Polymorphism in gene coding for ACE determines different development of myocardial fibrosis in rats

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In humans, the effect of angiotensin-converting enzyme (ACE) gene polymorphisms in cardiovascular disease is still controversial. In the rat, a microsatellite marker in the ACE gene allows differentiation of the ACE gene polymorphism among strains with different ACE levels. We tested the hypothesis that this ACE gene polymorphism determines the extent of cardiac fibrosis induced by isoproterenol (Iso) in the rat. We used a male F_2 generation (homozygous LL and BB ACE genotypes determined by polymerase chain reaction) derived from two rat strains [Brown-Norway (BB) and Lewis (LL)] that differ with respect to their plasma ACE activities. For induction of left ventricular (LV) hypertrophy (LVH) and cardiac fibrosis, rats were infused with Iso (5 $mg \cdot kg^{-1} \cdot day^{-1}$) or saline (control) for 10 days and euthanized at *day 1* after the last injection. The interstitial collagen volumetric fraction (ICVF), collagen I, and fibronectin content, but not collagen III content, were significantly higher in the homozygous BB rats than in homozygous LL rats. Differences in metalloprotease (MMP)-9, but not in MMP-2 activities as well as in cardiac cell proliferation, were also detected between LL and BB rats treated with Iso. LV ACE activity was higher in BB rats than LL rats and correlated with ICVF ($r = 0.61$, $P < 0.002$). No changes were observed in plasma ACE activities, ANG II plasma or LV levels, plasma renin activity, and ACE and ANG II type 1 receptor (AT1R) mRNA levels in the LV of rats with the two different ACE polymorphisms. Iso induced a similar degree of LVH [assessed by an increase in LV weight 100 per body weight, LV-to-right ventricle (RV) ratio, and LV protein content] in LL and BB rats. We concluded that rats in the F_2 generation with high plasma ACE activity developed more fibrosis but to a similar degree of LVH compared with rats with low plasma ACE activity.

renin angiotensin system; fibrosis; hypertrophy; angiotensin-converting enzyme; angiotensin-converting enzyme gene polymorphism

THE CELLULAR COMPARTMENT of the cardiac tissue is mainly represented by the cardiac myocytes, responsible for the contractile activity of the heart, and by interstitial cells, including resident fibroblasts, endothelial cells, vascular cells, and monocytes (47). Fibroblasts, the most abundant cell type in the heart, produce both extracellular matrix proteins (ECM) and metalloproteases (MMPs), thus playing a central role in the regulation of ECM (23, 48). The major ECM proteins are type I and III collagens, although other types of collagens as well as fibronectin are also present. Collagens are degraded by a family of MMPs capable of enzymatically digesting a wide variety of ECM proteins (41). The activity of MMPs is controlled at the transcriptional level as well as through activation and inhibition by other proteins, including tissue inhibitors of MMPs (12, 23). The fibrillar collagen network ensures the structural integrity of the adjoining myocytes, provides the means by which myocyte shortening is translated into ventricular pump function, and is essential for maintaining the alignment of the myofibrils within the myocytes through a collagen-integrin-cytoskeletal myofibril relation (6).

Changes of the ECM and cardiomyocyte hypertrophy exert profound effects on the contractile performance of the heart and thus on the long-term prognosis of heart disease. In turn, left ventricular (LV) hypertrophy (LVH) is also associated with the emergence of various cardiovascular diseases (28). The hypertrophic growth of cardiac myocytes may be paralleled by the occurrence of progressive cardiac fibrosis, which is the disproportionate increase in synthesis and/or inhibition of degradation of ECM proteins. Fibrosis has been classified into two groups: reparative and reactive fibrosis (50). Reparative (replacement) fibrosis or scarring accompanies myocyte death. Reactive fibrosis appears as "interstitial" or "perivascular" fibrosis and does not directly associate with myocyte death. An excessive fibroblast proliferation has also been associated with the disproportionate accumulation of ECM proteins, leading to interstitial fibrosis (49).

Hemodynamic factors and neurohormones derived mainly from the renin-angiotensin system (RAS) and adrenergic system may modulate both the extent of cardiac hypertrophy and fibrosis (4, 13, 22). Increased sympathetic activity has been observed as a central feature in patients with chronic heart failure (16, 44), and the extent of elevation of plasma norepinephrine correlates with the severity of LV dysfunction and mortality (10, 45). Adrenergic stimulation induces cardiac hypertrophy by either the α_1- or β-adrenergic pathway (2), and stimulation of the β-adrenoceptors by isoproterenol (Iso) is a well-known animal model of induced cardiac hypertrophy without systolic hypertension (2). In addition, β-adrenergic agonists not only stimulate the positive chronotropic and ino-

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tropic responses but also induce cardiac fibrosis (2). β-Adrenergic receptors have been identified on cardiac fibroblasts, and their activation leads to cell proliferation (25).

The angiotensin I-converting enzyme (ACE) is a zinc ectoenzyme involved in the metabolism of vasoactive and trophic peptides, such as angiotensins and kinins. In the cardiovascular system, ACE is mainly synthesized by the endothelial cells (38). Nevertheless, ACE is also produced by fibroblasts, smooth muscle cells, and inflammatory cells (9). Soluble ACE found in the heart is principally derived from the circulation. In addition, increased ACE expression at sites of cardiac injury has also been observed (9), and this tissue-specific overproduction of ACE could modulate the local metabolism of angiotensins and kinins. Pharmacological interferance of local generation of angiotensins within tissues has the potential to produce changes in tissue concentrations of angiotensins that are independent of the circulating level of renin and angiotensin.

In humans, the absence [deletion (D)] of a 287-bp marker in the ACE gene has been associated with greater plasma ACE levels (37) and exercise-related LV growth (29). The available evidence supports the notion that the DD-ACE polymorphism [as opposed to the insertion (I) polymorphism] adversely influences specific cardiovascular diseases but appears to be so in specific geographical areas and in particular patient subgroups (7). The identification of a microsatellite marker in the rat ACE gene has allowed the differentiation of ACE alleles among rat strains (17) and their association to different levels of plasma ACE activity (9).

