

# Role of Myocytes in Myocardial Collagen Production

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Excessive collagen deposition may cause abnormal stiffness of the heart during hypertrophy and heart failure. The potent vasoconstrictor angiotensin II seems, via an unknown mechanism, to stimulate collagen production. This study describes the in vitro and ex vivo effects of [Sar<sup>1</sup>]Ang II on collagen production by fibroblasts in culture and in beating, nonworking heart preparations. The effects of [Sar<sup>1</sup>]Ang II on isolated rat hearts or rat heart fibroblasts were determined by quantifying transcript levels of collagen phenotypes I and III through videodensitometry after Northern blot analysis with specific cDNA probes (collagen [P α<sub>2</sub>(I)] rat α<sub>2</sub>[I] probe for type I and human skin fibroblast α<sub>1</sub>[III] probe for type III). When [Sar<sup>1</sup>]Ang II was added in vitro to neonatal or adult 28-week-old Wistar-Kyoto rat heart fibroblasts, questionable stimulation in the mRNAs of types I and III occurred. In contrast, when 10<sup>-8</sup> mol/L [Sar<sup>1</sup>]Ang II was added to beating, nonworking Wistar-Kyoto rat heart preparation ex vivo, a 1.5- to 2.5-fold stimulation of collagen mRNAs of phenotypes I and III was observed. When neonatal fibroblasts were cocultured with neonatal myocytes in vitro, with 10<sup>-10</sup> mol/L [Sar<sup>1</sup>]Ang II added, there was no stimulation of either phenotype. However, significant stimulation of both collagen transcripts was recorded when 10<sup>-10</sup> mol/L [Sar<sup>1</sup>]Ang II was added to adult fibroblasts cocultured with either neonatal or adult myocytes. Our data suggest that factors produced by myocytes are necessary for upregulation of collagen genes in vitro and demonstrate that fibroblast-myocyte cross-talk is required for Ang II-induced collagen upregulation. (*Hypertension*. 2001;37:833-840.)

**Key Words:** angiotensin II ■ cardiac myocytes ■ collagen ■ fibroblasts ■ hypertrophy

Biosynthesis of the heart's collagen matrix has been shown to be regulated under various physiological and pathologic conditions. Excessive deposition of collagen may be responsible for abnormal tissue stiffness and altered cardiac function during hypertrophy and heart failure. Workers at our laboratory have shown that during the development of hypertrophy in hypertension, the alteration of collagens and their phenotypes occurs especially during the chronic phase of hypertrophy in both humans and rats.<sup>1</sup> Mukherjee and Sen<sup>2</sup> first showed the diverse effects of antihypertensive treatment on collagen metabolism. They showed that captopril causes regression of hypertrophy associated with reduction and normalization of blood pressure and reversal of the alteration of collagen phenotypes. The molecular mechanism of such a process is not known. Butt et al<sup>3</sup> observed that collagen production was stimulated by some of the growth factors tested (in order of potency, transforming growth factor [TGF]-1 > platelet-derived growth factor > insulin-like growth factor > basic fibroblast growth factor). Recently, evidence has been presented that defines a role of angiotensin II (Ang II), a potent vasoconstrictor, in stimulation of collagen gene expression and protein turnover in cultured cardiac fibroblasts.<sup>4-6</sup>

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In addition to having a potent vasoconstrictive effect, Ang II seems to work as an autocrine/paracrine factor that regulates the growth of local tissues such as blood vessel, kidney, and heart.<sup>7-10</sup> A close relationship has been suggested between Ang II generated at sites of injury and expression of the profibrotic cytokine represented by the TGF-β family of peptides. The binding of TGF-β<sub>1</sub> with its receptors on fibroblasts regulates their expression of type I collagen and tissue-induced metalloprotease and favors fibrous tissue formation.<sup>11,12</sup> Recently, Kim et al<sup>13</sup> showed that Ang II induces collagen type I and type III mRNA expression in vivo in rats when they were infused with 200 ng Ang II · kg<sup>-1</sup> · min<sup>-1</sup>. Stimulation could be observed only after 3 days of continuous infusion and gradually plateaued after 7 days. Zeydel et al<sup>14</sup> have shown that the deposition of type I collagen occurred only when the medium was supplemented with ascorbate and was independent of the age of the animals.

