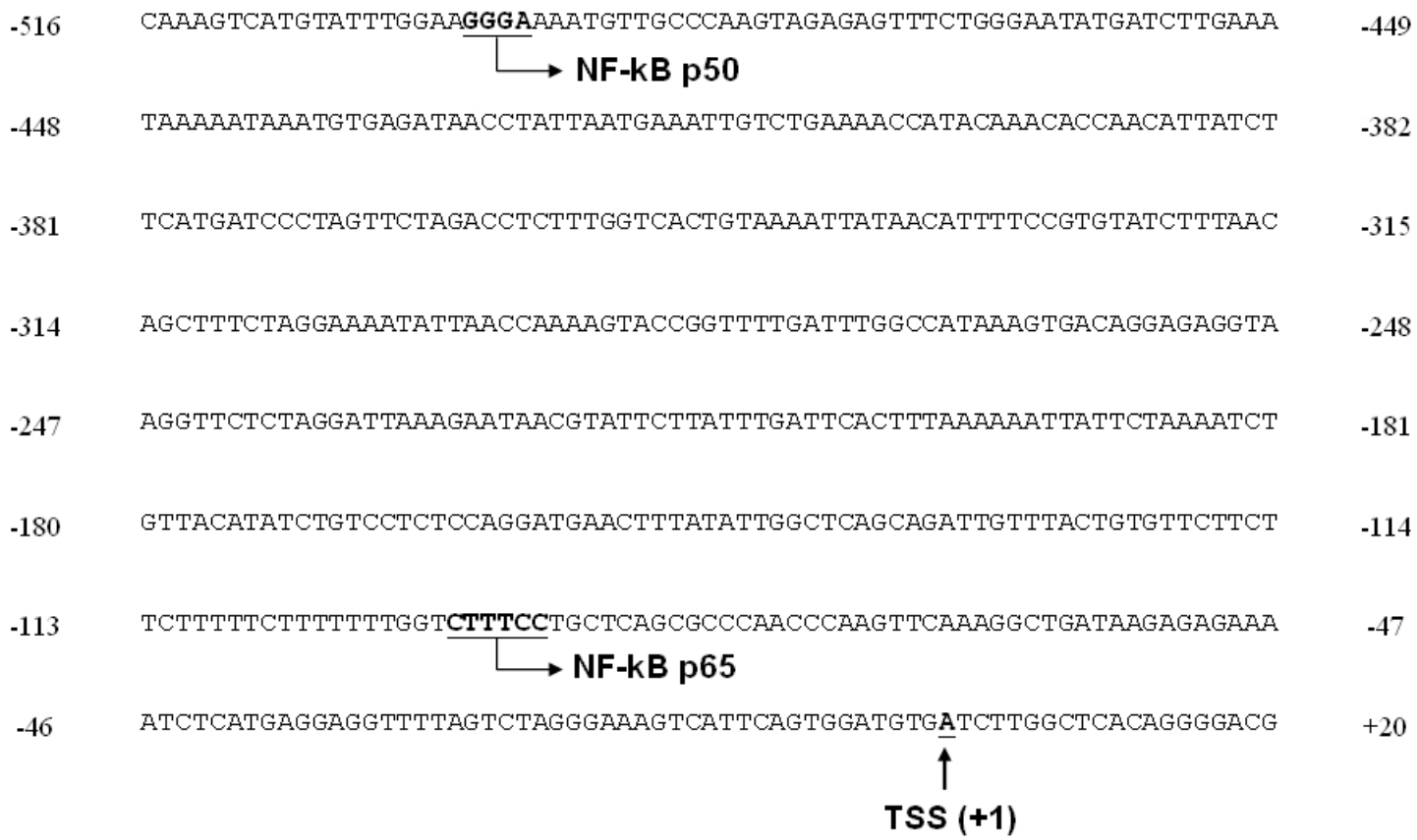


Figure 4-15. The upstream region of the ACE2 gene. The transcription start site (TSS) is marked by a downward arrow and is numbered as +1. The consensus sequence for putative NF-kB transcription factor binding sites is underlined. The promoter region is defined according to the position relative to the transcription start site (+1) in ACE2 mRNA sequence (GenBank No. AF_291820)

