

Angiotensin II Activates Signal Transducer and Activators of Transcription 3 via Rac1 in Atrial Myocytes and Fibroblasts

Implication for the Therapeutic Effect of Statin in Atrial Structural Remodeling

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Background—Recently, activation of the local renin-angiotensin system and mitogen-activated protein kinase pathways in atrial myocardium has been found to play an important role in atrial structural remodeling related to atrial fibrillation. Another important mediator of the angiotensin II (Ang II) effect is the Janus kinase/signal transducers and activators of transcription (STAT) pathway, which has never been characterized in the atrium.

Methods and Results—In cultured atrial myocytes and fibroblasts, Ang II induced tyrosine phosphorylation of STAT3 through a Rac1-dependent mechanism, which was inhibited by dominant-negative Rac1, losartan, and simvastatin. In atrial myocytes, activation of STAT3 by Rac1 was mediated by direct association of Rac1 with STAT3; however, in atrial fibroblasts, it was mediated by an indirect paracrine effect. Constitutively active STAT3 increased protein synthesis, and dominant-negative STAT3 abrogated Ang II–induced protein synthesis in atrial myocytes and fibroblasts. Rats infused long term with Ang II exhibited higher levels of activated Rac1, phospho-STAT3, collagen synthesis, and atrial fibrosis in the atria, all of which were attenuated by oral losartan and simvastatin. In human atrial tissues from patients with atrial fibrillation, Ang II and phospho-STAT3 levels were also elevated.

Conclusions—The Ang II/Rac1/STAT3 pathway is an important signaling pathway in the atrial myocardium to mediate atrial structural remodeling, and losartan and statin may be able to reverse Ang II–induced atrial structural remodeling in atrial fibrillation. (*Circulation*. 2008;117:344-355.)

Key Words: atrial fibrillation ■ angiotensin II ■ signal transduction ■ small GTPases ■ Rac1 protein

Atrial fibrillation (AF) is the most common sustained arrhythmia in clinical practice. A new pharmacological approach to AF therapy is to target the underlying substrates.¹ Structural remodeling is one of the most important substrates in AF and refers to the progressive architectural deterioration of the atria after repeated episodes of AF. The main changes include myocytic hypertrophy, myolysis, and interstitial fibrosis.² These structural abnormalities result in an increase of conduction heterogeneity, which facilitates the maintenance of AF.

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The molecular mechanism of atrial structural remodeling is largely unknown. The renin-angiotensin system is involved in the pathogenesis of various cardiovascular diseases.^{3–6} Recently, activation of the local renin-angiotensin system and mitogen-activated protein kinase pathways in atrial tissue has also been found to play an important role in atrial structural remodeling.^{7,8} Mitogen-activated protein kinases are important mediators of the effects of

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The online-only Data Supplement, consisting of an expanded Methods section and a figure, is available with this article at <http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.695346/DC1>.

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angiotensin II (Ang II) on tissue structure.^{9,10} Another important mediator of the Ang II effect is the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, which is involved in vascular atherosclerosis and ventricular hypertrophy.^{11–14} However, the status of Ang II/JAK/STAT signaling has never been characterized in the atrium, and its role in atrial structural remodeling is unknown.

The JAK/STAT pathway was initially discovered as a major cytokine signal transduction pathway,¹⁵ activated by interleukin-6, leukemia inhibitory factor, and cardiotrophin-1.^{16–20} Interleukin-6, leukemia inhibitory factor, and cardiotrophin-1 bind to glycoprotein 130 (gp130), which causes its autophosphorylation, which then activates the downstream JAK/STAT pathways.^{16–20} JAK/STAT can also be activated by G-protein-coupled receptors, such as the Ang II receptor, and this process requires membrane translocation and activation of the small GTPase, Rac1.^{21–24} Notably, statin inhibits Rac1 activation by inhibiting its geranylgeranylation and membrane translocation.

In the present study, we used in vitro atrial myocytes and fibroblasts, an in vivo animal model system, and human atrial samples to characterize the status of Ang II/JAK/STAT signaling in the atrial myocardium. We found that Ang II activated STAT3 via Rac1 in both atrial myocytes and fibroblasts, which may contribute to the structural changes in the atrium, and was blocked by both losartan and simvastatin.

