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Lentivirus-mediated overexpression of angiotensin-(1–7) attenuated ischaemia-induced cardiac pathophysiology

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Abstract

Myocardial infarction (MI) results in cell death, development of interstitial fibrosis, ventricular wall thinning and ultimately, heart failure. Angiotensin-(1–7) [Ang-(1–7)] has been shown to provide cardioprotective effects. We hypothesize that lentivirus-mediated overexpression of Ang-(1–7) would protect the myocardium from ischaemic injury. A single bolus of 3.5×10^8 transducing units of lenti-Ang-(1–7) was injected into the left ventricle of 5-day-old male Sprague–Dawley rats. At 6 weeks of age, MI was induced by ligation of the left anterior descending coronary artery. Four weeks after the MI, echocardiography and haemodynamic parameters were measured to assess cardiac function. Postmyocardial infarction, rats showed significant decreases in fractional shortening and $\mathrm{d}P/\mathrm{d}t$ (rate of rise of left ventricular pressure), increases in left ventricular end-diastolic pressure, and ventricular hypertrophy. Also, considerable upregulation of cardiac angiotensin-converting enzyme (*ACE*) mRNA was observed in these rats. Lentivirus-mediated cardiac overexpression of Ang-(1–7) not only prevented all these MI-induced impairments but also resulted in decreased myocardial wall thinning and an increased cardiac gene expression of *ACE2* and bradykinin B2 receptor (*BKR2*). Furthermore, *in vitro* experiments using rat neonatal cardiac myocytes demonstrated protective effects of Ang-(1–7) against hypoxia-induced cell death. This beneficial effect was associated with decreased expression of inflammatory cytokines (tumour necrosis factor-α and interleukin-6) and increased gene expression of *ACE2*, *BKR2* and interleukin-10. Our findings indicate that overexpression of Ang-(1–7) improves cardiac function and attenuates left ventricular remodelling post-MI. The protective effects of Ang-(1–7) appear to be mediated, at least in part, through modulation of the cardiac renin–angiotensin system and cytokine production.

It is a well-established fact that components of the renin–angiotensin system (RAS) play a critical role in the progression of heart failure. Pharmacotherapeutic interventions with either angiotensin-converting enzyme (ACE) inhibitors (Haywood *et al.* 1997) or angiotensin type 1 receptor blockers (Steckelings *et al.* 2005; Ferreira *et al.* 2009) have demonstrated significant protection against myocardial infarction and heart failure in experimental animal models as well as in patients. Several studies have suggested that the beneficial effects

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observed with angiotensin-converting enzyme inhibitors are not only due to the reduction in the formation of the detrimental angiotensin II (Ang II), but are also due to significant elevation in the levels of angiotensin-(1–7) (Ang-(1–7); Keidar *et al.* 2007). Correlative studies have also shown that ACE2 and Ang-(1–7) levels are increased in the heart following myocardial infarction in both humans and rats (Averill *et al.* 2003; Burrell *et al.* 2005).

A growing body of evidence indicates that Ang-(1–7) plays an active role in maintaining cardiovascular homeostasis, thus providing protection against heart diseases. This conclusion is supported by the following findings. Angiotensin-(1–7) has been shown to attenuate isoprenaline-induced cardiac hypertrophy (Santos *et al.* 2004). Chronic administration of Ang-(1–7) has been shown to improve coronary artery perfusion and endothelial function in a rat model for heart failure (Loot *et al.* 2002). Angiotensin-(1–7) was shown to reduce the incidence and the duration of postischaemic reperfusion arrhythmias in the isolated rat heart (Ferreira *et al.* 2001; De Mello, 2004). Intravenous infusion of Ang-(1–7) resulted in improved contractile function in rat hearts (Sampaio *et al.* 2003). Angiotensin-(1–7) has been demonstrated to attenuate cardiac remodelling significantly in terms of reducing myocyte hypertrophy and interstitial fibrosis (Santos *et al.* 2004; Tallant *et al.* 2005; Grobe *et al.* 2006, 2007*b*; Mercure *et al.* 2008; Ferreira *et al.* 2010; Giani *et al.* 2010). Furthermore, AVE0991, a synthetic mimetic of Ang-(1–7), has been shown to exert cardioprotective effects (Ferreira *et al.* 2007).

Although Ang-(1–7) exerts beneficial effects against a range of cardiovascular diseases, the *in vivo* efficacy of this peptide is severely hampered owing to rapid degradation by peptidases. The half-life for Ang-(1–7) is very short and dependent on species. For example, following intravenous administration the half-life of Ang-(1–7) in humans is approximately 30 min (Kono *et al.* 1986; Rodgers *et al.* 2006), whereas in rodents it is approximately 20 s (Iusuf *et al.* 2008). To overcome this limitation, we have overexpressed Ang-(1–7) in the rodent heart shortly after birth, as we previously demonstrated (Methot *et al.* 2001; Diez-Freire *et al.* 2006), to evaluate its cardioprotective effects against myocardial infarction. Also, we hypothesize that Ang-(1–7) may exert its beneficial effects against myocardial infarction by restoring the balance between the deleterious ACE–Ang II–angiotensin II type 1 receptor (AT1R) axis and the beneficial ACE2–Ang-(1–7)–Mas axis of the RAS.

Methods

Animals

Male Sprague–Dawley rats were used for the studies. All animals were conventionally housed in standard cages and maintained on a 12 h–12 h light–dark cycle with free access to standard rat chow and water. All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC) and conducted according to National Institutes of Health guidelines.

Characterization of lenti-Ang-(1–7) vector

Lentiviral vectors were created as previously described (Coleman *et al.* 2003). A lentiviral vector construct designed by Dr Tim Reudelhuber (Clinical Research Institute of Montreal, Canada) was used to produce the Ang-(1–7) (Methot *et al.* 2001). Lenti-Ang-(1–7) was driven by the human elongation factor promoter (EF1α). The plasmid for the Ang-(1–7) was provided by Dr Tim Reudelhuber, and contained a human prorenin signal peptide and the immunoglobulin fragment from the mouse IgG2b linked to a portion of the human prorenin prosegment. The human prorenin prosegment directs the translational complex (ribosomes, etc.) to bind to the endoplasmic reticulum and produce the protein within the lumen of the

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endoplasmic reticulum instead of within the cytosol of the cell. Thus, the presence of this sequence at the start of the protein results in placement of the whole protein into the secretory apparatus of the cell. The *Bgl*II site, located after the prorenin segment, was used to insert a furin cleavage site and the coding sequence for Ang-(1–7) followed by a stop codon. Thus, the transgene encodes a fusion protein capable of releasing the Ang-(1–7) peptide by taking advantage of the constitutive presence of the furin enzyme, which ultimately releases the peptide from the fusion protein, as previously described (Methot *et al.* 2001; Ferreira *et al.* 2010). Both IgG2b and Ang-(1–7) can be detected intracellularly as well, because there is always residual protein inside the endoplasmic reticulum/Golgi that has been synthesized and not yet secreted. The lenti-Ang-(1–7) vectors were titred using the HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (Beckman Coulter, Brea, CA, USA) following the manufacturer's instructions.

