Peptides 32 (2011) 1832–1839

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Identifying the regulatory element for human angiotensin-converting enzyme 2 (*ACE2*) expression in human cardiofibroblasts

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ARTICLE INFO

Article history:
Received 14 July 2011
Accepted 9 August 2011
Available online 16 August 2011

Keywords:
Angiotensin-converting enzyme 2
Regulatory element
Angiotensin II
Human cardiofibroblasts

ABSTRACT

Angiotensin-converting enzyme 2 (ACE2) has been proposed as a potential target for cardioprotection in regulating cardiovascular functions, owing to its key role in the formation of the vasoprotective peptides angiotensin-(1–7) from angiotensin II (Ang II). The regulatory mechanism of *ace2* expression, however, remains to be explored. In this study, we investigated the regulatory element within the upstream of *ace2*. The human *ace2* promoter region, from position −2069 to +20, was cloned and a series of upstream deletion mutants were constructed and cloned into a luciferase reporter vector. The reporter luciferase activity was analyzed by transient transfection of the constructs into human cardiofibroblasts (HCFs) and an activating domain was identified in the −516/−481 region. Deletion or reversal of this domain within *ace2* resulted in a significant decrease in promoter activity. The nuclear proteins isolated from the HCFs formed a DNA–protein complex with double stranded oligonucleotides of the −516/−481 domain, as detected by electrophoretic mobility shift assay. Site-directed mutagenesis of this region identified a putative protein binding domain and a potential binding site, ATTTGGA, homologous to that of an Ikaros binding domain. This regulatory element was responsible for Ang II stimulation via the Ang II–Ang II type-1 receptor (AT1R) signaling pathway, but was not responsible for pro-inflammatory cytokines TGF-β1 and TNF-α. Our results suggest that the nucleotide sequences −516/−481 of human *ace2* may be a binding domain for an as yet unidentified regulatory factor(s) that regulates *ace2* expression and is associated with Ang II stimulation.

1. Introduction

The renin–angiotensin system (RAS) is a critical hormone system that regulates blood pressure and is crucially involved in cardiovascular and renal diseases [15,36]. Most of the well-known cardiovascular effects of RAS are attributable to the angiotensin-converting enzyme–angiotensin II axis (ACE–Ang II axis), but angiotensin converting enzyme II (ACE2), a recently discovered ACE homolog found mainly in the heart, kidney and testis, also plays a key role in the pathophysiology of such diseases [4,16,34]. ACE2 regulates the effect of Ang II via cleavage of angiotensin I (Ang I) and Ang II to generate the nine- and seven-residue peptides, angiotensin-(1–9) (Ang-(1–9)) and angiotensin-(1–7) (Ang-(1–7)), respectively [5,18,38]. This mechanism effectively opposes the actions of ACE and antagonizes the effects of Ang II [18,40].

Results from experiments with *ace2* mutant mice suggest that ACE2 negatively regulates activated the RAS [10,47]. From *ace2* knock-out mice, the absence of ACE2 severely impaired cardiac functions, which led to increased blood pressure, abnormal cardiac contractility and adverse left ventricular remodeling post-myocardial infarction [3,9,42,47]. The high level of expression of ACE2 in the heart could protect experimental animals against Ang II-induced cardiac hypertrophy or fibrosis, and suggests a role for ACE2 in maintaining cardiovascular physiology [13,18,46]. It appears, therefore, that ACE2 and its role in the ACE2–Ang-(1–7) axis is a potential novel target for regulating cardiovascular homeostasis, and exploring the stimulation of ACE2 production may lead to future therapeutic applications [26,30].

The role of ACE2 in cardiac function is clearly important, but little is known about the regulatory elements of human *ace2* expression [2]. Although a binding site for hepatocyte nuclear factor 1 beta (HNF1β) has been identified within the promoter region (−818/−812) of *ace2*, the role of HNF1β on ACE2 regulation could not be verified [29]. In the previous study, we investigated the transcriptional and translational expression of human ACE2 in human

Abbreviations: Ang I, angiotensin I; Ang II, angiotensin II; AT1R, angiotensin II Type 1 Receptor; Ang-(1–7), angiotensin-(1–7); Ang-(1–9), angiotensin-(1–9); ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; EMSA, electrophoretic mobility shift assay; HCFs, human cardiofibroblasts; RAS, renin–angiotensin system; TGF-β1, transforming growth factor-beta1; TNF-α, tumor necrosis factor-alpha.

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doi:10.1016/j.peptides.2011.08.009

cardiac fibroblasts (HCFs) treated with the angiotensin peptides Ang II and Ang-(1–7), we have previously shown that stimulation of HCFs by Ang II significantly increased the expression of cardiac ACE2 [19]. In this study, we identified a regulatory element responsible for Ang II stimulation in human *ace2*.

2. Materials and methods

2.1. Chemicals and reagents

The goat polyclonal IgG, glyceraldehyde-3-phosphate-dehydrogenase antibody (V-18; #sc20357), horseradish peroxidase (HRP)-labeled secondary antibodies (donkey anti-goat IgG and goat anti-rabbit IgG; #sc2020 and #sc2004), and the rabbit polyclonal IgG, Ikaros antibody (H-100; #sc13039), were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal ACE2 antibody (#ab59351) was purchased from Abcam (Cambridge, MA, USA). Ang II (#H1705) was obtained from Bachem (Bubendorf, Switzerland). The Ang II type-1 receptor (AT1R) antagonist, valsartan (Val; #1708762), was obtained from U.S. Pharmacopeia (Rockville, MD, USA), and the mitogen-activated protein kinase kinase (MEK) inhibitor (PD98059; #P215), and poly-L-lysine (0.01% solution; #P4832) were obtained from Sigma–Aldrich (St. Louis, MO, USA). The luciferase reporter vectors, pGL3-Control Vector (#E1741) and pGL3-Basic Vector (which lacks a promoter; #E1751), and the Luciferase Assay System (#E1500) were purchased from Promega (Madison, WI, USA). All other reagents were obtained from Sigma–Aldrich.