To date, little is known about the association between the ACE gene polymorphism and cardiac fibrosis and/or LVH. Male rats with genetically determined plasma ACE expression [F_2 homozygous Brown-Norway (BB ACE) and Lewis (LL ACE) rats having high and low plasma ACE activities, respectively] were used to test the hypothesis that ACE gene polymorphism determines differential development of myocardial fibrosis and LVH induced by Iso.

METHODS

Experimental Design

The investigation conformed with 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. (8), in which two normotensive strains of rats, the Lewis and Brown-Norway 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 Rivers (Wilmington, MA). They were mated to produce F_1 hybrids (LB genotype). F_1 hybrids were mated to obtain the F_2 cohort as we previously described (32, 33). In these F_2 rats, genomic DNA was purified and the polymerase chain reaction (PCR) was used to amplify the microsatellite located at the 5′ end of the intron 13 inside the rat ACE gene that characterizes the different genotypes (LL, LB, or BB) (31, 32). However, Lewis and Brown-Norway rats have also been described in the literature as two strains that differ in their ability to mount type 1 and type 2 immune responses and in their susceptibility to autoimmune diseases (42).

Homozygous F_2 male rats (80–100 g) were injected subcutaneously with Iso (5 $mg \cdot kg^{-1} \cdot day^{-1}$, Sigma) or saline (control) for 10 days. Animals were divided into four experimental groups: *I*) C-LL: LL rats receiving saline injection ($n = 22$), *II*) C-BB: BB rats receiving saline injection ($n = 25$), *III*) Iso-LL: LL rats receiving Iso injection ($n = 25$), and *IV*) Iso-BB: BB rats receiving Iso injection ($n = 20$). After 10 days of treatment, animals were weighed and euthanized at *day 1* after the last injection. Blood was collected for ANG II measurement as previously described (33). Plasma sample was stored at −80°C and assayed within 1 wk. Another blood sample was collected for ACE activity (33). The heart was rapidly excised, and the 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 collagen studies, and the other was rapidly frozen in liquid N_2 and stored at −70°C until analyzed for ACE activity; fibronectin and collagen levels; metalloprotease (MMP) activities; and ACE, AT_1R, and GADPH mRNA levels.

Evaluation of Cardiac Fibrosis

Interstitial collagen volumetric fraction. Formalin-fixed heart sections were dehydrated, embedded in paraffin, sectioned 5-μm thick, and stained with hematoxylin-eosin and with the collagen-specific stain Sirius red F3BA in saturated aqueous picric acid (pH 2.0) for 90 min (18, 19). For each heart, 20 sections were examined and collagen was quantified by computer-assisted morphometry. The automated system included an image-analysis processor based on mathematical morphology software connected to a personal computer. Each field sent to the image analyzer was transmitted by a video camera connected to a microscope and transformed into a digital image (18, 33).

Collagen score. Under light microscopy one blind observer ranked the amount of collagen in the interventricular septum (IVS) and in the free LV wall (FLVW) from 1 (lowest amount) to 4 (highest amount).

Extracellular matrix protein levels. Proteins (100 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis on 8% (wt/vol) PAGE gels and transferred electrophoretically to nitrocellulose. Nonspecific binding sites were blocked with 7% (wt/vol) nonfat milk in PBS containing 0.05% (vol/vol) Tween-20 (PBST) for 30 min, and the blots were incubated with antifibronectin antibody (1:40,000 dilution in blocking solution, Biodesign International), anticollagen I antibody (1:50,000 dilution in blocking solution, Chemicon), or anticollagen III antibody (1:10,000 dilution in blocking solution, Biodesign International) overnight at 4°C. After being washed in PBST (3×10 min), the blots were incubated with horseradish peroxidase-linked anti-rabbit IgG antibodies [1:10,000 dilution in PBST containing 1% (wt/vol) nonfat milk, 1 h, room temperature]. The blots were washed again in PBST (3×10 min), and the bands were detected using ECL with exposure to film. Blots were quantified by laser scanning densitometry. Membranes were reprobed with an anti-α-actin monoclonal antibody (1:500, Dako) to confirm equal loading.

MMP activities. Assessment of MMP-2 and MMP-9 activities in the myocardium was performed by zymography (27). Protein was extracted from frozen tissue in lysis buffer containing phosphate-buffered saline (PBS, pH 7.4), 0.5% sodium deoxycolate, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet-P40 and aprotinin, and 1 μg/ml each leupeptin and pepstatin. Extracts (40 μg protein) were diluted 1× in nonreducing sample buffer [125 mM Tris·HCl (pH 6.8) 20% glycerol, 4% SDS, and 0.005% bromophenol blue] and run on 8% SDS-PAGE gels containing 0.1% gelatin. Gels were then washed twice with 2.5% Triton X-100 for 30 min at room temperature; incubated at 37°C for 18 h in 50 mM HEPES (pH 7.5), 0.2 M NaCl, 5 mM $CaCl_2$, and 20 μM $ZnCl_2$; and then subsequently stained with Coomasie blue R-250. Activated MMPs produced clear areas of gelatin lysis. Active bands were quantified by densitometry.

Assessment of cardiac cell proliferation. The tissue samples were put into 4% buffered formalin, fixed for 24 h, embedded in paraffin, and cut into step-serial sections of 5 μm. Sections were immunostained for Ki-67 or vimentin with monoclonal IgG antibodies (1/25, Dako or 1/100 Sigma, respectively) and with a biotinylated anti-mouse IgG (Dako). Sections were developed by using 3,3′-diaminobenzidine (Dako) as the cromogen, counterstained with hematoxylin,

and evaluated by microscopy. Each fibroblast stained immunohistochemically with vimentin, and Ki-67 was defined as labeled or not labeled, and afterward the percentage of positive fibroblasts was calculated.

Procollagen type I peptide and procollagen type III peptide serum levels. Procollagen type I peptide (PIP) is generally considered as a good circulating marker of extracellular collagen type I synthesis, whereas serum procollagen type III peptide (PIIIP) levels reflect simultaneously both synthesis of collagen type III molecules and degradation of collagen III fibers. Serum PIP and PIIIP levels were determined by RIA (21, 26). The interassay and intra-assay variations were 6% and 5%, respectively.

