Differential response of cardiac fibroblasts from young adult and senescent rats to ANG II

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ANG II exerts a wide range of cardiovascular effects that have a bearing on the pathophysiology of cardiac hypertrophy and heart failure (17, 19, 29, 33). Several studies have shown that ANG II promotes myocyte hypertrophy, increases myocardial collagen synthesis, and is mitogenic to neocardiomyocytes (1, 13, 20, 33). Several studies have shown that ANG II promotes myocyte hypertrophy, increases myocardial collagen synthesis, and is mitogenic to neocardiomyocytes (1, 13, 20, 33). Several studies have shown that ANG II promotes myocyte hypertrophy, increases myocardial collagen synthesis, and is mitogenic to neocardiomyocytes (1, 13, 20, 33).

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Cardiac fibroblasts from young adult and old rats and 2) to compare the effects of ANG II on production of collagen and transforming growth factor-β (TGF-β) in fibroblasts from young adult and aged rat heart.

MATERIALS AND METHODS

Isolation of cardiac fibroblasts from senescent rats. Cardiac fibroblasts from male Wistar rats (young, 2–3 mo; old, 24 mo) were isolated by enzymatic digestion of ventricular tissue, as described earlier (39). Briefly, the heart was excised, minced, and washed in PBS. The tissue was digested at 37°C in digestion medium containing a mixture of collagenase B [115 (young) or 150 (old) mg/100 ml; Boehringer-Mannheim], trypsin (50 mg/100 ml; Sigma Chemical, St. Louis, MO), and pancreatin (60 mg/100 ml; Sigma Chemical) for 10 min with constant shaking. Cells from fourth to tenth digestions, each of 10 min duration, were pelleted and plated on 35-mm plates in medium consisting of DMEM, medium-199, and FCS at 7:2:1. After incubation at 37°C in a CO2 incubator for 150 min, unattached cells were discarded, and attached cells (mostly fibroblasts) were washed and grown in the plating medium. Confluent cultures were passaged three or four times on 100-mm dishes using trypsin.

The fibroblastic nature of the cells was ascertained by immunocytochemistry using polyclonal anti-factor VIII (Dako), monoclonal anti-desmin (Sigma Chemical), and polyclonal anti-vimentin (Polysciences) antibodies for identification of endothelial cells, smooth muscle cells, and fibroblasts, respectively (43). A streptavidin alkaline phosphatase-based protocol with Vector Red (Vector Laboratories, Burlingame, CA) as a chromogen was used.

Isolation of RNA and quantification of renin, angiotensinogen, and AT1A/B receptor mRNA. Confluent cultures were serum deprived for 24 h and incubated in serum-free medium for another 24 h with 100 nM ANG II. The ANG II was replenished after 12 h. Total RNA was extracted as described by Chirgwin et al. (5) using guanidinium isothiocyanate. Absolute amounts of renin, angiotensinogen, and AT1A/B mRNA in RNA samples were determined using a multiplex RT-PCR titration assay, as described elsewhere (9). Fixed amounts of total RNA (250 ng/tube) were coreverse transcribed with 2:1 serial dilutions of angiotensinogen (100–1,563 fg), renin (12.5–0.195 fg), AT1A/B (762–11.91 fg), and elongation factor-1 (EF-1; 125–1.95 pg) competitor RNA. Coamplification was performed using Taq polymerase (2.5 U/reaction) for 15 cycles with angiotensinogen and renin primers (100 pmol each) or 12 cycles with AT1A/B primers (100 pmol), after which EF-1a primers (100 pmol) were added to the reaction, and a total of 35 amplification cycles was performed. The PCR products were separated by electrophoresis on 6% polyacrylamide gels, after which gels were stained for 45 min with Vistra Green (diluted 1:10,000). Intensities of PCR bands were quantified by fluorescent scanning (Storm 640; Molecular Dynamics, Sunnyvale, CA). Amounts of target mRNA in samples were determined as previously described (8).

Measurement of TGF-β levels in the medium. Confluent cultures were serum deprived for 24 h and incubated in serum-free medium for another 24 h with 100 nM ANG II. TGF-β levels in the medium were measured by a quantitative sandwich enzyme immunoassay technique, using commercial kits (R&D Systems) and the manufacturer’s protocol.

Measurement of net collagen production. Confluent cultures were serum deprived for 24 h and incubated in serum-free medium for another 24 h with 100 nM ANG II. Net collagen production (present in cell monolayer and medium) was determined by a hydroxypyroline-based assay, as described previously (43).

Statistical analysis. All data are expressed as means ± SD. To evaluate effects of ANG II on young and old rats, pairwise comparisons were made by Student’s t-test, and significance was determined at P < 0.05. Data were also analyzed by two-way ANOVA with age, drug, and age × drug interaction terms, and P < 0.05 was considered statistically significant.