2.2. Cell culture and treatment

Primary human cardiac fibroblasts (HCFs; #6300; ScienCell Research Laboratories, San Diego, CA, USA) were cultured according to our published protocol [19]. In brief, the HCFs were seeded in 100-mm Petri dishes (2×10^6 cells/dish) or 12-well plates (1×10^5 cells/well) that had been pre-coated with 0.01% poly-L-lysine (Sigma), and were cultured in Fibroblast Medium (#2301; ScienCell Research Laboratories), which included 2% fetal bovine serum (#0010; ScienCell Research Laboratories). The cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere and the culture medium as exchanged with fresh medium every 2 days. The cells at passages 3 or 4 were used in all experiments and were placed in serum-free medium for 24 h prior to their use in further experiments.

2.3. Human ace2 constructs

Human genomic DNA was used as the template to obtain the upstream of *ace2* using polymerase chain reaction (PCR) and DNA cloning. A 2.1-kb DNA fragment was obtained by PCR using primers based on the sequence for human *ace2* (GenBank ID: AY217547). The sequences for the forward (Hace2-proF) and reverse (Hace2-proR) primers were 5′-AACCCTCGAGTTTCATTTAGGA-3′ and 5′-GAGCTAAGCTTCGTCCCCTGTG-3′, respectively; XhoI and HindIII sites are indicated by underlined nucleic acids in the forward and reverse primers, respectively.

The DNA fragment was then cloned into the pGL3-Basic luciferase reporter vector at the XhoI and HindIII sites to generate the −2069/+20 construct. A series of deleted DNA fragments of the upstream region of *ace2* were obtained by PCR using the plasmid DNA of the −2069/+20 construct as template with the specific recognition primer pairs (Suppl. Table S1). These deleted DNA fragments were also cloned into the pGL3-Basic vector at the XhoI and HindIII sites to generate a series of deletion constructs to test the promoter activity of *ace2*. All of the constructs generated in this study were checked by restriction-mapping and sequencing to confirm their authenticity.

2.4. Transient transfection

Transient transfection was carried out according to our published protocol [32] with some minor modifications. Briefly, 2×10^5 HCFs were seeded in a 6-well culture plate one day before DNA transfection, and grown to approximately 70% confluence. The cells were washed with GIBCO Dulbecco's phosphate-buffered saline (D-PBS) (Invitrogen, Carlsbad, CA, USA) to remove the remaining medium, then 400 μl of cell growth medium containing 4 μg of plasmid DNA mixed with 6 μl of TurboFect Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was added gently. The DNA-transfected cells were then incubated at 37 °C and under 5% CO_2 in an incubator. After 24 h the cells were collected and lysed, and assayed for luciferase activity.

2.5. Luciferase reporter assay

The luciferase assay was performed according to the manufacturer's instructions of Luciferase Assay System (Promega). The DNA-transfected HCFs were rinsed twice with D-PBS (Invitrogen) and lysed with luciferase cell culture lysis reagent included in the kit (CCLR; Promega). Cell lysates were centrifuged at 4 °C for 2 min, and the supernatants were removed and mixed with the luciferase assay reagent (Promega). Luciferase activity was measured using a single tube luminometer (Lumat LB9507, Brethold Technologies, Bad Wildbad, Germany).

2.6. Nuclear extraction

Nuclear protein was extracted using a Nuclear Extraction kit (P/N 13938; Panomics, Redwood City, CA, USA) according to the manufacturer's protocol. HCFs (1×10^7 cells) were collected and washed twice with D-PBS, then centrifuged at $500 \times g$ for 5 min. The cells were resuspended in 1 ml of Working Reagent and the tubes were shaken at 200 rpm on ice for 10 min. The sample was centrifuged at $14{,}000 \times g$ for 3 min at 4 °C and the supernatant, consisting of cytoplasmic extract, was then removed. Forty microlitres of Buffer B Working Reagent was added to each pellet and then the sample was vortexed for 10 s. The mixture was incubated on ice for 60 min with gentle agitation by hand every 20 min. The nuclear extract was obtained as supernatant after centrifugation at $14{,}000 \times g$ for 5 min at 4 °C.

