Early expression of monocyte chemoattractant protein-1 correlates with the onset of isoproterenol-induced cardiac fibrosis in rats with distinct angiotensin-converting enzyme polymorphism

Miguel Copaja Soto,[*][¥] *Rodrigo Valenzuela,*[*][¥] *Adiela Saldaña,*[*] *María Paz Ocaranza,*[†] *Jorge E. Jalil,*[†] *Carlos Vio,*[#] *Paul Lijnen, Gamaliel E. Ordenes,*[§] *Raul Vivar Sanchez,*[*] *Sergio Lavandero,*[*][§] *Guillermo Díaz-Araya*[*]

Key words:
ACE gene polymorphism, cardiac fibrosis, isoproterenol, pro-fibrotic factors, pro-inflammatory factors

[*] Centro FONDAP Estudios Moleculares de la Célula, Universidad de Chile, Santiago, Chile.

[†] Departamento Enfermedades Cardiovasculares, Escuela de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile.

[#] Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile. Hypertension and Cardiovascular Rehabilitation Unit, Katholieke Universiteit von Leuven, Leuven, Belgium.

[§] Facultad de Medicina, Universidad de Chile, Santiago, Chile.

[¥] These authors contributed equally to the work.

Correspondence to:
Dr Guillermo Díaz-Araya
Facultad Ciencias Químicas y Farmacéuticas, Universidad de Chile, Olivos 1007, Santiago 8380294, Chile.
Tel: +56 2 978 2975
Fax: +56 2 737 8920
Email:
gadiaz@ciq.uchile.cl

Abstract

Introduction. Isoproterenol treatment of Brown Norway and Lewis rats (high and low plasma angiotensin-I-converting enzyme activity, respectively) results in similar cardiac hypertrophy but higher cardiac fibrosis in Brown Norway rats. **Materials and methods.** Rats were infused *in vivo* with isoproterenol for two or 10 days. Cardiac fibrosis and inflammation were evaluated histochemically. We measured the mRNAs of pro-fibrotic factors (transforming growth factor β_1, endothelin-1) and pro-inflammatory factors (monocyte chemoattractant protein-1). In studies with cardiac fibroblasts incubated with isoproterenol *in vitro*, we measured cell proliferation, angiotensin-I-converting enzyme and matrix metalloprotease 2 activities and deposition of collagen type I and fibronectin. **Results.** After treatment with isoproterenol for two days, there were large areas of myocardial injury and numerous inflammatory foci in the left ventricle, these being greater in Brown-Norway than in Lewis rats. After treatment with isoproterenol for 10 days, there were large areas of damage with extensive collagen deposition only in the left ventricle; both strains exhibited this damage which was, however, more severe in Brown-Norway than in Lewis rats. After treatment with isoproterenol for two, but not 10, days, greater amounts of monocyte chemoattractant protein-1 mRNA were found in Brown Norway than in Lewis rats. Cell proliferation, activities of angiotensin-I-converting enzyme and matrix metalloprotease 2, amounts of collagen type I and fibronectin were similar in cardiac fibroblasts from both strains; changes after isoproterenol (10 μM) were also similar in both strains. **Conclusion.** We conclude that the greater cardiac fibrosis in Brown Norway rats treated with isoproterenol correlates with the early and higher expression of proinflammatory factors.

Introduction

Components of the renin-angiotensin system (RAS), including angiotensin-converting enzyme (ACE) and angiotensin II (Ang II), have been implicated in the development of cardiac fibrosis.[1,2] ACE cleaves angiotensin I to produce Ang II, which then binds to its receptors to produce potent vasoconstriction, and, in the heart, modulating hypertrophy and fibrosis.[3] The identification of a microsatellite marker in the rat ACE gene has permitted distinction in ACE alleles between Brown-Norway (BB) and Lewis (LL) rats, with high and low plasma ACE activities, respectively.[4,5] We have shown that chronic administration of isoproterenol (ISO) produces microinfarct-like lesions in the rat myocardium and that these are associated with a similar degree of heart hypertrophy but more cardiac fibrosis in BB than in LL rats.[6] These results suggest that ACE may have a role in the development of cardiac fibrosis.

Cardiac fibrosis (defined as extracellular matrix [ECM] protein deposition) is mainly regulated by cardiac fibroblasts. These cells are the major source of extracellular connective tissue matrix, and the recruitment, accumulation and stimulation of these cells are thought to play important roles in both normal healing and the development of fibrosis.[1] These cells have a number of functional receptors, including adrenergic, Ang II, bradykinin and purinergic receptors, all coupled to G protein-linked signalling pathways, in a pattern that is distinct from those in cardiac myocytes.[7] In the heart, β_2-adrenergic receptors are mainly expressed in cardiac fibroblasts, indicating that ISO could directly modulate cardiac fibroblast function.[8]

Several lines of evidence have shown that tissue Ang II is involved in inflammatory and healing responses, regulating the expression of mediators

of repair at vascular and non-vascular sites of cardiac injury.[9] Ang II increases the expression of monocyte chemoattractant protein-1 (MCP-1), an important chemokine with strong influence on the amplification of inflammation processes, by promoting recruitment of macrophages to inflammatory sites.[9] However, Ang II also regulates the expression of the pro-fibrotic factors transforming growth factor (TGF)-β_1 and endothelin (ET)-1, as well as the expression of ECM proteins.[10,11] The role of these pro-inflammatory/pro-fibrotic factors on cardiac fibrosis induced by ISO in rats with different ACE polymorphisms is unknown. Elucidation of this role was the aim of this study.

Our results indicate that ISO-induced cardiac fibrosis correlates with increased levels of pro-inflammatory factors in Brown Norway rats only; although cardiac fibroblasts have an important role in ECM deposition, cardiac fibroblasts isolated from both rat strains responded *in vitro* in a similar manner to ISO in proliferation, collagen type I and fibronectin secretion, and matrix metalloprotease (MMP)-2 and ACE activities.