V. Discussion

5-1. The role of ACE2 in the cardiovascular system may be more complex

Angiotensin-converting enzyme II (ACE2) is a critical regulator of the renin–angiotensin system and the target of a number of highly effective therapeutic agents used to treat cardiovascular diseases. Through its ability to metabolize Ang II to Ang 1-7, it is able to regulate local Ang II levels thereby modulating its effects. Further evidence for a role of ACE2 in maintaining cardiovascular homeostasis *via* Ang II regulation is provided by studies conducted by Zisman *et al.* [2003] which detected increased ACE2 and Ang 1-7 forming activity in failing human hearts. The high level of the expression of ACE2 in heart together with its ability to hydrolyze angiotensin peptides have suggested a role for ACE2 in maintaining cardiovascular physiology from the outset. Interestingly, overexpression of ACE2 in cardiac myocytes did cause changes in cardiac conductivity, resulting in arrest and sudden death [Donoghue *et al.*, 2003b]. These contradictory data suggest that the role of ACE2 in the cardiovascular system may be more complex than is immediately apparent.

5-2. Ang II-AT1R modulated ACE2 upregulation was affirmed

In this study, we showed that the cardiac ACE2 was significantly upregulated at transcriptional and translational level in the HCF cells after Ang II treatment. The effect of Ang II-mediated ACE2 upregulation in the cardiac cells was confirmed by the experiments of blocking Ang II–AT1R signaling pathway, including AT1R itself and the downstream signaling of AT1R, NADPH oxidase and ERK-MAPK cascade (**Figure 4-3**). The influence of ROS on cardiac ACE2 upregulation was affirmed, suggesting that NADPH oxidase might induce ROS formation after Ang II treatment and then stimulate the ACE2 expression of HCF cells.

5-3. Cardiac ACE2 upregulation is associated with the modulation of Ang II to antagonize the effects of increased Ang II

Several reports showed the experimental results on the regulation of cardiac ACE2

expression associated with Ang II. For example, the studies mentioned that Ang II could reduce ACE2 expression in myocardial infarction or hypertensive rat model, and this effect on ACE2 expression by Ang II could be reversed by the treatment of AT1R blocker [Ishiyama *et al.*, 2004; Ferrario *et al.*, 2005a; Gallagher *et al.*, 2008; Koka *et al.*, 2008]. On the other hand, Yamamuro *et al.* [2008] reported that aldosterone but not Ang II reduced ACE2 mRNA expression in primary neonatal rat cardiomyocytes. However, above experimental results can not explain the clinical observations that the elevated Ang II paralleled to cardiac ACE2 upregulation was reported in the subjects with cardiovascular diseases, such as myocardial infarction, heart failure and atrial fibrillation [Zisman *et al.*, 2003; Goulter *et al.*, 2004; Burrell *et al.*, 2005; Epelman *et al.*, 2008]. These clinical data may raise a possibility that cardiac ACE2 upregulation is associated with the modulation of Ang II to antagonize the effects of increased Ang II.

5-4. The upregulated ACE2 might play a compensatory role in maintaining a steady state of RAS

In the present study, the effect of Ang II on the upregulation of ACE2 expression in the HCF cells was found. Our results provide the evidence showing that Ang II could markedly stimulate ACE2 upregulation in the cardiac cells through Ang II–AT1R signaling transduction pathway. We suggested that the different regulation of ACE2 by Ang II may cause by the influence of experimental models of heart physiological condition (health or disease, and progression of disease), species and cell types, and even the aging effect [Shivakumar *et al.*, 2003]. The upregulated ACE2 might play a compensatory role in counteracting the effect from the increased ACE activity and Ang II formation in hearts. This compensatory role, or called protective role, of ACE2 can maintain a steady state of RAS.