Measurements of transduction efficacy of lenti-Ang-(1–7)

Rat neonatal cardiac myocytes (RNCMs) were isolated according to the previously reported method (Qi *et al.* 2010). The RNCMs were used to determine the transduction efficacy of lenti-Ang-(1–7). The RNCMs were plated at 80% confluence in 24-well culture plates; they were then transduced with lenti-Ang-(1–7) at a concentration of 10 multiplicities of infection (MOI) in the presence of 8 μg ml^{-1} polybrene (Sigma-Aldrich, St Louis, MO, USA). After 6 h of viral transduction, the viral media was replaced with fresh growth medium. After 4 days, the secreted levels of Ang-(1–7) and murine IgG2b isotype were measured using a commercially available ELISA kit. The efficacy of the lentivirus in producing Ang-(1–7) was directly assessed by measuring the secreted levels of Ang-(1–7) in the media using an ELISA kit (Bachem, San Carlos, CA, USA) and indirectly tested by measuring the secreted levels of murine IgG2b isotype in the cell culture (Assaydesigns Plymouth Meeting, PA, USA). For the *in vivo* experiments, 10 weeks after intracardiac administration of the lenti-Ang-(1–7), lenti-Ang-(1–7) transduction in rat myocardium was examined by testing the expression of lentiviral vector. SYBR green real-time RT-PCR was used to quantify the lentiviral vector expression. To determine whether the transgene construct was integrated *in vivo*, the forward primer of the construct (5′-CATCACCCATCGAGAAACC-3′) was located in the IgG fragment and the reverse primer (5′-GGACCAAGCCTGGCCATGTCC-3′) was located in the human prorenin fragment of the construct. The glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) primers were as follows: forward primer, 5′-GCCAGCCTCGTCTCATAGACA-3′; and reverse primer, 3′-GTCCGATACGGCCAAATCC-5′. The SYBR green real-time PCR assays for each target gene were performed on cDNA samples.

Administration of lenti-Ang-(1–7)

Five-day-old male Sprague–Dawley rats received a single intraventricular injection of 3.5 × 10^8 transducing units of lenti-Ang-(1–7) in 30 μl 1× PBS, as described previously (Der Sarkissian *et al.* 2008). This method of gene transfer by our lentiviral vector has been established to produce efficient and long-term transduction of the heart (Der Sarkissian *et al.* 2008; Qi *et al.* 2010) with no adverse effects and a 100% survival rate. After viral administration, animals were returned to their mothers until weaning.

Myocardial infarction

At 6 weeks of age, rats were separated into the following four experimental groups: (a) control sham; (b) myocardial infarction (MI); (c) lenti-Ang-(1–7); and (d) MI + lenti-Ang-(1–7) (n = 4–7 animals per group). Myocardial infarction was induced by ligation of the left anterior descending coronary artery. At the time of operation, the rats were anaesthetized with isoflurane (2.0–2.5% in oxygen), after which the rats were intubated with an 18-gauge intravenous catheter and mechanically ventilated with the isoflurane–oxygen mixture using

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a Harvard ventilator (model 683; Harvard Apparatus, Holliston, MA, USA). After the chest area had been shaved and cleaned, rats underwent a left thoracotomy. The thorax was entered via the left fourth and fifth intercostal space and the pericardium incised to expose the heart. The exposed heart was ligated at the proximal left anterior descending coronary artery, 2–3 mm from its origin between the pulmonary artery conus and the left atrium, using a Ethicon 7–0 (0.5 metric) polypropylene suture (Suturedirect, Mettawa, IL, USA). Successful cessation of coronary blood flow was confirmed by elevation of the ST segment on ECG and cyanosis of the anterior LV wall; if necessary, the procedure was repeated by placement of a second or third ligature. The heart was returned to its normal position, and the thorax was sutured after evacuation of fluid and air. All the animals received buprenorphine hydrochloride (Buprenex; 0.02 mg kg^{-1} every 12 h I.M.; Reckitt and Colman Pharmaceuticals, Richmond, VA, USA) and were closely monitored for signs of discomfort. Sham-operated rats underwent identical surgical procedures to those described above except that the suture was not tightened around the coronary artery. In the present study, the operation-related mortality was approximately 25% at 24 h after the ligation procedure. Figure 1*A* summarizes the experimental protocol.

Echocardiography

Four weeks after coronary artery ligation surgery, cardiac function was evaluated using a Hewlett Packard Sonos Model 5500 with a 12 Hz transducer (Agilent Technologies, Andover, MA, USA). Rats were anaesthetized with 2% isoflurane in oxygen using a vaporizer during the assessment. Images were obtained from the parasternal short axis. M-mode echocardiography was performed by using a parasternal short-axis view at the level of the papillary muscles. All measurements were based on the average of three consecutive cardiac cycles. Left ventricular (LV) internal diameter at end diastole (LVIDd) and end systole (LVIDs) was obtained. The fractional shortening (FS) was calculated according to the following formula:

$$\mathrm{FS}=[(\mathrm{LVIDd}-\mathrm{LVIDs})/\mathrm{LVIDd}]\times 100.$$

Haemodynamic measurements

Rats were anaesthetized with a ketamine–xylazine–acepromazine rodent cocktail (100, 20 and 10 mg kg^{-1}, respectively, I.M.; JA Webster, Jacksonville, FL, USA). The rats were placed in supine position, and the body temperature was maintained at 37°C using a heated pad. Left ventricular function was measured by inserting a 22-gauge needle filled with heparin saline (20 i.u. ml^{-1}) into the left ventricular chamber. The data were recorded after stabilization of the trace using a liquid pressure transducer, which was interfaced to a PowerLab signal transduction unit (ADInstruments, Colorado Springs, CO, USA). Data were analysed using the Chart program supplied along with the PowerLab system. The parameters measured included heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and maximal positive and negative rate of rise of the left ventricular pressure ($\mathrm{d}P/\mathrm{d}t_{max}$ and $\mathrm{d}P/\mathrm{d}t_{min}$).

