Angiotensin converting enzyme 2 (ACE2) activity in fetal calf serum: implications for cell culture research

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Abstract Cell culture experiments often employ the use of culture media that contain fetal calf serum (FCS). The angiotensin peptides angiotensin II and angiotensin 1–7 have opposing effects with angiotensin converting enzyme 2 (ACE2) being the enzyme predominantly responsible for generating angiotensin 1–7 from angiotensin II. The effect of FCS on angiotensin peptides has not previously been described. We have shown that FCS has ACE2 enzyme activity capable of degrading angiotensin II and generating angiotensin 1–7. Researchers should be aware that FCS possesses ACE2 activity and that heat-treating FCS to 56 °C only partially inhibits this enzyme activity, whereas heat-treating to 70 °C completely abolishes ACE2 activity.

Keywords ACE2 activity · Culture medium · Angiotensin 1–7

Introduction

There have been considerable advances over the last decade in our understanding of the renin–angiotensin system (RAS; Burrell et al. 2004; Lambert et al. 2008; Warner et al. 2007). Since the discovery of the angiotensin converting enzyme (ACE) homologue, ACE2 in 2000 it has become widely accepted that the RAS is composed of two axes, with ACE, angiotensin II (Ang II) and Ang II type I receptor (AT1) representing one axis and ACE2, angiotensin 1–7 (Ang-(1–7)) and the Ang-(1–7) receptor *mas* representing a counterbalancing, ‘alternative’ axis (Warner et al. 2007). ACE2 has a central role in the RAS since it degrades Ang II to generate Ang-(1–7) (Ferrario et al. 2005; Santos et al. 2006; Warner et al. 2007). Ang-(1–7), the effector peptide of the ‘alternative’ axis, has been shown both in-vivo and in-vitro to have numerous effects that directly oppose those of Ang II (Fig. 1). These include antihypertensive, antiarrhythmic, cardioprotective anti-inflammatory, anti-fibrotic, anti-thrombotic and antitrophic effects in a number of different cell types (Benter et al. 1995; Ferreira et al. 2001, 2002; Freeman et al. 1996; Grobe et al. 2006; Iwata et al. 2005; Kucharewicz et al. 2000, 2002; Strawn et al. 1999; Tallant et al. 2005). Ang-(1–7) is degraded by ACE into the inactive peptide Ang-(1–5). Thus, ACE both generates Ang II and inactivates Ang-(1–7). The opposing effects of ACE and ACE2 on Ang II and Ang-(1–7) production has lead researchers to suggest that these two

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Fig. 1 The renin–angiotensin system. The renin–angiotensin system is shown with peptides (*clear boxes*) being metabolised by enzymes (*gray boxes*) into the two main active components, Ang II and Ang-(1–7). These two hormones have opposing effects and act through distinct receptors AT₁ and MAS (*speckled boxes*). ACE2 is the principle enzyme responsible for the generation of Ang-(1–7) from Ang II. Ang-(1–7) is metabolised by ACE into the non-active peptide fragment Ang-(1–5)

enzymes control the balance between the two axes of the RAS.

The effects of Ang II on hepatic stellate cells (HSC) has been extensively studied (Bataller et al. 2005, 2003a, b, 2000; Wei et al. 2001; Yokohama et al. 2006; Zhang et al. 2003), yet little information exists on the effect of Ang-(1–7) on these cells. Most studies using cell culture use culture medium supplemented with fetal calf serum (FCS) to encourage cell growth. Our laboratory has been studying the role of the RAS in hepatic fibrosis using primary rat HSC culture. We were particularly interested to know whether Ang II was stable in such media in order to determine the optimum frequency of dosing of this peptide. We also wished to determine whether Ang-(1–7) was present in the culture media before or after Ang II was administered since this could significantly affect our results.

Although numerous papers have been published describing Ang II effects on a variety of cell culture systems using FCS as a constituent of the culture medium, to date no one has described whether any of the components in culture medium could influence the balance between Ang II and Ang-(1–7).