2.7. Electrophoretic mobility shift assay (EMSA)

EMSA was performed using an EMSA Gel Shift kit (P/N 13009; Panomics). The double-stranded oligonucleotides comprising the sequence −516/−481 of *ace2* were labeled with biotin. Nuclear extracts of HCFs were incubated in the Reaction Buffer for 5 min, before adding the biotin-labeled DNA probe. After incubating for 30 min at 15 °C, the mixture was separated by electrophoresis in a 6% polyacrylamide gel operating at 120 V, with 0.5 × TBE as the running buffer, for 1 h. In competition assays, 66-fold molar excess of unlabeled double-stranded oligonucleotide was added to the binding reaction 5 min before the labeled oligonucleotides. After electrophoresis, the DNA–protein complexes were transferred to positively charged nylon membranes (BrightStar®-Plus; Ambion, Austin, TX, USA) by semi-dry electroblotting (Hoefer™; Amersham Biosciences, Uppsala, Sweden) and immobilized using a Spectroline Spectrolinker UV Crosslinker (Spectronics Corporation, New York, NY, USA). The membrane was blocked in 1× Blocking Buffer, incubated with streptavidin-horseradish peroxidase for 15 min and

incubated in 1× Detection Buffer for 5 min. Working Substrate Solution (200 μl Solution I, 200 μl Solution II, and 1.6 ml Solution III) was added to develop the results (all of the aforementioned solutions were included in the Panomics Gel Shift kit). The developed bands were visualized by exposing the membrane to X-ray film (Super Rx Medical X-Ray Film; Fujifilm, Kanagawa, Japan).

2.8. RNA isolation, reverse transcription and real-time (RT) PCR

Extraction of total RNA and reverse transcription were performed as described [25]. Briefly, total cellular RNA was extracted using TRIzol Plus RNA Purification System (Invitrogen) according to the manufacturer's protocol. The cDNA was synthesized using ReverTra Ace Set (Toyobo, Osaka, Japan). Semi-quantitative real-time (RT) PCR was performed using SYBR Green Realtime PCR Master Mix Plus (Toyobo) with 20 pM of each primer and 5 μl cDNA, in a total volume of 25 μl and monitored using Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. Specificity of the real-time PCR was confirmed by routine agarose gel electrophoresis and melting-curve analysis, according to a published method [20]. Expression of the GAPDH (GenBank ID: NM_002046.3) gene was used as an internal standard. The primers for ACE2 (GenBank ID: AF291820) and GAPDH (GenBank ID: NM002046.3) were the same as those used in previous studies [8,33] and were: ACE2 forward, hACE2-F, 5′-CATTGGAGCAAGTGTTGGATCTT-3′, and, ACE2 reverse, hACE2-R, 5′-GAGCTAATGCATGCCATTCTCA-3′; GAPDH forward, hGAPDH-F, 5′-ACAGTCAGCCGCATCTTCTT-3′, and, GAPDH reverse, hGAPDH-R, 5′-GTTAAAAGCAGCCCTGGTGA-3′.

2.9. Protein extraction and Western blotting

Cellular protein extraction, electrophoresis, and Western blotting were performed as described [19]. The cultured HCFs (approximately 4×10^5 cells) were washed with 1× PBS and lysed by adding 100 μl of PRO-PREP™ protein extraction solution (Intronbio, Gyeonggi-do, Korea) according to the manufacturer's instructions. The lysate was centrifuged at 12,000 × *g* at 4 °C for 10 min and the supernatant was collected for analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined using a Bio-Rad Protein Assay kit (Bio-Rad; Hercules, CA, USA) with bovine serum albumin as a standard. Aliquots containing 30 μg protein were electrophoresed on 8% SDS-PAGE gels and then transferred electrophoretically to polyvinylidene fluoride membranes (Immobilon-P™; Millipore, Bedford, MA, USA) by semi-dry electroblotting (Hoefer™). Briefly, nonspecific binding sites were blocked by incubating the membranes in 5% non-fat milk in Tris-buffered saline. Primary antibodies against proteins were diluted 1:1000 for ACE2 and for GAPDH. The secondary antibodies were applied using a dilution of 1:2000. Substrates were visualized using enhanced chemiluminescence detection (Western Lightning Plus-ECL, Enhanced Chemiluminescence Substrate; PerkinElmer, Boston, MA, USA) and exposing the membranes to X-ray film (Fujifilm). The bands on the film were detected at the anticipated location, based on size. Band intensity was quantified by densitometric analysis using Scion Image software (Scion, Frederick, MD, USA). The amount of ACE2 was expressed relative to the amount of GAPDH (as the internal standard) in each sample.

2.10. Statistics

All values were 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.

3. Results

3.1. Expression levels of deletion constructs in the ace2 promoter

To examine the transcriptional activity of *ace2*, a 2.1 kb fragment of the upstream region of human *ace2* was cloned into the upstream of the luciferase coding gene in the pGL3-Basic vector to generate the −2069/+20 construct. This construct was transiently transfected into HCFs, and the resulting expression of luciferase was monitored by measuring luciferase activity. Luciferase activities from HCFs transfected with the pGL3-Basic vector were compared with those transfected with the pGL3-Control vector, which was used to monitor DNA transfection efficiency. Transfection of the HCFs with the −2069/+20 construct showed a significant increase (8.9 ± 2.0-fold increase) in luciferase expression compared to the baseline levels for pGL3-Basic vector transfection.

Based on these results, we obtained 11 serially deleted constructs (starting at −1493, −1110, −916, −786, −664, −627, −516, −481, −355, −253, and −161) using the designed primer pairs (Suppl. Table S1) and the plasmid DNA of the −2069/+20 construct as the template by PCR (Fig. 1A). These serial deletion fragments of the *ace2* promoter were used to drive the downstream gene expression of the reporter gene, luciferase, in order to determine which region contained critical regulatory activity of *ace2* expression. The results showed that luciferase expression of the serial deletion constructs was essentially unchanged from position −2069 to position −627 within the *ace2* promoter. Deletion of the construct to position −516, however, resulted in a significant increase in promoter activity; a further 5′ deletion construct to position −481 resulted in markedly decreased promoter activity (Fig. 1B). These results indicate the presence of a significantly activating domain between position −516 and −481.