To date, no data on the effects of Ang II on collagen gene expression are available from cardiac fibroblasts of adult (>20 weeks old) rats or from isolated whole rat heart preparation. However, research on older animals is important because altered cardiac function and heart failure do not

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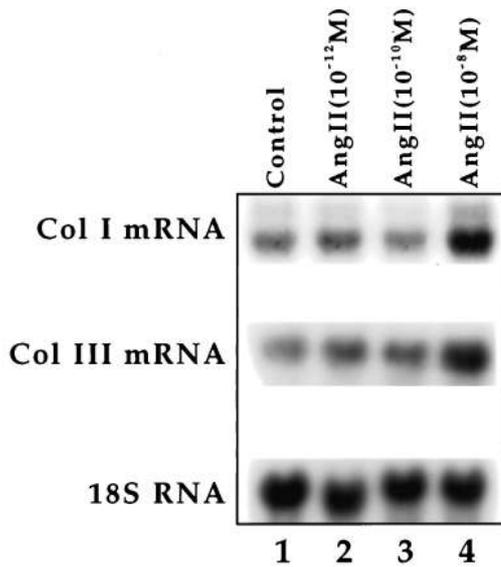
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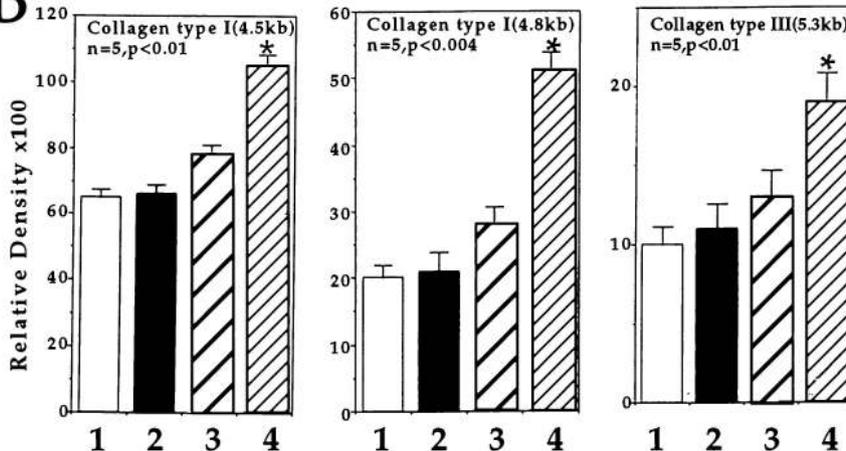
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**Figure 1.** Effect of Ang II perfusion on the beating, nonworking heart preparation ex vivo. A, Autoradiograph showing the 2 transcripts (4.5 and 4.8 kb) of collagen (Col) type I mRNA and the 5.3-kb transcript of collagen type III mRNA. Isolated beating, nonworking rat hearts were perfused for 2 hours in the absence (control) and presence (treated) of  $10^{-12}$ ,  $10^{-10}$ , and  $10^{-8}$  mol/L [ $\text{Sar}^1$ ]Ang II. An 18S RNA oligoprobe was used to normalize our results. B, Quantification of the different collagen transcripts. Dose-dependent significant stimulation of all of the collagen transcripts was found when Ang II was added to the ex vivo preparation.

B



usually occur in young animals. Thus, the objectives of the present study were to examine, in parallel, the effect of [ $\text{Sar}^1$ ]Ang II on collagen phenotypes in vitro with fibroblasts in culture and ex vivo with an isolated beating, nonworking heart preparation in 28-week-old rats. Our data showed a distinct and significant difference between ex vivo and in vitro results with fibroblasts alone in culture and combinations of fibroblasts and myocytes, the 2 major types of cells present in the heart, from both neonatal and adult rats, in which a significant stimulation of collagen transcripts was observed; this suggests the participation of myocytes in modulation of collagen production.

## Methods

### Materials

All tissue culture reagents used in this study were purchased from Life Technologies, Inc. All radiochemicals were obtained from DuPont-New England Nuclear. All restriction enzymes were from Boehringer Mannheim GmbH. All other chemicals and reagents were from Sigma Chemical Co. All rats used in this study were obtained from Taconic Farms. The investigation conforms with the "Guide for the Care and

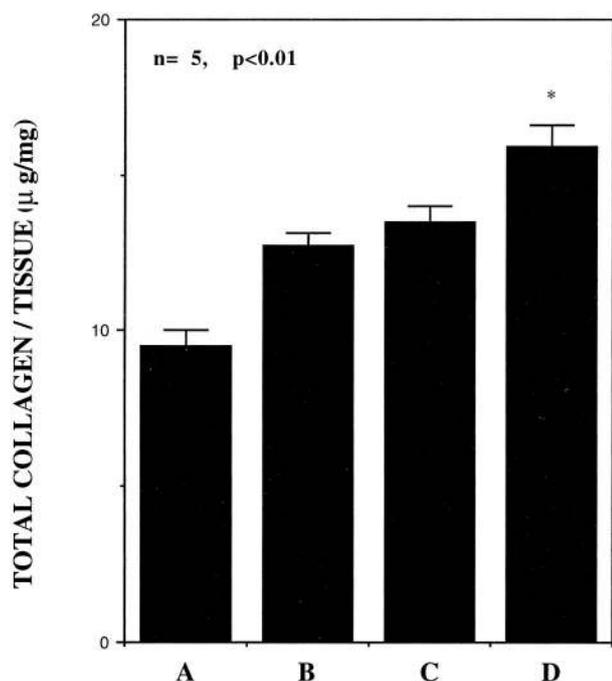
Use of Laboratory Animals" (US National Institutes of Health, NIH publication No. 85-23, revised 1996).