FCS can be purchased as a heat-inactivated preparation where the serum has been heated to 56 °C for 30 min. The theoretical advantage of this

treatment being the reduction of complement when studying immunological mechanisms in cell culture.

The experiment described below is important to all researchers using culture medium containing FCS because Ang II and Ang-(1–7) are active peptides of the RAS capable of stimulating cytokine production as well as influencing cellular proliferation, extracellular matrix deposition, and cellular contractility. Many cells have been shown to produce Ang II in cellular culture and the presence of ACE2 can influence the respective concentrations of Ang-(1–7) and Ang II in-vitro. These two peptides have opposing effects and the relative concentrations of these peptides as determined by ACE2 may influence experiments by changing the cytokine profile of the cellular milieu (Fig. 1).

This paper describes how we investigated whether FCS has ACE2 activity and discusses possible implications for researchers working with cell culture systems.

Materials and methods

All experiments were performed on culture media in the absence of cells, thus removing possible influences of locally generated angiotensin peptides and angiotensinases. Culture media (M199, Invitrogen Australia Pty Ltd, Victoria, Australia), was reconstituted on the day of the experiment and supplemented with 10% (v/v) fetal calf serum (catalogue No: 10099-141, FCS, Invitrogen), and penicillin and streptomycin (1% v/v, Invitrogen). The media was adjusted to a pH of 7.0 and filtered through 0.22 µm bottle top filters (Millipore corporation, Massachusetts, USA). Known concentrations of purified Ang II (1,2,10 and 20 nmol/L, Auspep Pty. Ltd, Parkville, Melbourne, Australia) were added to 13 mL sterile tubes (Becton Dickinson Pty Ltd, NJ, USA) containing 5 mL of saline, saline with EDTA (5 mmol/L), culture media or culture media with EDTA (5 mmol/L). The tubes were gently mixed and left at room temperature for 1 h prior to being stored at –80 °C until the day of Ang II and Ang-(1–7) radioimmunoassay.

For ACE2 activity, individual components of standard culture media were placed in 13 mL sterile tubes. In addition, standard culture medium with EDTA (5 mmol/L), standard culture medium with 0.1% (w/v) bovine serum albumin (BSA, premium

grade, Trace Biosciences NZ Ltd.) and standard culture medium with M199 and penicillin-streptomycin (1% v/v) were tested for ACE2 activity. Additional ACE2 activity assays were performed on 100% FCS and 100% FCS treated for 30 min at 56 and 70 °C. Different batches were tested with origins in Australia, Mexico and the United States of America (Invitrogen, catalogue numbers 10099-141, 26140-111 and 10437-077, respectively) and expressed as a percentage relative to Australian FCS. For comparison, adult bovine serum from cattle aged 12–24 months was also tested (Invitrogen catalogue No. 16170-078, source New Zealand).

Data were expressed as mean $\pm$ standard error of mean (SEM) unless otherwise stated. Analysis of variance (ANOVA) with Bonferroni post-test was used to analyse the data, with a *p* value less than 0.05 considered to be statistically significant.

Procedures

Radioimmunoassay for angiotensin peptides

Radioimmunoassay for Ang II and Ang-(1–7) was performed on all samples in duplicate as described previously (Herath et al. 2007; ProSearch International Australia Pty Ltd, Malvern, Victoria, Australia). Briefly, antibodies to Ang II and Ang-(1–7) were raised in rabbit and guinea pig respectively, by immunising animals against the natural peptide sequences, N-terminally conjugated to either porcine thyroglobulin (Ang-(1–7)) or BSA (Ang II). Ang II and Ang-(1–7) tracers were produced separately by mono-iodinating peptides with ^{125}I by Chloramine-T oxidation (Greenwood et al. 1963) and purifying with high-performance liquid chromatography (HPLC, Millipore Waters, Milford, MA, USA). Samples to be tested were then incubated at 4 °C with the iodinated peptide tracer and the respective antibody. For Ang-(1–7) the antibody–antigen complex was separated using secondary antibody precipitation (goat anti-guinea pig IgG, ProSearch International Australia Pty Ltd) enhanced with polyethylene glycol (PEG-6000, BDH, Poole, Dorset, UK). Ang II antibody–antigen complexes were isolated using dextran 10-coated charcoal absorption and centrifugation. Radioactivity in the pellets was measured using a multi-well gamma counter (LKB-Wallac 1261, LKB