Histological analysis

Following the haemodynamic measurement, the hearts were harvested. The ventricles were separated from atria and rinsed in PBS, weighed, and cut into three different sections made perpendicular to the long axis. The basal and apex sections were snap frozen in liquid nitrogen and stored at −80°C for subsequent quantitative real-time RT-PCR measurements. The middle section was used to measure the infarct size and cardiac remodelling. Cardiac remodelling was assessed by determining ventricular hypertrophy, which was calculated by normalizing the wet weights of rat heart ventricles to that of the body mass. Cross-sections

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of the ventricles were then fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 4 μm. Ventricular sections were either stained with Haematoxylin and Eosin to determine myocyte diameter or with Picro-Sirius Red to measure left ventricular wall thickness. Myocyte diameter was observed at ×40 magnification and quantified using the ImageJ program from National Institutes of Health as previously described (Grobe *et al.* 2006). Twenty separate images from different (non-overlapping) regions of the left ventricular free wall alone were examined. Left ventricular wall thickness was also measured using the ImageJ program and represented as a percentage of control LV wall thickness.

Hypoxia–reoxygenation model and cell viability assay

Culture medium to grow RNCMs was changed every 24 h. After 2 days of culture, Ang-(1–7) (final concentration 1 μM) and/or A779 (final concentration 1 μM) were added into the medium 1 h before exposing the cells to hypoxic or normoxic control media. Cells were exposed to hypoxia for 4 h using a Hypoxia glove box (Banthrax Corp., Springboro, OH, USA). A hypoxic mixture of gases (1% oxygen, 5% CO_2 and 94% nitrogen) was flushed through the system for 2 h prior to treatment, followed by a steady flow of the hypoxic gas mixture for maintenance. The temperature of the glove box was sustained at 37°C and monitored using a thermometer placed inside the glove box. Cells plated in 96-well dishes were inserted into the chamber via an air-lock port flushed with the hypoxic gas mixture. Cells were removed from the glove box through the port following the hypoxic treatment. One hour after exposure to hypoxia, cell viability was tested using CellQuanti-BlueTM Cell Viability Assay Kits (BioChain, Hayward, CA, USA). Twenty-four hours after exposure to hypoxia, cell lysate was collected to isolate RNA.

RNA isolation and PCR

Tissues or cells were homogenized and total RNA was isolated using RNAqueous 4 PCR kit (Ambion, Foster City, CA, USA) according to the manufacturer's instructions. Two hundred nanograms of RNA was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA).

The *AT1R*, *AT2R*, collagen I, collagen III, transforming growth factor-β (*TGF-β*), tumour necrosis factor-α (*TNF-α*), *ACE2*, *ACE*, interleukin-1 (*IL-1*), interleukin-1 (*IL-10*) and interleukin-6 (*IL-6*) were analysed by quantitative real-time PCR using Taqman probe (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR was run using the ABI Prism 7000 sequence detection system. All cDNA samples were assayed in triplicate. Data were normalized to *GAPDH*.

Statistical analysis

Results are expressed as means ± SEM. Data were analysed by one-way ANOVA with Bonferroni correction for multiple comparisons. Values of $P < 0.05$ were considered statistically significant. All of the data were analysed using GraphPad Prism 5 software (Graphpad Prism Institute Inc., La Jolla, CA, USA).

Results

Efficacy of the lentiviral vector to overexpress Ang-(1–7)

In previous experiments, we have established that intracardiac administration of lentiviral vector results in predominant long-term transduction in the heart (Der Sarkissian *et al.* 2008) and systemic administration of lentiviral vector expressing Enhanced Green Fluorescence Protein (EGFP) has no physiological consequence (Shenoy *et al.* 2010). In the present study, the transduction efficacy of lenti-Ang-(1–7) was tested *in vitro* and *in vivo*. For the *in vitro*

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study, 4 days after transduction of rat neonatal cardiac myocytes with 10 MOI of lenti-Ang-(1–7), robust expression of Ang-(1–7) (0.25 ng ml^{-1}; Fig. 1*C*) and IgG2b isotype (12.5 ng ml^{-1}; data not shown) were detected in the culture media. Furthermore, for the *in vivo* studies, integration of lentiviral vector in the rat hearts was determined by real-time RT-PCR for all groups at the conclusion of the study. The lentiviral vector construct was detected only in lenti-Ang-(1–7) and MI + lenti-Ang-(1–7) groups, as shown in Fig. 1*B*. These data demonstrate that lenti-Ang-(1–7) was present in the heart and effective in secreting Ang-(1–7) throughout the 10 weeks of the study.

Effects of Ang-(1–7) overexpression on cardiac function post-MI

Echocardiographic analyses were performed 4 weeks postmyocardial infarction. Myocardial infarction caused a significant reduction in fractional shortening in the MI group compared with the control group. Lenti-Ang-(1–7) administration attenuated this decrease in fractional shortening induced by myocardial infarction (Fig. 2). Left ventricular systolic pressure, dP/dt_{max} and dP/dt_{min} were also significantly decreased in the MI group compared with the control group (Table 1). In addition, significant increases in left ventricular end-diastolic pressure and heart rate were observed in the MI group compared with the control group. Lentiviral vector-mediated Ang-(1–7) treatment was able to restore all of these cardiac parameters to control values. Lenti-Ang-(1–7) alone did not change any of these variables in healthy hearts (Table 1).

Effects of Ang-(1–7) overexpression on ventricular remodelling post-MI

Lentiviral vector-mediated Ang-(1–7) overexpression attenuated MI-induced cardiac hypertrophy as evaluated by both the ventricular weight to body weight ratio and measurement of cardiomyocyte diameter (Fig. 3). No significant change in the body weights were observed among the different study groups (Fig. 3*C*). Wall thinning was observed in the MI group. Angiotensin-(1–7) overexpression significantly prevented the LV wall thinning and tended to reduce the infarction area, as shown in Fig. 3*D*.