5-5. Ang 1-7 provides counter-regulatory effects to Ang II-induced deleterious effects on the cardiac functions

Nowadays, we also demonstrated that the cardiac ACE2 of HCF cells could be significantly upregulated after Ang 1-7 treatment (**Figure 4-5**). Ang 1-7, another critical angiotensin peptide in RAS, could be converted from Ang II by ACE2 enzyme catalysis, and several studies revealed that Ang 1-7 provides counter-regulatory effects to Ang II-induced

deleterious effects on the cardiac functions [Ferrario *et al.*, 1997; Iwata *et al.*, 2005; Grobe *et al.*, 2007; Pan *et al.*, 2007]. The elevated Ang 1-7 expression in failing heart tissue and ischemia zone of myocardial infarction might cause by the increase of ACE2 expression [Averill *et al.*, 2003; Santos *et al.*, 2005]. Clark *et al.* [2001] indicated that Ang 1-7 can downregulate AT1R transcription and translation in vascular smooth muscle cells. Recently, it is reported that Mas receptor can interact with the AT1R and antagonize AT1R through the formation of a hetero-oligomeric complex to block the effects of Ang II in cultured mammalian cells [Kostenis *et al.*, 2005]. Furthermore, Sampaio *et al.* [2007] showed that Ang 1-7 negatively modulates the activation of AT1R-dependent c-Src and the downstream targets of ERK-MAPK and NADPH oxidase by Mas receptor in endothelial cells.

5-6. Ang 1-7–enhanced ACE2 expression might be independent to the Ang II–AT1R signaling transduction pathway

In this study, the presence of Ang 1-7 significantly increased the ACE2 expression in HCF cells the same as Ang II stimulation. However, the AT1R expression, including at the transcriptional and translational level, in HCF cells did not influence by the Ang 1-7 treatment. We proposed that the different AT1R regulation in the cardiofibroblasts and endothelial cells is because of cell specificity. To distinguish the signaling pathway in the ACE2 regulation by Ang II and Ang 1-7 becomes more important for the study of ACE2 regulation. HCF cells pretreated with AT1R blocker, Valsartan, could determine the existence of Ang 1-7–AT1R pathway. The results show that HCF cells pretreated with Valsartan have no marked effect on Ang 1-7 induced ACE2 expression. Our result hints that the pathway of Ang 1-7–enhanced ACE2 expression in the cardiac cells might be independent to the Ang II–AT1R signaling transduction pathway.

5-7. ERK-MAPK cascade could be the main pathway to stimulate ACE2 expression

Nie *et al.* [2009] showed that Ang1-7 stimulated ERK1/2 phosphorylation alone and significantly enhanced Ang II-induced phosphorylation of ERK1/2 in mouse bone marrow-derived dendritic cells. Studies in mammalian (including human, rat and mouse) cells demonstrated that Ang 1-7 inhibit a lot of processes stimulated by Ang II, such as

vasoconstriction, cell growth and proliferation, proarrhythmia, prothrombogenic actions, and fibrogenic responses as well as activation of the MAPK family [Tallant *et al.*, 1999; Iyer *et al.*, 2000; Ferreira and Santos, 2005; Tallant *et al.*, 2005b; Su *et al.*, 2006]. All of those processes stimulated by Ang II are mediated by AT1R, and Ang 1-7 takes the inhibitory effects *via* a specific receptor, the G-protein-coupled receptor Mas, whose relationship with Ang 1-7 was established in mouse kidney and Chinese hamster ovary cells [Santos *et al.*, 2003a]. In this study, we suggest that the induction of phosphorylation of ERK1/2 by Ang II and Ang 1-7 alone are mediated by AT1R and Mas receptor, respectively. The ACE2 expression regulating by NADPH oxidase signaling pathway could even go through ERK-MAPK cascade. It could be explained that ERK-MAPK cascade was the main pathway to stimulate ACE2 expression. As found in human and rat cells, ACE2 can directly cleave Ang II to form Ang 1-7 [Donoghue *et al.*, 2000a], the coexistence of Mas receptor and ACE2 discovered in the present study suggests that there has an ACE2–Ang 1-7–Mas axis in human cardiofibroblast as well as ACE–Ang II–AT1R axis.