3.2. Identification of the regulatory domain within the ace2 promoter

To further identify the regulatory sequences within the −516/−481 region that enhance *ace2* expression, two constructs were created from the −2069/+20 construct: one in which the −516/−481 domain was internally deleted and the other in which it was reversed (Fig. 2A). The −516/−481 deleted construct (−2069 to −516/−481 to +20) and the reversed construct (−2069 to −481/−516 to +20) were then transiently transfected into HCFs and the promoter activity of *ace2* was assessed. The results showed that both the deleted and the reversed sequence domain significantly reduced downstream luciferase expression (Fig. 2B).

3.3. Identification of the regulatory element for ace2

We showed that the −516/−481 domain of *ace2* contains major regulatory sequences, but the main regulatory element needed to be clarified. The nucleotide sequence of −516/−481 region was therefore analyzed using the database TFSEARCH [37] to find possible transcription factor binding elements. The results show a potential Ikaros binding site 5′-ATTTGGA-3′ with 95% calculated score. PCR site-directed mutagenesis was used to generate seven mutant sequences of ATTTGGA to further identify the regulatory element of *ace2* (Fig. 3A). The designed primer pairs used to PCR amplify and construct a series of site-directed mutant constructs are shown in Supplementary Table S2. Compared to the original −516/+20 construct, luciferase expression was significantly decreased in all mutant constructs (Fig. 3B). This indicates that

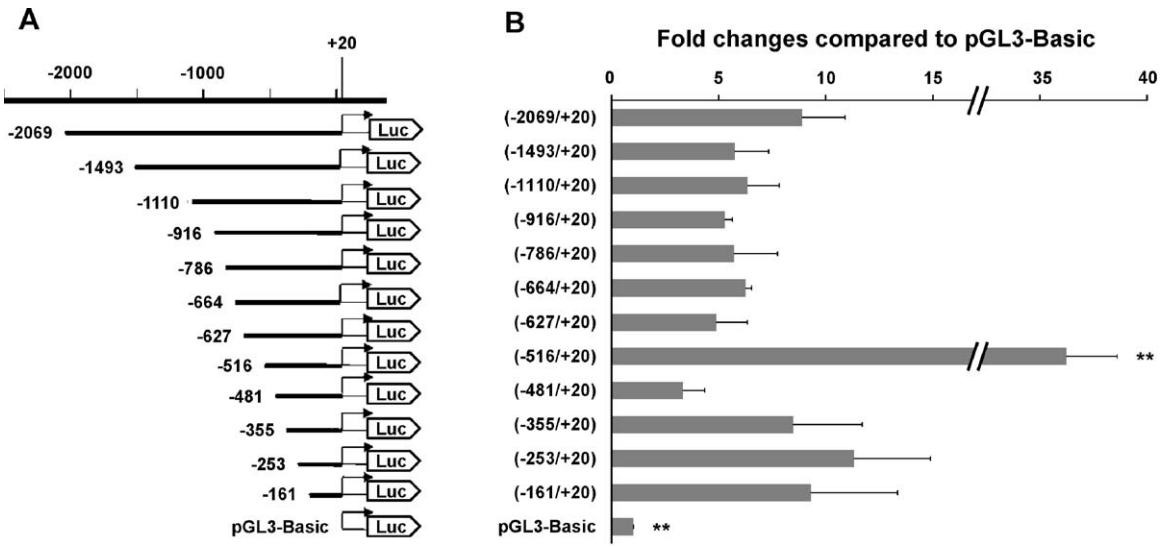

Fig. 1. Composition and promoter activity of the constructs on the expression of the reporter enzyme, luciferase, in HCFs. (A) The constructs were comprised of serially deleted portions of the upstream region of *ace2*, fused to firefly luciferase cDNA in the vector pGL3-Basic. The position of the promoter fragments relative to transcription start site (+1) is indicated. (B) The constructs were transfected into HCFs. Cells were lysed 24 h later and luciferase activities were measured. Relative luciferase activity of each construct (*i.e.*, compared to that of the control, pGL3-Basic vector) is shown. All values are expressed as the mean ± SD from three independent experiments; $^{**}p < 0.01$ compared to the −2069/+20 construct.

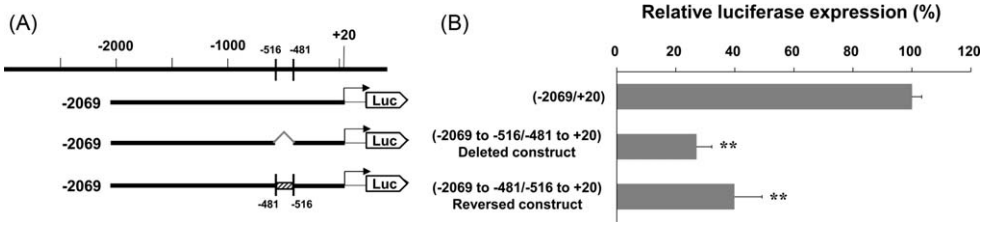

Fig. 2. Analyses of the promoter activity of the deleted and reversed domain within the upstream region of *ace2*. (A) Schematic representation of the deleted (−2069 to −516/−481 to +20) and reversed (−2069 to −481/−516 to +20) domain in the −2069/+20 construct. (B) The constructs were transfected into HCFs. Cells were lysed 24 h later and luciferase activities were measured. Relative luciferase activity of each construct (*i.e.*, compared to that of the control −2069/+20 construct) is shown. All values are expressed as the mean ± SD from three independent experiments; $^{**}p < 0.01$ compared to the −2069/+20 construct.

the sequence ATTTGGA is indeed a main regulatory element in the −516/−481 domain of the *ace2* promoter.