Methods

Culture of Atrial Myocytes and Fibroblasts and Transient Transfection

Both atria from week-old neonatal Wistar rats were cut into chunks and subjected to trypsin (0.125%) digestion. More detailed methods are provided in the online-only Data Supplement. Because of the low transfection efficiency in neonatal atrial myocytes ($\approx 10\%$), we used HL-1 atrial myocytes for all other in vitro atrial myocyte experiments that required transient transfection. The HL-1 atrial cell line was derived from adult mouse atria,²⁵ which were obtained from Louisiana State University in New Orleans, La. The culture and maintenance of HL-1 atrial myocytes were as described previously.²⁵ Transient transfection of wild-type STAT3,²⁶ constitutively active STAT3 (STAT3C),²⁶ dominant-negative STAT3 (STAT3Y705F),²⁶ constitutively active Rac1 (RacV12),²⁴ and dominant-negative Rac1 (RacN17)²⁴ for atrial fibroblasts was performed with Lipofectamine (Invitrogen, Carlsbad, Calif) and Lipofectamine 2000 (Invitrogen) for HL-1 cells.

Rat Model of Continuous Ang II Infusion

Ang II ($2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) was infused for 6 hours, 3 days, and 14 days into Wistar rats (weight $300 \pm 20 \text{ g}$). In addition to normal diet, the rats were also given pure water (vehicle), losartan ($10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), or simvastatin ($5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$)²⁷ every day during Ang II infusion. Atrial samples were collected subsequently for the experiments. Further details are provided in the online-only Data Supplement. The experimental protocol of neonatal rat and adult rat studies described in the present report conformed to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of the National Taiwan University College of Medicine.

Human Atrial Samples

Consecutive patients with valvular heart disease who underwent mitral or aortic valve replacement were recruited. Patients taking

angiotensin-converting enzyme inhibitors or angiotensin receptor blockers were excluded. Ultimately, 6 patients with chronic persistent AF and 6 without a history of AF were recruited. All patients underwent echocardiography before surgery. Samples of right atrial appendages were obtained during open heart surgery.

Protein Extracts, Western Blot Analyses, and Immunoprecipitation

Preparations for protein extracts and Western blot analyses have been described previously.²⁸ The primary and secondary antibodies used in the present study and the method of immunoprecipitation are provided in the online-only Data Supplement.

Measurement of Ang II Concentration and Activated Rac1 Assay

Ang II concentration was measured by ELISA, as described previously.⁷ The detection of active, GTP-bound Rac1 was performed with a p21-activated kinase-PBD (PAK-PBD) pull-down assay as described previously.²⁹

RNA Extraction and Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction

Real-time quantitative reverse-transcription polymerase chain reaction (ABI-Prism 7900 Sequence Detection System, Applied Biosystems, Foster City, Calif) was performed with SYBR green dye. The primer sequences for mouse and rat procollagen type I α -1 (COL1A1), atrial natriuretic peptide (ANP; NPPA) and GAPDH are provided in the online-only Data Supplement.

Incorporation of [³H]-Leucine and [³H]-Proline

The cells were pulsed with [³H]-leucine ($1 \mu\text{Ci}/\text{well}$ in $10 \mu\text{L}$ of culture medium) for HL-1 atrial myocytes, and [³H]-proline for neonatal atrial fibroblasts. After 24 hours, the cells were harvested, and radioactivity was determined with a direct beta counter (Matrix 96, Packard Instrument Co, Meriden, Conn). More detailed methods are described in the online-only Data Supplement.

Statistical Analysis

All data are expressed as mean \pm SD. Binary data for patient characteristics were compared with the Fisher exact test and continuous data with unpaired Student *t* test. A probability value < 0.05 was considered statistically significant. Continuous data from > 2 groups were compared with 1-way ANOVA. Bonferroni correction was applied for multiple comparisons after 1-way ANOVA.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Activation of JAK/STAT Pathways by Ang II in Atrial Myocytes and Fibroblasts

In both atrial myocytes and fibroblasts, incubation with Ang II significantly induced tyrosine 705 phosphorylation of STAT3 in a time-dependent (Figure 1A and 1B) and concentration-dependent (Figure 1C and 1D) manner; however, the time course was not the same for the 2 cell types. In atrial myocytes, phospho-STAT3 increased 5 minutes after incubation with Ang II, intensified over 2 hours, and was sustained for 24 hours (Figure 1A), whereas in atrial fibroblasts, phospho-STAT3 increased 2 hours later and was also sustained for 24 hours (Figure 1B). The effect could be observed when the concentration of Ang II was $> 10^{-7} \text{ mol/L}$ (Figure 1C and 1D). We did not detect a significant change in phospho-STAT1 in either cell type after Ang II treatment (Figure 1A and 1B).

