Experimental Physiology

Interplay of angiotensin II and angiotensin(1–7) in the regulation of matrix metalloproteinases of human cardiocytes

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Angiotensin II (Ang II) is a critical effector in the renin–angiotensin system (RAS), which modulates cardiovascular homeostasis, and the matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) related metabolism of extracellular matrix (ECM). Angiotensin(1–7) [Ang(1–7)] is another bioactive peptide in the RAS and is considered to have opposite effects to Ang II. However, the modulation of MMPs and TIMPs by Ang(1–7) is largely unclear in cardiocytes, and the antagonistic effects of Ang(1–7) on Ang II-mediated expression of MMPs and TIMPs have yet to be identified. In the present study, we examined the transcript expression of MMPs and TIMPs in human cardiac fibroblasts (HCF) and myocytes (HCM) after Ang II or Ang(1–7) stimulation, and analysed the antagonistic effects of Ang(1–7) to Ang II. The results show that Ang II decreased transcript expression of MMP-1, MMP-2, TIMP-1, TIMP-2 and TIMP-3, but upregulated MMP-9 expression in the HCF cells. Transcript expression of MMP-9 and TIMP-2 was downregulated by Ang(1–7) in the same cells. In the HCM cells, Ang II induced MMP-1 and MMP-9 overexpression but MMP-2 was downregulated. All of the examined MMPs and TIMPs, except MMP-9, were markedly decreased by Ang(1–7). In the studies of antagonistic effects of Ang(1–7) to Ang II, Ang(1–7) counteracted the effects of Ang II-mediated regulation on MMP-9 and TIMP-1 in the HCF cells compared with the control group. The regulations of all examined MMPs by Ang II were reversed to basal expression by Ang(1–7) in the HCM cells. Our results suggest that Ang(1–7) and Ang II have opposite and antagonistic effects on regulation of transcription of MMPs and TIMPs in primary cultures of human cardiocytes. These effects lead to increased ratios of MMPs to TIMPs after Ang II stimulation and decreased ratios of MMPs to TIMPs after Ang(1–7) stimulation; effects which may partly depend of the type of cardiac cells. These results suggest a potential role for Ang(1–7) in attenuatating cardiac damage in Ang II-induced ECM remodelling.

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Cardiac remodelling resulting from abnormal metabolism of extracellular matrix (ECM) proteins is a common phenomenon or an adaptive response in the pathogenesis of several heart diseases, such as atrial fibrillation, cardiomyopathy, heart failure, hypertension and myocardial infarction (Deschamps & Spinale, 2005; Diez, 2007; Lin *et al.* 2007). The structural remodelling may induce disturbances in cardiac conduction and may contribute to the loss of contractile force, which finally leads to heart dysfunction (Allessie *et al.* 2002; Spach *et al.* 2004).

The matrix metalloproteinases (MMPs), a family of zinc-dependent proteolytic enzymes, have the capability to degrade the ECM and have been implicated in the physiological remodelling of tissues in cardiovascular diseases (Galis & Khatri, 2002; Spinale, 2002). The activity of MMPs is controlled by naturally endogenous inhibitors called the tissue inhibitors of matrix metalloproteinases (TIMPs), which regulate and maintain matrix homeostasis. Many bioactive molecules, such as cytokines/chemokines, bioactive peptides and neurohormones, contribute to the regulation

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of MMPs and TIMPs (Rouet-Benzineb *et al.* 2000; Papakonstantinou *et al.* 2001; Chen *et al.* 2004; Xie *et al.* 2004; Brown *et al.* 2007). In particular, angiotensin II (Ang II), an important component of the renin–angiotensin system (RAS), is a key factor that modulates the expression of MMPs and TIMPs (Papakonstantinou *et al.* 2001; Deschamps *et al.* 2005; Castoldi *et al.* 2007).

Angiotensin(1–7) [Ang(1–7)] is also a critical peptide fragment in the RAS, which is generated from Ang II or angiotensin(1–9) by the forming enzyme, angiotensin-converting enzyme 2 (ACE2; Ferreira & Santos, 2005). The potential role of Ang(1–7) as a cardioprotective peptide with vasodilator, anti-growth and anti-proliferative actions has been recognized, and it antagonizes the effects of Ang II (Ferrario, 1992; McEwan *et al.* 1998; Santos *et al.* 2000). In a previous study, we found that atrial fibrosis may be associated with downregulation of ACE2 in the porcine atria with atrial fibrillation (Pan *et al.* 2007). Additionally, it has been reported that Ang(1–7) can downregulate both transcription and translation of the Ang II type 1 receptor (AT_1R) which is largely responsible for many of the pathological effects of Ang II, including marked cardiac fibrosis (Studer *et al.* 1994; Clark *et al.* 2001). Thereby, the ACE2–Ang(1–7) axis could provide further protection against the development of cardiovascular diseases, and these accumulating lines of evidence suggest that Ang(1–7) may serve to counterbalance the actions of Ang II (Ferrario *et al.* 1997; Zhu *et al.* 2002; Su *et al.* 2006; Pan *et al.* 2007).

In contrast to the actions of Ang II on MMPs and TIMPs, the effects of Ang(1–7) in the regulation of MMPs and TIMPs are still largely obscure. Based on the opposite physiological effects of Ang II and Ang(1–7), we proposed, first, that Ang(1–7) regulates MMPs and TIMPs in cardiac cells in an opposite way to Ang II and, second, that Ang(1–7) antagonizes the effects of Ang II on cardiac regulation of MMPs and TIMPs. Therefore, we determined the transcript expression of MMP-1, -2 and -9 and TIMP-1, -2 and -3 in human cardiac fibroblasts (HCF) and myocytes (HCM) after treatment with Ang II or Ang(1–7) (opposite effects) alone or after pre-incubation with Ang(1–7) and treatment with Ang II (antagonistic effects).

Methods

Chemicals and reagents

Angiotensin II (no. H1705), Ang(1–7) (no. H1715) and (D-Ala^7)Ang(1–7), an Ang(1–7) antagonist (A779; no. H2888) were purchased from Bachem (St Helens, UK). Valsartan, a selective AT_1R blocker (no. 1708762) was from USP (Rockville, MD, USA). Anti-MMP-1 (IM35T) antibody was purchased from Calbiochem (San Diego, CA, USA). Antibodies against GAPDH (sc-20357), horseradish peroxidase-labelled secondary antibodies against goat immunoglobulin G (sc-2020) and mouse immunoglobulin G (sc-2005) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were purchased from Sigma-Aldrich (Poole, UK).