Methods
Experimental design
The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication no. 85-23, revised 1985). We used an experimental ACE genetic model previously described by Challah *et al.*,[4] in which two normotensive strains of rats, the Brown Norway and Lewis strains, have contrasting levels of plasma ACE. F_0 Brown Norway males (BB ACE genotype) and F_0 Lewis females (LL ACE genotype) were from Charles River (Wilmington, MA, USA). Rats were mated to produce F_1 hybrids (LB genotype). F_1 hybrids were mated to obtain the F_2 cohorts, which were used in this study.[6]

Treatment
Homozygous F_2 male rats (80–100 g) were injected subcutaneously with ISO (5 mg·kg^{-1} day^{-1}; Sigma) or saline (control) for 10 days. Animals were divided into four experimental groups: (1) LL-C: LL rats receiving saline injection; (2) BB-C: BB rats receiving saline injection; (3) LL-ISO: LL rats receiving ISO injection; and (4) BB-ISO: BB rats receiving ISO injection. After two or 10 days of treatment, animals were weighed and killed by decapitation at day three before the following injection or at day 11 (24 h after the last injection). The blood was collected into pre-chilled tubes containing heparin for the determination of plasma ACE activity. The heart was rapidly excised, and

the left ventricle (LV) plus septum and right ventricle (RV) were weighed (LVW and RVW, respectively). The LV was sectioned transversely into two slices. One was fixed in 4% formalin for histochemical studies, and the other was rapidly frozen in liquid N_2 and stored at −70°C until used for measurement of ACE activity and mRNA (ET-1, TGF-β_1, MCP-1, ACE, and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]).

Determination of plasma and cardiac fibroblast ACE activity
Plasma and cellular ACE activities were assayed by following the release of His-Leu from the synthetic substrate Z-Phe-His-Leu (Bachem Biosciences Inc., King of Prussia, PA, USA) with or without enalapril according to the fluorometric assay previously described.[5] A sample of plasma or homogenised cardiac fibroblast membranes was then incubated at 37°C in a shaking waterbath with Z-Phe-His-Leu for 30 minutes or 2 hours for plasma and cardiac fibroblast membranes, respectively. The reaction was stopped by addition of 100 μl of cold 10% trichloracetic acid and 280 mM NaOH. Phthalaldehyde (0.1%) was added to the samples, which were then incubated for 10 minutes at 37°C before reaction was stopped with 3 M HCl. The fluorescence at 486 nm was measured with an excitation of 364 nm (Fluorescence Spectrophotometer 650-10, Perkin Elmer, Waltham, MA, USA). Plasma ACE activity was expressed as nmol of His-Leu liberated per minute per ml of plasma (U/ml plasma); cellular ACE activity was expressed as nmol of His-Leu liberated per minute per mg protein at 37°C (U/mg protein).

Evaluation of cardiac hypertrophy
The degrees of LV hypertrophy (LVH) and RV hypertrophy (RVH) were quantified by the relative LV weight ratio (RLVW, LV weight 100/body wt) and the relative RV weight ratio (RRVW, RV weight 100/body wt), respectively.

Tissue processing and staining
The equatorial regions of each LV were routinely processed and paraffin embedded. Sections (5 μm) were stained with hematoxylin and eosin for histopathological analysis. Sections (5 μm-thick) were also stained with Masson's trichrome for determination of interstitial collagen.

Histopathological analysis
Semi-quantification of myocardial injury and inflammatory lesions was performed by a trained pathologist who had no knowledge of the experimental groups during the evaluation of the tissues. Two categories were used to score the level of myocardial injury and inflammatory lesions in

Table 1

List of sense and antisense primers used in RT-PCR of rats with distinct ACE gene polymorphism.

Primers	Sequence (5′ → 3′)	Annealing temperature [°C]	Size [bp]
TGF-β_1	Sense 5′-AAGCCCTGTATTCCGTCTCC-3′		
	Antisense 5′-CAACGCCATCTATGAGAAAACC-3′	52	290
ET-1	Sense 5′-CTAGGTCTAAGCGATCCTTG-3′		
	Antisense 5′-TTCTGGTCTCTGTAGAGTTC-3′	50	319
MCP-1	Sense 5′-CAGGTCTCTGTCACGCTTCT-3′		
	Antisense 5′-GTGCTTCAGGTGGTTGTGG-3′	53	450
GAPDH	Sense 5′-CCATCACCATCTTCCAGGAG-3′		
	Antisense 5′-CCTGCTTCACCACCTTCTTG-3′	60	541

several sections from each heart. A score of zero represented no damage. A score of one represented clear area of myocardial necrosis and inflammatory infiltrate.

Determination of MCP-1, TGF-β_1 and ET-1 mRNAs by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the LV with Trizol (Invitrogen, Carlsbad, CA, USA). RNA pellets were suspended in distilled and DEPC water, and their concentrations were measured by UV spectroscopy. RNA integrity was assessed from the intensity of the staining with ethidium bromide of 18 and 28S ribosomal RNA after agarose electrophoresis. The amounts of tissue TGF-β_1, MCP-1, and ET-1 mRNAs were determined by RT-PCR. For reverse transcription, 1 μg RNA was incubated with or without reverse transcriptase (GIBCO-BRL, Gaithersburg, MD, USA) in a mixture containing random primers, deoxynucleotides, and RNAs in reverse transcription buffer. For amplification of the resulting cDNA, 1 μM of each specific primer pair, 0.2 mM deoxynucleotides, 1.5 mM MgCl$_2$, and 1.5 U Taq polymerase (GIBCO-BRL) were added to 3 μl of each RNA sample in a final volume of 50 μl. All the sequences of the sense and antisense primers are described in table 1. After PCR, the amplification products were fractionated on a 1.5% (w/w) agarose gel and visualised by staining with ethidium bromide. Band intensities were quantified by computerised densitometry and normalised with respect to GAPDH mRNA.