Angiotensin-(1–7)-mediated protection post-MI is associated with restoration of the balance between the ACE–Ang II–AT1R axis and the ACE2–Ang-(1–7)–Mas axis

In an effort to determine possible cardioprotective mechanisms mediated by lenti-Ang-(1–7), a portion of the left ventricular tissue from the non-infarcted area was harvested to quantify the cardiac RAS genes. As shown in Table 2, mRNA levels of *ACE* were significantly elevated in the MI group compared with the control group. Overexpression of Ang-(1–7) completely prevented this increase in cardiac *ACE* gene expression. In contrast, cardiac *ACE2* and *Mas* receptor levels tended to decrease in the MI group compared with the control group and the animals treated with lenti-Ang(1–7) alone. Overexpression of Ang-(1–7) led to a significant increase in cardiac *ACE2* and near normalization of the *Mas* receptor levels following MI. When data were expressed as a ratio of *ACE2*/*ACE*, the MI group was significantly lower (0.007 ± 0.002) than the control (0.482 ± 0.017), lenti-Ang-(1–7) alone (0.568 ± 0.042) and MI + lenti-Ang-(1–7) groups (0.723 ± 0.138). The *Mas*/*AT1R* ratios displayed a similar pattern, but the MI group failed to reach significance (P value = 0.08). Lenti-Ang-(1–7) administration also increased the mRNA levels of the bradykinin receptor B2 (*BKR*) in the MI + lenti-Ang-(1–7) group. The mRNA levels of *IL-6*, *IL-10* and *AT2R* were undetectable (data not shown).

Angiotensin-(1–7) increases the viability of RNCMs after exposure to hypoxia

The CellQuanti-Blue™ reagent utilizes the redox dye resazurin. Viable cells can convert this redox dye into a highly fluorescent product (resorufin) and cause an increase in fluorescence intensity, which is absent in non-viable cells. Therefore, the fluorescence

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intensity observed in this assay is a true measure of the viable cells. The RNCMs were exposed to hypoxia for 4 h and cultured in normoxic conditions for another 1 h, after which time the fluorescence was measured. Fluorescence intensity was significantly decreased following exposure to hypoxia. Pretreatment with Ang-(1–7) prevented this decrease, suggesting that Ang-(1–7) provides protection to RNCMs from hypoxia-induced cell death (Fig. 4). A Mas receptor antagonist, A779, was also used to study whether it could block the protective effects of Ang-(1–7); however, this treatment did not significantly attenuate the beneficial effects of Ang-(1–7). Angiotensin-(1–7) was also overexpressed in RNCMs using 10 MOI of lenti-Ang-(1–7). Hypoxia reduced the cell viability to 50% of the control normoxic group, which was attenuated by overexpression of Ang-(1–7) (cell viability was restored to 75% of the normoxic control RNCMs; data not shown). Gene expression was also analysed 24 h after the exposure to hypoxia. Angiotensin-(1–7) treatment in the RNCMs exposed to hypoxia resulted in a significant increase in *AT2R*, *ACE2* and *IL-10* expression levels (Table 3). The Ang-(1–7) treatment induced an increased in *IL-10* expression, which was also observed in the cells maintained in the normoxic environment. Inflammatory cytokines (*TNF-α* and *IL-6*) also were significantly increased in the hypoxic RNCMs, and these increases were prevented by Ang-(1–7) treatment. There was also a tendency for Ang-(1–7) to reduce the level of these cytokines in the normoxic conditions.

Discussion

To our knowledge, this is the first study to demonstrate that lentivirus-mediated overexpression of Ang-(1–7) in cardiac tissue provides protection against myocardial infarction. Angiotensin-(1–7) is not a gene product but an active metabolite of angiotensin II degradation. Here, we used a synthetic gene construct to secrete Ang-(1–7), packaged it into a lentivirus and overexpressed in the rat heart. Overexpression of Ang-(1–7) provided cardiac protection by preserving fractional shortening, attenuating the increase in left ventricular end-diastolic pressure, and preventing the dysfunctional changes in d*P*/d*t*. Also, Ang-(1–7) prevented MI-induced ventricular hypertrophy and LV wall thinning. These findings suggest that the beneficial effects of Ang-(1–7) are the result of direct actions on the cardiac tissue. Overexpression of cardiac Ang-(1–7) in control rats did not alter normal cardiac morphology and function, which has also been observed previously (Mercure *et al.* 2008).

The half-life for Ang-(1–7) is very short (seconds to minutes) and dependent on species and route of administration (Kono *et al.* 1986; Yamada *et al.* 1998; Rodgers *et al.* 2006; Iusuf *et al.* 2008). Considering the short half-life of Ang-(1–7), it is quite difficult to maintain a therapeutic level of Ang-(1–7) when the peptide is administrated into patients or rodents by intravenous infusion or subcutaneous injection. Without achieving a certain level of Ang-(1–7) in the plasma or tissues, Ang-(1–7) may not exert its cardioprotective effects. Therefore, a gene therapy approach, like the one described here, could be used to mediate long-term Ang-(1–7) production after a single administration of viral vector containing Ang-(1–7). Viral vectors can elicit efficient transduction and long-term expression. We have recently demonstrated that use of an recombinant adeno-associated virus, serotype 9 (rAAV9) serotype is not only cardiospecific, but preferentially targets the myocytes (Qi *et al.* 2010). Utilization of such a specific viral vector to target the heart may even enhance the effects observed in the present study. However, one of the limitations of the present study is that we did not evaluate whether there is any cell specificity with the lentiviral vector.

Left ventricular remodelling characterized by cardiomyocyte hypertrophy and interstitial fibrosis represents an independent risk factor for heart failure following MI. In the present study, MI was associated with the development of ventricular hypertrophy and LV wall thinning, which was significantly prevented by lentivirus-mediated overexpression of Ang-

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(1–7). The observation of an antihypertrophic effect of Ang-(1–7) is consistent with previous findings (Santos *et al.* 2004; Tallant *et al.* 2005; Grobe *et al.* 2006, 2007*b*; Mercure *et al.* 2008; Ferreira *et al.* 2010; Giani *et al.* 2010). In a recent study in mice, it was demonstrated that increased circulating, but not cardiac, levels of Ang-(1–7) reduced the cardiac hypertrophy induced by MI (Wang *et al.* 2010). The authors concluded that elevated levels of circulating Ang-(1–7) were more important than the cardiac tissue level in preventing the pathophysiological remodelling following an MI. Although our results suggest that elevated cardiac Ang-(1–7) does offer beneficial effects against MI, at this time the significance of circulating *versus* tissue Ang-(1–7) is yet to be completely elucidated. Further evaluation using different species and experimental models is required to clarify the role of circulating *versus* tissue-specific levels of Ang-(1–7) in rendering the observed cardioprotective effects. Blood and tissue levels of Ang-(1–7) were not determined in the present study. Although the major source of the viral vector producing Ang-(1–7) was in cardiac tissue, we do not know whether circulating levels of Ang-(1–7) were increased and, if so, whether this increase was responsible for the cardioprotective effect. However, it is clear from our data that the peptide, Ang-(1–7), whether in the blood or tissues, does indeed provide a significant degree of cardiac protection in this MI model.