Cell culture

Primary human cardiac myocytes (HCM, no.6200) and human cardiac fibroblasts (HCF, no. 6300) were purchased from ScienCell Research Laboratories (San Diego, CA, USA). All of the cells were seeded in 100 mm Petri dishes coated with 0.01% poly-L-lysine (no. P4832; Sigma-Aldrich) and cultured in commercial media (no. 6201 or no. 2301; ScienCell Research Laboratories), according to the manufacturer's instructions. The cells were incubated in a humidified air contained 5% CO_2 atmosphere at 37°C and culture media were changed every 2 days. 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. For assay of the transcript expression of MMPs and TIMPs, 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. Valsartan is a selective AT_1R blocker, and A779 is an antagonist of the Ang(1–7) receptor, Mas. The cells pretreated with receptor blockers, valsartan and A779, were used to confirm the receptor specific-effects of Ang II and Ang(1–7), respectively. To examine the antagonistic effects of Ang(1–7) on Ang II, the cells were pretreated with Ang(1–7) (1 μM) or phosphate-buffered saline (PBS; as a control) for 6 h and then challenged with Ang II (1 μM) for 24 h. Angiotensin II, Ang(1–7), valsartan and A779 applied in the present studies were used at a concentration of 1×10^{-6} M. Each experiment was performed independently three times.

Total RNA extraction

Total cellular RNA of cells was extracted as recommended by the manufacturer of TRIzol™ (GIBCO BRL, Rockville, MD, USA). For each treatment, approximately 4×10^5 cells were used to prepare the RNA in 20 μl of diethylpyrocarbonate (DEPC)-treated water, and stored at −80°C. The RNA was quantified by measuring absorbance at 260 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.

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Semi-quantitative RT-PCR was performed as described in our previous report (Chen *et al.* 2007). For cDNA synthesis, 5 μg RNA was supplemented in a total reaction

volume of 20 μl with ReverTra Ace set (no. PU-TRT-200; Purigo, Taipei, Taiwan) composed of 1× RT buffer, 1 mM dNTPs, 0.5 nM oligo-dT, 40 units μl^{-1} RNase inhibitor and 100 units μl^{-1} ReverTra Ace (reverse transcriptase). After incubation for 20 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.

The primer pairs used for RT-PCR were: MMP-1, 5′-GAC CTA CAG GAT TGA AAA TTA CAC GCC AG-3′ (forward) and 5′-CCG CAA CAC GAT GTA AGT TGT ACT CTC-3′ (reverse); MMP-2, 5′-CAC AGC CAA CTA CGA TGA TGA CC-3′ (forward) and 5′-TCA CAG TCC GCC AAA TGA ACC-3′ (reverse); MMP-9, 5′-CCA ACT ACG ACA CCG ACG AC-3′ (forward) and 5′-CGC CAC GAG GAA CAA ACT GTA TC-3′ (reverse); TIMP-1, 5′-ACC TCG TCA TCA GGG CCA AGT TCG-3′ (forward) and 5′-TGC ATT CCT CAC AGC CAA CAG TGT AG-3′ (reverse); TIMP-2, 5′-TCA TTG CAG GAA AGG CCG AGG G-3′ (forward) and 5′-TGT CAG AGC TGG ACC AGT CGA AAC-3′ (reverse); TIMP-3, 5′-CCT TCT GCA ACT CCG ACA TCG TGA T-3′ (forward) and 5′-CAT CAT AGA CGC GAC CTG TCA GC-3′ (reverse); glyceraldehyde-3-phophate dehydrogenase (GAPDH), 5′-TGG CGC TGA GTA CGT CGT G-3′ (forward) and 5′-TTC AGC TCA GGG ATG ACC TT-3′ (reverse). The PCR reaction contained 2 μl cDNA, 2 μl of each primer (10 μM), 5 μl of 10× PCR buffer, 2 μl of 10 mM dNTP, 1 μl of 5 units μl^{-1} Taq polymerase (Promega, Madison, WI, USA) and 36 μl distilled water in a total volume of 50 μl. Thermal cycler (MiniCycler™; MJ Research, Waltham, MA, USA) conditions were as follows: one cycle of 5 min at 95°C, 22∼38 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 45 s, and one cycle of 5 min at 72°C. The resulting PCR products were visualized on 2% agarose gels stained with ethidium bromide. The stained images were recorded by an image analyser (Kodak DC290 Digital camera System™; Eastman Kodak, Rochester, NY, USA), and the band intensity was quantified using densitometric analysis by Scion Image™ (Scion, Frederick, MD, USA). The relative transcript expression of MMPs and TIMPs was calculated as the ratio to GAPDH expression.

Protein extraction

The cultured cardiac cells (about 4×10^5 cells) were washed with 1× PBS and lysed by adding 100 μl of ice-cold lysis buffer containing 10 mM Tris-HCl, pH 7.4, 10% glycerol, 1 mM calcium chloride, 150 mM sodium chloride, 1 mM phenylmethylsulphonyl fluoride and 1% (v/v) Triton X-100. The cell lysates were then centrifuged at 12 000*g* at 4°C for 10 min, and the supernatant was collected for sodium dodecyl sulphate-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 as a standard. Aliquots containing 30 μg protein were resolved on 10% slab SDS-PAGE gels.

Immunoblotting

The extracted proteins of the cardiac cells were electrophoresed on SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (POLYSCREEN™; PerkinElmer, Boston, MA, USA). Briefly, non-specific binding sites were blocked by incubating membranes in 5% non-fat milk. Primary antibodies against proteins were diluted as follows: 1:1000 for MMP-1 and GAPDH. The secondary antibodies were applied using a dilution of 1:2000. Substrates were visualized using enhanced chemiluminesce (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™). The amount of MMP-1 is expressed relative to the amount of GAPDH in respective samples.

Statistics

All values are expressed as means ± S.D. Data were compared with one-way ANOVA followed by modified Student's unpaired *t* tests to evaluate differences among multiple groups. A value of $P < 0.05$ was considered statistically significant.

Results

Regulation of MMPs and TIMPs by Ang II and Ang(1–7) in the HCF cells

Angiotensin II-induced cellular responses at the transcriptional level of MMPs and TIMPs in the cultured HCF cells were examined by RT-PCR assay (Fig. 1*A*). The results show that compared with the control group, Ang II significantly downregulated the expression of MMP-1, MMP-2, TIMP-1, TIMP-2 and TIMP-3 by 23 ($P < 0.05$), 26 ($P < 0.05$), 19 ($P < 0.05$), 26 ($P < 0.05$) and 51% ($P < 0.01$), respectively, but upregulated the expression of MMP-9 by 86% ($P < 0.01$) in the HCF cells. All of these Ang II-modulated effects on MMPs and TIMPs in the HCF cells were blocked by pretreatment with valsartan, an antagonist of AT_1R. In contrast to Ang II-induced responses, the Ang(1–7)-modulated effects were restricted to the regulation of MMP-9 and TIMP-2 in the HCF cells. The relative transcript expression of MMP-9 and TIMP-2 was decreased by 39 ($P < 0.01$) and 20% ($P < 0.05$),

respectively, when the HCF cells were treated with Ang(1–7). The Ang(1–7)-induced MMP-9 and TIMP-2 expression was abolished by pretreatment with A779, a selective Ang(1–7) receptor blocker. Angiotensin II increased the ratio of MMP-9/TIMP-1 ($P < 0.01$) and MMP-9/TIMP-3 ($P < 0.01$). Angiotensin(1–7), however, reduced the MMP-9/TIMP-1 ratio ($P < 0.05$) and MMP-9/TIMP-3 ratio ($P < 0.05$) in the HCF cells (Fig. 1*B*). Additionally, the changes in the ratios of MMPs/TIMPs triggered by Ang II or Ang(1–7) were reversed to their basal levels by addition of their antagonists. Treatment with the antagonists alone did not influence the transcript expression of MMPs and TIMPs in the HCF cells (Fig. 1*C*).