### Preparation of Fibroblasts in Culture

Rat cardiac fibroblasts were isolated from normal 28-week-old Wistar-Kyoto (WKY) rats according to the method of Sil and Sen.<sup>15</sup> The flasks containing pure fibroblasts were then maintained at 37°C until the cells were confluent; they were subsequently passaged. The cells were used at 70% to 75% confluency and kept in serum-free medium for 24 hours before each experiment.

### Beating, Nonworking Rat Heart Preparation

For the beating, nonworking heart preparation, normal 28-week-old WKY rats were heparinized with 500 U heparin and anesthetized with 20 mg/kg pentobarbital IP. The heart was taken out and washed with modified Krebs' buffer (pH 7.35). The buffer was bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub> for 30 minutes. The heart was then cannulated through the aorta and was perfused with buffer at 37°C at the rate of 135 mL/min. The heart was kept beating with a Grass electrical stimulator (frequency 8 Hz, duration 15 ms, 2.5 V). The positive and negative terminals were connected to the atria of the heart. A constant pressure of 75 mm Hg was maintained throughout the experimental period. [ $\text{Sar}^1$ ]Ang II was perfused with a small peristaltic pump and allowed to mix with the buffer. Proper concentrations of [ $\text{Sar}^1$ ]Ang II were carefully maintained. For a typical



**Figure 2.** Estimation of total collagen ex vivo due to the effect of Ang II perfusion on the beating, nonworking heart preparation (A, control; B,  $10^{-12}$  mol/L Ang II; C,  $10^{-10}$  mol/L Ang II; D,  $10^{-8}$  mol/L Ang II). Total collagen was estimated by measuring the amount of hydroxyproline in the tissue as described in the text. Perfusion with  $10^{-8}$  mol/L [Sar<sup>1</sup>]Ang II significantly stimulated the total collagen level.

experiment, hearts were perfused with the buffer alone for 30 minutes and then perfused with [Sar<sup>1</sup>]Ang II for 2 hours. At the end of the experiment, the hearts were removed and kept frozen in liquid nitrogen.

### Preparation of Rat Myocytes in Culture

Neonatal rat myocytes were isolated and cultured on laminin-coated glass coverslips placed in the standard 6-well plates according to the procedure described by Sil and Sen.<sup>15</sup> Adult myocytes were prepared on laminin-coated cover glasses according to the method of Bugaisky and Zak<sup>16</sup> with some modifications.

### Experiment With Myocytes on Coverslips Added to Neonatal and Adult Fibroblasts in Culture

Both neonatal and adult fibroblast cells ( $2 \times 10^6$  per well of standard 6-well plates) were incubated for 24 hours at 37°C in the absence (control) or presence (treated) of [Sar<sup>1</sup>]Ang II. Six-well plates, each containing neonatal and adult rat fibroblasts, respectively, were kept for incubation with neonatal and adult myocytes separately on coverslips or coculture inserts for 2 to 10 hours without the addition of [Sar<sup>1</sup>]Ang II. In the treated cultures (experimental), fibroblasts were incubated in the presence of  $10^{-10}$  mol/L [Sar<sup>1</sup>]Ang II with coverslips and inserts that contained myocytes. The cellular combination of fibroblasts and myocytes was incubated for 2 to 4 hours alone and then with  $10^{-10}$  mol/L [Sar<sup>1</sup>]Ang II for 20 hours at 37°C. The same amount of Ang II was replenished every 6 hours during the experiment.

### Extraction of RNA and Northern Hybridization

Total RNA from fibroblast cells (in 6-well plates with or without myocytes) was isolated using the RNeasy minikit following the Qiagen protocol. RNA from each sample was then run on a 1% agarose-formaldehyde gel with DEPC-treated 1[times] MOPS as a running buffer, transblotted onto a GeneScreen membrane, and crosslinked in a UV crosslinker. The membrane was then subjected

to prehybridization for 6 hours, followed by hybridization at 42.5°C for 16 hours. The hybridization of the same membrane was performed with 3 different radiolabeled probes: (1) collagen  $\alpha_2(\text{I})$  type I rat cDNA probe, (2) collagen  $\alpha_1(\text{I})$  type III human skin fibroblast cDNA probe, and (3) 18S rRNA oligoprobe. Hybridization and washing were performed according to Sambrook et al.<sup>17</sup> The membrane then was subjected to autoradiography. For estimation, the intensity of the bands was quantified with videodensitometry. The 18S RNA oligoprobe was used to normalize our results.

### Quantification of Collagen

Total collagen was quantified from each rat heart by measuring hydroxyproline with a modified Stagemann procedure.<sup>18</sup> The amount of hydroxyproline in unknown samples was calculated with a standard curve. Collagen content was estimated by multiplying the hydroxyproline content by a factor of 8.2 and was expressed as micrograms of collagen per milligram of heart tissue.