Determination of Systemic and Local Components of RAS

Plasma renin activity. This enzyme activity was measured in duplicate by using the GammaCoat Plasma Renin Activity ^{125}I-labeled RIA kit (DiaSorin) (33).

Plasma and LV ACE activities. Both ACE activities were determined as previously described (expressed as U/ml or U/mg protein, respectively) (4). Protein concentration was measured according to Bradford (3).

Plasma and LV Ang II levels. Plasma and LV ANG II levels were determined as previously reported (33) and based on the procedure described by Admiraal et al. (1). Angiotensins were separated by reverse phase HPLC according to Nussberger et al. (31). The concentrations of ANG II were measured by RIA by using an antibody generated by Dr. A. H. J. Danser (Erasmus Universität, Rotterdam). This ANG II antibody was previously characterized (31, 33). ANG II recovery from tissue was 65% and from plasma 78%. The coefficients of variation for interassay and intra-assay variances were 13% and 6.5% for ANG II, respectively (33).

Determination of ACE and AT1R mRNA levels. Total RNA was isolated from the left ventricle as previously described (32). RNA pellets were suspended in distilled water, and their concentrations were quantified by UV spectroscopy, assuming 40 μg/ml for 1 absorbance unit. RNA integrity was assessed from the intensity of the staining with ethidium bromide of 18 and 28S ribosomal RNA after agarose electrophoresis. The expression of tissue ACE was done by RT-PCR. For reverse transcription, 1 μg RNA was incubated with or without reverse transcriptase (GIBCO-BRL; Gaithersburg, MD) in a mixture containing random hexamers, deoxynucleotides, and RNAsin 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 50 μl. ACE sequences of the sense and antisense primers were 5′-CGCTACAACTTCGACTG-GTGG-3′ and 5′-TATTTCCGGGATGTGGCCAT-3′, yielding an amplification fragment of 881 bp (32). Angiotensin receptor type 1 (AT_1R) sequences of the sense and antisense primers were the following: 5′-CACCTATGTAAGATCGCTTC-3′ and 5′-GCA-CAATCGCCATAATTATCC-3′. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) sequences of the sense and antisense primers were the following: 5′-CCA TCA CCA TCT TCC AGG AG-3′ and 5′-CCT GCT TCA CCA CCT TCT TG-3′. Amplification conditions for ACE and AT1R PCR were as follows: denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and elongation at 72°C for 1 min for 28 cycles, with a final elongation step at 72°C for 7 min. Amplification conditions for GADPH were the following: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1 min for 16 cycles, with a final elongation step at 72°C for 10 min. After PCR, the amplification products were fractionated on a 1.5% (wt/wt) agarose gel and visualized by staining with ethidium bromide. Band intensities were quantified by computerized densitometry and normalized with respect to GADPH mRNA.

Evaluation of Cardiac Hypertrophy

The degrees of LVH and RV hypertrophy (RVH) were quantified by the relative LV weight ratio (RLVH, LV weight 100/body wt) and the relative right ventricular weight ratio (RRVH, RV weight 100/body wt), respectively. The ratio of LV weight to RV weight and LV protein (LVP) content at *day 1* after the last Iso or saline injection were also determined. LVP concentration was determined by Bradford's method (3).

Statistical Analysis

Data are expressed as means ± SE. Differences between strains were estimated by one-way ANOVA followed by Student-Newman-Keuls. Analysis of CVF and LV ACE activity as a function of strain, and its interaction with Iso treatment was analyzed by two-way ANOVA (SPSS 10.0) and the subsequent Student-Newman-Keuls test. Correlations between parameters of interest were determined from linear regression. Statistical significance was set at $P < 0.05$.

RESULTS

Genetic Determination of Plasma ACE Activity

Using a microsatellite marker in the ACE gene, we identified rats expressing B and L ACE polymorphism alleles in the progenies. The PAGE results of the amplified fragments in the BN and LL rats showed a clear difference in both homozygous genotypes (data not shown). Both F_0 inbred strains were homozygous, and the distribution of the ACE polymorphism in the F_1 and F_2 progenies followed a Mendelian segregation.

We next determined plasma ACE activity in the different progenies. Plasma ACE activities were 1.3-fold higher in both F_0 ($n = 14$) and F_2 ($n = 14$) homozygous BB rats compared with either F_0 ($n = 14$) or F_2 ($n = 14$) homozygous LL rats. These levels were, however, not different between F_0 and F_2 progenies in either LL or BB rats. F_1 and F_2 heterozygous LL and LB rats exhibited plasma ACE activity levels intermediate between F_0 and F_2 homozygous male rat and were not different between both progenies. No differences were detected between the LV ACE activities (0.54 ± 0.20 vs. 0.45 ± 0.16 U/mg protein) and lung ACE activities (609 ± 47 vs. 577 ± 41 U/mg protein) in the F_2 homozygous LL and BB rats, respectively.

Cardiac Hypertrophy Development in Rats with Distinct ACE Polymorphism

It is well known that β-adrenergic agonists stimulate LVH development. We therefore examined the effect of Iso on LVH

Table 1. *Effect of isoproterenol on development of cardiac hypertrophy in rats with distinct ACE gene polymorphisms*

	LL		BB	
	C	Iso	C	Iso
BW	136±4	131±4	131±3	132±3
LVW	0.35±0.01	0.48±0.02†	0.31±0.02	0.47±0.01†
RLVW	259±4	364±10†	234±10	353±6†
LVP	3.66±0.48	6.22±0.95†	2.99±0.39	5.89±0.62†
RRWW	71±4	95±4†	68±4	94±2†