Regulation of MMPs and TIMPs by Ang II and Ang(1–7) in the HCM cells

The transcript expression of MMPs and TIMPs was analysed in the cultured HCM cells after Ang II or Ang(1–7) treatment (Fig. 2*A*). The transcripts of MMP-1 (+28%, $P < 0.05$) and MMP-9 (+54%, $P < 0.01$) but not of MMP-2 were enhanced after Ang II stimulation. These effects were abolished by pretreated with valsartan. The Ang II-induced effects in the HCM cells, except MMP-2, were abolished by pretreatment with valsartan. However, all analysed TIMPs (TIMP-1, -2 and -3) stayed unchanged after Ang II stimulation. With the exception of MMP-9, all of the determined MMPs and TIMPs were downregulated in the Ang(1–7)-treated HCM cells compared with those in the control conditions. The relative transcript expression of MMP-1 was decreased by 17% ($P < 0.05$), and MMP-2 expression was reduced by 30% ($P < 0.05$). Moreover, the levels of TIMP-1, TIMP-2 and TIMP-3 in the HCM cells were all downregulated by Ang(1–7) treatment by 15 ($P < 0.05$), 21 ($P < 0.05$) and 20% ($P < 0.05$), respectively. The regulation effects of Ang(1–7) in the HCM cells were reversed by pretreatment with A779. In the measurement of MMPs/TIMPs ratios (Fig. 2*B*), Ang II increased

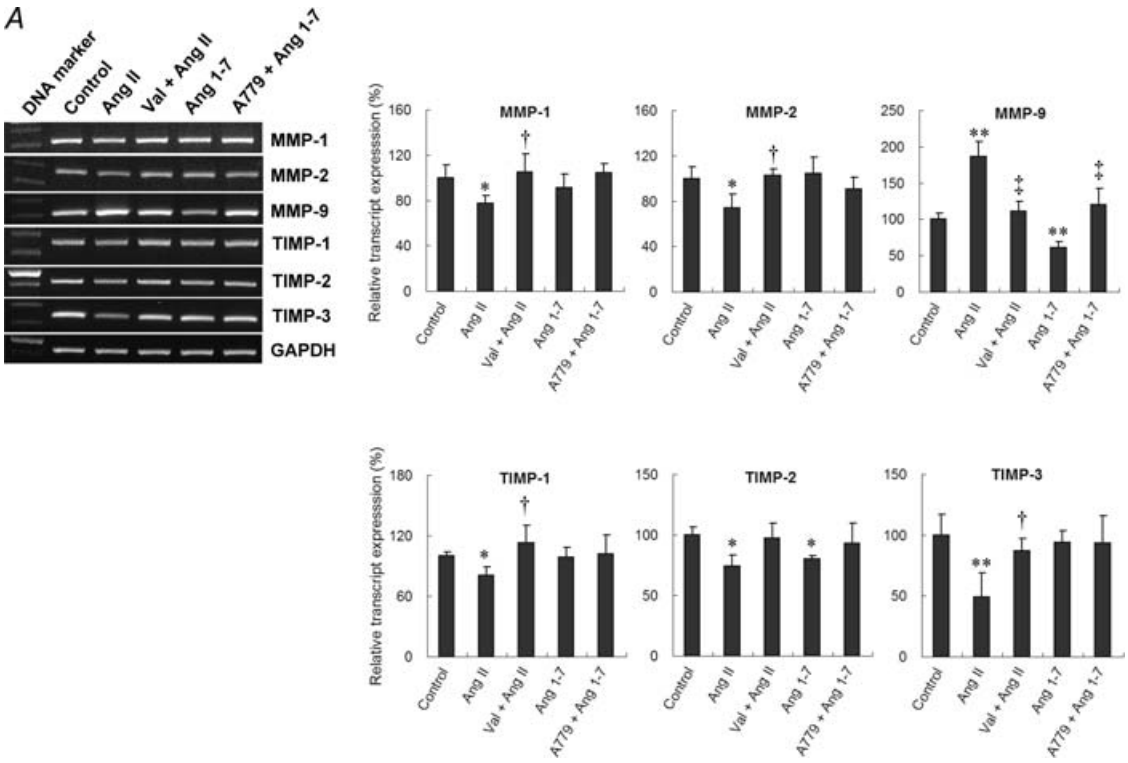

Figure 1. The transcript expression of MMPs and TIMPs in cultured HCF cells with Ang II and Ang(1–7) stimulation

A, expressed transcripts of MMPs and TIMPs in the treated HCF cells were determined by semi-quantitative RT-PCR. The length of the PCR products for MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2, TIMP-3 and GAPDH were 308, 362, 419, 336, 404, 231 and 405 bp, respectively. Amplification of GAPDH was determined as an internal control. The relative transcript expression of MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2 and TIMP-3 was normalized to the level of expressed transcript of the GAPDH gene. *B*, the changes in MMPs/TIMPs ratios with Ang II and Ang(1–7) treatment. *C*, the influence of valsartan (Val) or A779 treatment alone on the regulation of expression of MMPs and TIMPs. Bar graphs of all values are expressed as the means ± S.D. from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group; †$P < 0.05$ and ‡$P < 0.01$ compared with the original agonist group (Ang II or Ang(1–7) treated only), respectively.