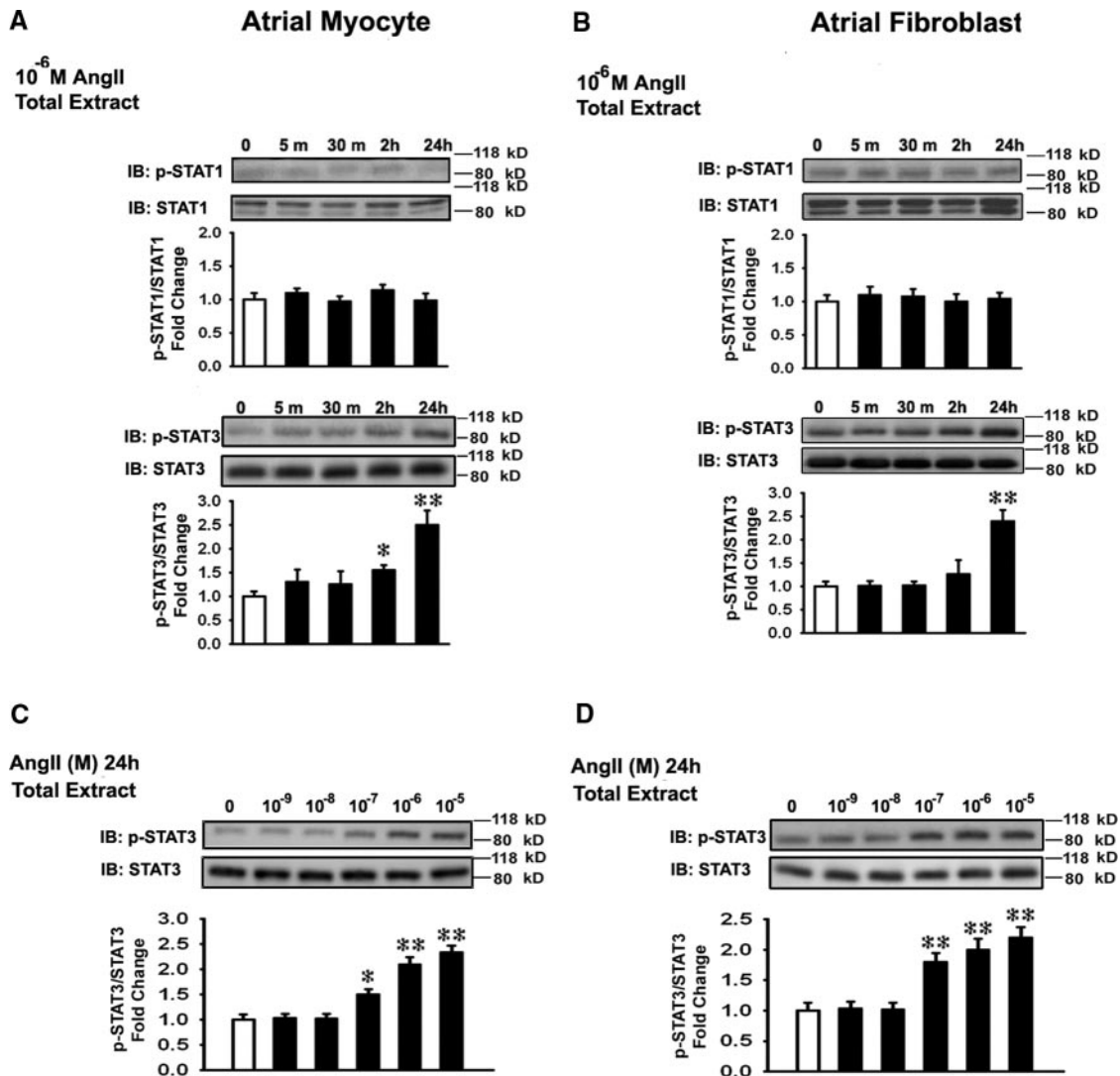


Figure 1. Ang II induces STAT3 tyrosine phosphorylation in atrial myocytes and fibroblasts. Upper panels show representative immunoblotting (IB); Bottom, quantification by densitometry. A and B, Effect of Ang II ($1 \mu\text{mol/L}$) for indicated times on tyrosine phosphorylation of STAT1 and STAT3 in atrial myocytes and atrial fibroblasts, respectively. C and D, Effect of Ang II (24 hours) at indicated concentrations on tyrosine phosphorylation of STAT3 in atrial myocytes and atrial fibroblasts, respectively. $n=3$ per experiment; data are mean \pm SD. * $P<0.05$ (trend toward increase after Bonferroni correction), ** $P<0.01$ vs untreated cells. p-STAT indicates phosphorylated STAT.