### Statistical Analysis

Results are expressed as mean  $\pm$  SEM. Data were analyzed by 2-way ANOVA, and the differences between groups were determined by the least-square means test (SUPERNOVA). Differences were considered statistically significant at  $P < 0.05$ .

## Results

### Effect of [Sar<sup>1</sup>]Ang II on Beating, Nonworking Heart Preparation Ex Vivo

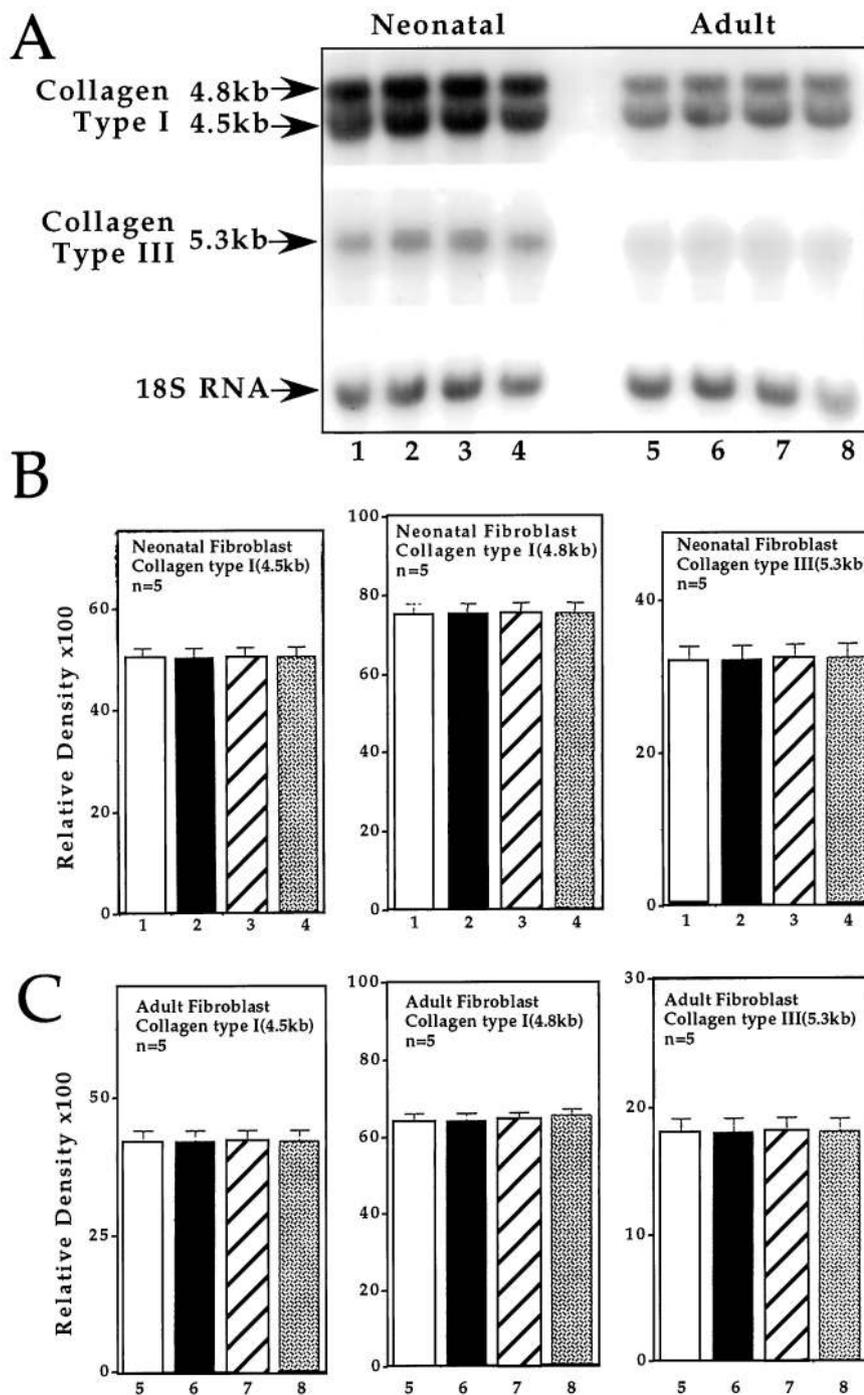
As shown in Figure 1A, both the transcripts of collagen type I and the 5.3-kb transcript of collagen type III were significantly upregulated when the hearts were perfused with [Sar<sup>1</sup>]Ang II for 2 hours. After quantification with videodensitometry and normalization of results with 18S RNA, both transcripts of collagen type I were found to be upregulated more in the presence of  $10^{-8}$  mol/L [Sar<sup>1</sup>]Ang II than with either  $10^{-12}$  or  $10^{-10}$  mol/L Ang II (Figure 1B). The stimulation was 1.5-fold ( $n=5$ ,  $P < 0.01$ ) for the 4.5-kb transcript and 2.5-fold ( $n=5$ ,  $P < 0.004$ ) for the 4.8-kb transcript of collagen type I mRNA, when estimated over the respective control measurements. The stimulation of the collagen type III transcript was 1.8-fold ( $n=5$ ,  $P < 0.01$ ) over the control.