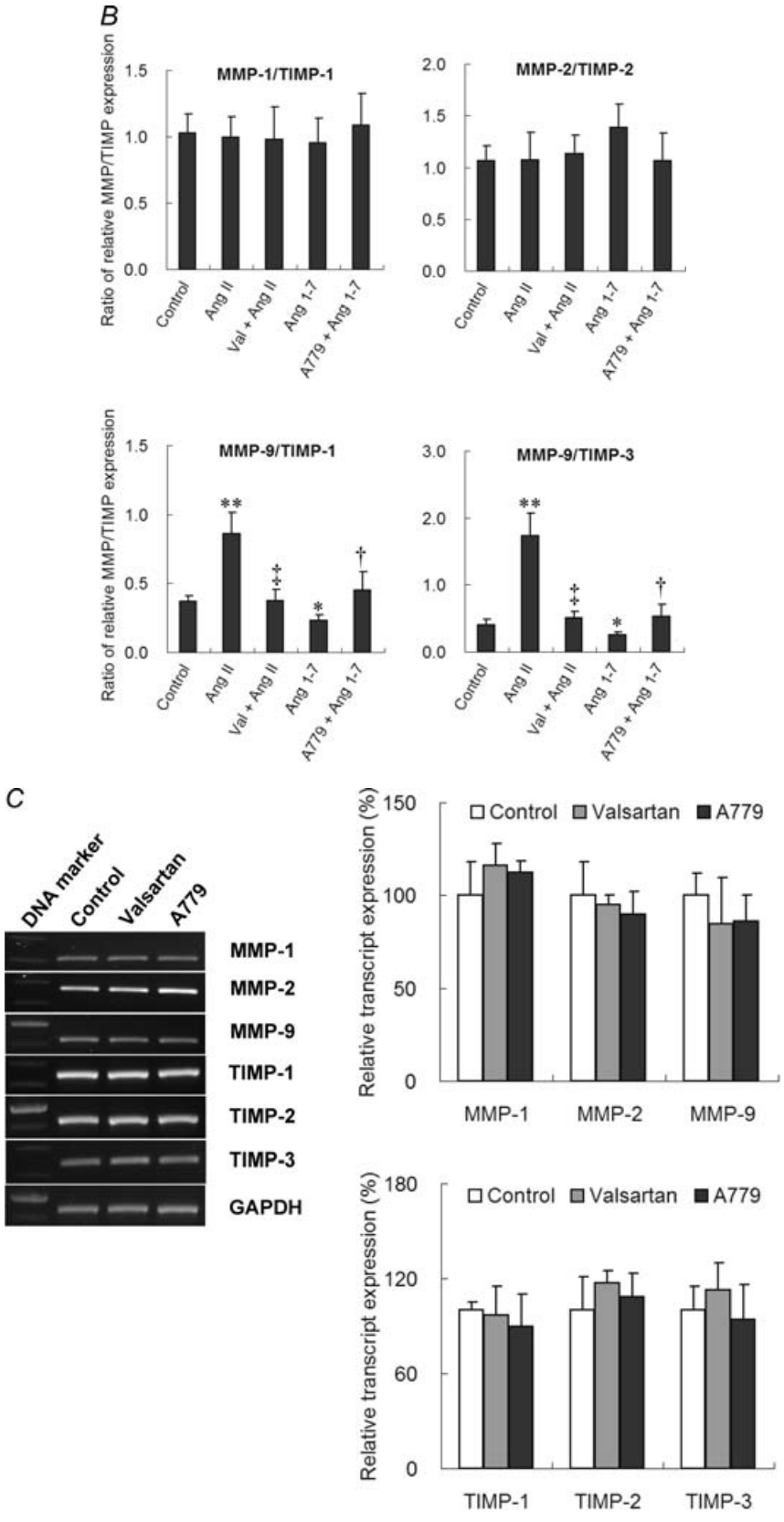

Figure 1. – Continued

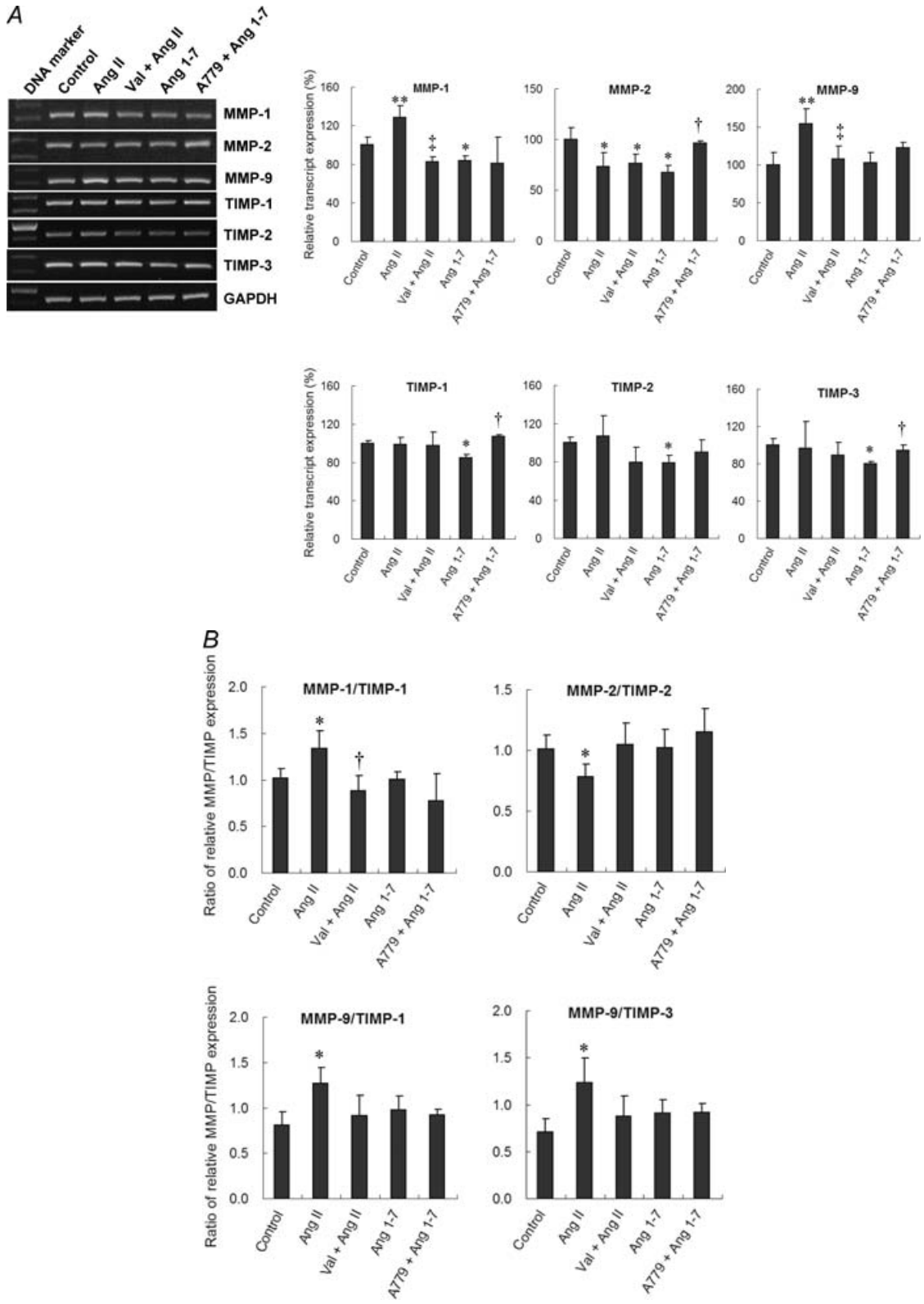

Figure 2. The transcript expression of MMPs and TIMPs in cultured HCM cells with Ang II and Ang(1–7) stimulation

A, expressed transcripts of MMPs and TIMPs in the treated HCM cells were determined by semi-quantitative RT-PCR, as in Fig. 1. *B*, the changes of MMPs/TIMPs ratios with Ang II and Ang(1–7) treatment. *C*, the influence of valsartan (Val) or A779 treatment alone on regulation of expression of MMPs and TIMPs. Bar graphs of all values are expressed as the means ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group; †$P < 0.05$ and ‡$P < 0.01$ compared with the original agonist group (Ang II or Ang(1–7) treated only), respectively.