To determine whether cellular regulatory factors are produced in HCFs that are capable of interacting with the −516/−481 domain, we used the synthetic and biotin-labeled double-stranded oligonucleotides of the −516/−481 sequences to react with the nuclear extracts prepared from HCFs by EMSA. As shown in Fig. 4A, one distinctive DNA–protein complex was observed when the −516/−481 double-stranded DNA was incubated with nuclear extracts of HCFs. This DNA–protein complex is specific to the −516/−481 sequences because it was readily eliminated by an excess of unlabeled competitor and was partially abolished when an Ikaros antibody was used to pretreat the nuclear extracts of HCFs.

For further confirmation that the sequence ATTTGGA within the −516/−481 domain of the *ace2* promoter was a significant bind-

A

	Sequences (-516/-481)	Relative TF binding score
(-516/+20)	CAAAGTCATGTATTTGGAAGGGAAAATGTTGCCCAA	100
M1(-516/+20)	CAAAGTCATGTACTCGAAAGGGCAAATGTTGCCCAA	76.6
M2(-516/+20)	CAAAGTCATGTACTCGGAAGGGCAAATGTTGCCCAA	74.6
M3(-516/+20)	CAAAGTCATGTATTCGAAAGGGCAAATGTTGCCCAA	76.6
M4(-516/+20)	CAAAGTCATGTACTTGAAAGGGCAAATGTTGCCCAA	78.3
M5(-516/+20)	CAAAGTCATGTACTTGGAAGGGCAAATGTTGCCCAA	74.7
M6(-516/+20)	CAAAGTCATGTATTTGAAAGGGCAAATGTTGCCCAA	76.6
M7(-516/+20)	CAAAGTCATGTATTCGGAAGGGCAAATGTTGCCCAA	83.7

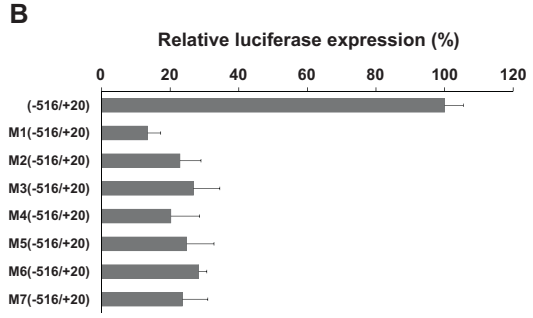

Fig. 3. Identification of the regulatory element within the −516/−481 domain. The full sequence, −516/−481, was analyzed for putative binding elements using TFSEARCH. The sequence, ATTTGGA, was identified as a potential binding element. (A) Using PCR site-directed mutagenesis at the ATTTGGA site, seven mutant constructs (M1–M7) were generated. The location of the mutations is indicated in red typeface. The relative element binding score was calculated according to its TFSEARCH score, relative to a score of 100 for the full sequence, −516/−418. (B) The constructs were transfected into HCFs. Cells were lysed 24 h later and luciferase activities were measured. Relative luciferase activity of each construct (*i.e.*, compared to that of the control −516/+20 construct) is shown. All values are expressed as the mean ± SD from three independent experiments.

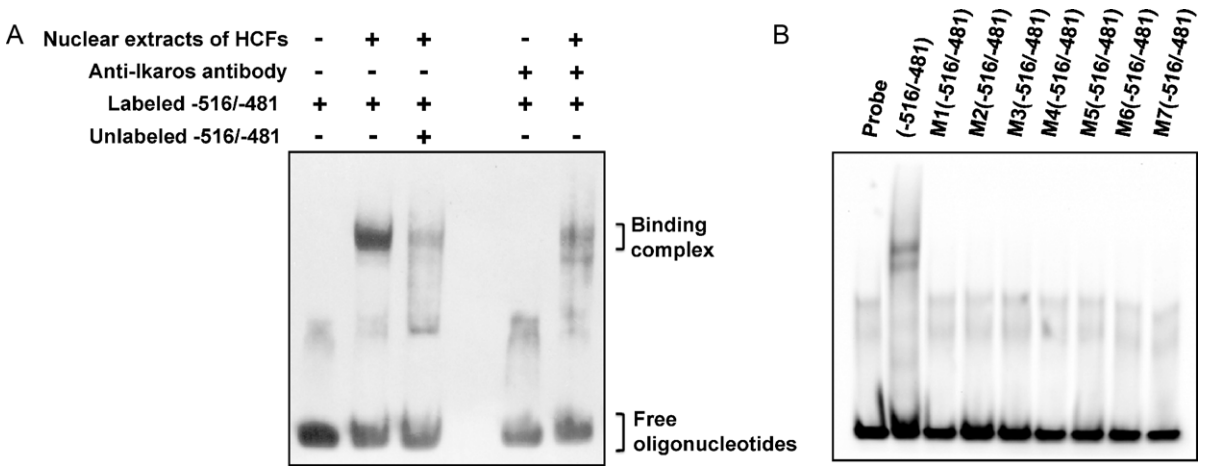

Fig. 4. Interaction of nuclear extracts from HCFs with (−516/−481) and mutant (M1–M7) oligonucleotides by EMSA. Binding complexes were separated using 6% non-denaturing PAGE. (A) Unlabeled and labeled (biotinylated) double-stranded oligonucleotides, −516/−481, were mixed with nuclear extracts from HCFs. A 66× molar excess of the unlabeled oligonucleotide, −516/−481, was used for competitive binding. (B) Nuclear extracts from HCFs were mixed with labeled oligonucleotides (−516/−481) and labeled mutant oligonucleotides (M1–M7). "Probe" indicates labeled oligonucleotides (−516/−481) alone, *i.e.*, in the absence of nuclear extract.