Cardiac fibroblast isolation and culture

Hearts were rapidly extracted and cardiac fibro - blasts from BB and LL male adult rats (130±20 g) were isolated by cardiac retrograde aortic perfusion as described previously,[12] with a few modifications. Briefly, the heart was digested with a solution containing collagenase-hyaluronidase (1:1); the resulting cells were centrifuged at 1,000 rpm for 5 minutes, and then resuspended in Dulbecco's modified Eagle's medium (DMEM)-F12 containing 10% foetal bovine serum and seeded in non-treated culture dishes for 2 hours. The cells were then washed with phosphate-buffered saline to eliminate debris and non-adherent cells. Passage two fibroblasts were washed twice with Moscona's solution and cultured in serum-free DMEM-F12 (SFM), supplemented with ascorbic acid (50 μg/ml) and glutamine (50 μg/ml) for 24 hours. They were then incubated with or without 10 μM ISO in SFM for 24–48 hours. Cardiac fibroblasts derived from each rat were used in an independent experiment. Fibroblasts from both rat strains were morphologically characterised by microscopy.

Cardiac fibroblast proliferation

Cardiac fibroblasts were seeded into 96-well plates (5,000 cells per well), incubated overnight and growth-arrested using SFM for 24 hours. SFM was then replaced with 0.2 ml DMEM-F12 containing the appropriate stimulus. This time was designated 'Day 0'. Medium and stimulus were replaced after 24 hours by DMEM-F12 without phenol red and quantified by Cell Titer® (Molecular Probes, Seattle, OR, USA), following the manufacturer's instructions.

Collagen and fibronectin protein levels

Serum-starved cardiac fibroblasts were treated with 10 μM ISO or vehicle. At the indicated times, culture medium was collected and concentrated using Centricon plus-20 tubes (Millipore, Billerica, MA, USA). Non-reducing SDS-PAGE sample buffer was added directly to the concentrated medium and samples were subjected to electrophoresis. Proteins prepared using non-reducing sample buffer (25 μg per lane) were separated by SDS-PAGE on 12% (w/v) gels and transferred electrophoretically to nitrocellulose. The blots were incubated with anti-fibronectin antibody (1:10,000; BIODESIGN International, Maine, USA) or anti-collagen type I antibody

(1:5,000; Chemicon, Tamecula, CA, USA). After washing, blots were incubated with anti-rabbit IgG antibodies linked to horseradish peroxidase (1:10,000). Bands were detected using ECL, and quantified by laser scanning densitometry. Membranes were stained with ponceau red to confirm equal loading.

MMP activities
Assessment of MMP-2 activity was performed by zymography. Concentrated culture media (20 μg protein) were treated with non-reducing sample buffer and resolved on 12% SDS-PAGE gels containing 0.1% gelatin. Gels were washed twice with 2.5% triton X-100 for 30 minutes at room temperature, incubated at 37°C for 18 hours in 50 mM HEPES (pH 7.5) containing 0.2 M NaCl, 5 mM $CaCl_2$ and 20 μM $ZnCl_2$ and subsequently stained with coomassie blue R-250. Activated MMPs produced clear areas and bands were quantified by densitometry.

Statistical analysis
Results were expressed as mean±SEM. Differences between strains were estimated by one-way ANOVA followed by Student-Newman-Keuls assessment for the number of independent experiments indicated (n). The limit of statistical significance was set at $p < 0.05$.

Results
Cardiac hypertrophy
Figure 1 shows that the relative LV weight ratio did not change after two days of ISO treatment. The LV ratio was, however, remarkably increased after 10 days of ISO treatment when compared with that in normal rats.

Myocardial histopathology
Hearts from control rats were histologically normal. No myocardial lesions were identified in hearts from rats receiving saline solution after two days (figure 2A LL-C, BB-C) or 10 days (figure 2B LL-C, BB-C) of treatment. In contrast, myocardial alterations were observed in two-day samples in both rats (figure 2 LL-ISO, BB-ISO). Myocardial changes were characterised by degeneration and necrosis of cardiomyocytes and replacement by inflammatory cells (predominantly histiocytes), distributed mainly in interstitial space. The semi-quantitative scores for myocardial injury in animals treated for two days are shown in table 2. After two days of treatment, the score for myocardial alterations was 0 for all LL-C and BB-C rats, while it was 1 for only fifty percent of LL-ISO rats (three rats); in contrast, it was 1 for all BB-ISO rats (five animals).
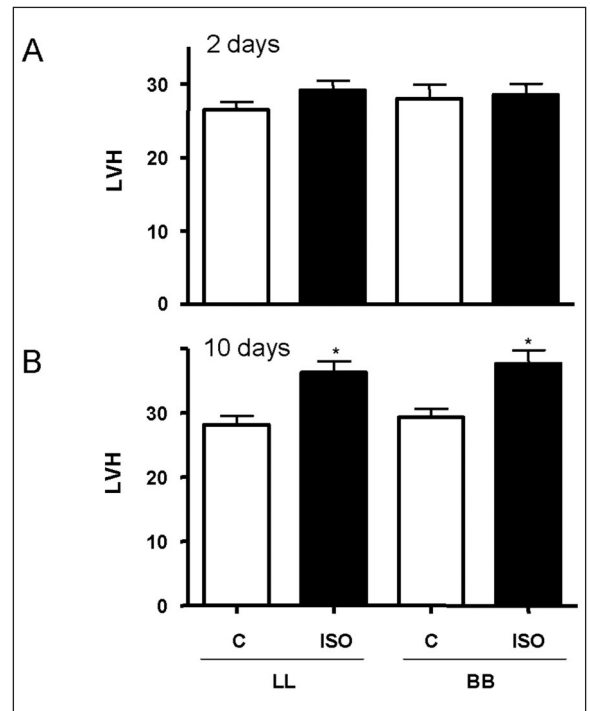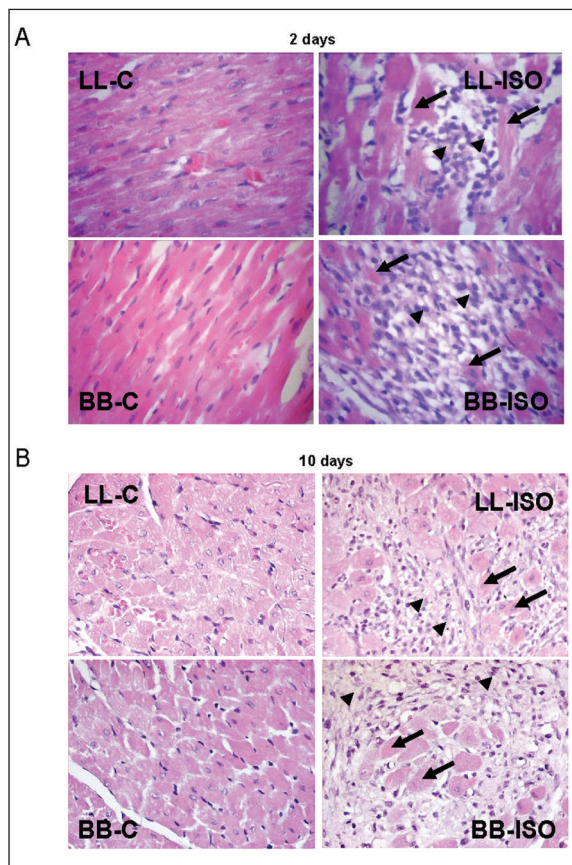