Values are means ± SE; n = 20–27 rats, except for left ventricular (LV) protein, which was 9–12 rats. Homozygous F_2 Lewis (LL) and Brown-Norway (BB) rats were injected with saline (C) or isoproterenol (Iso, 5 mg·kg^{-1}·day^{-1}) per 10 days. One day after the last injection, body weight (BW, g) was determined. Animals were then euthanized, and LV weight (LVW, g), relative LV weight (RLVW, mg LV/100 g BW), LV protein content (LVP, mg) and relative right ventricular (RV) weight (RRVW) were assessed as described in METHODS. ACE, angiotensin-converting enzyme. †$P < 0.001$ vs. C. No statistical differences in LVW, RLVW, LVP, and RRVW were found between LL-Iso and BB-Iso groups.

in rats with distinct ACE polymorphisms. Table 1 shows that LVW were significantly higher in the Iso groups compared with the control groups ($P < 0.001$). No differences were observed in the body weight between all groups, but the morphometric indexes (RLVW and RRVW) of LL-Iso and BB-Iso groups were also increased with respect to their controls ($P < 0.001$) (Table 1). LVP content increased 1.7- and 2-fold above the control rats after the last Iso injection in the LL-Iso and BB-Iso groups, respectively (Table 1). Neither RLVW, LVP, nor RRVW were significantly different between the BB-Iso rats and the LL-Iso rats (Table 1). Consequently, the presence of different ACE gene polymorphisms did not induce differential LVH or RVH development in this experimental model.

Cardiac Fibrosis in Rats with Distinct ACE Gene Polymorphism

Collagen is an integral part of the normal ECM of the heart, and remodeling of collagen within the myocardial interstitium includes abnormal accumulation or degradation of collagen structures and/or specific collagen types. The predominant collagens in the adult heart are type I and III. Histochemical staining for collagen and subsequent image analysis were used to estimate the interstitial collagen volumetric fraction in the subendocardium and myocardium (Fig. 1*A*). As shown in Fig. 1*B*, we found that interstitial collagen volumetric fraction values in the control rats did not differ significantly between LL and BB genotypes. The interstitial collagen volumetric

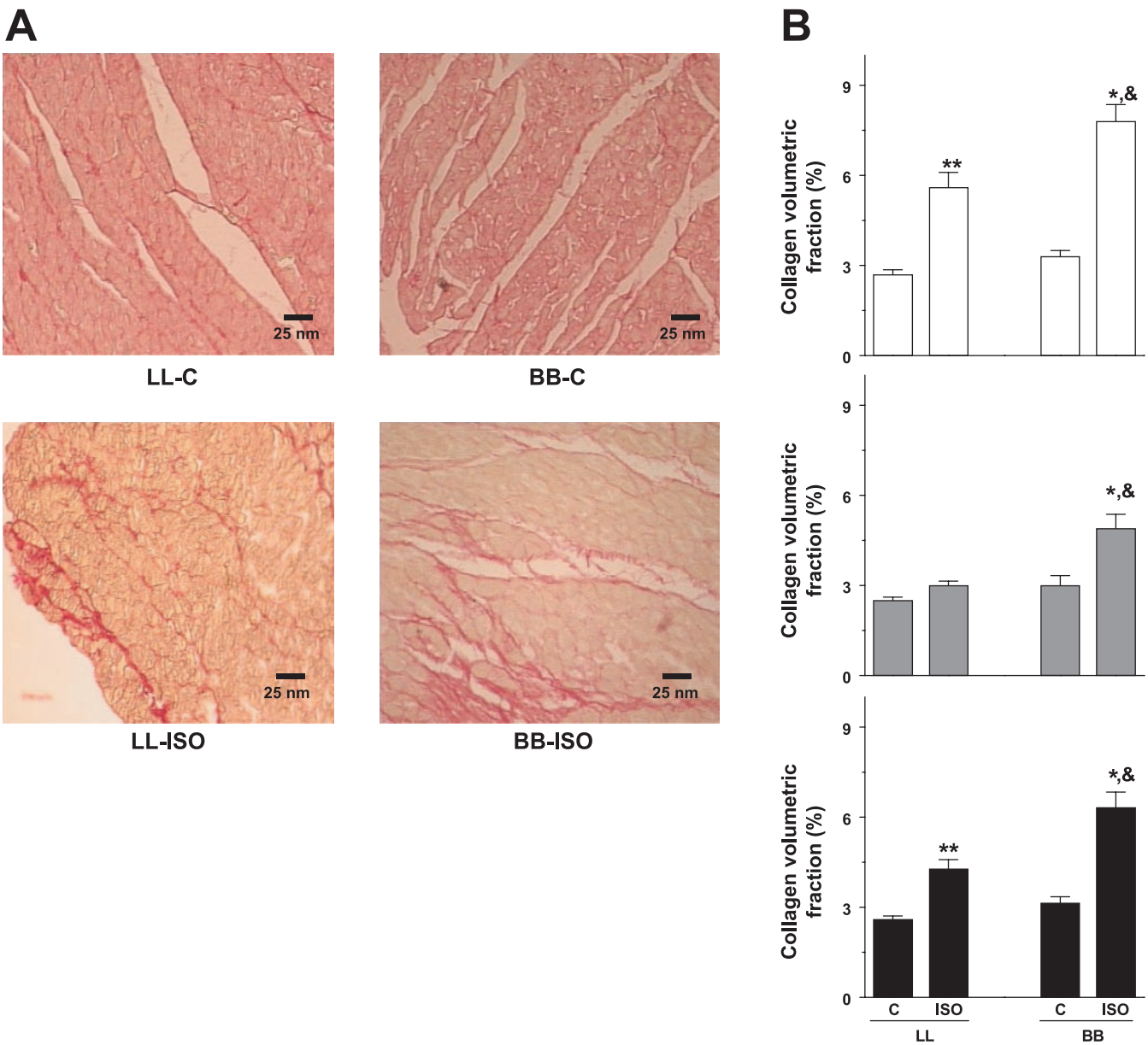

Fig. 1. Effect of isoproterenol (Iso) on left ventricular (LV) interstitial collagen volumetric fraction (ICVF) in rats with distinct angiotensin-converting enzyme (ACE) genotypes. Lewis (LL) and Brown-Norway (BB) rats were injected with saline (C) or Iso (5 mg·kg^{-1}·day^{-1}) for 10 days. One day after the last injection, LV picrosirius red sections were prepared and photographed (*A*). Subendocardial, myocardial, and total (subendocardial + myocardial) ICVF were quantified by computer-assisted morphometry (*B*). Results are means ± SE; $n = 10$–14 per group. *$P < 0.05$ vs. C, **$P < 0.01$ vs. C, &$P < 0.01$ vs. Iso-LL.