It has been previously reported that in the event of heart failure, the activity of the ACE–Ang II–AT1R axis is increased, while that of the ACE2–Ang-(1–7)–Mas axis is decreased (Ferrario *et al.* 1997; Ferreira *et al.* 2007). We too observed a similar phenomenon in our present study. Cardiac *ACE* mRNA levels were significantly upregulated following MI, while the expression level of *AT1R* tended to be increased, although this was not statistically significant. In contrast, gene expression of *ACE2* tended to decrease in the rat heart post-MI. All these changes indicate a disrupted intracardiac RAS. However, cardiac overexpression of Ang-(1–7) significantly prevented these changes, thus restoring the balance between the deleterious ACE–Ang II–AT1R axis and the beneficial ACE2–Ang-(1–7)–Mas axis. Studies from our laboratory have demonstrated that overexpression of *ACE2* provided protection against ischaemia-induced left ventricular dysfunction (Der Sarkissian *et al.* 2008) and inhibited cardiac collagen production (Grobe *et al.* 2007*a*). Results from the present study extend the findings of those experiments and suggest that the protective effects observed with *ACE2* overexpression could possibly be mediated via generation of Ang-(1–7). We recognize that we were not able to measure the protein associated with the mRNAs studied, but we do believe that the results obtained support the hypothesis that the ACE2–Ang-(1–7)–Mas pathway of the RAS is cardioprotective. We have recently reported that lenti-Ang-(1–7) significantly reduced pulmonary fibrosis and right ventricular pressure in rats treated with blemomycin (Shenoy *et al.* 2010). In that study, protein levels of AT1R were determined. The increases in AT1R in the lungs of bleomycin-treated animals were significantly reduced with lenti-Ang-(1–7) treatment. Collectively, this study supports the premise that elevation of the ACE–Ang II–AT1R axis of the RAS is detrimental in cardiovascular disease states and that stimulation of the ACE2–Ang-(1–7)–Mas pathway is beneficial.

Myocardial infarction causes local hypoxia in the infarct zone, leading to death of cardiomyocytes. The *in vitro* hypoxia study mimics this hypoxic phase of myocardial infarction. Our *in vitro* experiments demonstrated that Ang-(1–7) treatment protected cardiac myocytes from hypoxia-induced cell death. However, the protective effect of Ang-(1–7) was not attenuated by A779. There are other receptor types that have been suggested to bind to Ang-(1–7), and this may be the case for this particular parameter (Santos *et al.* 2003; Gurzu *et al.* 2005; Silva *et al.* 2007). Alternatively, it is possible that the dose of A779 used in the present study was not high enough to completely block the Ang-(1–7) receptor. It was also demonstrated that Ang-(1–7)-induced vasodilatation in isolated aortic rings of Sprague–Dawley rats was abolished by the Ang-(1–7) antagonist D-Pro^{7}-Ang-(1–7), but not

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by A779 (Silva *et al.* 2007). The beneficial effect of Ang-(1–7) observed in the present study was associated with an upregulation of *ACE2*, *AT2R* and *IL-10* (anti-inflammatory cytokine) and downregulation of the proinflammatory cytokine genes (*IL-6* and *TNF-α*). Upregulation of the *ACE2* gene in the myocyte culture is consistent with our *in vivo* findings. The changes in components of the RAS were also exemplified in the observed alterations of the *ACE2*/*ACE* and *Mas*/*AT2R* ratios.

The AT2R also has been reported to exert cardioprotective effects (Oishi *et al.* 2003, 2006). Direct AT2R stimulation improves systolic and diastolic function postmyocardial infarction (Kaschina *et al.* 2008). In fact, a direct correlation has previously been demonstrated between AT2R expression and Ang-(1–7)-forming activity in failing human heart ventricles from patients with primary pulmonary hypertension (Zisman *et al.* 2003). Furthermore, AT2Rs have been reported to interact functionally with Ang-(1–7) through the Mas receptor (Castro *et al.* 2005; Gurzu *et al.* 2005). It appears from our *in vitro* data that Ang-(1–7) may mediate its protective actions, in part, via upregulation of the AT2R. Whether this same effect takes place *in vivo* is not clear from the results of the present study, because the levels of AT2R were undetectable, and the use of gene therapy in a neonatal cardiac tissue may complicate the interpretation of the results. However, possible interactions between ACE2, Ang-(1–7) and AT2R may not be ruled out.

Proinflammatory cytokines (e.g. TNF-*α* and IL-6), anti-inflammatory cytokine (e.g. IL-10) and profibrotic cytokines (e.g. TGF-*β*1) play a critical role in mediating homeostasis within the heart in response to cardiac injury. Interleukin-6 appears to play an important role in the pathophysiology of congestive heart failure patients, as plasma levels of IL-6 have been shown to be elevated in these patients (Tsutamoto *et al.* 1998). Also, mRNA and protein levels of TNF-*α* have also been reported to be elevated in animal models, as well as in patients with advanced heart failure (Testa *et al.* 1996; Torre-Amione *et al.* 1996). In fact, IL-6 and TNF-*α* are biomarkers for heart failure (Boffa *et al.* 2009). In contrast, a recent report has demonstrated that treatment with the anti-inflammatory cytokine IL-10 significantly improved LV function in rats with heart failure after experimental MI (Stumpf *et al.* 2008). Consequently, anti-inflammatory therapy is currently being investigated for the treatment of myocardial infarction. In line with these findings, our *in vitro* studies revealed that co-treatment with Ang-(1–7) prevented the hypoxia-induced elevation in mRNA levels of *IL-6* and *TNF-α*. Also, treatment with Ang-(1–7) elevated the levels of the anti-inflammatory cytokine, *IL-10*, in both normoxic and hypoxic conditions. Thus, it appears that the protective effect of Ang-(1–7) is mediated by modulating the levels of various pro- and anti-inflammatory cytokines. We observed similar effects on these cytokines with Ang-(1–7) in our recently published findings in models of lung diseases (Shenoy *et al.* 2010). Likewise, it also has been reported that Ang-(1–7) reduced proinflammatory genes in the diabetic spontaneously hypertensive rat (Al-Maghrebi *et al.* 2009). Although not statistically significant, the expressions of *AT1R*, *TGF-β*, collagen I and collagen III all tended to increase in the MI group, and Ang-(1–7) overexpression tended to reduce these increases. We have previously demonstrated that Ang-(1–7) reduces fibrosis in models of lung diseases (Shenoy *et al.* 2010), which may be mediated by reducing TGF-*β* and collagen production. Support for this possibility is the observed reduction in TGF-*β* and the collagen production reported in cardiac fibroblasts after exposure to hypoxia (Grobe *et al.* 2007*a*). It also appears from our *in vivo* data that Ang-(1–7) may mediate its protective actions, in part, via upregulation of BKR. It has previously been demonstrated that Ang-(1–7) could prevent the degradation of bradykinin and desensitization of the BKR (Wiemer *et al.* 2002), as well as stimulate bradykinin release (Heitsch *et al.* 2001).