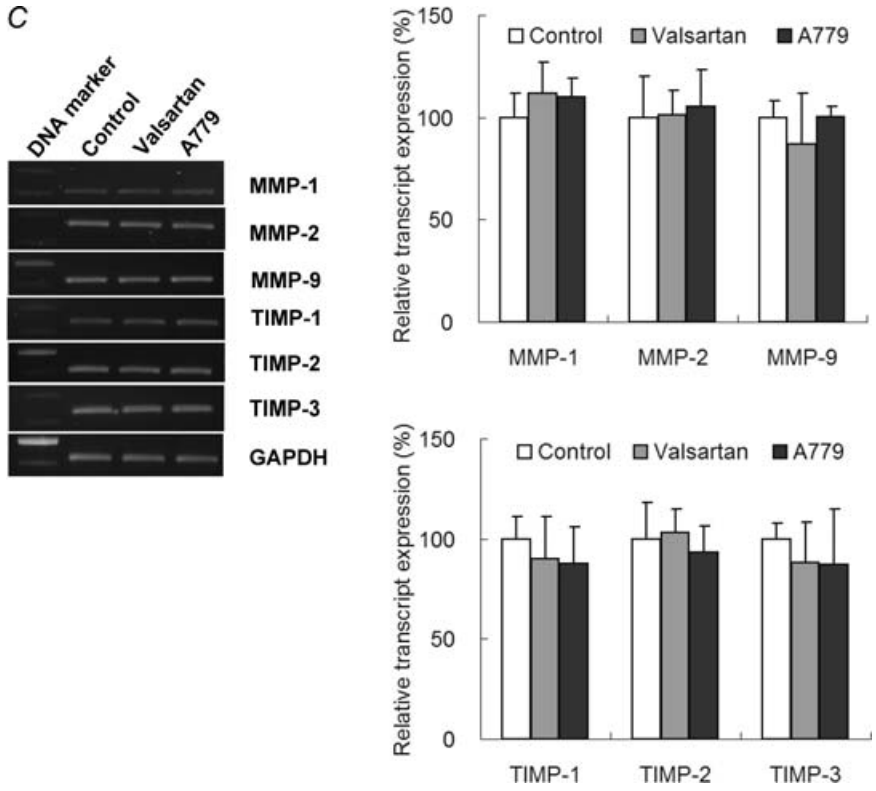

Figure 2. – Continued

MMP-1/TIMP-1 ($P < 0.05$), MMP-9/TIMP-1 ($P < 0.05$) and MMP-9/TIMP-3 ratios ($P < 0.05$), but decreased the ratio of MMP-2/TIMP-2 ($P < 0.05$). However, no difference was found in these ratios after Ang(1–7) treatment. Treatment with the antagonists alone did not influence the transcript expression of MMPs and TIMPs in the HCM cells (Fig. 2*C*).

Antagonistic effects of Ang(1–7) on Ang II-induced expression of MMPs and TIMPs

For evaluating the potential antagonistic role of Ang(1–7) in the Ang II-modulated expression of MMPs and TIMPs, the HCM and HCF cells were pretreated with Ang(1–7) and then challenged with Ang II. The results of antagonistic effects of Ang(1–7) to Ang II in the HCF and HCM cells are illustrated in Figs 3 and 4, respectively.

In the HCF cells, all of examined MMPs and TIMPs transcripts were regulated by Ang II, but Ang(1–7) counteracted the effects of Ang II on MMP-9 and TIMP-1 expression (Fig. 3*A*). The antagonistic effect of Ang(1–7) on Ang II-modulated MMP-1 protein expression was confirmed (Fig. 3*B*), and the antagonistic profile of Ang(1–7) on Ang II-mediated MMP-1 translational expression was similar at the transcription level (Fig. 3*A*). Angiotensin II upregulated the MMP-9/TIMP-1 ($P < 0.01$) and MMP-9/TIMP-3 ($P < 0.01$) ratios, but only the ratio of MMP-9/TIMP-1 was antagonistically reversed by the Ang(1–7) treatment (Fig. 3*C*).

In the HCM cells, Ang(1–7) abolished the effects of Ang II-induced MMP-1, MMP-2 and MMP-9 expression compared with the control group (Fig. 4*A*). As shown in Fig. 4*A*, the expression levels of TIMP-1, -2 and -3 were not affected by Ang II treatment with/without pretreatment with Ang(1–7). The antagonistic effect of Ang(1–7) on Ang II was examined at the translational level, and protein expression of MMP-1 mediated by Ang II stimulation was significantly reversed to basal levels in the HCM cells pretreated with Ang(1–7). (Fig. 4*B*). Angiotensin II increased the ratio of MMP-1/TIMP-1 and MMP-9/TIMP-1, but decreased the MMP-2/TIMP-2 ratio. Only the ratio of MMP-1/TIMP-1 returned to the basal level with Ang(1–7) pretreatment (Fig. 4*C*).

Discussion

We examined the opposite and antagonistic effects of Ang(1–7) on Ang II-mediated expression of MMPs and TIMPs in the cultured human cardiocytes, HCF and HCM cells. In this study, several opposing effects, dependent on the cell type, were found between Ang II and Ang(1–7) in regulation of MMPs and TIMPs (Figs 1 and 2). In HCF, Ang II increased and Ang(1–7) decreased MMP-9 expression while in HCM, Ang II increased and Ang(1–7)