Table 2. *Effect of isoproterenol on cardiac collagen and fibronectin levels in rats with distinct ACE genotypes*

	LL		BB	
	C	Iso	C	Iso
IVS collagen	1.00±0.01	2.90±0.27*	1.00±0.01	3.00±0.26*§
FLVW collagen	1.00±0.01	2.85±0.21*	1.00±0.03	2.39±0.16*
LV collagen I	1.00±0.26	1.20±0.49	1.42±0.26	3.50±0.74*‡
LV collagen III	0.86±0.24	0.88±0.17	0.85±0.28	0.99±0.21
LV fibronectin	1.00±0.16	1.26±0.26†	1.32±0.30	2.77±0.03†‡
RV collagen I	1.00±0.37	1.43±0.43	0.74±0.17	0.86±0.23

Values are means ± SE; n = 6–9 rats per group. Homozygous LL and BB rats were injected with saline (C) or Iso (5 mg·kg^{-1}·day^{-1}) per 10 days. One day after the last injection, collagen scores were determined in the interventricular septum (IVS) and free LV wall (FLVW) as described in METHODS. LV and RV collagen I, LV collagen III, and LV fibronectin levels were also determined by Western blot as described in METHODS. Results of relative protein levels obtained by laser-scanning densitometry of the immunoblots. Membranes were reprobed with an anti-α-actin antibody to check equal loading. *$P < 0.05$ vs. C; †$P < 0.001$ vs. C; ‡$P < 0.05$ vs. LL-Iso; §$P < 0.01$ vs. FLVW (Iso-BB group).

fractions were significantly increased in treated Iso rats (both LL or BB genotypes, $P < 0.0001$), both in the subendocardium (Fig. 1*B*, *top*), myocardium (*middle*), and total (*bottom*, subendocardium + myocardium). Furthermore, we observed that inducibility of the interstitial collagen volumetric fraction by Iso is significantly higher in the BB than in the LL genotype in both tissues (Fig. 1*B*). The results also indicate that collagen scores in the interventricular septum (IVS) and in the LV (FLVW) are increased in response to Iso in both genotypes ($P < 0.05$). As a result, in Iso-treated BB rats, the amount of collagen was higher in the IVS compared with the FLVW. Quantitation of different types of collagen and fibronectin is presented in Table 2. A significant increase in collagen type I, but not in collagen type III, was found in the LV from the BB-Iso group, relative to the LL-Iso group ($F = 4.5$, $P < 0.04$). Changes in fibronectin content induced by Iso were similar to those found in collagen I (Table 2). No significant changes in the RV collagen I levels were found among the different groups (Table 2), indicating that Iso did not stimulate RV fibrosis. In LL-C, BB-C, and LL-Iso rats, the number of Ki-67-positive cells was small (Fig. 2*A*). In contrast, we observed a robust increase in Ki-67-positive cells in the LV of Iso-treated BB rats (Fig. 2*B*). No such increase was observed in LL animals (Fig. 2*B*). Most of Ki-67-positive cells detected were also vimentin-positive cells (data not shown), indicating that they can be cardiac fibroblasts.

Taken together, these results clearly demonstrate that rats with high plasma ACE activity and treated with Iso have more accumulation of collagen I and fibronectin and proliferation of fibroblasts in the LV than those animals with low plasma ACE activity.