Figure 1
Effect of ISO on LVH in rats with different ACE polymorphisms. LVH at two days (A), and at 10 days (B) of ISO treatment was measured as mentioned in methods. Data are mean±SEM; n = 6–7 in duplicate; *p < 0.01 vs. control.

Strong and markedly different myocardial alterations were observed after 10 days between both rat strains (figure 2 LL-ISO, BB-ISO). The semi-quantitative scores for myocardial injury in animals treated for 10 days are shown in table 2. After 10 days of treatment, the score for myocardial damage was 0 for LL-C and BB-C rats; meanwhile it was similar between BB-ISO and LL-ISO rats (score 0–1 with similar number of rats). However, in BB-ISO rats the myocardial changes were characterised by large areas with isolated cardiomyocytes individually or in groups, surrounded by ECM protein and non-myocyte cells, and low presence of inflammatory areas. These damage areas looked as if wound healing was finished, as evidenced by lower infiltration of inflammatory cells, which were replaced by excessive ECM protein deposition (figure 3 BB-ISO vs. LL-ISO and respective controls). In contrast, in LL-ISO group, areas with granulation tissue and extensive infiltration of inflammatory cells were observed; however, low collagen deposition was observed by low trichrome Masson stain (figure 3 LL-ISO).

Myocardial fibrosis
After two days there was neither collagen deposition nor a difference between both experimental groups (data not shown). The myocardial interstitial collagen deposition of rats receiving

Figure 2
Effect of ISO on LV histology of ACE polymorphic rats. Lewis (LL) and Brown Norway (BB) rats were injected with saline (C) or ISO (5 mg·kg⁻¹·day⁻¹) for two (upper panel) or 10 (lower panel) days. Histological view of LV sections (transversal cross-section) of rat ventricular myocardium, stained with hematoxylin and eosin, was photographed. Arrows indicate isolated cardiomyocytes surrounded by infiltrative cells (arrowheads) and connective tissue. At two days in LL-ISO rat, focal and isolated lesions were found, meanwhile in BB-ISO rat, multifocal and confluent lesions were found. At 10 days in LL-ISO rat, multifocal and confluent lesions were found, meanwhile in BB-ISO rat, a focal and isolated lesion were found.

vehicle or ISO was, however, clearly different only after 10 days of treatment (figure 3). In the BB-ISO group, there was a strong increase in interstitial collagen with respect to its control, and the LL-ISO group showed as blue stain with trichromo Masson. At 10 days, the perivascular fibrosis was similar for both groups receiving ISO (data not shown).

Gene expression of pro-inflammatory and pro-fibrotic mediators

Consistent with the histological evidence of inflammatory changes induced by ISO in the heart, the amounts of MCP-1 mRNA were significantly increased at two days of treatment only in the BB-ISO group (figure 4). The contents of TGF-β_1 and ET-1 mRNA were also increased after two days of ISO treatment (figure 4); there were,

however, no statistical differences between the two rat strains. There were no changes in the amounts of MCP-1, TGF-β_1, and ET-1 mRNAs in both strains at 10 days (figure 4).

Effect of ISO on proliferation, collagen and fibronectin secretion, and ACE and MMP-2 activities of cardiac fibroblasts derived from rats with distinct ACE polymorphism

Cardiac fibroblasts cultured in SFM (control) did not proliferate over a 24 hour period. Incubation with ISO *in vitro* increased (p < 0.01) cardiac fibroblast proliferation obtained from both rat strains (34±7% for LL-ISO and 26±8% for BB-ISO) (figure 5A).

Under basal conditions, the amounts of collagen type I and fibronectin in cardiac fibroblasts from both rat strains were similar. However, incubation with ISO (10 μM) decreased the amount of collagen type I, but increased fibronectin in cardiac fibroblasts from both rat strains (figures 5B and 5C), although there was no statistical difference between the two ISO groups. Finally, in cardiac fibroblasts isolated from both rat strains, MMP-2 and ACE activities were unchanged in all conditions examined (figures 5D and 5E).

Discussion

The main findings of the present study were: (1) great and numerous inflammation areas observed early (two days) in BB-ISO rats; (2) increased MCP-1 mRNA levels were observed early (two days) in BB-ISO rats; (3) ISO increased TGF-β_1 mRNA levels, but there was no statistical difference between the two rat strains; and (4) cardiac fibroblasts derived from LL or BB rats exhibited a similar response to ISO *in vitro* with regard to cell proliferation, ECM protein deposition, and MMP-2 and ACE activities.