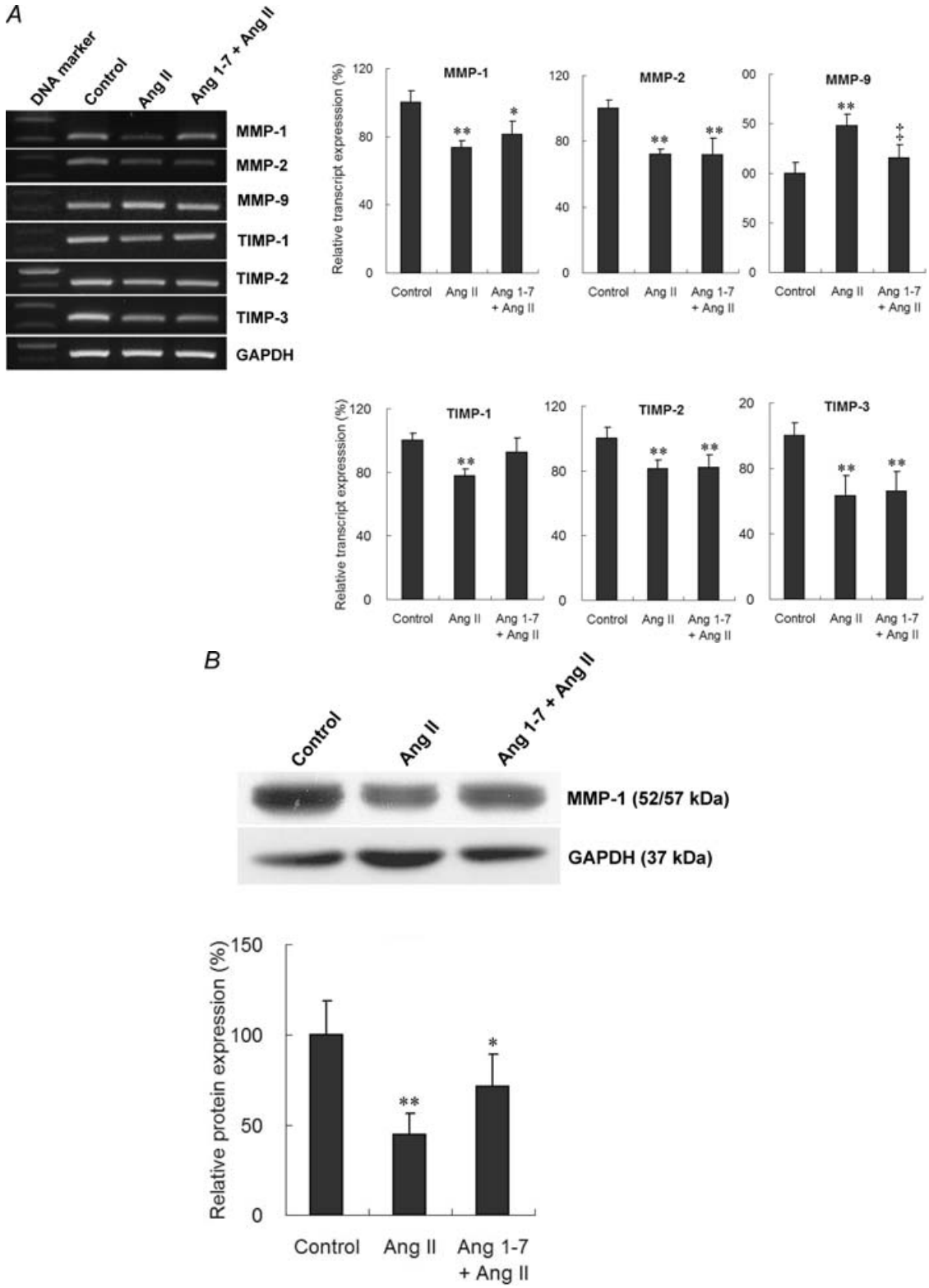

Figure 3. Antagonistic effects of Ang(1–7) on Ang II-dependent regulation of MMPs and TIMPs in HCF cells

A, the determination and quantification of gene expression by RT-PCR were as in Fig. 1. The relative transcript expression of MMPs and TIMPs were calculated with the values of the control group as 100%, as in Fig. 1. *B*, antagonistic effects of Ang(1–7) on Ang II-induced MMP-1 regulation was examined by Western blotting. *C*, the alteration of MMPs/TIMPs ratios by antagonistic effects of Ang(1–7). Bar graphs of all values are expressed as the means ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group; †$P < 0.05$ and ‡$P < 0.01$ compared with the original agonist group (Ang II or Ang(1–7) treated only), respectively.

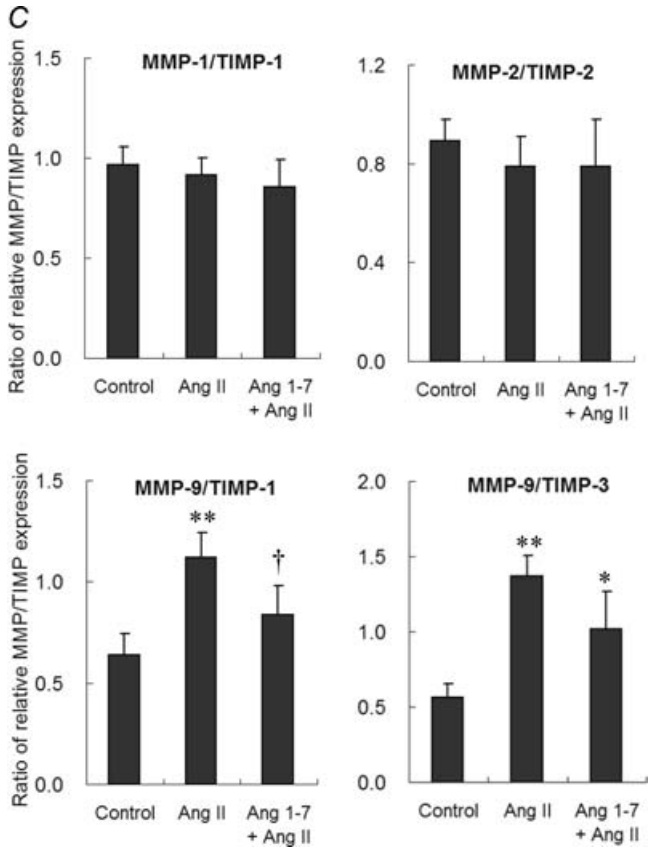

Figure 3. – Continued

decreased MMP-1 expression. Additionally, Ang II and Ang(1–7) mediated opposite changes on the MMP-9/TIMP-1 and MMP-9/TIMP-3 ratios in HCF. Moreover, Ang(1–7) showed a cell-dependent antagonistic effect on several Ang II-mediated changes in MMPs and TIMPs (Figs 3 and 4). Angiotensin(1–7) antagonized Ang II-induced modulation of MMP-9 and TIMP-1 in HCF and of MMP-1 and MMP-9 in HCM, leading to abolition of the increased MMP-9/TIMP-1 ratio in HCF and MMP-1/TIMP-1 ratio in HCM.

According to substrate specificity and primary structure, the MMP family can be subdivided into five groups that include collagenases, gelatinases, stromelysins and membrane-type MMPs (MT-MMPs). The examined MMPs of this study, MMP-1 (collagenase I), MMP-2 (gelatinase A) and MMP-9 (gelatinase B), have been demonstrated within mammalian myocardium (Gunja-Smith *et al.* 1996; Spinale *et al.* 2000). Numerous studies have also demonstrated abnormal expression and activity of MMP-1, -2 and -9 in diseased myocardium, in conditions such as atrial fibrillation, cardiomyopathy, heart failure and myocardial infarction (Danielsen *et al.* 1998; Thomas *et al.* 1998; Spinale *et al.* 2000; Nakano *et al.* 2004).

The activated MMPs can be inhibited by interaction with their endogenous inhibitors, the TIMPs. Four different TIMP species have been identified and bind non-covalently to active MMPs in a 1:1 stoichiometric ratio. There is a certain degree of specificity in the activity of different TIMPs toward distinct members of the MMP family (Birkedal-Hansen *et al.* 1993; Denhardt *et al.* 1993). It is reported that TIMP-1 and TIMP-3 preferentially bind to MMP-9, and that TIMP-2 binds to MMP-2 (Ward *et al.* 1991; Murphy *et al.* 1994; Sternlicht & Werb, 2001). The ratio of expression of MMPs/TIMPs is a useful index for evaluating the balance between the proteolytic enzymes and their endogenous inhibitors, which might raise a view in the status of ECM metabolism or structural remodelling of tissues. Several studies suggest that increased MMP expression and activation coupled with a decreased TIMP expression occur early in the postmyocardial infarction period and contribute to postmyocardial infarction remodelling (Li *et al.* 1998; Peterson *et al.* 2000). A reduction in relative myocardial TIMP level and/or alterations in MMP/TIMP ratio has been reported in the failing human heart (Li *et al.* 1998; Spinale *et al.* 2000). Schwartzkopff *et al.* (2002) provided the evidence that an imbalance in the MMP-1/TIMP-1 ratio plays an important role in left ventricular remodelling and enlargement in the early stages of heart failure. The disturbance of MMP-9/TIMP-1 ratio may promote the process of atrial structural remodelling during chronic atrial fibrillation (Nakano *et al.* 2004; Zhu *et al.* 2005). Plasma from patients with congestive heart failure showed an increase in MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios (Wilson *et al.* 2002). Taken together, these results suggest that an imbalance between MMPs and TIMPs within the failing myocardium would result in abnormal ECM metabolism and continued myocardial remodelling.