Instruments, Turku, Finland) and compared to known standards of synthetic peptide (Ang-(1–7); Auspep Pty. Ltd, Parkville, Victoria, Australia, Ang II; MRC, Nat Inst for Biological Standards and Controls, London UK). Results were expressed in pmol/L. The cross-reactivity to Ang II in the Ang-(1–7) assay was 0.04%. Cross reactivity to Ang-(1–7) in the Ang II assay was 0.01%.

Measurement of ACE2 activity

Enzyme activity for ACE2 was measured using specific quenched fluorogenic substrates (QFS, Auspep, Parkville, Australia) as described previously (Burchill et al. 2008; Herath et al. 2007; Lew et al. 2008). Briefly, the QFS comprised a fluorophore, 7-methoxycoumarin-4-acetyl (MCA), and a quencher, *N*-2,4-dinitrophenyl (DNP) separated by a short peptide chain (alanine, proline and lysine). The proline–lysine bond is cleaved by ACE2 allowing the quencher to separate from the fluorophore resulting in increased fluorescence emission of MCA. The assay was performed with 50 μM QFS in a final volume of 200 μL ACE2 assay buffer (100 mM Tris–HCl, 1 M NaCl, pH 6.5).

The fluorescence of the samples was measured at 37 °C using a FLUOstar Optima plate reader (BMG LABTECH, Offenburg, Germany) with excitation and emission wavelengths of 320 and 405 nm, respectively. The test samples were analysed in the absence and presence of 100 μM EDTA, a known inhibitor of ACE2; the difference in fluorescence representing the enzyme activity within the test sample. Samples were tested in triplicate. Results were expressed as nano moles of substrate cleaved per minute per millilitre of plasma.

Measurement of ACE activity

Enzyme activity for ACE was measured by Austin pathology (Austin Health, Melbourne, Australia) using the synthetic substrate 3-(2-furylacryloyl)-L-phenylalanyl-glycyl-glycine (FAPGG) which is hydrolysed by ACE to glycylglycine and FAP [3-(2-furylacryloyl)-L-phenylalanine]. The hydrolysed chromogen undergoes a small bathochromic shift. The absorbance at 340 nm of the chromophore FAPGG is monitored kinetically and its rate of decrease is proportional to

the activity of the ACE enzyme in the sample. Results were expressed as U/L.

Results

Ang II radioimmunoassay demonstrated that addition of increasing doses of Ang II to saline lead to the expected sequential increase in the detectable Ang II concentration. For the purposes of this experiment Ang II in saline and EDTA was used as the control group (Fig. 2).

When Ang II was added to culture media containing FCS there was a $90 \pm 3.5\%$ reduction in Ang II concentration for all doses measured (1, 2, 10, 20 nmol/L; $p < 0.01$, $p < 0.001$, $p < 0.05$, $p < 0.001$, respectively). This effect was abolished by the addition of EDTA (5 mmol/L) to the culture medium. When Ang II was added to culture medium there was an associated increase in detectable Ang-(1–7) which was observed for all concentrations of Ang II ($p < 0.001$, $p < 0.001$, $p < 0.01$, $p < 0.01$, respectively) and showed a concentration dependent effect. Addition of EDTA inhibited the reduction in Ang II ($p < 0.01$, $p < 0.01$, $p < 0.01$, $p < 0.001$) and resulted in a $98\% \pm 0.9$ reduction in Ang-(1–7) production from Ang II ($p < 0.001$, $p < 0.001$,

$p < 0.05$, $p < 0.01$). There was no significant differences between saline with EDTA compared to media with EDTA across all doses of Ang II in both the Ang II and Ang-(1–7) assays ($p > 0.05$).