### Estimation of Total Collagen Ex Vivo

Ex vivo hearts compared with the control show an increase in collagen content of  $\approx 33.8\%$ ,  $\approx 42.06\%$ , and  $\approx 67.50\%$  over control when perfused with  $10^{-12}$ ,  $10^{-10}$ , or  $10^{-8}$  mol/L [Sar<sup>1</sup>]Ang II, respectively ( $n=5$ ,  $P < 0.01$ ). Control heart measurement was 9.51 g/mg, whereas hearts perfused with  $10^{-12}$ ,  $10^{-10}$ , or  $10^{-8}$  mol/L [Sar<sup>1</sup>]Ang II resulted in the values of 12.73, 13.51, and 15.93 g/mg, respectively (Figure 2).

### Effect of [Sar<sup>1</sup>]Ang II on Neonatal and Adult Rat Fibroblasts in Culture

When the neonatal and adult (28-week-old) WKY rat heart fibroblasts in serum-free media were incubated for 24 hours at 37°C in the absence (control) or presence (treated) of the 3 different concentrations of [Sar<sup>1</sup>]Ang II ( $10^{-10}$ ,  $10^{-8}$ , and  $10^{-6}$  mol/L, respectively;  $n=5$ ), there was no change over the control in either of the 2 collagen type I transcripts for  $10^{-10}$  or  $10^{-6}$  mol/L Ang II (Figure 3). There was, however, a questionable increase in both collagen type I transcripts for  $10^{-8}$  mol/L Ang II. For the same membrane, with a collagen type III cDNA probe ( $n=5$ ), no stimulation was observed (as



**Figure 3.** Effect of [Sar<sup>1</sup>]Ang II on neonatal and adult rat fibroblasts in culture. A, Autoradiograph showing the 2 transcripts (4.5 and 4.8 kb) of collagen type I mRNA and the 5.3-kb transcript of collagen type III mRNA. Lanes 1 and 5, controls (neonatal and adult); lanes 2 and 6, fibroblasts treated with 10<sup>-10</sup> mol/L [Sar<sup>1</sup>]Ang II; lanes 3 and 7, fibroblasts treated with 10<sup>-8</sup> mol/L [Sar<sup>1</sup>]Ang II; lanes 4 and 8, fibroblasts treated with 10<sup>-6</sup> mol/L [Sar<sup>1</sup>]Ang II. An 18S RNA oligoprobe was used to normalize our results. B and C, Quantification of the collagen transcripts. Ang II could not stimulate either collagen type I or type III transcripts in neonatal and adult rat cardiac fibroblasts.

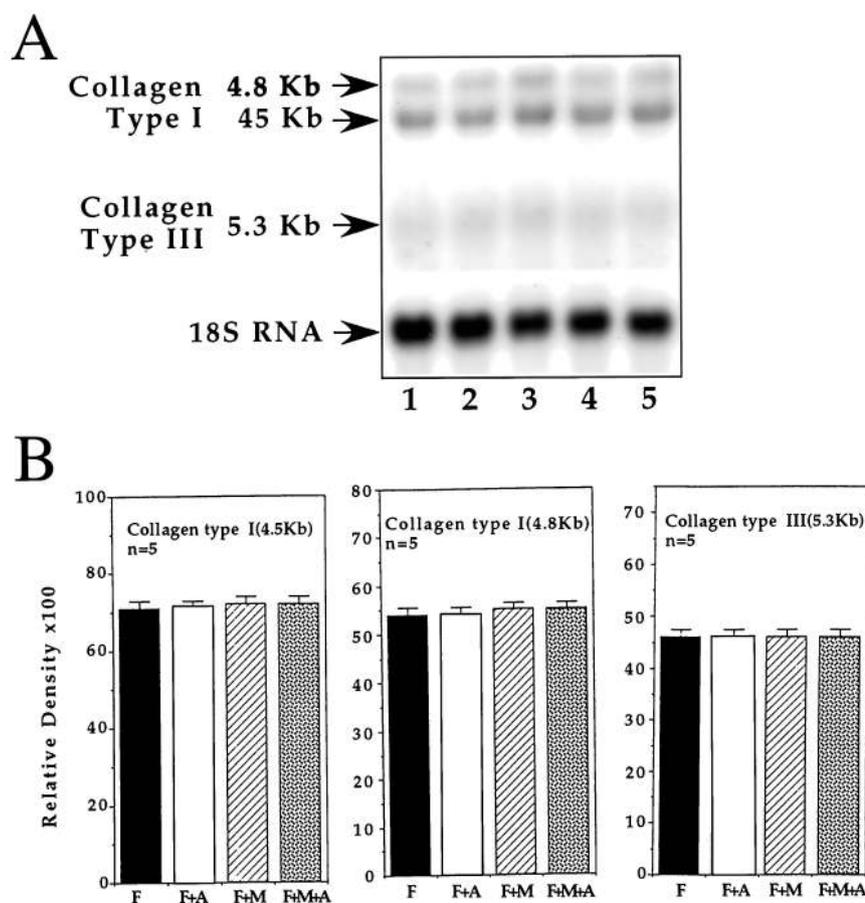
shown in Figure 3). Incubation of fibroblasts for periods of 2 to 24 hours at 37°C in the presence of 10<sup>-12</sup> to 10<sup>-6</sup> mol/L [Sar<sup>1</sup>]Ang II (n=5) also did not show any stimulation for either of the collagen transcripts (result not shown).