We further examined the phosphorylation of JAK1, JAK2, and tyrosine kinase 2 (TYK2; Figure 2A and 2B). In atrial myocytes, Ang II did not induce tyrosine phosphorylation of JAK1, JAK2, and TYK2 (Figure 2A); however, in fibroblasts, Ang II ($1 \mu\text{mol/L}$) significantly increased tyrosine phosphorylation of JAK1 within 2 hours and of JAK2 within 5 minutes after stimulation, both of which were sustained for 24 hours (Figure 2B).

Activation of STAT3 by Ang II Was Mediated Through Ang II Type 1 Receptor and Small-GTPase Rac1 Activation

A trend was present toward Rac1 activation within 5 minutes after Ang II stimulation in atrial myocytes (Figure 3A) and within 30 minutes in atrial fibroblasts (Figure 3B), which in both cases was sustained for 24 hours and was blocked by the Rac1 membrane translocation inhibitor simvastatin (1

$\mu\text{mol/L}$; Figure 3A and 3B). Transfection with constitutively active Rac1 (RacV12) induced tyrosine phosphorylation of STAT3, and both dominant-negative Rac1 (RacN17) and simvastatin (24 hours, $1 \mu\text{mol/L}$) inhibited Ang II–induced STAT3 tyrosine phosphorylation in atrial myocytes (Figure 3C) and fibroblasts (Figure 3D). Levels of overexpression of RacV12 and RacN17 were determined by measuring the protein level of Rac1 (RacV12, RacN17) by Western blot (Figure 3C and 3D). Together, these results indicated that Ang II–induced STAT3 activation was Rac1-dependent (blocked by RacN17), and the inhibitory effect of simvastatin on Ang II–induced STAT3 activation was through Rac1 inhibition (simvastatin inhibited both Rac1 activity and Ang II–induced STAT3 phosphorylation).

With regard to the mechanism of Rac1-induced STAT3 activation, we found that Ang II (24 hours, $1 \mu\text{mol/L}$) significantly increased the association between Rac1 and