ing element, seven mutant double-stranded oligonucleotides, M1 through M7, were synthesized and used for EMSA (Fig. 4B). The results show that unlike the −516/−481 double-stranded oligonucleotides, the M1 through M7 double-stranded oligonucleotides could not form a DNA–protein complex with the nuclear extracts of HCFs (Fig. 4B). This result is consistent with the other results from the promoter activity.

3.4. Effect of Ang II on the transcriptional activation of ace2

The effect of Ang II on the transcriptional activation of *ace2* were investigated by transient transfection of HCFs with a −516/+20 construct, and a −481/−516/+20 construct (in which the sequence of the −516/−418 region was reversed) and treated with 0, 0.1, 1 and 10 μM of Ang II. The results show that the relative luciferase expression from cells transfected with the −516/+20 construct was significantly increased by Ang II stimulation in a dose-dependent manner (Fig. 5A), and this increased luciferase expression could be abolished by pretreatment with valsartan (AT1R inhibitor) or PD98059 (MEK inhibitor) (Fig. 5B). In contrast, increased luciferase expression was not observed with Ang II treatment of HCFs transfected with the −481/−516/+20 construct (Fig. 5A). These results indicate that Ang II can up-regulate the transcription of *ace2*.

To examine the expression regulation of endogenous ACE2 in HCFs, the expression of *ace2* and its protein production with and without treatment with 1 μM of Ang II was investigated. The results showed that upon Ang II stimulation, the relative levels of expressed ACE2 mRNA (Fig. 6A) and protein (Fig. 6B) increased by 2.97- and 1.80-fold, respectively.

3.5. Effect of pro-inflammatory factors on the transcriptional activation of ace2

We examined the effects of the pro-inflammatory cytokines, transforming growth factor-β1 (TGF-β1) and tumor necrosis factor-α (TNF-α), on the transcriptional activity of *ace2* in HCFs. The −516/+20 construct was transiently transfected into HCFs and the cells were treated with different dosages of TGF-β1 or TNF-α (0, 1, 5 and 10 ng/ml). Neither TGF-β1 (Fig. 7A) nor TNF-α (Fig. 7B) significantly affected luciferase expression: compared to expression levels in the absence of added pro-inflammatory factors, at the highest concentration of added cytokine (10 ng/ml), ACE2 mRNA expression and protein expression decreased to 88% and 95%, respectively, with TGF-β1 treatment, and increased to 121% and 113%, respectively, with TNF-α treatment. These variations were not statistically significant.

4. Discussion

To investigate the molecular mechanism by which Ang II regulates the expression of ACE2, we examined the promoter activity its gene, *ace2*. Using sequence deletion and site-directed mutation analyses, we identified a region upstream of *ace2*, at −516/−481 domain, that is required for Ang II-activated transcription. We also demonstrated that the sequence ATTTGGA is the Ang II responsive element.

From this study, the results of the promoter activity assay are consistent with those that show that cardiac ACE2 was significantly up-regulated at both transcriptional and translational levels in HCFs after Ang II stimulation—presumably via the Ang II-AT1R signaling pathway. Several reports have shown that elevated Ang II levels were observed in conjunction with cardiac ACE2 up-regulation in subjects with cardiovascular disease (*e.g.*, myocardial infarction, heart failure and atrial fibrillation) both in the clinic and in animal experiments [1,6,8,24,48]. This raises the possibility that cardiac ACE2 up-regulation is associated with the modulation of the effect of Ang II, by an antagonist for example, which diminishes the effect of increased Ang II. Based on the results of Ang II-stimulated ACE2 up-regulation in HCFs, we suggest that the regulation of ACE2 by Ang II may be largely dependent on pathological and/or physiological conditions, and that up-regulated ACE2 may play a compensatory role in counteracting the effects from the increased ACE activity and Ang II production in the heart. This compensatory or protective role of ACE2 may serve to maintain homeostasis within the RAS.

In addition to the angiotensin peptides in the RAS, inflammation plays a key role in the initiation, progression, and clinical outcome of cardiovascular diseases. Substantial evidence suggests the involvement of the inflammatory and immune systems in adverse remodeling of cardiac failure and hypertrophy [35,39,43]. In this study, we attempted to evaluate whether the expression of ACE2 could be modulated by pro-inflammatory factors in HCFs. We examined the effects of two pro-inflammatory cytokines, TGF-β1 and TNF-α on the expression ACE2. Increased doses of TGF-β and TNF-α did not cause significant change in ACE2 expression, however, nor in the promoter activity of *ace2*. This result confirms a previous report that ACE2 expression was not affected by TNF-α, IL-