**Effect of [Sar<sup>1</sup>]Ang II on Neonatal Rat Fibroblasts Cocultured With Neonatal or Adult Rat Myocytes**

As shown in Figure 4, in the absence or presence of 10<sup>-10</sup> or 10<sup>-8</sup> mol/L [Sar<sup>1</sup>]Ang II (n=5), neonatal or adult rat myocytes cocultured with neonatal fibroblasts did not show any stimulation for either of the collagen phenotypes.

**Effect of [Sar<sup>1</sup>]Ang II on Adult Rat Fibroblasts Cocultured With Neonatal and Adult Rat Myocytes**

Fibroblasts from 28-week-old WKY rats cocultured with either neonatal or adult rat cardiomyocytes could upregulate the transcript levels of both collagen type I and type III quite significantly after incubation with Ang II at the concentration of 10<sup>-10</sup> mol/L. The results of these experiments are shown in Figures 5A and 6A. Both transcripts of collagen type I and type III transcripts were stimulated, more so when fibroblasts cocultured with myocytes were kept for 4 hours and then incubated with 10<sup>-10</sup> mol/L [Sar<sup>1</sup>]Ang II for an additional 24



**Figure 4.** Effect of Ang II on neonatal rat fibroblasts together with neonatal rat myocytes in culture. A, Autoradiograph showing the 2 transcripts (4.5 and 4.8 kb) of collagen type I mRNA and the 5.3-kb transcript of collagen type III mRNA. Lane 1, fibroblasts alone; lane 2, fibroblasts treated with Ang II ( $10^{-10}$  mol/L); lane 3, fibroblasts combined with myocytes on coverslips; lanes 4 and 5, fibroblasts combined with myocytes on coverslips and treated with Ang II ( $10^{-10}$  mol/L). B, Quantification of the different collagen transcripts. No significant stimulation was noted due to the addition of neonatal myocytes to neonatal fibroblasts.

hours. Figures 5B and 6B show the quantification of 4.5-kb (1.5-fold over the control,  $n=5$ ,  $P<0.001$ ) and 4.8-kb (1.7-fold over the control,  $n=5$ ,  $P<0.005$ ) transcripts of collagen type I and the 5.3-kb (1.4-fold over the control,  $n=5$ ,  $P<0.005$ ) transcript of collagen type III after videodensitometry and appropriate normalization with an 18S RNA oligoprobe. We incubated the fibroblasts with myocytes in coculture plates for different lengths of time (2 to 6 hours) before the addition and incubation of  $10^{-10}$  mol/L [ $\text{Sar}^1$ ]Ang II for 24 hours, but we found that 4 hours of prior contact between fibroblast and myocytes results in the maximum upregulation of the collagen transcripts.