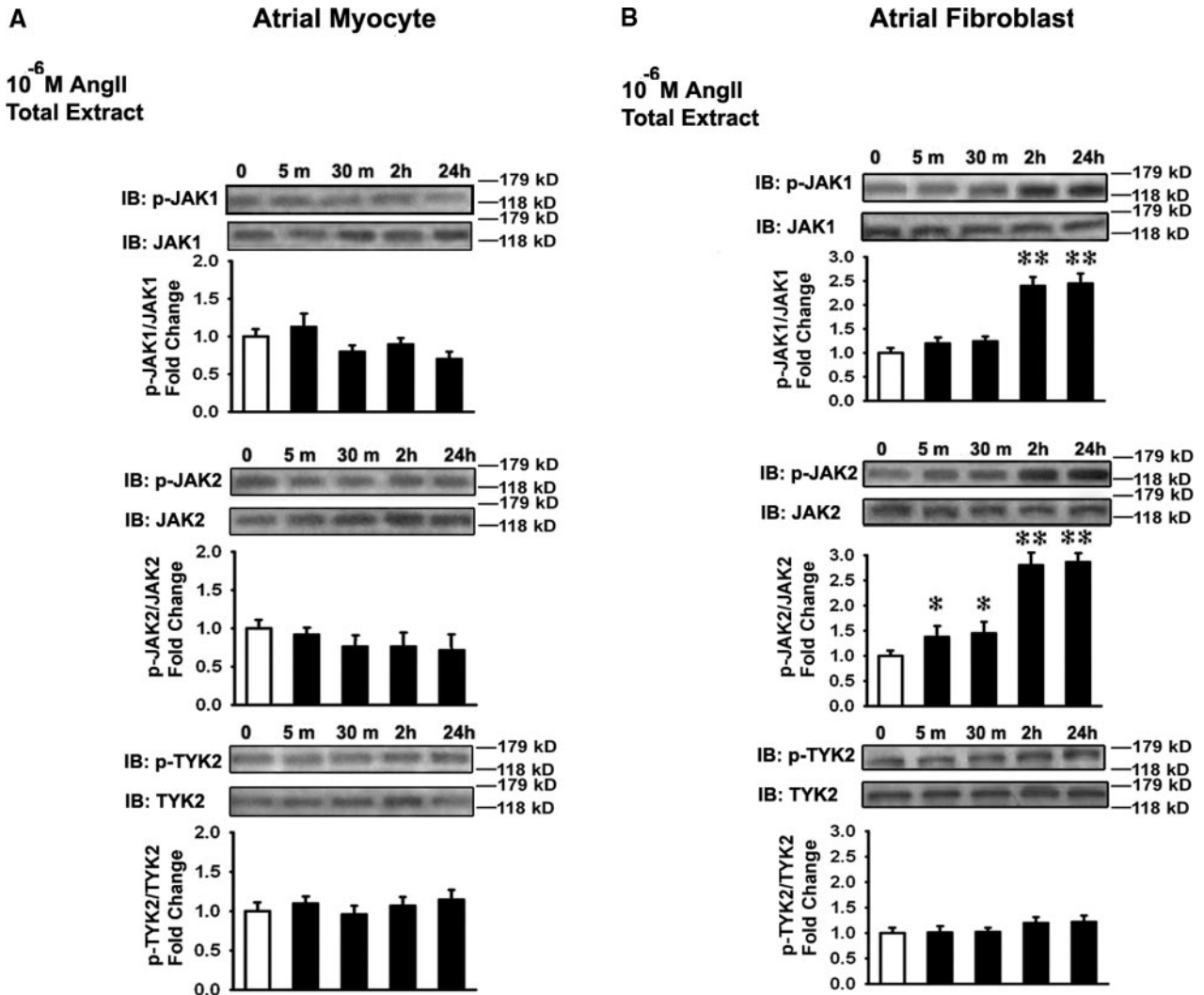


Figure 2. Ang II induces JAK1 and JAK2 tyrosine phosphorylation in atrial fibroblasts but not in atrial myocytes. Upper panels show representative immunoblotting (IB); Bottom, quantification by densitometry. A and B, Effect of Ang II ($1 \mu\text{mol/L}$) for indicated times on tyrosine phosphorylation of JAK1, JAK2, and TYK2 in atrial myocytes and atrial fibroblasts, respectively. $n=3$ per experiment; data are mean \pm SD. * $P<0.05$ (trend toward increase after Bonferroni correction), ** $P<0.01$ vs untreated cells. p- indicates phosphorylated.

STAT3 in the immunoprecipitation assay in atrial myocytes (Figure 4A and 4C, lane 1 versus lane 2), which indicates the possibility of direct activation of STAT3 by Rac1.²¹ The association was inhibited by simvastatin (Figure 4A and 4C, lane 3). In atrial fibroblasts, Ang II did not increase the association between Rac1 and STAT3 (Figure 4B and 4D, lane 1 versus lane 2). Inhibition of general transcription by actinomycin D abolished Ang II–induced STAT3 phosphorylation in atrial fibroblasts (Figure 4F) but not in atrial myocytes (Figure 4E), which indicates the need for transcription (possible synthesis of a paracrine factor) to activate the JAK/STAT pathway in atrial fibroblasts.^{23,24}

Although the mechanisms of Rac1-induced STAT3 activation were different in atrial myocytes and fibroblasts, simvastatin inhibited Ang II–induced tyrosine phosphorylation of STAT3 both in atrial myocytes (Figure 4G, lane 4) and in fibroblasts (Figure 4H, lane 4). Furthermore, Ang II–induced STAT3 phosphorylation was inhibited by the angiotensin receptor blocker losartan (Figure 4G and 4H,

lane 3). Moreover, the inhibitory effect was even greater when simvastatin and losartan were combined (Figure 4G and 4H, lane 5).