In animals, the effects of ISO on hearts are linked to hypertrophy and cardiac fibrosis.[13-16] These pathological effects can be divided into early and late phases, similar to that of the cardiac remodelling observed in rats with myocardial infarcts. Our study showed that ISO injection induced both phases of cardiac remodelling in both rat strains, an early inflammatory process (figure 2), and late hypertrophy (figure 1) and fibrotic process (figure 3). Our histopathology study showed that at two days of treatment in both rat strains, ISO induced an acute inflammatory process characterised by myocardial changes with degeneration and necrosis of cardiomyocytes and replacement by inflammatory cells, distributed mainly in interstitial space, classified at score 0–1 for LL-ISO group and only at

Table 2
Score of ISO-induced inflammatory and cardiac damage in rats with distinct ACE gene polymorphism.

| Two days of treatment				
Score	LL-C	LL-ISO	BB-C	BB-ISO
0	4	3	4	0
1	0	3	0	5
Total	4	6	4	5
Ten days of treatment				
Score	LL-C	LL-ISO	BB-C	BB-ISO
0	4	4	7	4
1	1	4	0	3
Total	5	8	7	7

Key: Values are number of rats in this score. Homozygous F_2 Lewis (LL) and Brown Norway (BB) rats were injected with saline (C) or ISO (5 mg/kg/day) for two or 10 days. One day after the last injection, LV cross-sections were evaluated for inflammatory or cardiac damage as described in methods.
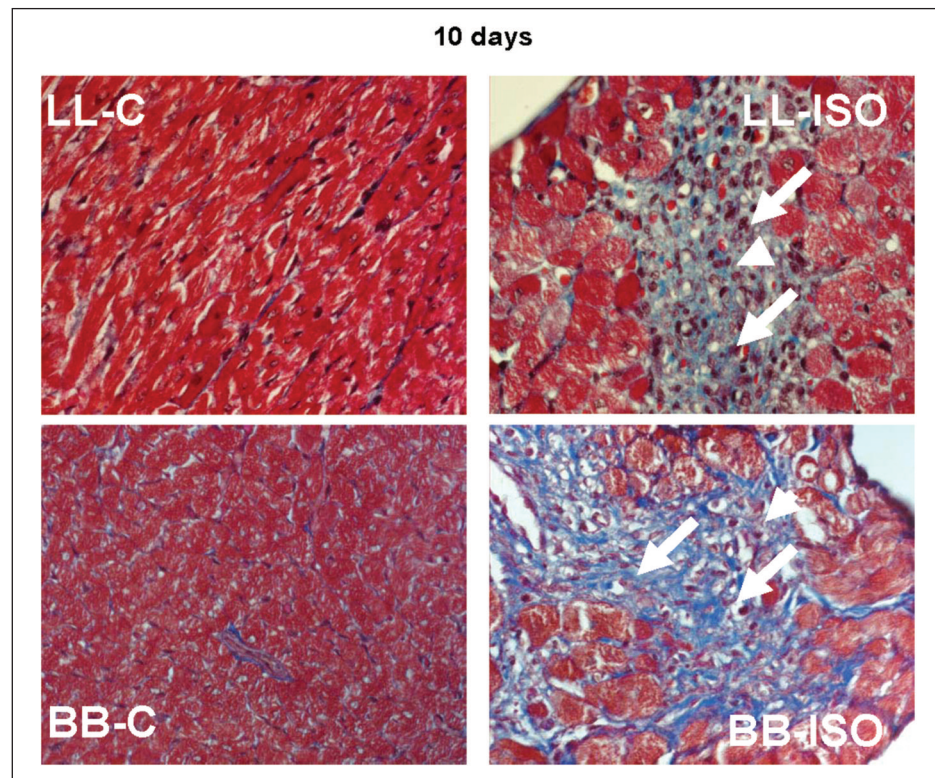
Figure 3
Effect of ISO on LV interstitial fibrosis of rats with different ACE polymorphism. Lewis (LL) and Brown Norway (BB) rats were injected with saline (C) or ISO (5 mg·kg^{-1}·day^{-1}) for 10 days. Histological view of LV sections (transversal cross-section) of rat ventricular myocardium, stained with trichrome Masson's, was photographed. Arrows indicate collagen deposition (blue), and arrowheads infiltrative inflammatory cell (brown). In LL-ISO rat, low blue stain was found, meanwhile in BB-ISO rat, intense blue stain was observed.