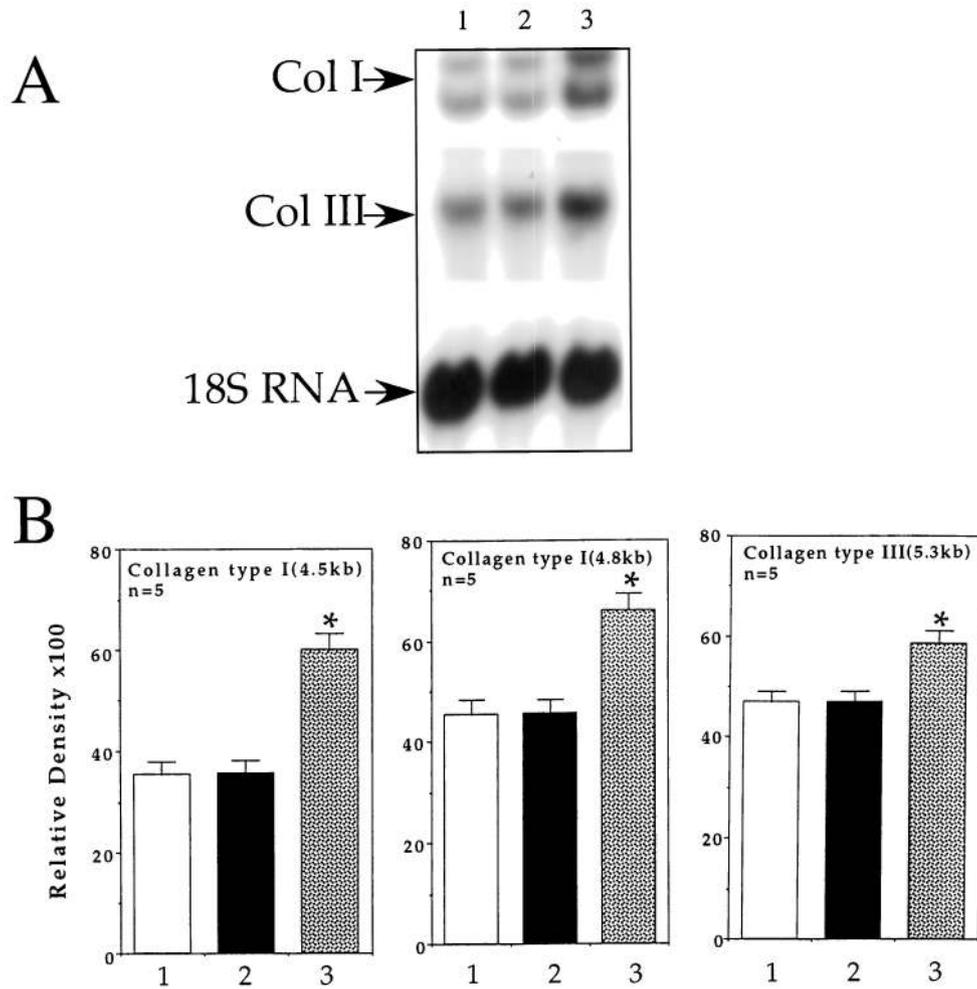
### Discussion

In the present study, we demonstrated that when Ang II was added to neonatal and adult rat fibroblasts in culture in vitro in a dose-dependent fashion, no change occurred in the transcript levels of collagen type I or type III (except some questionable increase for collagen I transcripts with  $10^{-8}$  mol/L [ $\text{Sar}^1$ ]Ang II). In contrast, in the beating, nonworking adult heart preparation, the addition of  $10^{-8}$  mol/L [ $\text{Sar}^1$ ]Ang II showed a significant stimulation ( $\approx 2$ -fold) of collagen mRNAs for both phenotypes (Figure 1). This observation suggests that the effects of Ang II in vitro and ex vivo are different and that Ang II may have an indirect effect on fibroblasts for collagen production. To understand the cellular mechanism for collagen upregulation by fibroblasts, we sought to probe into the involvement of some other cardiac

cell types in the collagen gene action. When neonatal as well as adult myocytes were cocultured along with adult fibroblasts and incubated in the presence of  $10^{-10}$  mol/L [ $\text{Sar}^1$ ]Ang II, a significant upregulation in both collagen phenotypes was observed (Figures 5 and 6). However, when neonatal and adult myocytes were grown on coverslips and added to neonatal fibroblasts in culture in the presence of Ang II, there was no stimulation of either collagen phenotype.

It has been suggested that excessive collagen deposition is a potential cause of stiffness of the heart during the chronic phase of hypertrophy, especially during its transition to heart failure. The molecular mechanism for this abnormal collagen formation during the chronic phase is not clearly understood. Ang II, a potent vasoconstrictor, is believed to play a role in the stimulation of collagen production in vivo. Our study shows that although Ang II had a significant effect in the stimulation of collagen production in vivo and ex vivo, it had no effect when added to pure fibroblasts in vitro except with cardiomyocytes (although fibroblasts are reported to be the sole producer for collagen in heart).

Collagen biosynthesis is regulated at different levels of transcription and translation. Type I and type III are the 2 major types of collagen present in the myocardium in both normal and diseased myocardial tissue. Thus, the quality rather than the quantity of the collagen would be an important marker to define the pathophysiology during the chronic phase of hypertrophy and heart failure. Therefore, a ratio of type I and type III is an important marker for determination of