Recently, it has been shown that Rac1 transgenic mice (racET) develop a significant dilatation of the atria, and downstream activation of p21-activated kinase (PAK) by Rac1 is important for the development of structural atrial changes.³⁰ On activation by Rac1, PAK was translocated to the cytoskeletal fraction from the cytosolic fraction.³⁰ Therefore, we also studied whether Ang II activated PAK through Rac1. In atrial fibroblasts, incubation with Ang II significantly increased the level of PAK in cytoskeletal fractions in a time-dependent manner (online-only Data Supplement Figure 1B). Ang II–induced PAK translocation was inhibited by simvastatin ($1 \mu\text{mol/L}$; online-only Data Supplement Figure 1D). In atrial myocytes, basal expression of PAK was substantially lower than that of atrial fibroblasts, and Ang II did not induce the translocation of PAK to cytoskeleton fractions (online-only Data Supplement Figure 1A and 1C).

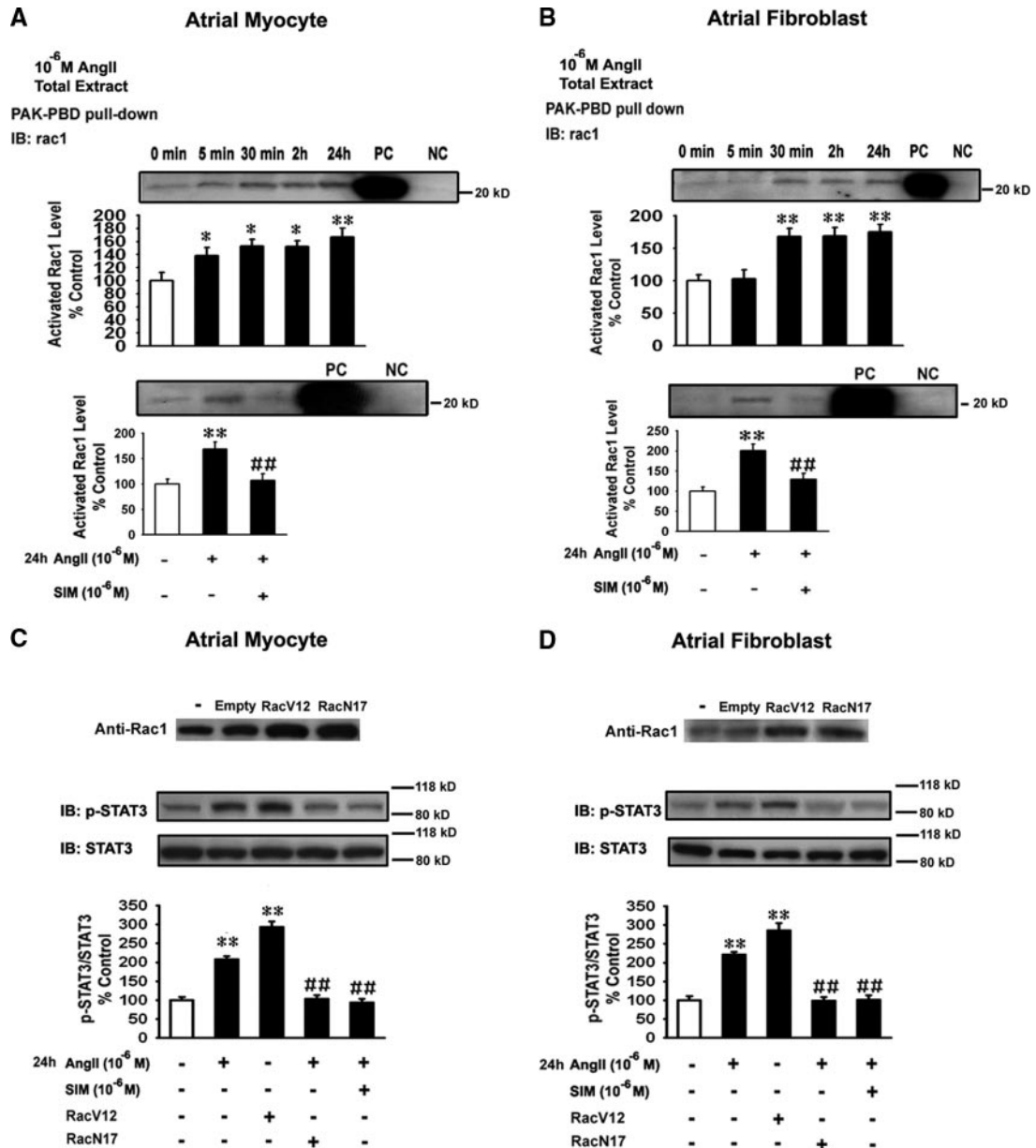


Figure 3. Ang II-induced tyrosine phosphorylation of STAT3 is Rac1-dependent and is inhibited by simvastatin (SIM). A and B, Effect of Ang II (1 $\mu\text{mol/L}$) and simvastatin (1 $\mu\text{mol/L}$, 24-hour pretreatment) on activation of Rac1 in atrial myocytes and atrial fibroblasts, respectively. Activated Rac1 levels were measured by p21-activated kinase-PBD (PAK-PBD) pull-down assay. Samples were incubated with GTP γ S or GDP for positive (PC) and negative (NC) controls. C and D, Effect of Ang II (1 $\mu\text{mol/L}$, 24 hours), constitutively active Rac1 (RacV12), dominant-negative Rac1 (RacN17), and