score 1 for BB-ISO group. In the chronic phase (10 days), the damage area looked a finished inflammatory process in BB-ISO rats with low granulation tissue and extensive ECM protein deposition; meanwhile, in LL-ISO rats, there appeared to be an active inflammatory process with extensive granulation tissue and without excessive deposition of ECM protein. In concordance with these morphological observations, our study showed a strong increase in MCP-1 mRNA levels only in the BB-ISO group in the early phase (two days). Although our study did not determine in which cardiac cell type the expression was enhanced, MCP-1 expression has been localised in cardiac cells.[17] Kralisch *et al.*[18] have shown that ISO increases MCP-1 mRNA levels in fibroblasts. Whether higher ACE activity in BB rats regulates the expression of MCP-1 is unclear; however, others have demonstrated that ACE
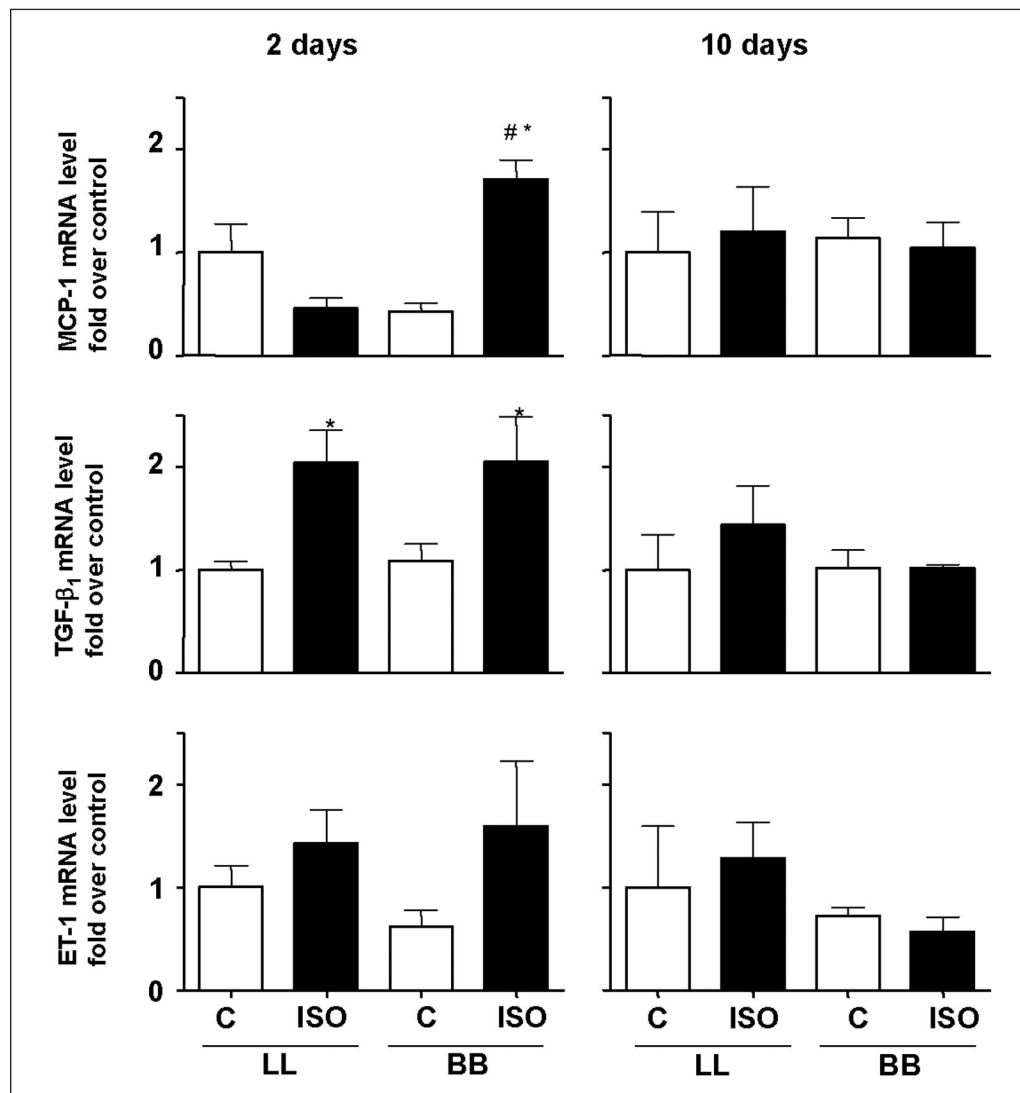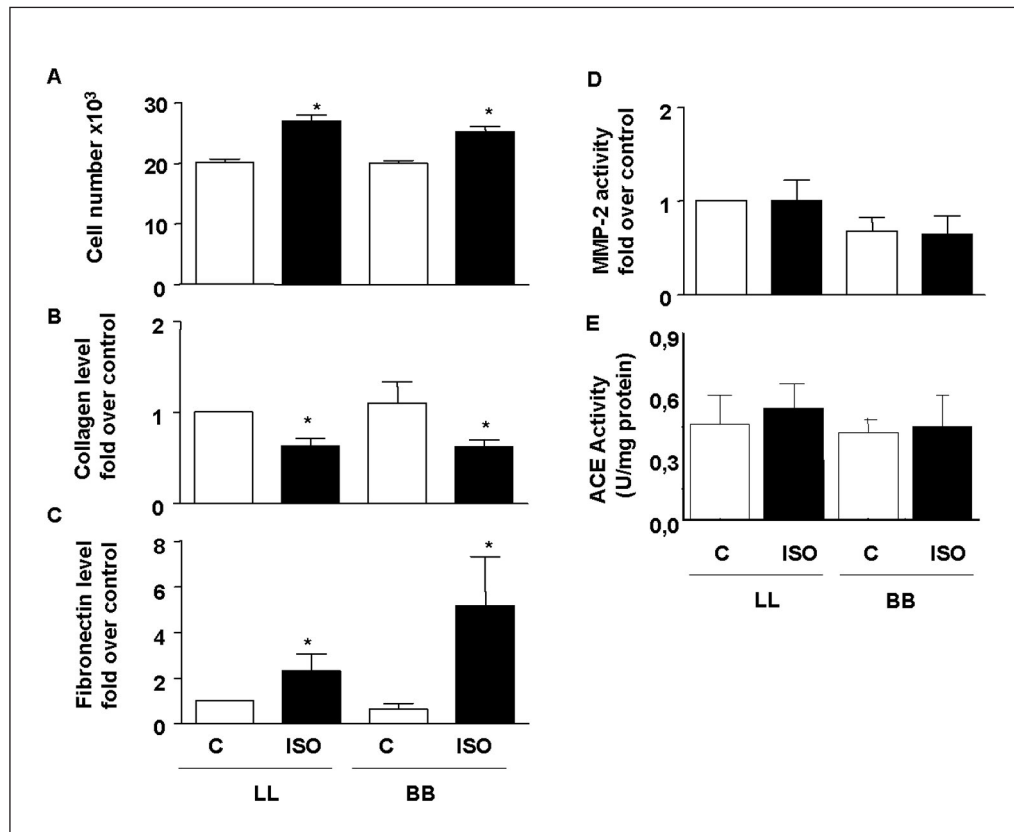
Figure 4
Effect of ISO on mRNA levels of MCP-1, TGF-β and ET-1, in LV tissue of rats with different ACE polymorphism. Lewis (LL) and Brown Norway (BB) rats were injected with saline (C) or ISO (5 mg·kg^{-1}·day^{-1}) for two or 10 days. One day after the last injection, mRNA of LV tissue was extracted and analysed by RT-PCR. Data are mean±SEM expressed as multiples of the control value; n = 5–6; *p < 0.05 vs. control, #p < 0.05 vs. genotype.