Only the highest concentration of Ang II added to saline or saline with EDTA could be detected by the Ang-(1–7) radioimmunoassay confirming a very low cross-reactivity for this assay ($<0.05\%$, Fig. 3).

Measurement of ACE2 activity in the components of culture media demonstrated that only FCS and media containing 10% FCS had significant activity. Importantly, BSA (0.1% w/v) in saline or with M199 did not show any ACE2 activity (Fig. 4).

ACE2 activity was measured in separate batches of FCS with different countries of origin. Australian FCS without heat treatment showed an ACE2 activity of $2,516 \pm 252$ pmol min⁻¹ mL⁻¹ (Fig. 5). Heat treatment to 56 °C for 30 min resulted in an average reduction in ACE2 activity of $27\% \pm 4.2$ whereas heat treatment to 70 °C completely abolished ACE2 activity for all samples tested.

In contrast to FCS adult bovine serum (from New Zealand) had much lower ACE2 activity compared to untreated FCS from all countries of origin tested ($p < 0.01$).

The average molar conversion of Ang II to Ang-(1–7) was $35\% \pm 9.8$ implying that Ang-(1–7)

Fig. 2 Ang II radioimmunoassay. Ang II was detected in saline by radioimmunoassay at expected concentrations. However, there was a dramatic reduction in detectable Ang II when Ang II was added to culture medium. This effect was reversed by the addition of EDTA (5 mmol/L)

Fig. 3 Ang-(1–7) radioimmunoassay. Increasing doses of Ang II (1, 2, 10, 20 nmol/L) were added to saline and culture media with and without EDTA (5 mmol/L). Addition of Ang II to culture media resulted in substantial and dose-dependent generation of Ang-(1–7) which was abolished by the addition of EDTA (5 mmol/L)

Fig. 4 ACE2 activity of culture media components. ACE2 activity was only detected in undiluted fetal calf serum (FCS) and medium containing 10% FCS. Bovine serum albumin (BSA) had no detectable ACE2 activity

produced by ACE2 degradation of Ang II was further metabolised. It is known that Ang-(1–7) is enzymatically cleaved by ACE to produce the inactive peptide Ang-(1–5; Fig. 1). To ascertain whether FCS contained ACE activity, an ACE assay was performed on

FCS (Austin Health Biochemistry department, Melbourne, Australia). Figure 6 shows that untreated FCS (catalogue No: 10099-141, source Australia) has an ACE concentration of 197 ± 3.0 U/L compared to 56 °C heat treated FCS which had an ACE

Fig. 5 ACE2 activity of FCS with and without heat treatment. ACE2 activity was measured in untreated fetal calf serum (FCS) and FCS heat treated for 30 min at 56 and 70 °C in batches of FCS from Australia, United States of America (USA) and Mexico. Heat treatment at 56 °C was unable to

completely abolish ACE2 activity with an average reduction in ACE2 activity of 27% compared to untreated FCS. On the other hand, heating FCS to 70 °C completely abolished ACE2 activity

Fig. 6 ACE activity of FCS. ACE activity was measured in untreated Australian FCS and Australian FCS heat treated for 30 min at 56 and 70 °C. Heat treatment to 56 and 70 °C resulted in a reduction in ACE concentration by 37 and 86%, respectively ($p < 0.001$)

concentration of 123.5 ± 0.5 U/L representing a 37% reduction in ACE concentration ($p < 0.001$). Heat treatment to 70 °C resulted in a 86% reduction in ACE concentration of untreated FCS ($p < 0.001$).