simvastatin (1 $\mu\text{mol/L}$, 24-hour pretreatment) on tyrosine phosphorylation of STAT3 in HL-1 atrial myocytes and atrial fibroblasts, respectively. The level of overexpression was measured by immunoblotting of Rac1. $n=3$ per experiment; data are mean \pm SD. * $P<0.05$ (trend toward increase after Bonferroni correction), ** $P<0.01$ vs untreated cells; ### $P<0.01$ vs Ang II-treated cells. IB indicates immunoblotting; p-, phosphorylated.

Overexpression of Constitutively Active and Dominant-Negative STAT3 in Atrial Myocytes and Fibroblasts

To investigate the functional significance of STAT3 activation in atrial myocytes and atrial fibroblasts, we overexpressed STAT3C²⁶ and STAT3Y705F²⁶ in a murine atrial cell line, HL-1, and neonatal atrial fibroblasts. The level of overexpression was evaluated by STAT3 luciferase reporter and by measuring the levels of FLAG-tagged proteins (STAT3C, STAT3Y705F; Figure 5A and 5B). After transfection, protein levels of STAT3C and STAT3Y705F were

high (Figure 5A and 5B). Transfection of STAT3C and STAT3Y705F increased and decreased STAT3 reporter activities, respectively (Figure 5A and 5B).

Atrial myocyte hypertrophy can induce conduction heterogeneity³¹ and is one of the structural changes in AF.^{31,32} We used [³H]-leucine uptake and ANP expression to represent protein synthesis and a cellular marker of hypertrophy of atrial myocytes, respectively. We found an increase in myocyte [³H]-leucine uptake and ANP expression by overexpressing STAT3C and a decrease by overexpressing STAT3Y705F (Figure 5C). Ang II also increased [³H]-