The expression of AT_1R has no obvious variation and seems to be present on almost all myocardial cells, whereas the angiotensin type 2 (AT_2) receptor is little expressed and has so far been localized to coronary endothelial cells and fibroblasts in the heart (Ohkubo *et al.* 1997; Regitz-Zagrosek *et al.* 1998). Additionally, our study shows that the expression of Mas receptor was relatively higher in cardiac fibroblasts than in cardiac myocytes (data not shown). Thereby, the sensitivity of both cell types to Ang II and Ang(1–7) may be different. In our study, MMPs and TIMPs of HCF and HCM cells were regulated differently by Ang II and Ang(1–7). For example, MMP-1 expression was downregulated by Ang II in the HCF cells, but upregulated in the HCM cells. Moreover, all of the TIMPs were downregulated in the HCF cells by treatment with Ang II, whereas TIMPs of the HCM cells remained unchanged with Ang II treatment. Furthermore, Ang(1–7) significantly decreased MMP-9 expression in the HCF cells, but MMP-9 expression was not influenced by Ang(1–7) in the HCM cells.

Angiotensin(1–7), an endogenous angiotensin peptide, is considered to play an opposing function to Ang II;

however, the mechanisms of Ang(1–7) in the RAS pathways largely remain to be explored (Ferrario *et al.* 1997; Machado *et al.* 2001). The balance of local Ang II and Ang(1–7) may play a critical role in cardiac remodelling or fibrosis, thereby underlining the importance of understanding their effects in the regulation of ECM. In the present study, we demonstrated that Ang(1–7) counteracts Ang II-mediated MMP-1 and/or MMP-9 modulation in primary cardiac cell cultures. These findings might result from Ang(1–7)-mediated downregulation of the expression of AT_1 receptor or partly through the Ang(1–7) receptor, Mas protein (Clark *et al.* 2001). The alteration of MMPs is correlated with the pathogenesis of several heart diseases (Mukherjee *et al.* 2006; Batlle *et al.* 2007). Angiotensin II-stimulated alternations of MMP expression returned to the control level by antagonistic effects of Ang(1–7), which might stabilize cardiac structure to protect it from tissue remodelling and functional degeneration during heart diseases.

It is clear now that Ang(1–7) has an antifibrotic effect in the heart, which is important in protecting it from cardiac remodelling and heart failure. Iwata *et al.* (2005) mentioned that Ang II-induced increases in extracellular proteins, growth factors and cardiac hypertrophy could be reversed by Ang(1–7), which interacts with specific receptors to exert potential antifibrotic and antitrophic effects. In addition, Ang(1–7) infusion could prevent deoxycorticosterone acetate-salt- and isoproterenol-induced cardiac hypertrophy, myocardial fibrosis and cardiac dysfunction in rat models (Grobe *et al.* 2006; Ferreira *et al.* 2007*b*). Similarly, co-infusion of Ang(1–7) significantly attenuates myocyte hypertrophy and interstitial fibrosis induced by chronic infusion of Ang II (Grobe *et al.* 2007). The selective ligand of the Mas receptor, AVE-0991, has actions similar to those attributed to Ang(1–7), which indicates that AVE-0991 can attenuate postischemic heart failure (Ferreira *et al.* 2007*a*). Furthermore, the Mas receptor knockout model has revealed that the changes in cardiac function could partly be explained by a marked change in collagen expression in Mas-deficient mice, which indicates that the Ang(1–7)–Mas axis plays a key role in maintenance of the structure and function of the heart (Santos *et al.* 2006). These results suggest that Ang(1–7) may play an important role in the heart in regulating cardiac remodelling, and these

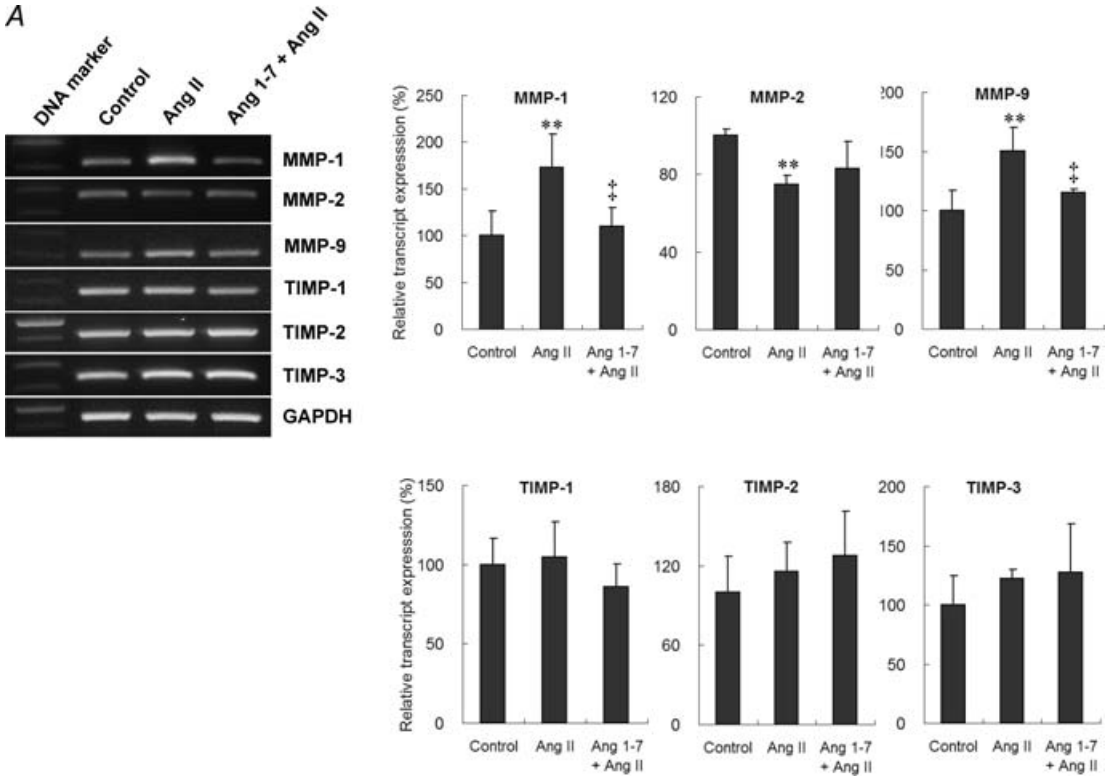

Figure 4. Antagonistic effects of Ang(1–7) on Ang II-dependent regulation of MMPs and TIMPs in HCM cells

A, the determination and quantification of gene expression by RT-PCR were as in Fig. 1. The relative transcript expression of MMPs and TIMPs were calculated with the values of the control group as 100%, as in Fig. 2. *B*, antagonistic effects of Ang(1–7) on Ang II-induced MMP-1 regulation was analysed by immunoblotting. *C*, the alteration of MMPs/TIMPs ratios by antagonistic effects of Ang(1–7). Bar graphs of all values are expressed as the means ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group; †$P < 0.05$ and ‡$P < 0.01$ compared with the original agonist group (Ang II or Ang(1–7) treated only), respectively.