In summary, our data demonstrate that lentivirus-mediated overexpression of Ang-(1–7) preserves cardiac function and attenuates cardiac remodelling postmyocardial infarction.

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Our findings suggest that the beneficial effects of Ang-(1–7) may be a result of a restoration of the RAS balance and a modulation of various pro- and anti-inflammatory cytokines. Our observations support the concept that targeting of the cardiac ACE2–Ang-(1–7)–Mas axis using a gene therapy approach could provide a novel therapeutic strategy for the treatment of myocardial infarction and its associated complications. These observations are supported by a recent article which demonstrated that myocardial inhibition of ACE2 activity was associated with a significant increase in MI size and a reduction in LV fractional shortening in a rodent model of MI induced by coronary artery ligation (Kim *et al.* 2010).

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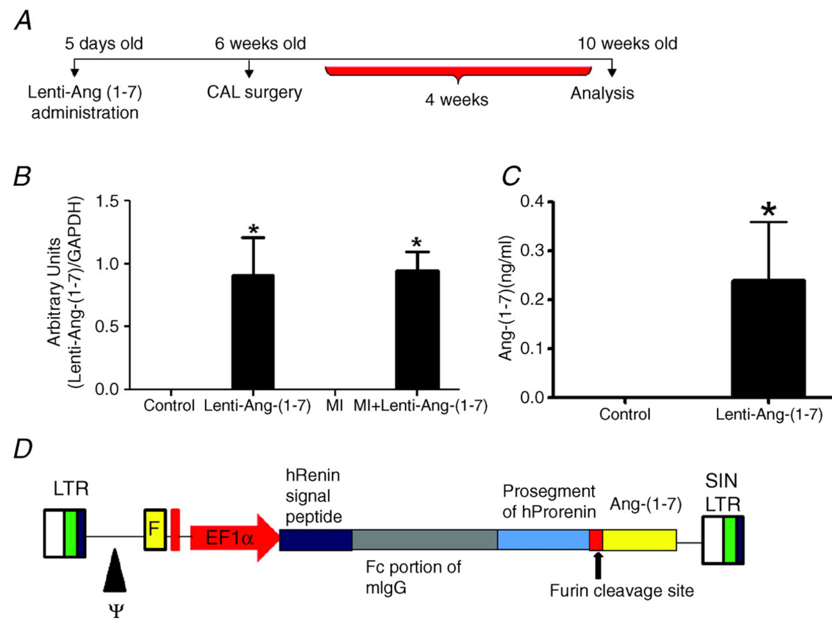

Figure 1. Schematic representation of study protocol

A, illustration of the experimental protocol for myocardial infarction. Lenti-Ang-(1–7) was administered into the left ventricular chamber of 5-day-old Sprague–Dawley pups. Myocardial infarction (MI) was induced by coronary artery ligation (CAL) when the rats were 6 weeks old. Four weeks after the surgery, animals were subjected to echocardiography followed by haemodynamic heart function assessment and harvesting of tissues; $n = 4–7$ per group. *B*, transduction efficiency of lenti-Ang-(1–7) in rat hearts ($n = 4–7$ per group). The bar graph shows semi-quantitative real-time RT-PCR to detect the expression of lenti-Ang-(1–7) viral vector in the heart. *$P < 0.001$, lenti-Ang-(1–7) and MI + lenti-Ang-(1–7) *versus* control and MI. *C*, lenti-Ang-(1–7) induced a significant increase in the levels of Ang-(1–7) after 4 days of transduction in the rat neonatal cardiac myocytes. *$P < 0.05$, lenti-Ang-(1–7) *versus* control, $n = 3$. *D*, schematic representation of structural components of the lenti-Ang-(1–7) viral vector. Angiotensin-(1–7) is released by the action of endogenous furin during the secretion process. Abbreviations: LTR, Long terminal repeat; , Psi packaging signal; F, central polypurine tract DNA flap; hEF1α, Human elongation factor 1 α promoter; hRenin signal peptide, human renin signal peptide; Fc portion of mIgG, immunoglobulin fragment from mouse IgG2b; SIN LTR, Self-inactivating LTR.

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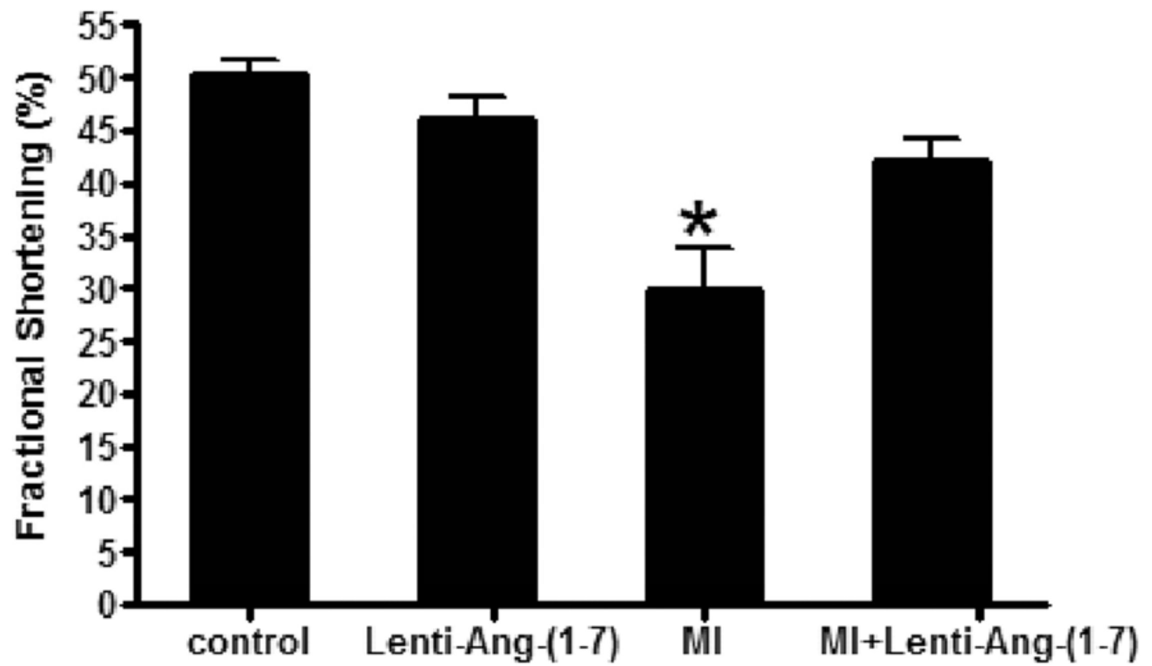