Discussion

This study has demonstrated for the first time that FCS has ACE2 activity and that Ang II is readily

degraded into Ang-(1–7) in culture medium containing FCS (10% v/v). Ang II added to saline and EDTA failed to generate any appreciable quantity of Ang-(1–7). However, when Ang II was added to media containing FCS there was a simultaneous reduction in Ang II and increase in detectable Ang-(1–7). This suggests that media contains an enzyme capable of degrading Ang II into Ang-(1–7). ACE2 is a recently described enzyme of the RAS capable of generating Ang-(1–7) from Ang II. It is a metallo-proteinase which is chelated by EDTA. When EDTA was added to media containing FCS the Ang-(1–7) generating effect was inhibited and the Ang II concentrations were not reduced.

Furthermore ACE2 activity was measured in samples of undiluted FCS and found to be very high. This activity persisted despite heat inactivation to 56 °C. To give an idea of the magnitude of the ACE2 activity, serum from healthy human volunteers has a mean ACE2 activity of 4.44 ± 0.56 pmol min⁻¹ mL⁻¹ (Lew et al. 2008), whereas the undiluted FCS from Australia had an ACE2 activity of $2,516.1 \pm 252$ pmol min⁻¹ mL⁻¹ which represents a 567-fold higher ACE2 activity in FCS compared to human serum. Serum from adult cattle showed significantly reduced ACE2 activity demonstrating that the high activity levels of ACE2 is a phenomenon predominantly of FCS. Furthermore, the lack of ACE2 activity

within BSA supports the use of BSA as an alternative constituent to FCS before collection of supernatants intended for RAS peptide analysis.

Heat inactivated FCS is commercially available and is produced by heating at 56 °C for 30 min. ACE2 activity of FCS persisted despite heat treatment to 56 °C with an average activity of 73% of untreated FCS for all samples measured. In contrast, heat treatment for 30 min at 70 °C completely abolished ACE2 activity in FCS. It is noteworthy that these findings are in keeping with the knowledge that the N-domain of somatic ACE, which is denatured at 70.5 °C as compared to its C-domain with melting temperature of 55.3 °C (Voronov et al. 2002) more closely resembles ACE2 with ~60% sequence similarity (Tipnis et al. 2000). Although heat treatment to 70 °C eliminated ACE2 activity it is unknown whether FCS treated in this way effects cell culture. Further work is required in a variety of cell types to determine if heat treatment to 70 °C is a viable option when studying RAS peptides in cell culture.

FCS was also shown to possess ACE activity that was only partially inhibited by heat treatment to 56 °C. In order to give an indication of the magnitude of the ACE activity in untreated FCS, healthy humans have serum ACE activity of 31.23 ± 4.08 U/L ($n = 13$). In diseased states such as cirrhosis ACE increases to 129.4 ± 29.14 U/L ($n = 9$, unpublished data). Therefore, untreated Australian FCS had a sixfold higher ACE activity compared to serum from healthy humans. This data demonstrated that FCS contains ACE in addition to ACE2 and that the relative proportions of Ang II and Ang-(1–7) will be determined by the ratio of these two enzymes.

When designing cell culture experiments researchers should be aware that FCS has ACE and ACE2 activity, and that this may significantly affect the levels of RAS peptides to which cells are exposed. Ang II and Ang-(1–7) have been shown to have a number of opposing effects on cell physiology and growth. Thus the outcome of cell culture work involving Ang II treatment may well be confounded by the formation of biologically active Ang-(1–7) by ACE2 present in FCS and this may affect the reproducibility and interpretation of cell culture experiments. Although heat-treated FCS (56 °C for 30 min) is commercially available, ACE2 is not completely inactivated by this treatment and further

heat treatment of FCS at 70 °C is required for complete inhibition of ACE2 activity.