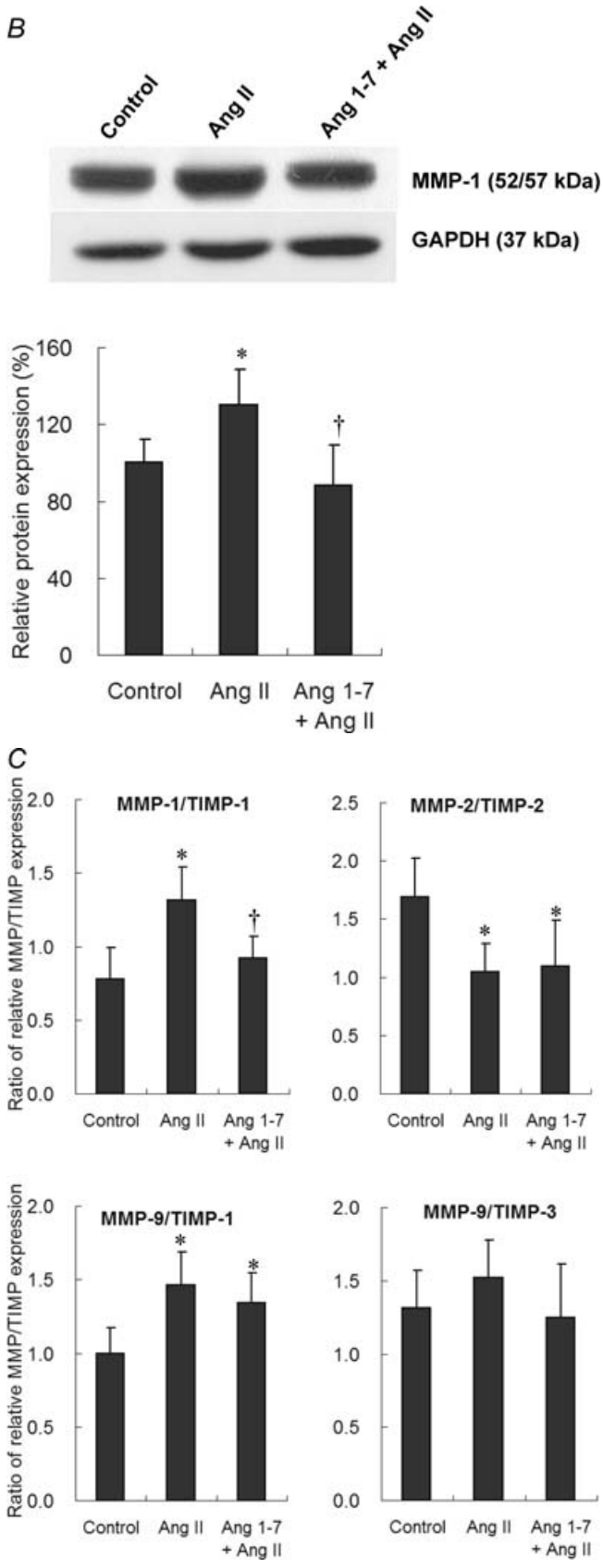

Figure 4. – Continued

findings indicate an antiremodelling role for Ang(1–7) in cardiac tissue, which may be mediated through an Ang(1–7) receptor.

A number of studies have indicated that Ang II not only promotes the synthesis of ECM proteins, but also modulates the MMPs and TIMPs at transcriptional, translational and activity levels (Brilla *et al.* 1994; Coker *et al.* 2001; Stacy *et al.* 2007). Our study showed that Ang II decreased expression of MMP-1, MMP-2 and all examined TIMPs, but upregulated MMP-9 expression in the HCF cells. However, only MMP-1 and MMP-9 were markedly upregulated by Ang II in the HCM cells. Angiotensin II may modulate the expression and activity of MMPs differently in cardiac cells, such as cardiac fibroblasts and cardiac myocytes. Angiotensin II decreases collagenase activity in the cultured rat cardiac fibroblasts (Brilla *et al.* 1994; Taniyama *et al.* 2000; Chen *et al.* 2004), and decreases gelatinase activity in adult murine cardiac fibroblasts (Stacy *et al.* 2007). The activity and content of gelatinase were increased by Ang II in ventricular myocytes isolated from pigs and neonatal rats (Rouet-Benzineb *et al.* 2000; Coker *et al.* 2001). In study of the regulation of TIMPs, TIMP-1 was increased in neonatal rat cardiac fibroblasts by stimulation of Ang II (Peng *et al.* 2002).

In the present study, the results show that cultured human cardiocytes challenged with Ang(1–7) could significantly decrease transcript expression of MMP-9 in HCF cells, and MMP-1 as well as MMP-2 in HCM cells. Additionally, the HCM cells stimulated with Ang(1–7) could cause all of examined TIMPs expression in a decreasing trend, but only decreased TIMP-2 expression was observed in the HCF cells. Several lines of evidence suggest that Ang(1–7) could inhibit Ang II-induced signalling transduction, particularly in the mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathway, in several types of mammalian cells (Zhu *et al.* 2002; Tallant & Clark, 2003; Su *et al.* 2006). It is believed that MAPK and PKC signalling cascades are critical pathways which might be involved in regulation of the expression of MMPs (Nagase & Woessner, 1999; Xie *et al.* 2004). In addition, Ang(1–7) could counteract the effects of Ang II through downregulation of the AT_1R (Clark *et al.* 2001). The biological effects of Ang(1–7) are mainly mediated by the Mas receptor, a G protein-coupled receptor (Santos *et al.* 2003). However, Ang(1–7)-induced responses might depend on the Ang(1–7) concentration of treatments. Clark *et al.* (2001) mentioned that Ang(1–7) can bind to AT_1R without stimulating subsequent cellular responses in vascular smooth muscle cells (VSMC). Based on these lines of evidence, it is considered that the regulation of MMPs and TIMPs might also be directly or indirectly influenced by Ang(1–7) stimulation.

In conclusion, this study demonstrates that in primary cultures of human cardiocytes, Ang(1–7) and Ang II have opposite and antagonistic effects on regulation of

the transcription of MMPs and TIMPs. These effects are partial, and dependent on cell type, but lead to increased ratios of MMPs/TIMPs after Ang II stimulation and decreased ratios of MMPs/TIMPs after Ang(1–7) stimulation. We suggest that these effects may depend on cell type, upstream regulatory elements of the genes, cell or tissue-specific factors, and the expression and localization of angiotensin peptide receptors (AT_1R and Mas receptor). These results suggest a potential role for Ang(1–7) in attenuatating the cardiac damage in Ang II-induced ECM remodelling.

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