RESULTS

Highly enriched cultures of cardiac fibroblasts were prepared from 2- to 3-mo-old or 24-mo-old rats by passing three or four times the cells that adhered to the culture dish during the preplating procedure after isolation. Cardiac myocytes were ruled out on morphological grounds. The cultures were negative for factor VIII and desmin, ruling out contaminating endothelial cells and smooth muscle cells. The cells formed a monolayer, had typical rat cardiac fibroblast morphology, and stained positive for vimentin, confirming their fibroblastic nature (=99% purity). Importantly, cultures (young and old) took about the same time to become confluent so that the cells from both age groups remained in culture for the same number of days at each passage. This precluded any influence of age-related differences in time to confluence on cellular metabolism and response to stimuli. To compare the profibrogenic effects of ANG II on fibroblasts from young adult and old rats, cultures at passage three or four were used to evaluate direct transcriptional control of ANG II over renin, angiotensinogen, and the AT1 receptor. Quantitative RT-PCR was employed to measure transcript levels with precision. Figure 1A shows the separation of multiplex PCR products for the AT1A/B receptor. Effects of ANG II on TGF-β production and net collagen production were also assessed.

Dostal et al. (10, 11) had demonstrated that cardiac fibroblasts from neonatal rats express renin mRNA. This study extends their observations and shows that cardiac fibroblasts from adult rats (both young and old) also express the transcript for renin. ANG II, at 100 nM, did not have any effect on renin or angiotensinogen mRNA levels in either young or old rats (Fig. 1, B and C). However, ANG II exerted differential effects on AT1 mRNA expression in young and old rats (Fig. 1D), inducing a nearly twofold increase in young rats (P < 0.01) and a 2.5-fold decrease in old rats (P < 0.001).

ANG II has been shown to increase TGF-β mRNA levels and bioactivity in neonatal and adult rat cardiac fibroblasts (14, 22). In the present study, experiments were carried out to compare the effects of ANG II on TGF-β production in young and old rats. ANG II, at 100 nM, caused a more than fourfold increase in TGF-β production in young rats (P < 0.001). However, ANG II had no effect on TGF-β production in old rats (Fig. 2).

Because cardiac fibroblasts are the main source of collagens, the effect of ANG II on net collagen production (collagen deposition) was determined. The cell monolayer and medium were pooled and used for determination of collagen-associated hydroxyproline content. Results presented in Fig. 3 show that ANG II
significantly enhanced net collagen production in young (P < 0.001) and old (P < 0.01) rats, but the extent of stimulation in old rats was only 14.2% compared with the 31% increase in young rats. Analysis using two-way ANOVA indicates that the age/ANG II interaction was significant for AT1 mRNA expression and TGF-β (P < 0.001) but not net collagen production.

**DISCUSSION**

The presence of RAS components in cardiac fibroblasts, ANG II-induced hyperplasia of neonatal rat cardiac fibroblasts, and stimulation of AT1 receptors resulting in increased gene expression for extracellular matrix proteins in adult rat cardiac fibroblasts suggest a role for RAS in regulating cardiac fibroblast activity. However, information regarding regulation of RAS in cardiac fibroblasts is sparse. Atrial natriuretic peptide and isoproterenol are reported to be positive regulators of renin and/or angiotensinogen mRNA expression in cardiac fibroblasts.

![Fig. 1. A: separation of multiplex PCR products for the angiotensin type 1A/1B (AT1A/B) receptor. PCR products were separated as described under MATERIALS AND METHODS. EF-1, elongation factor-1; Com, competitor. B–D: effect of ANG II on expression of renin, angiotensinogen, and AT1 mRNA in cardiac fibroblasts. Confluent cultures at passage 3 or 4 were serum deprived for 24 h and incubated in serum-free medium for another 24 h with 100 nM ANG II. The ANG II was replenished after 12 h. Isolation of RNA and quantification by RT-PCR of renin (B), angiotensinogen (C), and AT1 mRNA were carried out as described under MATERIALS AND METHODS. All data are expressed as means ± SD. Values in parentheses indicate the sample size. Student's t-test was used for pairwise comparison to evaluate ANG II effects on young (Y) and old (O) rats. *Y vs. Y + ANG II, P < 0.01; *O vs. O + ANG II, P < 0.01. By 2-way ANOVA, age × ANG II interaction for AT1 mRNA expression was significant (P < 0.001; D).](http://ajpheart.physiology.org/)

![Fig. 2. Measurement of transforming growth factor-β (TGF-β) levels in medium. Confluent cultures at passage 3 or 4 were serum deprived for 24 h and incubated in serum-free medium for another 24 h with 100 nM ANG II. TGF-β levels were measured in the medium by a quantitative sandwich enzyme immunoassay technique, using commercial kits (R&D Systems) and the manufacturer's protocol. Values were computed on a per dish basis and expressed as means ± SD of 5 separate determinations [expressed as a percentage of control (=100)]. Values ranged from 64.4 to 141.4 (Y), 375.2 to 488.6 (Y + ANG II), 79.8 to 191.8 (O), and 71.4 to 161 (O + ANG II) pg/ml medium. Student's t-test was used for pairwise comparison to evaluate ANG II effects on Y and O. *Y vs. Y + ANG II, P < 0.001. *O vs. O + ANG II (not significant). By 2-way ANOVA, age × ANG II interaction for TGF-β production was significant (P < 0.001).](http://ajpheart.physiology.org/)
neonatal cardiac fibroblasts (11, 23). Negative feedback regulation of renin and angiotensinogen expression by ANG II in neonatal cardiac fibroblasts (11), in contrast to upregulation of these transcripts by ANG II in cardiac myocytes (27), would point to cell-specific differences in the regulation of RAS components within the myocardium. Given the marked alterations in cardiac structure and morphology associated with aging, and the role of fibroblasts in myocardial remodeling, it would be reasonable to expect changes in the regulation of RAS activity in cardiac fibroblasts from the senescent myocardium.