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References

- Bataller R, Gines P, Nicolas JM et al (2000) Angiotensin II induces contraction and proliferation of human hepatic stellate cells. *Gastroenterology* 118:1149–1156. doi: [10.1016/S0016-5085\(00\)70368-4](https://doi.org/10.1016/S0016-5085(00)70368-4)
- Bataller R, Gabele E, Schoonhoven R et al (2003a) Prolonged infusion of angiotensin II into normal rats induces stellate cell activation and proinflammatory events in liver. *Am J Physiol Gastrointest Liver Physiol* 285:G642–G651
- Bataller R, Sancho-Bru P, Gines P et al (2003b) Activated human hepatic stellate cells express the renin-angiotensin system and synthesize angiotensin II. *Gastroenterology* 125:117–125. doi: [10.1016/S0016-5085\(03\)00695-4](https://doi.org/10.1016/S0016-5085(03)00695-4)
- Bataller R, Gabele E, Parsons CJ et al (2005) Systemic infusion of angiotensin II exacerbates liver fibrosis in bile duct-ligated rats. *Hepatology* 41:1046–1055. doi: [10.1002/hep.20665](https://doi.org/10.1002/hep.20665)
- Benter IF, Ferrario CM, Morris M et al (1995) Antihypertensive actions of angiotensin-(1–7) in spontaneously hypertensive rats. *Am J Physiol* 269:H313–H319
- Burchill L, Velkoska E, Dean RG et al (2008) Acute kidney injury in the rat causes cardiac remodelling and increases angiotensin-converting enzyme 2 expression. *Exp Physiol* 93:622–630. doi: [10.1113/expphysiol.2007.040386](https://doi.org/10.1113/expphysiol.2007.040386)
- Burrell LM, Johnston CI, Tikellis C et al (2004) ACE2, a new regulator of the renin-angiotensin system. *Trends Endocrinol Metab* 15:166–169. doi: [10.1016/j.tem.2004.03.001](https://doi.org/10.1016/j.tem.2004.03.001)
- Ferrario CM, Trask AJ, Jessup JA (2005) Advances in biochemical and functional roles of angiotensin-converting enzyme 2 and angiotensin-(1–7) in regulation of cardiovascular function. *Am J Physiol Heart Circ Physiol* 289:H2281–H2290. doi: [10.1152/ajpheart.00618.2005](https://doi.org/10.1152/ajpheart.00618.2005)
- Ferreira AJ, Santos RA, Almeida AP (2001) Angiotensin-(1–7): cardioprotective effect in myocardial ischemia/reperfusion. *Hypertension* 38:665–668
- Ferreira AJ, Santos RA, Almeida AP (2002) Angiotensin-(1–7) improves the post-ischemic function in isolated perfused rat hearts. *Braz J Med Biol Res* 35:1083–1090. doi: [10.1590/S0100-879X2002000900009](https://doi.org/10.1590/S0100-879X2002000900009)
- Freeman EJ, Chisolm GM, Ferrario CM et al (1996) Angiotensin-(1–7) inhibits vascular smooth muscle cell growth. *Hypertension* 28:104–108
- Greenwood FC, Hunter WM, Glover JS (1963) The preparation of I-131-labelled human growth hormone of high specific radioactivity. *Biochem J* 89:114–123
- Grobe JL, Mecca AP, Mao H et al (2006) Chronic angiotensin-(1–7) prevents cardiac fibrosis in DOCA-salt model of hypertension. *Am J Physiol Heart Circ Physiol* 290:H2417–H2423. doi: [10.1152/ajpheart.01170.2005](https://doi.org/10.1152/ajpheart.01170.2005)