5-8. Distinguishing the signaling pathway in the ACE2 regulation between Ang II and Ang 1-7

To distinguish the signaling transduction pathway in the ACE2 regulation by Ang II and Ang 1-7 and realize the particular signaling cascade become more important for the study of a positive feedback-like loop (i.e., ACE2 catalyzes Ang II to be Ang 1-7 and Ang 1-7 can upregulate ACE2 production) in the cardiac ACE2 expression controlled by angiotensin peptides (**Figure 4-16**).

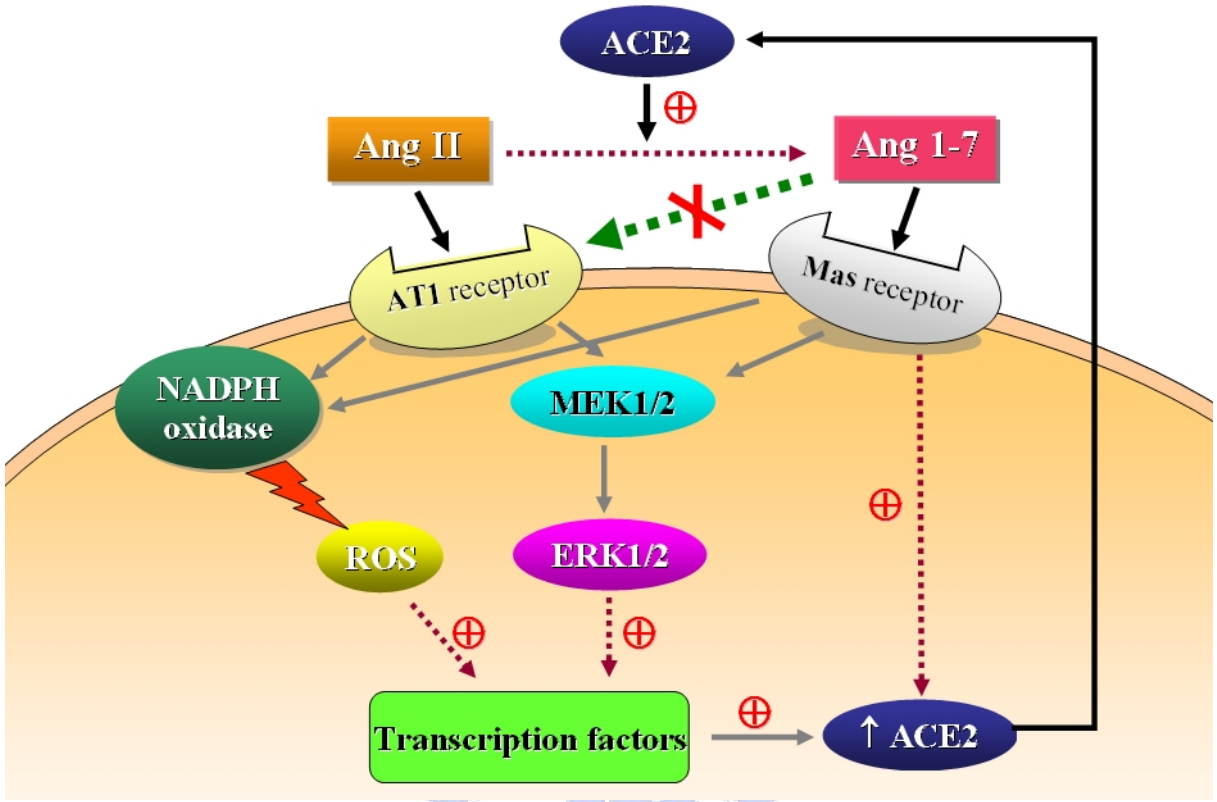


Figure 4-16. Schematic representation of interplay of Ang II and Ang 1-7 on the cardiac ACE2 regulation. ACE2, angiotensin-converting enzyme II; Ang II, angiotensin II; Ang 1-7, angiotensin 1-7; AT1R, angiotensin II type I receptor; ERK1/2, extracellular signal-regulated kinases 1/2; MEK1/2, mitogen-activated/ERK kinase 1/2; ROS, reactive oxygen species.

VI. Conclusions

Cardiovascular diseases are predicted to be the most common cause of death worldwide by 2020. In the general population, heart failure is chiefly the end stage of hypertensive, coronary and valvular cardiovascular disease. It is a major and growing problem in most affluent countries because of aging populations of increased size, and the prolongation of the lives of cardiac patients by modern therapy. Whatever the reason for the increasing rate of hospitalization for heart failure, it is clear that this condition is imposing a major burden on the health care system. Heart failure is a multifactorial quantitative trait controlled by both genetic and environmental factors. While much is known about environmental factors that can contribute to high blood pressure, such as diet and physical activity, less is known about the genetic factors that are responsible for predisposition to cardiovascular disease. Thus, the molecular and genetic mechanisms underlying heart failure and other cardiovascular diseases remain obscure.

A major risk factor for heart disease is high blood pressure. One important regulator of blood pressure homeostasis is the renin–angiotensin system (RAS). Recently, a homologue of ACE, termed ACE2, has been identified; it is predominantly expressed in the vascular endothelial cells of the kidney and heart. Unlike ACE, ACE2 functions as a carboxypeptidase, cleaving a single residue from Ang I, generating Ang 1-9, and a single residue from Ang II to generate Ang 1-7. These *in vitro* biochemical data suggest that ACE2 may modulate the RAS and thus affect blood pressure regulation. Nevertheless, the *in vitro* role of ACE2 in the human cardiovascular system and the RAS is not known.