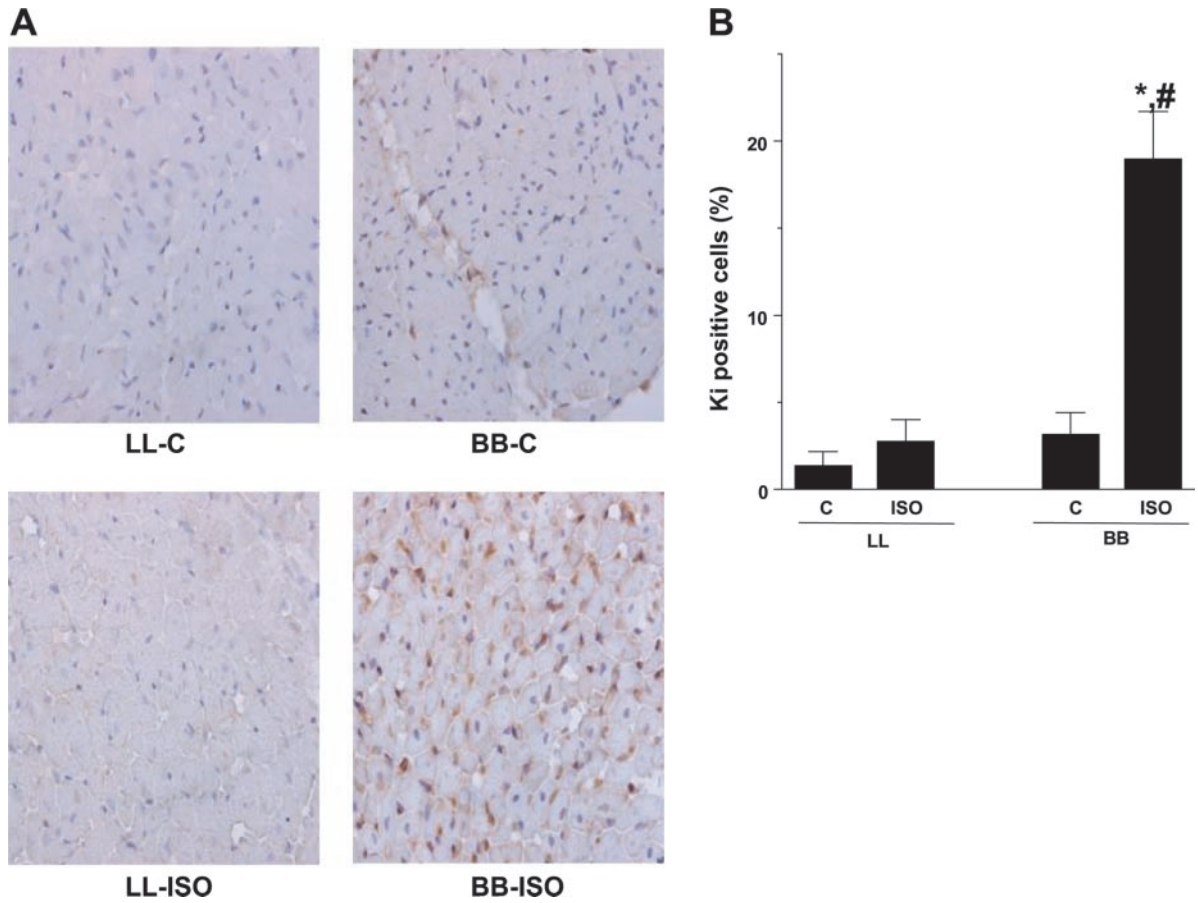

Fig. 2. Effect of Iso on cardiac cell proliferation in rats with distinct ACE genotypes. LL and BB rats were injected with saline (C) or Iso (5 mg·kg^{-1}·day^{-1}) for 10 days. One day after the last injection, Ki-67-positive cardiac cells were evaluated, photographed (*A*) and quantified (*B*) as indicated in METHODS. Results are means ± SE; n = 6–9 per group. *$P < 0.05$ vs. C and #$P < 0.05$ vs. LL-Iso.

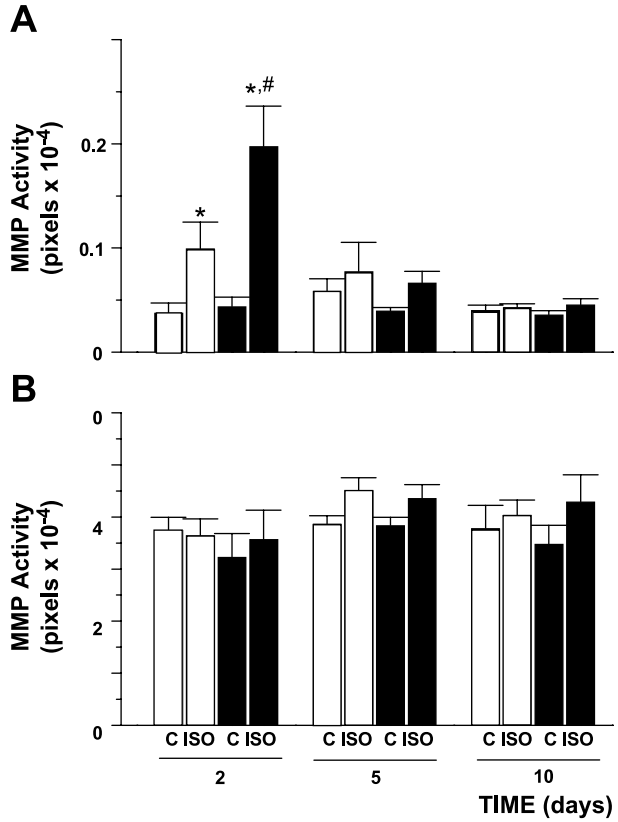

Fig. 3. Effect of Iso on LV metalloprotease activities in rats with distinct ACE genotypes. LL and BB rats were injected with saline (C) or Iso (5 mg·kg^{-1}·day^{-1}) for 1, 5, and 10 days. After the last injection, the activities of metalloproteases (MMP)-9 (*A*) and MMP-2 (*B*) in the LV were determined as described in METHODS. Results are means ± SE; $n = 3$–4 per group. $^{*}P < 0.05$ vs. C and $\#P < 0.05$ vs. LL-Iso.

Because collagen deposition is a function of both synthesis and degradation, and both collagens and their partially degraded fragments are substrates for MMPs, the observed increase in collagen accumulation suggests earlier injury with subsequent myofibroblast-based collagen accumulation at some sites while ongoing degradation at later sites of injury. On the contrary, tissue inhibitors of MMPs (or TIMPs) may prevent collagen breakdown and lead to collagen accumulation at sites of repair. Collectively, these results suggest the existence of a heterogenous response based on time-dependent injury and repair. After treatment of BB or LL rats with Iso or its vehicle for 2, 5, or 10 days, the gelatinolytic activity of MMP-2 and MMP-9 in the LV was examined by gelatin zymography. Figure 3*A* indicates that the gelatinolytic activity of MMP-9 (92 kDa) showed a significative increase at *day 2* in both the LL-Iso and BB-Iso groups, relative to controls. Furthermore, MMP-9 activity at *day 2* was significantly higher in the BB-Iso than LL-Iso ($P < 0.05$). No changes in MMP-2 (72 kDa) activity were observed between the different groups (Fig. 3*B*).

Collectively, these data demonstrate the presence of a different development of cardiac fibrosis in rats with distinct ACE polymorphisms.

Because PIP and PIIIP have been described as circulating markers of extracellular collagen type I synthesis (PIP) and both synthesis of collagen type III molecules and degradation of collagen III fibers (PIIIP), we next evaluated the effect of Iso on PIP and PIIIP serum levels in both BB and LL rats. Figure 4 depicts the effect of Iso administration on these parameters. Iso was associated with an increase in the serum concentration of PIP in both BB rats (1.3 ± 0.4 vs. 2.3 ± 0.3, $P < 0.05$) and LL rats (2.0 ± 0.6 vs. 3.2 ± 0.4, $P < 0.05$). No statistically significant difference was found between BB-Iso and LL-Iso rats (Fig. 4). In contrast, serum PIIIP levels did not change in the Iso groups compared with their controls (Fig. 4).