The present study has for the first time developed a model of cultured cardiac fibroblasts isolated from aged rats to examine senescence-associated changes in the expression of profibrogenic factors in response to ANG II. The use of such a model permitted evaluation of age-associated changes in cellular responses to ANG II in the absence of complicating systemic effects such as hemodynamic stress.

The present findings demonstrate, for the first time, that cardiac fibroblasts from senescent rats express the transcripts for renin, angiotensinogen, and the AT1 receptor. Previously, expression of renin mRNA has been demonstrated in neonatal rat cardiac fibroblasts (11) but not cardiac fibroblasts from adult rats. The question of the cardiac origin of renin is not settled yet. Literature on the subject indicates that both renin uptake from the plasma and local renin synthesis may be important (12). The relative contributions of uptake vs. local synthesis of renin and the factors that regulate these processes remain to be evaluated. This study examined the possibility that ANG II may regulate RAS component expression in cardiac fibroblasts (Fig. 1, B-D). This is particularly relevant in old rats, since the intracardiac ANG II-forming pathway has been reported to be activated in the senescent myocardium (16), raising the possibility of enhanced ANG II effects on fibroblasts. It was observed that ANG II does not have any effect on the expression of angiotensinogen or renin in either young or old rats. It has been reported that ANG II does not have any appreciable effect in vitro on the expression of angiotensinogen, renin, and ACE genes but significantly downregulates AT1 expression in neonatal rat cardiac fibroblasts (27). In the present study, ANG II caused a significant upregulation of AT1 mRNA expression in young rats but downregulated AT1 expression in old rats (Fig. 1D). Although the expression of AT1 receptor was determined only at the mRNA level, the differential effect of ANG II was striking. The effect of ANG II on AT2 expression in cardiac fibroblasts was not assessed in the present study. Although there are two major ANG II receptor subtypes, AT1 and AT2, traditional ANG II effects are attributed to AT1, and the precise role of AT2 is unclear (3, 31).

ANG II was found to stimulate TGF-β production in young rats, whereas TGF-β production in old rats remained unaffected in response to ANG II (Fig. 2). Another significant finding was that the stimulatory effect of ANG II on net collagen production is less marked in old compared with young rats (Fig. 3). Besides its stimulatory effect on collagen synthesis, ANG II has been shown to inhibit collagenase activity in cultured adult rat cardiac fibroblasts (4). Because net collagen production is related to turnover, the relative contributions of synthesis and degradation to the observed ANG II effects in young and old rats remain to be determined.

The downregulation of AT1 expression and the lack of an effect on TGF-β production in old rats in response to ANG II may have important implications in relation to the extent of fibrosis in the aged myocardium. It is recognized that ANG II acts via the AT1 receptor to stimulate collagen synthesis in adult cardiac fibroblasts (6, 43). Furthermore, it has been suggested that TGF-β may mediate the stimulatory effect of ANG II on collagen synthesis (22). In light of these observations, increased production of ANG II in the senescent heart (16) would be expected to exert stimulatory paracrine effects on collagen synthesis in fibroblasts. However, contrary to expectation, collagen synthesis per se has been shown to be depressed in the aged heart (2, 28), in which reduced expression and activity of matrix metalloproteinases are suggested to contribute to myocardial fibrosis (34). Findings of the present study raise the possibility that downregulation of AT1 expression by ANG II and lack of a stimulatory effect of ANG II on TGF-β production in old rats may blunt the effect of augmented local production of ANG II on net collagen formation, thereby limiting the extent of ANG II-mediated fibrosis in the aged heart. The postulation is consistent with the less marked stimulatory effect of ANG II...
ANG II on net collagen production in old compared with young rats (Fig. 3). Importantly, such a mechanism would be protective, since increasing myocardial fibrosis is an important cause of contractile dysfunction and arrhythmias during aging (21). It is also interesting because several age-associated changes, such as the decline in sarcoplasmic reticulum Ca\(^{2+}\)-ATPase activity (26) and diminished capacity of the heart to undergo compensatory hypertrophy in response to hemodynamic overload (41), have adverse effects and contribute to higher incidence of heart failure in the aged.

In summary, a new model has been set up to study alterations in the expression of profibrogenic factors in cardiac fibroblasts from the aged myocardium in the absence of systemic effects. The findings reveal significant modification of the response of old rats to ANG II. The downregulation of AT1 by ANG II, the inability of ANG II to enhance TGF-\(\beta\) production, and attenuation of the stimulatory effect of ANG II on net collagen production in cardiac fibroblasts from the aged rat may represent a compensatory mechanism to limit fibrogenesis in the context of increased local production of ANG II in the aged heart.

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