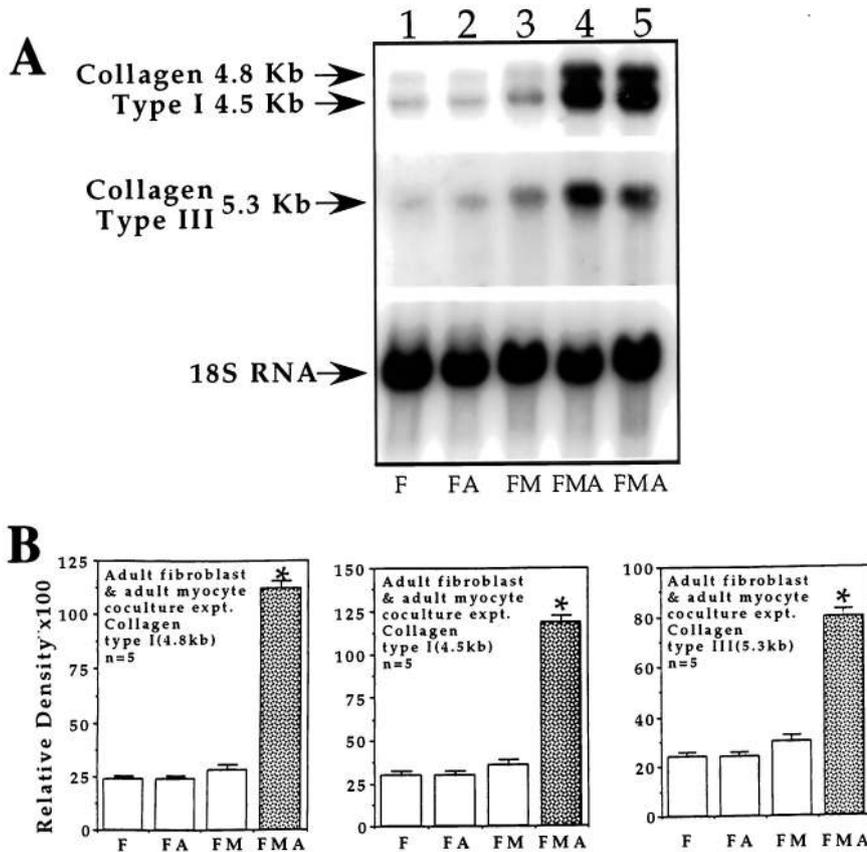
**Figure 5.** A, Effect of Ang II ( $10^{-10}$  mol/L) on collagen (Col) I and III transcripts of adult rat fibroblasts, cocultured with neonatal rat myocytes. Lane 1, fibroblasts only; lane 2, fibroblasts with Ang II; lane 3, fibroblasts cocultured with myocytes for 12 hours, at which point Ang II was added. B, Quantification of the different collagen transcripts. Significant stimulation of all of the transcripts were observed after the addition of neonatal myocytes to adult fibroblasts.

the quality of collagen and therefore prediction of the stiffness of the heart muscle. Our *in vitro* study shows that regardless of the time of exposure of fibroblasts alone to  $[Sar^1]Ang II$ , the fibroblasts did not alter collagen production, whereas a 2-hour perfusion of  $10^{-8}$  mol/L  $[Sar^1]Ang II$  showed significant stimulation of collagen production at the transcription as well as translation levels in the whole-heart model.

Several studies *in vivo* have suggested that Ang II may also be a critical factor in mediation of cardiac hypertrophy.<sup>19–22</sup> These observations are consistent with, although they do not prove, the notion that Ang II may act as an endogenous growth factor for the myocardium. Thus, Ang II, directly or in combination with other growth factors, may play an important role in the development of vascular hypertrophy and elevated arterial resistance in hypertension. It is thought that many factors play a direct role in abnormal collagen deposition, such as Ang II and TGF- $\beta$ , as well as an indirect role, such as prostaglandin  $A_2$ , bradykinin, NO, and endothelin-1, as either a stimulator or an inhibitor.<sup>3</sup> All of these other factors work indirectly through Ang II, and Ang II in turn stimulates collagen

formation. Therefore, the results from our study are important in understanding Ang II-mediated collagen deposition because the myocyte-generated factor plays an important role in stimulation of collagen formation in conjunction with Ang II. Our preliminary data suggest that the factor from myocytes is soluble. When the supernatants from the myocyte cultures (neonatal and adult) were added to the fibroblasts, similar upregulation of collagen was observed (data not shown). Furthermore, results from the present study opened new doors to understanding the molecular mechanism for collagen production. The nature of such factors is not known, nor can we comment on the nature of the factors produced by the myocytes, but we know that identification and characterization of the factors and development of an inhibitor would be important steps in the control of the deposition of excessive fibrous tissue during the chronic phase of hypertrophy and its transition to heart failure.

Cardiac hypertrophy and heart failure remain major health problems in the United States. The chronically failing heart is associated with alteration in tissue composition, including fibrous tissue deposition that appears in



**Figure 6.** A, Effect of Ang II ( $10^{-10}$  mol/L) on collagen type I and type III transcripts of adult rat fibroblasts, cocultured with adult rat myocytes. Lane 1, fibroblasts alone (F); lane 2, fibroblasts treated with Ang II (FA); lane 3, fibroblasts added with myocytes on coverslips (FM); lanes 4 and 5, fibroblasts added with myocytes on coverslips and treated with Ang II (FMA). B, Quantification of the different collagen transcripts. Significant stimulation of all of the collagen transcripts were observed due to the addition of adult myocytes to adult fibroblasts.

both left and right ventricles.<sup>23</sup> Such adverse accumulation of extracellular matrix is believed to be in part responsible for myocardial stiffness and eventually affects the contractile behavior of the heart.<sup>24–29</sup> Therefore, understanding the cellular and molecular mechanisms responsible for the deposition of collagen (fibrous tissue) is an essential step in designing cardioprotective agents that could prevent the deposition of collagen and, therefore, fibrosis during heart failure.

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