Effect of Iso on Some RAS components in Rats With Distinct ACE Polymorphisms

The main rate-limiting step controlling the activity of the circulating RAS is the release of active renin from juxtaglomerular cells of the afferent glomerular arterioles in the kidney. Hormones, neurotransmitters, autacoids, and drugs such as Iso that raise the intracellular production of cAMP stimulate renin mRNA levels and renin secretion. As shown in Table 3, plasma renin activity was increased in the LL-Iso (114%) and BB-Iso rats (99%) after Iso treatment compared with the untreated

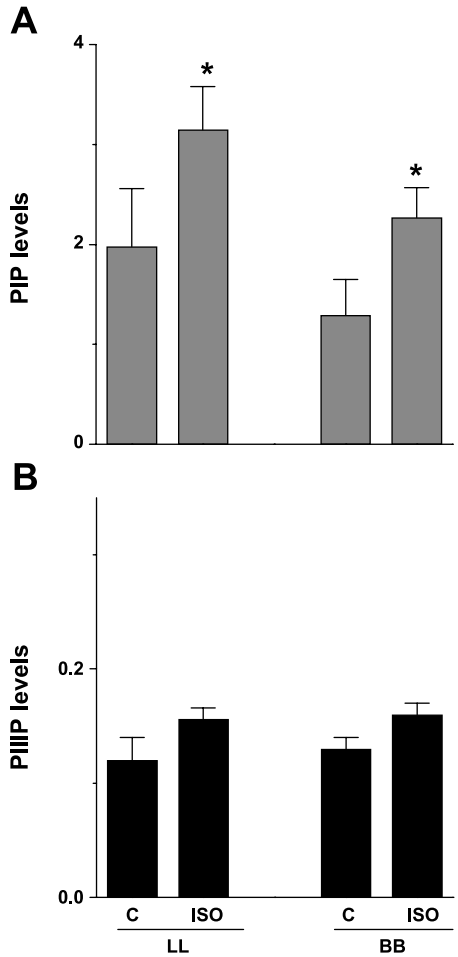

Fig. 4. Effect of Is on the serum procollagen type I peptide (PIP) and procollagen type III amino-terminal peptide (PIIIP) levels in rats with distinct ACE genotypes. LL and BB rats were injected with saline (C) or Iso (5 mg·kg^{-1}·day^{-1}) for 10 days. One day after the last injection, serum was obtained and carboxy-terminal PIP (*A*) and PIIIP (*B*) concentrations were determined by RIA. Results (in μg/mg protein for PIP and U/ml for PIIIP) are means ± SE; $n = 9$–11 animals per group). $^{*}P < 0.05$ vs. C.

Table 3. *Effect of isoproterenol on some components of the circulting and tissue renin-angiotensin system in rats with distinct ACE genotypes*

		LL		BB	
	n	C	Iso	C	Iso
Plasma renin	10	1.66±0.23	3.56±0.82*	1.89±0.31	3.76±0.61*
Plasma ACE	11	119±8	109±6	242±9§	240±1
Plasma ANG II	6	238±31	175±31	422±21§	390±30‡
LV ACE	11	0.54±0.20	2.97±0.40†	0.45±0.16	5.79±0.84†‡
LV ANG II	6	2.68±0.25	2.43±0.23	1.60±0.12§	1.67±0.76
LV ACE mRNA	6	1.86±0.19	1.46±0.2	2.04±0.36	2.52±0.36
LV AT_1R mRNA	6	7.34±2.02	7.95±1.73	10.1±1.67	8.9±0.21
Lung ACE	11	609±47	764±44†	577±41	732±26†

Values are means ± SE; *n*, number of animals per group. Homozygous LL and BB rats were injected with saline (C) or Iso (5 $mg \cdot kg^{-1} \cdot day^{-1}$) per 10 days. One day after the last injection, plasma renin activity (in $ng \cdot ml^{-1} \cdot h^{-1}$) was determined by RIA. ACE activities in plasma (in U/ml), LV (U/mg prot) and lung (U/mg prot) were determined by spectrofluorimetry. 1U = mmol His-Leu per min. ACE, AT_1R and GAPDH mRNA levels were determined by RT-PCR. Plasma and LV ANG II levels were determined by RIA previous separation by HPLC. *$P < 0.05$ vs. C. †$P < 0.01$ vs. C, ‡$P < 0.05$ vs. LL-Iso, §$P < 0.05$ vs. LL-C.

groups. However, there was no difference between the BB and LL ACE polymorphisms in this parameter. Plasma ACE activities were not different between the Iso and control groups in both F_2 rats with either of the ACE polymorphisms (Table 3). The LV and lung ACE activities increased significantly after treatment with Iso in both the BB-Iso and LL-Iso groups (Table 3, $P < 0.05$). However, LV ACE activity was higher in the BB-Iso group than in the LL-Iso group (Table 3; $F = 9.4$, $P < 0.004$). LV ACE and AT_1R mRNA levels were not significantly different in the BB-Iso and LL-Iso groups compared with control rats (LL-C and BB-C groups) (Table 3).

Plasma ANG II levels were significantly higher ($P < 0.05$) in the BB-C rats than in the LL-C rats (Table 3), indicating that there were differences between the ACE genotypes. However, Iso did not increase plasma ANG II levels in either LL or BB rats compared with their respective controls (Table 3). No significant differences were observed in the LV ANG II concentrations among all groups (Table 3).

Finally, Fig. 5 shows that interstitial collagen volumetric fraction and LV ACE activities were significantly correlated ($r = 0.66$, $P < 0.002$).

DISCUSSION

In the present work we investigated the effects of chronic administration of Iso on myocardial fibrosis in homozygous males of two inbred strain (F2) of BB × LL rats, which differ with respect to plasma ACE activities and circulating ANG II levels (BB > LL) but not in cardiac or lung ACE activities. The main finding of this study was that Iso stimulates higher LV fibrosis in BB than in LL rats while inducing a similar degree of cardiac hypertrophy.