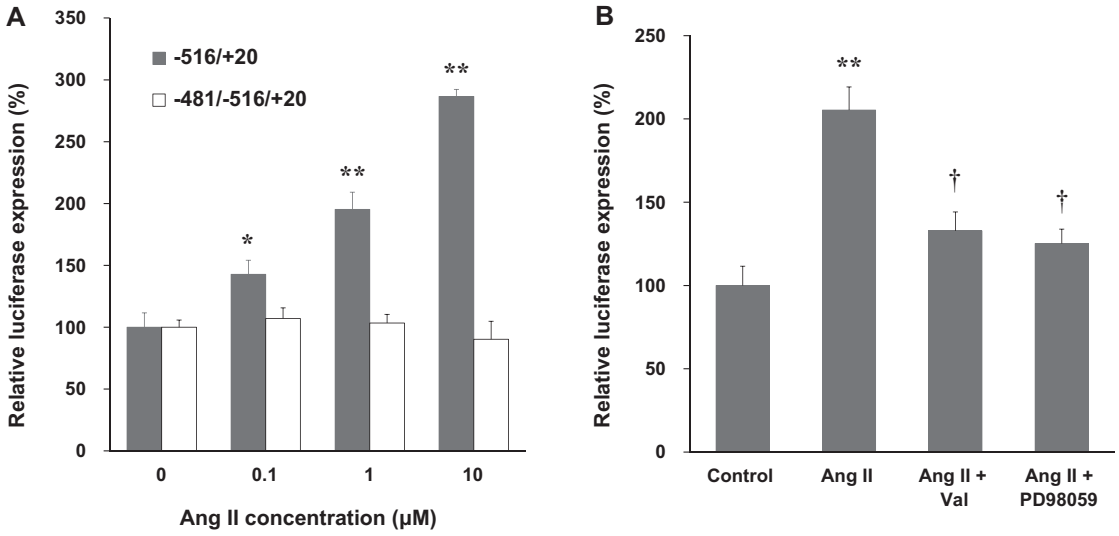

Fig. 5. The effects of Ang II stimulation on ACE2 expression in HCFs. (A) HCFs were transfected with the (−516/+20) and reversed (−481/−516/+20) constructs, then treated with various concentrations of Ang II. Cells were lysed 24 h later and luciferase activity was measured. Relative luciferase activity (*i.e.*, compared to luciferase activity in the absence of added Ang II) for each sample is shown. All values are expressed as the mean ± SD from three independent experiments; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the group (control) with no added Ang II. (B) The signaling pathway of Ang II-induced ACE2 expression in HCFs was also investigated. HCFs transfected with the −516/+20 construct were pre-treated with 1 μg/ml of valsartan (AT1R inhibitor) or PD98059 (MEK inhibitor) for 1 h, then treated with 1 μg/ml of Ang II. The cells were lysed 24 h after addition of Ang II and luciferase activity was measured. Relative luciferase activity (*i.e.*, compared to luciferase activity in the absence of added Ang II) for each sample is shown. All values are expressed as the mean ± SD from three independent experiments; $^{**}p < 0.01$ compared to the group without added Ang II; $^{\dagger}p < 0.01$ compared to the group with only Ang II added.

1β and chronic hypoxia in human cardiac myofibroblasts [11]. It has been shown that Ang II can induce TGF-β1 and TNF-α expression in cardiac cells via the Ang II/AT1R signaling pathway [14,27,28]. We therefore suggest that Ang II-stimulated ACE2 up-regulation may occur via a TGF-β1/TNF-α independent pathway—although the results of ACE2 modulation by angiotensin and the cytokines reported here may be dependent on the specific experimental models used.

The sequence ATTTGGA is a potential binding domain for the transcriptional factor Ikaros. Ikaros was originally found to function as a key regulator of lymphocyte differentiation [7,21]. Subsequent studies demonstrated the role of Ikaros in normal hematopoiesis [22], and in the migration and invasion of extravillous trophoblasts in early placentation [41]. In a recent study, it was reported that Ikaros primes the lymphoid transcriptional program in hematopoietic stem cells, and that loss of Ikaros may