inhibitors prevent the increase in the amounts of MCP-1 and TGF-β_1 mRNAs caused by Ang II.[19] The importance of the inflammatory cell response in leading to fibrous tissue formation has been shown before.[20,21] A key role is played by MCP-1, which controls not only the accumulation of macrophages, but also fibroblast proliferation, the induction of TGF-β_1 and the appearance of fibrosis.[20] Omura et al.[22] have shown that ISO induces a marked increase in TGF-β_1 in vivo, and this can stimulate the differentiation of fibroblasts to myofibroblasts. Although pro-fibrotic factors (TGF-β_1, ET-1) can stimulate ECM protein deposition, they are by themselves not sufficient to induce fibrosis. We can, however, suggest that these factors together with inflammatory factors are implicated in the greater extent of cardiac fibrosis in the BB-ISO group compared with the LL-ISO group.

We have previously described that, at two days of treatment, ISO increases both cell proliferation of non-cardiomyocyte cells and MMP-9 activity in BB-ISO rats more than in LL-ISO rats.[6] These findings are coincident with an inflammatory process in which MCP-1 seems to have a key role. The higher MMP-9 activity in the BB-ISO group suggests that this MMP activity could come from infiltration by inflammatory cells.

Extensive myocardial alterations were observed after 10 days of ISO injection in both BB and LL rats. In BB-ISO rats, however, the myocardial changes were characterised by large areas with isolated cardiomyocytes individually or in groups surrounded by extensive deposition of ECM protein (mainly collagen) and non-myocyte cells. These damage areas resembled the final stage of

Figure 5

Effect of ISO on cardiac fibroblasts. Cell proliferation (A), collagen type I (B) and fibronectin (C) secretion, MMP-2 (D) and ACE (E) activities were measured in cardiac fibroblasts derived from rats with different ACE polymorphism. Cardiac fibroblast proliferation was measured in LL and BB treated for 24 hours with 10 μM of ISO (solid bar). Collagen type I and fibronectin secretion, and MMP-2 activity were measured in culture medium as indicated in methods. ACE activity was measured in cell extracts as indicated in methods. Data are mean±SEM expressed as multiples of the control values (n = 3–5); *p < 0.05 *vs.* control.

wound healing, as evidenced by extensive collagen deposition. Finally, after 10 days of ISO treatment, there were no changes in either pro-fibrotic nor pro-inflammatory markers. These findings suggest that an early increase of these markers could be important in the onset of cardiac fibrosis.

Our previous studies *in vivo* have shown that administration of ISO stimulated cardiac fibroblast proliferation more in the BB rats than in the LL rats. Similar β-adrenergic dependent-growth rates were, however, observed *in vitro* in cardiac fibroblasts derived from BB and LL rats (figure 5), suggesting that other proliferative signals may operate *in vivo*. These results are consistent with previous reports showing ISO-induced proliferation of human cardiac fibroblasts.[8,23] Cardiac ECM protein deposition in the heart results from a balance between synthesis and degradation. The present data showed that cardiac fibroblasts *in vitro* derived from two rat strains had similar levels of ECM protein deposition, and they responded in a similar mode to ISO in collagen type I and fibronectin levels, and MMP-2 activity.

Finally, there were no significant differences between ACE activities in cardiac fibroblasts derived from BB and LL rats, and ISO treatment did not modify these activities. Cardiac fibroblasts show low ACE activity.[24,25] Studies *in vivo* have shown that ACE immunoreactivity is present mainly in endothelial cells.[26] Our earlier studies *in vivo*[6] showed a substantial increase in plasma and LV ACE activities in BB-ISO rats compared with LL-ISO and control rats. *In vivo*, ISO induces microinfarction-like lesions that resemble the scar zone observed after myocardial infarction; this zone is accompanied by the appearance of levels of ACE immunoreactivity in fibrotic tissue scars and it is localised on myofibroblasts.[27] Thus, the present data suggest that the differential increase in LV ACE activity induced by ISO in BB rats *in vivo* cannot be explained by an induction of ACE in cardiac fibroblasts. However, the higher inflammatory process in the BB-ISO group also suggests that high ACE activity could come from infiltration by inflammatory cells. ACE is also involved in the metabolism of vasoactive and trophic peptides, such as angiotensins and kinins.[28] Whether in these ACE polymorphic rats the higher ACE

plasma activity induced by ISO influences the metabolism of kinins and its relationship with the development of LV fibrosis remain unknown. Although an increase in cardiac ACE activity, as shown in BB-ISO rats, could mean a decrease of kinin levels, it has recently been demonstrated that endogenous Ang II modulates kinin B1 receptor expression in hypertensive rats;[29] this could have strong consequences on inflammatory process. Thus, an imbalance between local kinin levels and receptor number in the heart could have consequences in mediating inflammatory responses. Finally, it remains to be confirmed in other cardiac cells expressing high ACE activity, such as cardiac myofibroblasts and/or pro-inflammatory cells from these polymorphic rats, whether ISO induces a selective increase in its activity.

In summary, our study confirms that constant β-adrenergic stimulation serves as a stimulus to trigger LVH and fibrosis, and that they are increased in Brown-Norway as compared to Lewis rats. Further studies need to be performed to delineate the mechanism whereby ISO induces the expression of the genes for pro-inflammatory cytokines in BB-ISO rat hearts.