Figure 2. Effect of angiotensin-(1–7) gene transfer and myocardial infarction on ventricular function

Echocardiographic analyses of rat myocardium 4 weeks after the MI demonstrates a significant improvement in fractional shortening in lenti-Ang(1–7)-treated animals compared with the MI group. * $P < 0.05$, MI *versus* all other groups, $n = 4$–7 animals per group.

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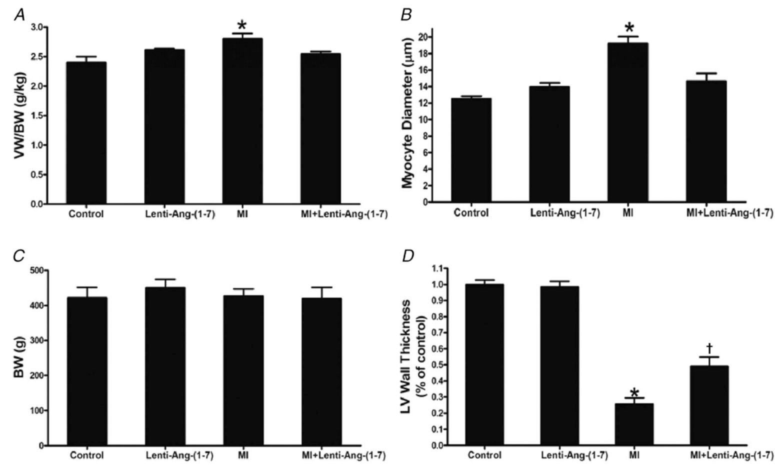

Figure 3. Effect of Ang-(1–7) gene transfer on body weight and left ventricular remodeling
Lentiviral delivery of Ang-(1–7) attenuates MI-induced cardiac hypertrophy as evaluated by the ventricular weight (VW, in grams) to body weight (BW, in kilograms) ratio (*A*) and measurement of myocyte diameter in the LV free wall peri-infarct area (*B*). Body weight was not significantly different among groups (*C*). $*P < 0.05$, MI *versus* all other groups. *D*, quantification of the LV wall thickness as a percentage of control left ventricular wall thickness. Myocardial infarction induced a significant reduction in LV wall thickness, and Ang-(1–7) attenuated the LV wall thinning. $*P < 0.05$, MI *versus* control, lenti-Ang-(1–7) and MI + lenti-Ang-(1–7); $\dagger P < 0.05$, MI + lenti-Ang-(1–7) *versus* control and lenti-Ang-(1–7).

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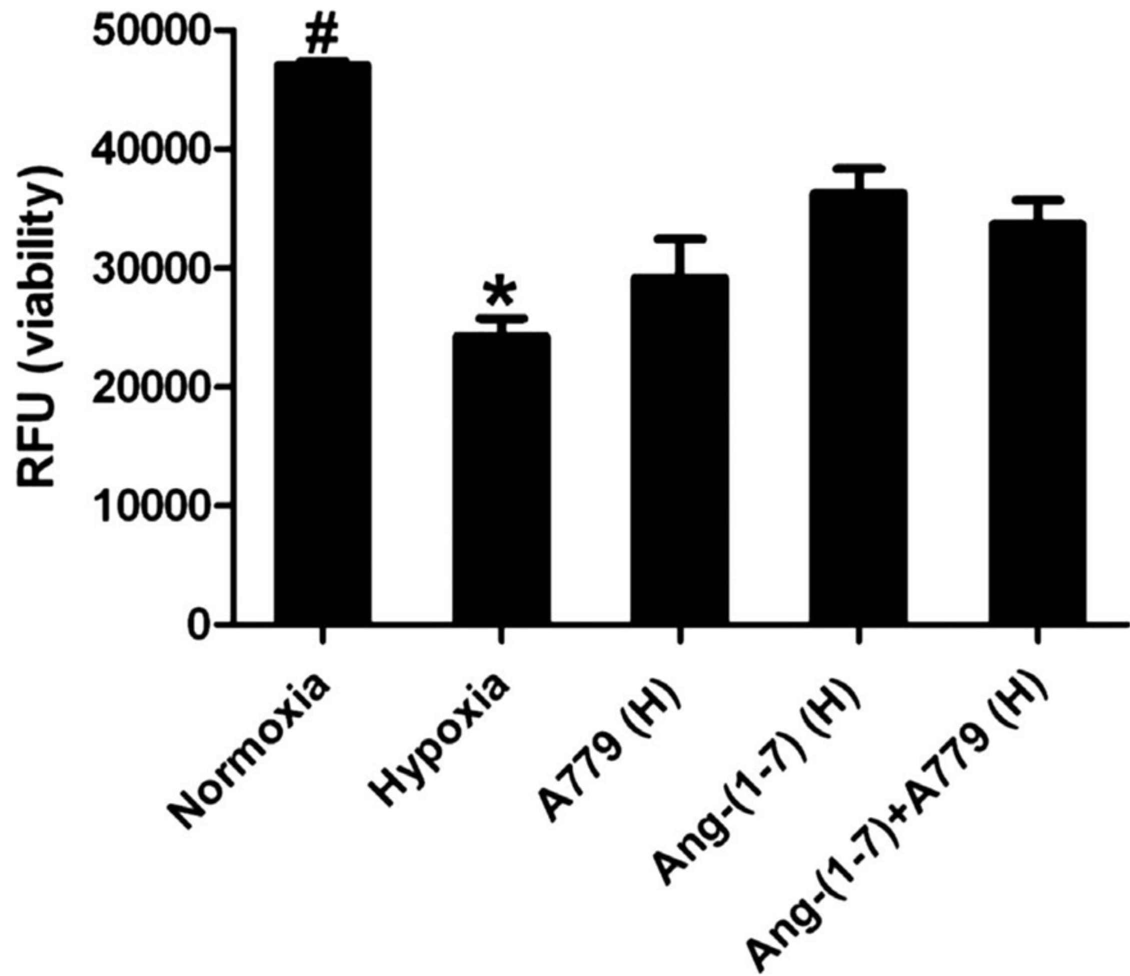