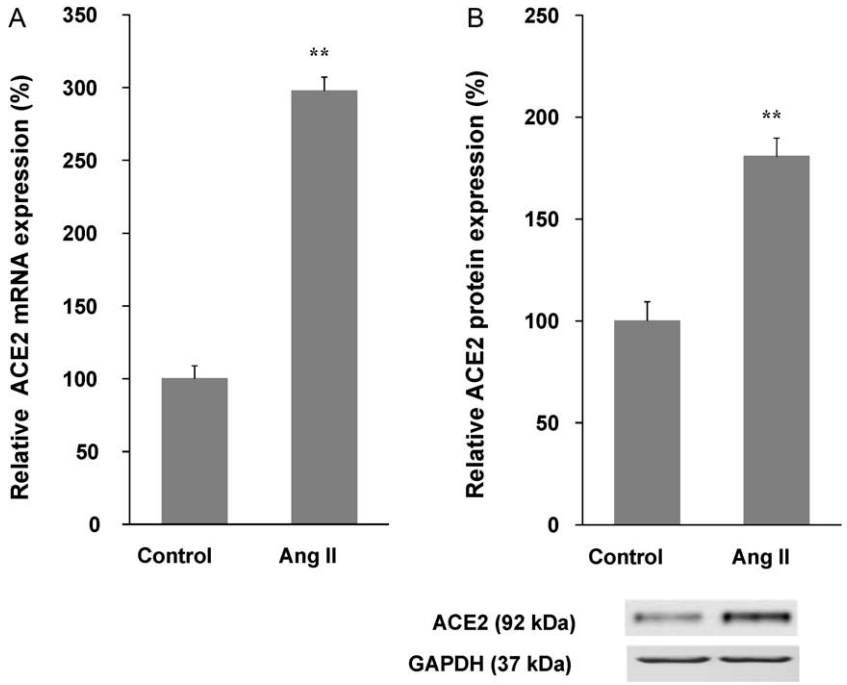

Fig. 6. The effect of Ang II stimulation on endogenous ACE2 expression in HCFs. HCFs were treated with 1 μM of Ang II for 24 h, and the cells were then analyzed for ACE2 mRNA using semi-quantitative RT-PCR (A), and for protein, using Western blotting (B). Relative expression of ACE2 mRNA and protein (*i.e.*, compared to expression without added Ang II) for each sample is shown. All values are expressed as the mean ± SD from three independent experiments; $^{**}p < 0.01$ compared to the group without added Ang II.

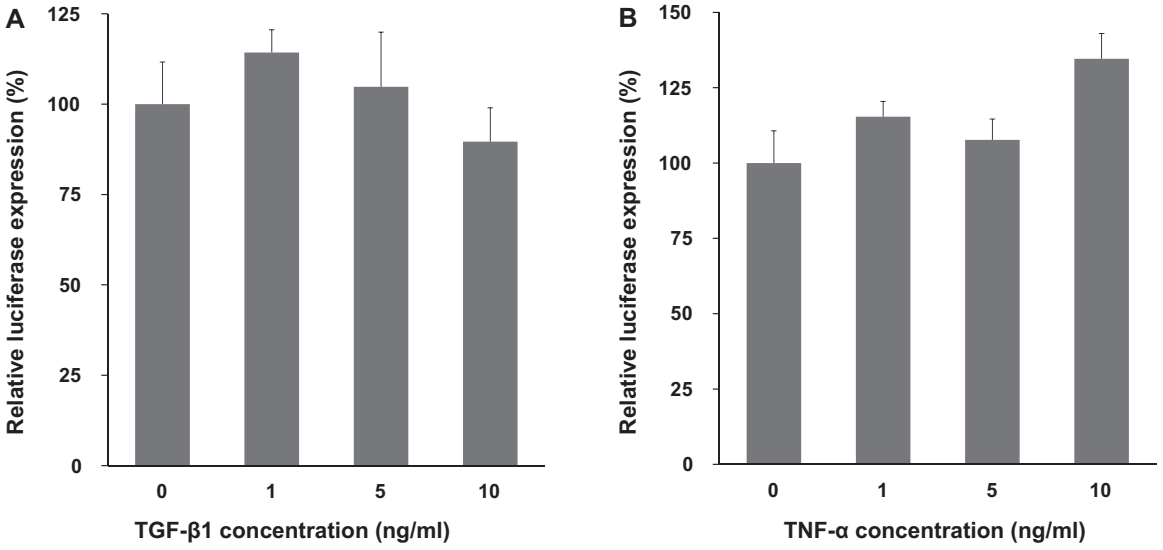

Fig. 7. The promoter activity of *ace2* in HCFs treated with pro-inflammatory factors. HCFs were transfected with the −516/+20 construct, and then treated with various concentrations of TGF-β1 (A) and TNF-α (B). The transfected HCFs were lysed 24 h after treatment and the luciferase activity was measured. Relative luciferase activity for each sample (*i.e.*, compared to luciferase activity without added TGF-β1 or TNF-α) is shown. All values are expressed as the mean ± SD from three independent experiments.

confer aberrant self-renewing properties on myeloid progenitors [44]; yet despite the clearly important biological role of Ikaros, its mechanism of action remains elusive. Consensus DNA recognition sequences for Ikaros have been unusually difficult to define because of several encoded Ikaros isoforms [23] and because multiprotein complexes containing Ikaros family members have not been purified to homogeneity [31]. From sequence analysis (using TFSEARCH) the potential binding domain of Ikaros was found in the regulatory region of *ace2*, but was not found in *ace* gene. This may explain why some factors have been shown to regulate *ace* and *ace2* differently [12,17,45].

We report here for the first time the characterization of the regulatory element of human gene, *ace2*, and provide insight into the molecular mechanism controlling cardiac ACE2 expression in HCFs. We have identified the −516/−481 sequence domain within the upstream region of *ace2* as a putative protein binding domain for modulation of ACE2 expression, which is associated with the Ang II signaling pathway. Furthermore, a potential regulatory element, ATTTGGA, within the −516/−481 promoter region of *ace2* is responsible for Ang II stimulation, and this is unaffected by the pro-inflammatory cytokines, TGF-β1 and TNF-α. Our results suggest that the −516/−481 domain of *ace2* is involved in modulating ACE2 expression, and may be a binding domain for Ikaros, or other unidentified regulatory factor(s). Investigating the regulatory role of Ikaros on *ace2* and other potential regulatory factor(s) would lead to a greater understanding of the molecular mechanisms that regulate ACE2 expression.

Acknowledgements

This work was supported by the grants of NSC 95-2313-B-009-002-MY3 and NSC 98-2313-B-009-002-MY3 from the National Science Council, Taiwan. This work was also supported in part by the UST-UCSD International Center of Excellence in Advanced Bio-engineering sponsored by the Taiwan National Science Council I-RiCE Program under Grant Number: NSC-99-2911-I-009-101.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.peptides.2011.08.009.

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