Acknowledgements

References

1. Weber KT, Brilla CG. Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. *Circulation* 1991;**38**:1849-65.
2. Dzau VJ. Tissue renin-angiotensin system in myocardial hypertrophy and failure. *Arch Int Med* 1993;**153**:937-42.
3. de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev* 2000;**52**:415-72.
4. Challah M, Villard E, Philippe M *et al*. Angiotensin I-converting enzyme genotype influences arterial response to injury in normotensive rats. *Arterioscler Thromb Vasc Biol* 1998;**18**:235-43.
5. Ocaranza MP, Piddo AM, Faundez P, Lavandero S, Jalil JE. Angiotensin I-converting enzyme gene polymorphism influences chronic hypertensive response in the rat Goldblatt model. *J Hypertens* 2002;**20**:413-20.
6. Ocaranza MP, Diaz-Araya G, Carreno JE *et al*. Polymorphism in gene coding for ACE determines different development of myocardial fibrosis in rats. *Am J Physiol* 2004;**286**:H498-H506.
7. Meszaros JG, Gonzalez AM, Endo-Mochizuki Y, Villegas S, Villarreal F, Brunton LL. Identification of G protein-coupled signaling pathways in cardiac fibroblasts: cross talk between Gq and Gs. *Am J Physiol* 2000;**278**:C154-C162.
8. Turner NA, Porter KE, Smith WH, White HL, Ball SG, Balmforth AJ. Chronic beta2-adrenergic receptor stimulation

9. Sun Y, Zhang J, Lu L, Bedigian MP, Robinson AD, Weber KT. Tissue angiotensin II in the regulation of inflammatory and fibrogenic components of repair in the rat heart. *J Lab Clin Med* 2004;**143**:41-51.
10. Villarreal FJ, Kim NN, Ungab GD, Printz MP, Dillmann WH. Identification of functional angiotensin II receptors on rat cardiac fibroblasts. *Circulation* 1993;**88**:2849-61.
11. Gray MO, Long CS, Kalinyak JE, Li HT, Karliner JS. Angiotensin II stimulates cardiac myocyte hypertrophy via paracrine release of TGF-β$_1$ and endothelin-1 from fibroblasts. *Cardiovasc Res* 1998;**40**:352-63.
12. Claycomb WC, Palazzo MC. Culture of the terminally differentiated adult cardiac muscle cell: a light and scanning electron microscope study. *Dev Biol* 1980;**80**:466-82.
13. Stanton HC, Brenner G, Mayfield ED Jr. Studies on iso-proterenol-induced cardiomegaly in rats. *Am Heart J* 1969;**77**:72-80.
14. Laks M, Morady MF, Swan HJ. Myocardial hypertrophy produced by infusion of subhypertensive doses of norephine-phrine in the dog. *Chest* 1973;**64**:75-8.
15. Boluyt MO, Long X, Eschenhagen T *et al*. Isoproterenol infusion induces alterations in expression of hypertrophy-associated genes in rat heart. *Am J Physiol* 1995;**269**:H638-H647.
16. Sun Y, Weber KT. Animal models of cardiac fibrosis. *Methods Mol Med* 2005;**117**:273-90.
17. Hohensinner PJ, Kaun C, Rychli K *et al*. Monocyte chemoattractant protein (MCP-1) is expressed in human cardiac cells and is differentially regulated by inflammatory mediators and hypoxia. *FEBS Lett* 2006;**580**:3532-8.
18. Kralisch S, Klein J, Lossner U *et al*. Isoproterenol stimulates monocyte chemoattractant protein-1 expression and secretion in 3T3-L1 adipocytes. *Regul Pept* 2006;**135**:12-16.
19. Amann B, Tinzmann R, Angelkort B. ACE inhibitors improve diabetic nephropathy through suppression of renal MCP-1. *Diabetes Care* 2003;**26**:2421-5.
20. Kuwahara F, Kai H, Tokuda K *et al*. Hypertensive myocardial fibrosis and diastolic dysfunction - another model of inflammation? *Hypertension* 2004;**43**:739-45.
21. Weber KT. From inflammation to fibrosis: a stiff stretch of highway. *Hypertension* 2004;**43**:716-19
22. Omura T, Kim S, Takeuchi K, Iwao H, Takeda T. Transforming growth factor beta 1 and extracellular matrix gene expression in isoprenaline induced cardiac hypertrophy: effects of inhibition of the renin-angiotensin system. *Cardiovasc Res* 1994;**12**:1835-42.
23. Colombo F, Noel J, Mayers P, Calderone A. Beta adrenergic stimulation of rat cardiac fibroblast promotes protein synthesis via the activation of phosphatidylinositol 3-kinase. *J Mol Cell Cardiol* 2001;**33**:1091-106.
24. Petrov VV, Fagard RH, Lijnen PJ. Transforming growth factor-ß1 induces angiotensin-converting enzyme synthesis in rat cardiac fibroblasts during their differentiation to myofibroblasts. *J Renin Angiotensin Aldosterone Syst* 2000;**1**:342-52.
25. Lijnen PJ, Petrov VV. Role of intracardiac renin-angiotensin-aldosterone system in extracellular matrix remodeling. *Methods Find Exp Clin Pharmacol* 2003;**25**:541-64.
26. Falkenhahn M, Franke F, Bohle RM *et al*. Cellular distribution of angiotensin-converting enzyme after myocardial infarction. *Hypertension* 1995;**25**:219-26.
27. Sun Y, Weber KT. Angiotensin converting enzyme and myofibroblasts during tissue repair in the rat heart. *J Mol Cell Cardiol* 1996;**28**:851-8.
28. Tschope C, Schultheiss HP, Walther T. Multiple interactions between the renin-angiotensin and the kallikrein-kinin systems: role of ACE inhibition and AT1 receptor blockade. *J Cardiovasc Pharmacol* 2002;**39**:478-87.
29. Fernandes L, Ceravolo GS, Fortes ZB *et al*. Modulation of kinin B1 receptor expression by endogenous angiotensin II in hypertensive rats. *Regul Pept* 2006;**136**:92-7.