There are almost no studies concerning the relationship between ACE polymorphisms and cardiac fibrosis. In humans, the DD genotype of the ACE gene is closely associated with the presence of nephropathy in diabetic subjects (20) and probably with the development of interstitial fibrosis. In agreement, our results support a role of ACE genotype in the development of cardiac fibrosis. In the LV tissue, we found that administration of high dosages of Iso was followed by increases in interstitial collagen volumetric fraction, collagen I and fibronectin contents, cardiac cell proliferation (mainly fibroblasts), and the activity of MMP-9 but with no changes in the collagen III content or MMP-2 activity. All these fibrotic markers were dependent on the ACE genotype, being higher in BB rats than in LL rats. This remarkable difference in cardiac fibrosis between both strains can be explained either by a different fibroblast proliferation and/or activation of MMP-9.

Moreover, we did not find a correlation between the amount of LV fibrosis and serum PIP concentrations (a circulating marker of collagen type I synthesis) as previously reported to occur during experimental hypertension (11). PIIIP levels, the other serological marker of collagen III turnover, were also similar in the four experimental groups, and this observation agrees with our results on the levels of LV collagen type III. In summary, the systemic markers of fibrosis described above were not associated with LV fibrosis induced by Iso.

The mechanism whereby Iso increases cardiac ACE activity in LL and BB rats was not addressed in detail in our study. There is some evidence that trophic cardiac responses to sympathetic stimulation in vivo might depend on the circulatory or cardiac RAS. Because no RV fibrosis was observed in either of the two strains, our results suggest that local factors, rather than systemic ones are more likely to be involved in this response.

Iso administration had differential effects on cardiac and systemic RAS components. Similar activation of plasma renin was observed after Iso stimulation in both ACE genotypes, whereas there was no effect on plasma ACE activity or circulating ANG II levels. Iso increased LV ACE activity in rats with either ACE genotype. However, whereas the effect was more pronounced in rats with the BB ACE genotype, these results were not associated with changes in LV ACE mRNA, LV AT_1R mRNA, or LV ANG II levels. These data also confirm our previous observations (32) in Sprague-Dawley rats, where Iso induced a differential expression of LV, lung, and plasma ACE. Taken together, our findings support a role for the cardiac, rather than circulatory RAS, in the cardiac fibrotic response to β-adrenoceptor stimulation.

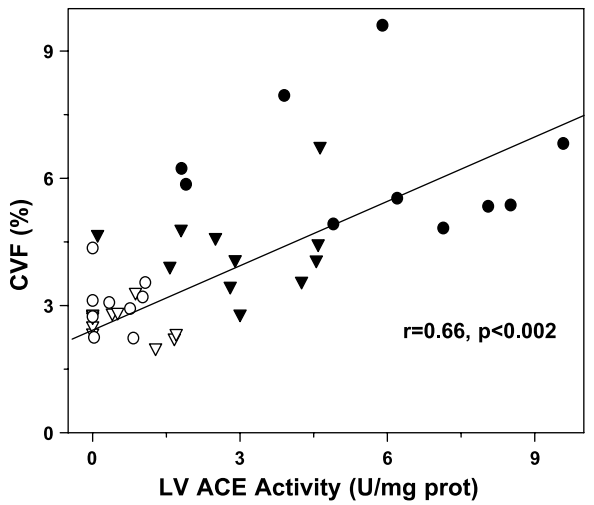

Fig. 5. Correlation between LV ACE activity and ICVF. Open triangle, C-LL; solid triangle, Iso-LL; open circle, C-BN; and solid circle, Iso-BN.

ACE is involved in the metabolism of vasoactive and trophic peptides, such as angiotensins and kinins (46). Because there was no correlation between LV ACE activity and LV ANG II levels in rats with different ACE genotypes, further studies will be needed to determine whether higher ACE activity induced by Iso influences the metabolism of ANG-(1,7) and/or kinins and its relationship with the development of LV fibrosis. Some of these peptides have antifibrotic properties in cardiac tissue.

Questions regarding the mechanism(s) by which collagen I, fibronectin, and collagenase production are regulated essentially focus on the cardiac fibroblast. Different studies have shown that profibrotic agents (i.e., ANG II, endothelin, and TGF-β) and antifibrotic substances (i.e., kinins) regulate collagen and collagenase gene expression and protein synthesis, as well as collagenolytic activity and cardiac fibroblast proliferation (14, 27, 30, 43). Previous studies (35) have shown that Iso also increases TGF-β expression and cardiac fibroblast proliferation, whereas whether or not Iso modulates cardiac kinin and/or ANG-(1,7) levels remains unexplored. This last hypothesis needs to be tested because our results suggest that a relationship exists between LV ACE activity and interstitial collagen volumetric fraction, and there are no differences in the LV ANG II levels between all groups.

Overexpression of extracellular matrix components during either reparative fibrosis (observed in LVH induced by Iso) or adaptive fibrosis (observed in early pressure overload) may have a significant impact in both systolic and diastolic function, as well as electrical activity and intramyocardial perfusion of the heart (5, 19). Future studies should explore whether the difference in cardiac fibrosis between Iso-BB and Iso-LL rats affects differently LV systolic and/or diastolic function.

As in previous studies (2, 18), we also found that Iso stimulated the development of LVH. The present study extends these findings by showing that a similar degree of LVH was observed in rats with high or low plasma ACE activity. In humans, some experimental evidence suggested an association between the ACE I/D polymorphism and LVH (40). However, recent careful studies have found that this association was inconsistent when additional populations were investigated (24, 36, 39).

Some limitations of the present work were the role of other neurohormonal substances were not evaluated here, such as ANG-(1,7) and kinins. Particularly, it would have been of interest to study aldosterone levels, because these are increased by Iso administration (15), and this hormone has also been proposed to stimulate reparative fibrosis.

In conclusion, we have shown that Iso induced more myocardial fibrosis, but a similar degree of LVH, in rats with the BB ACE genotype (high-plasma ACE activity) than in those with the LL ACE genotype (low-plasma ACE activity). This differential fibrotic response correlated with the levels of LV ACE activity, increased cardiac proliferation, and MMP-9 activity, and in the long term it could determine a significant difference in myocardial remodeling.

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