In the present study, human cardiofibroblast (HCF) cells were used to test the regulatory effects of Ang II and Ang 1-7 on the ACE2 expression at transcriptional and translational level. To summarized, our results demonstrate that cardiac ACE2 was markedly upregulated in the HCF cells stimulated with Ang II, which might explain why the elevated ACE2 expression could be found at the initial stage in failing heart of human. AT1R signaling transduction pathway is involved in the cardiac ACE2 upregulation by Ang II stimulation to confirm the AT1R-dependent effect. Besides, the upregulated ACE2 may increase Ang 1-7 formation from Ang II and then the ACE2 expression was further enhanced by formatted Ang 1-7 *via* Mas receptor. Cardiac ACE2 upregulation with Ang II and Ang 1-7 stimulation is further confirmed by promoter assay and confocal laser scanning microscopy. The analyzed data showed that human cardiac ACE2 promoter activity was markedly upregulated with

transfection of pGL3 (-516/+20) constructs. This constructs was further incubated with Ang II and Ang 1-7 to verify the role in Ang II– and Ang 1-7–mediated ACE2 promoter activity. The result showed that human cardiac ACE2 promoter activity was significantly upregulated with Ang II stimulation. This result indicated that ACE2 promoter activity was parallel to the transcriptional and translational expression of ACE2. In addition, the ACE2 florescence imaging also show the consistent results with our previous data.

To sum up, we propose a positive feedback-like loop on the cardiac ACE2 regulation for heart to maintain a steady state of RAS. These effects indicate a protective role of ACE2–Ang 1-7 axis to counteract Ang II-induced deleterious effects on heart. Our results may present a novel target for developing potential therapeutic strategies in improving serious cardiovascular disease induced by the dysfunction of RAS.

VII. References

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