- Herath CB, Warner FJ, Lubel JS et al (2007) Upregulation of hepatic angiotensin-converting enzyme 2 (ACE2) and angiotensin-(1–7) levels in experimental biliary fibrosis. *J Hepatol* 47:387–395. doi:[10.1016/j.jhep.2007.03.008](https://doi.org/10.1016/j.jhep.2007.03.008)
- Iwata M, Cowling RT, Gurantz D et al (2005) Angiotensin-(1–7) binds to specific receptors on cardiac fibroblasts to initiate antifibrotic and antitrophic effects. *Am J Physiol Heart Circ Physiol* 289:H2356–H2363. doi:[10.1152/ajpheart.00317.2005](https://doi.org/10.1152/ajpheart.00317.2005)
- Kucharewicz I, Chabielska E, Pawlak D et al (2000) The antithrombotic effect of angiotensin-(1–7) closely resembles that of losartan. *J Renin Angiotensin Aldosterone Syst* 1:268–272. doi:[10.3317/jraas.2000.041](https://doi.org/10.3317/jraas.2000.041)
- Kucharewicz I, Pawlak R, Matys T et al (2002) Antithrombotic effect of captopril and losartan is mediated by angiotensin-(1–7). *Hypertension* 40:774–779. doi:[10.1161/01.HYP.0000035396.27909.40](https://doi.org/10.1161/01.HYP.0000035396.27909.40)
- Lambert DW, Hooper NM, Turner AJ (2008) Angiotensin-converting enzyme 2 and new insights into the renin-angiotensin system. *Biochem Pharmacol* 75:781–786. doi:[10.1016/j.bcp.2007.08.012](https://doi.org/10.1016/j.bcp.2007.08.012)
- Lew RA, Warner FJ, Hanchapola I et al (2008) Angiotensin-converting enzyme 2 catalytic activity in human plasma is masked by an endogenous inhibitor. *Exp Physiol* 93:685–693. doi:[10.1113/expphysiol.2007.040352](https://doi.org/10.1113/expphysiol.2007.040352)
- Santos RA, Castro CH, Gava E et al (2006) Impairment of in vitro and in vivo heart function in angiotensin-(1–7) receptor MAS knockout mice. *Hypertension* 47:996–1002. doi:[10.1161/01.HYP.0000215289.51180.5c](https://doi.org/10.1161/01.HYP.0000215289.51180.5c)
- Strawn WB, Ferrario CM, Tallant EA (1999) Angiotensin-(1–7) reduces smooth muscle growth after vascular injury. *Hypertension* 33:207–211
- Tallant EA, Ferrario CM, Gallagher PE (2005) Angiotensin-(1–7) inhibits growth of cardiac myocytes through activation of the mas receptor. *Am J Physiol Heart Circ Physiol* 289:H1560–H1566. doi:[10.1152/ajpheart.00941.2004](https://doi.org/10.1152/ajpheart.00941.2004)
- Tipnis SR, Hooper NM, Hyde R et al (2000) A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem* 275:33238–33243. doi:[10.1074/jbc.M002615200](https://doi.org/10.1074/jbc.M002615200)
- Voronov S, Zueva N, Orlov V et al (2002) Temperature-induced selective death of the C-domain within angiotensin-converting enzyme molecule. *FEBS Lett* 522:77–82. doi:[10.1016/S0014-5793\(02\)02888-0](https://doi.org/10.1016/S0014-5793(02)02888-0)
- Warner FJ, Lubel JS, McCaughan GW et al (2007) Liver fibrosis: a balance of ACEs? *Clin Sci (Lond)* 113:109–118. doi:[10.1042/CS20070026](https://doi.org/10.1042/CS20070026)
- Wei H, Lu H, Li D et al (2001) The expression of AT1 receptor on hepatic stellate cells in rat fibrosis induced by CCl4. *Chin Med J (Engl)* 114:583–587
- Yokohama S, Tokusashi Y, Nakamura K et al (2006) Inhibitory effect of angiotensin II receptor antagonist on hepatic stellate cell activation in non-alcoholic steatohepatitis. *World J Gastroenterol* 12:322–326
- Zhang Y, Yang X, Wu P et al (2003) Expression of angiotensin II type 1 receptor in rat hepatic stellate cells and its effects on cell growth and collagen production. *Horm Res* 60:105–110. doi:[10.1159/000072520](https://doi.org/10.1159/000072520)