Figure 4. Effects of Ang-(1–7) on cell viability

Angiotensin-(1–7) (1 μM) and/or A779 (1 μM) were added 1 h before exposure to hypoxia. The bar graph shows means + SEM, $n = 6$. * $P < 0.05$, hypoxia *versus* Ang-(1–7) with exposure to hypoxia (H); #$P < 0.05$, normoxia *versus* all other groups. Abbreviation: RFU, Relative Flourescence Unit.

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Table 1

Haemodynamic data 4 weeks after myocardial infarction (MI)

	Control	Lenti-Ang-(1–7)	MI	MI + lenti-Ang-(1–7)
Left ventricular systolic pressure (mmHg)	108.6 ± 4.2	91.2 ± 2.9	83.6 ± 6.9 *	110.7 ± 4.6
Heart rate (beats min^{-1})	260.8 ± 15.3	247 ± 14.8	336.1 ± 29.6 *	262.9 ± 11.3
dP/dt_{max} (mmHg s^{-1})	8417.1 ± 337.4	7188.1 ± 1329.0	4479.4 ± 1419.3 *	7154.5 ± 1369.2
dP/dt_{min} (mmHg s^{-1})	5166.2 ± 453.9	4768.0 ± 1260.0	3115.6 ± 441.9 *	4690.5 ± 816.8
Left ventricular end-diastolic pressure (mmHg)	4.0 ± 1.3	3.8 ± 1.3	12.5 ± 2.7 *	4.9 ± 0.7

Abbreviations: dP/dt_{max} and dP/dt_{min}, maximal and minimal peak rate of left ventricular pressure, respectively.

* $P < 0.05$, MI *versus* all other groups; n = 4–7 per group.

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Table 2

Quantitative real-time PCR data

mRNA	Control	Lenti-Ang-(1–7)	MI	MI + lenti-Ang-(1–7)
AT1R	1.00 ± 0.33	0.73 ± 0.10	1.60 ± 0.21	0.81 ± 0.10
TGF-β	0.59 ± 0.21	0.57 ± 0.24	0.98 ± 0.01	0.83 ± 0.04
BKR	0.33 ± 0.08	0.33 ± 0.07	0.28 ± 0.02	0.74 ± 0.11 *
ACE2	0.76 ± 0.03	0.56 ± 0.05	0.43 ± 0.21	1.17 ± 0.22 †
ACE	1.64 ± 0.29	0.98 ± 0.07	3.48 ± 0.62 ‡	1.62 ± 0.34
MAS	0.99 ± 0.40	1.06 ± 0.12	0.29 ± 0.04	0.85 ± 0.31
TNF-α	0.64 ± 0.09	0.82 ± 0.06	0.67 ± 0.09	1.00 ± 0.08
COLI	0.27 ± 0.08	0.39 ± 0.17	0.95 ± 0.22	0.53 ± 0.24
COLIII	0.45 ± 0.08	0.88 ± 0.06	1.13 ± 0.17	0.71 ± 0.07

[*] $P < 0.05$, MI + lenti-Ang-(1–7) *versus* all other groups;

[†] $P < 0.05$, MI + lenti-Ang-(1–7) *versus* lenti-Ang-(1–7) and MI; and

[‡] $P < 0.05$, MI *versus* all other groups; $n = 4$–7 animals per group. Abbreviations: Angiotensin II Type 1 Receptor (AT1R), Transforming Growth Factor *β* (TGF-*β*), Angiotensin Converting Enzyme 2 (ACE2), Angiotensin Converting Enzyme (ACE), Bradykinin receptor B2 (BKR), Mas Receptor (Mas), Tumor Necrosis Factor *α* (TNF*α*), Collagen Type I (CoL I), and Collagen Type III (CoL III).

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Table 3

Quantitative real-time PCR data for *in vitro* experiment

mRNA	Normoxia	Hypoxia	Normoxia + Ang-(1–7)	Hypoxia + Ang-(1–7)
AT1R	1.19 ± 0.26	1.41 ± 0.32	0.83 ± 0.18	1.04 ± 0.20
AT2R	0.89 ± 0.10	1.17 ± 0.07	0.97 ± 0.24	1.42 ± 0.18 *
ACE	0.97 ± 0.05	1.00 ± 0.04	0.96 ± 0.08	0.99 ± 0.05
ACE2	0.42 ± 0.13	0.29 ± 0.10	0.28 ± 0.16	0.95 ± 0.20 †
MAS	0.91 ± 0.05	1.23 ± 0.04	1.36 ± 0.24	1.12 ± 0.04
BKR	0.76 ± 0.13	0.92 ± 0.14	0.52 ± 0.10	0.51 ± 0.12
IL-10	0.03 ± 0.004	0.01 ± 0.001	1.05 ± 0.14‡	1.30 ± 0.23‡
TNF-α	0.44 ± 0.08	1.67 ± 0.26§	0.18 ± 0.05	0.40 ± 0.05
IL-6	0.97 ± 0.04	1.35 ± 0.04§	0.75 ± 0.12	0.88 ± 0.05

* $P < 0.05$, hypoxia + Ang-(1–7) *versus* normoxia and normoxia + Ang-(1–7);

† $P < 0.05$, hypoxia + Ang-(1–7) *versus* all other groups;

‡ $P < 0.05$, normoxia + Ang-(1–7) and hypoxia + Ang-(1–7) *versus* corresponding controls; and

§ $P < 0.05$, hypoxia *versus* all other groups. Abbreviations: Angiotensin II Type 1 Receptor (AT1R), Angiotensin II Type 2 Receptor (AT2R), Transforming Growth Factor *β* (TGF-*β*), Angiotensin Converting Enzyme 2 (ACE2), Angiotensin Converting Enzyme (ACE), Bradykinin receptor B2 (BKR), Mas Receptor (*Mas*), Tumor Necrosis Factor *α* (TNF*α*), Collagen Type I (CoL I), and Collagen Type III (CoL III).