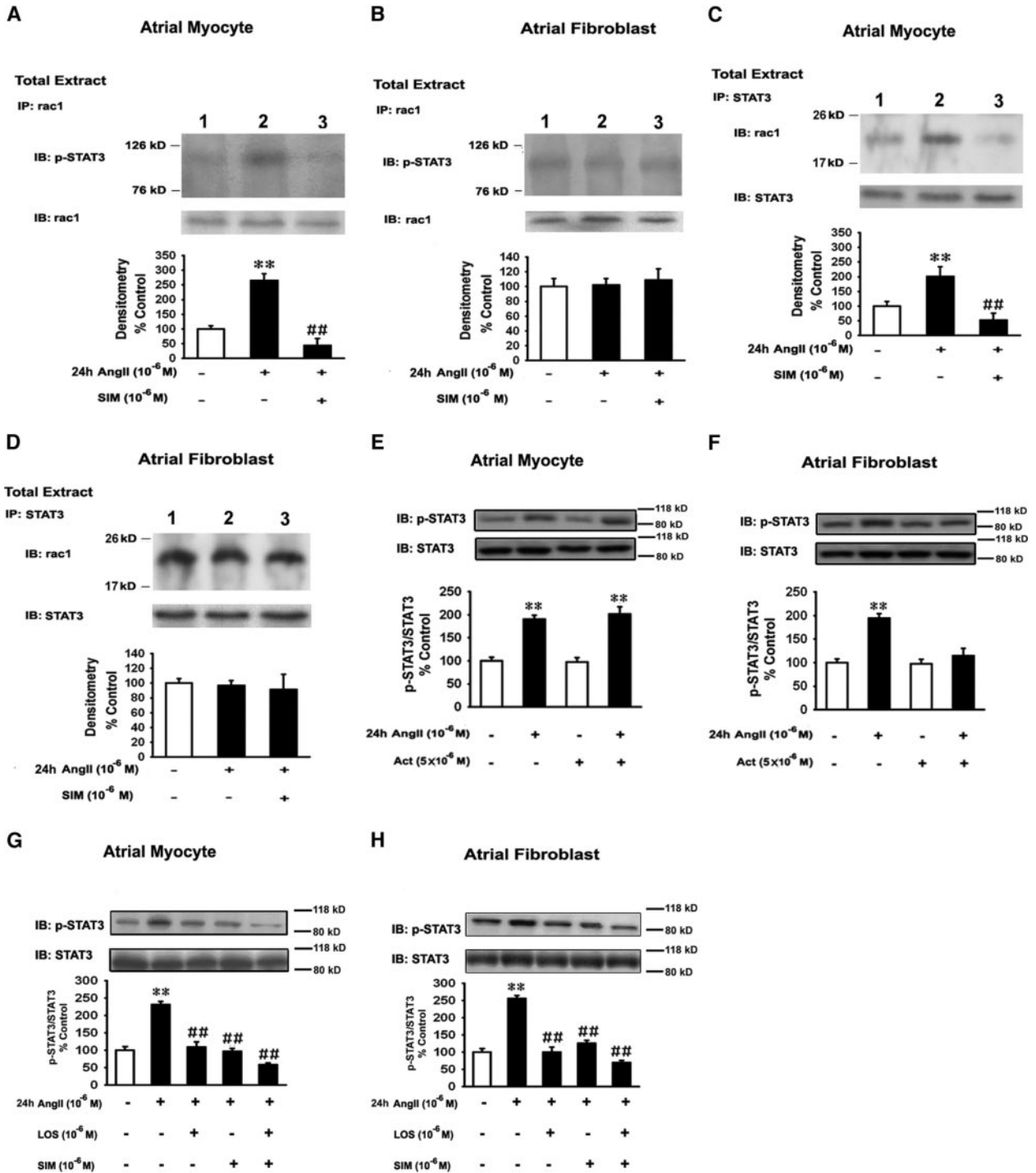


Figure 4. Activation of STAT3 by Rac1 is mediated by direct association of Rac1 with STAT3 in atrial myocytes, whereas it is mediated by transcription (possible synthesis of an indirect paracrine factor) in atrial fibroblasts. A and B, Cell lysates were incubated with anti-Rac1, and immunocomplexes were collected by incubation with protein A. Immunoprecipitates were then immunoblotted with anti-p-STAT3 and anti-Rac1 in atrial myocytes (A) and atrial fibroblasts (B), respectively. Ang II (1 μ mol/L, 24 hours) induced increased association of phosphorylated STAT3 and Rac1 in atrial myocytes (lane 2). The association was inhibited by 24 hours of pretreatment with simvastatin (1 μ mol/L; lane 3). B, In atrial fibroblasts, Ang II did not increase the association of Rac1 with p-STAT3. No significant differences were present in levels of p-STAT3 in the protein complex immunoprecipitated by Rac1 antibody (lanes 1 to 3). C and D, Cell lysates were immunoprecipitated with anti-STAT3 and then immunoblotted with anti-Rac1 and anti-STAT3, which was the reverse of experiments shown in A and B. E and F, Inhibition of general transcription by actinomycin D (5 μ mol/L, 24 hours) attenuated Ang II-induced STAT3 tyrosine phosphorylation in atrial fibroblasts (F) but not in atrial myocytes (E). G and H, Both the Ang II type 1 receptor blocker losartan (1 μ mol/L; cotreatment with Ang II) and simvastatin (1 μ mol/L; 24-hour pretreatment) suppressed Ang II (1 μ mol/L, 24 hours)-induced tyrosine phosphorylation of STAT3 in atrial myocytes (G) and fibroblasts (H). The inhibitory effect of combining losartan and simvastatin was greater than that of either alone. n=3 per experiment; data are mean \pm SD. **P<0.01 vs untreated cells; ##P<0.01 vs Ang II-treated cells. IB indicates immunoblotting; IP, immunoprecipitation; SIM, simvastatin; LOS, losartan; Act, actinomycin D; and p-, phosphorylated.

Atrial Myocyte

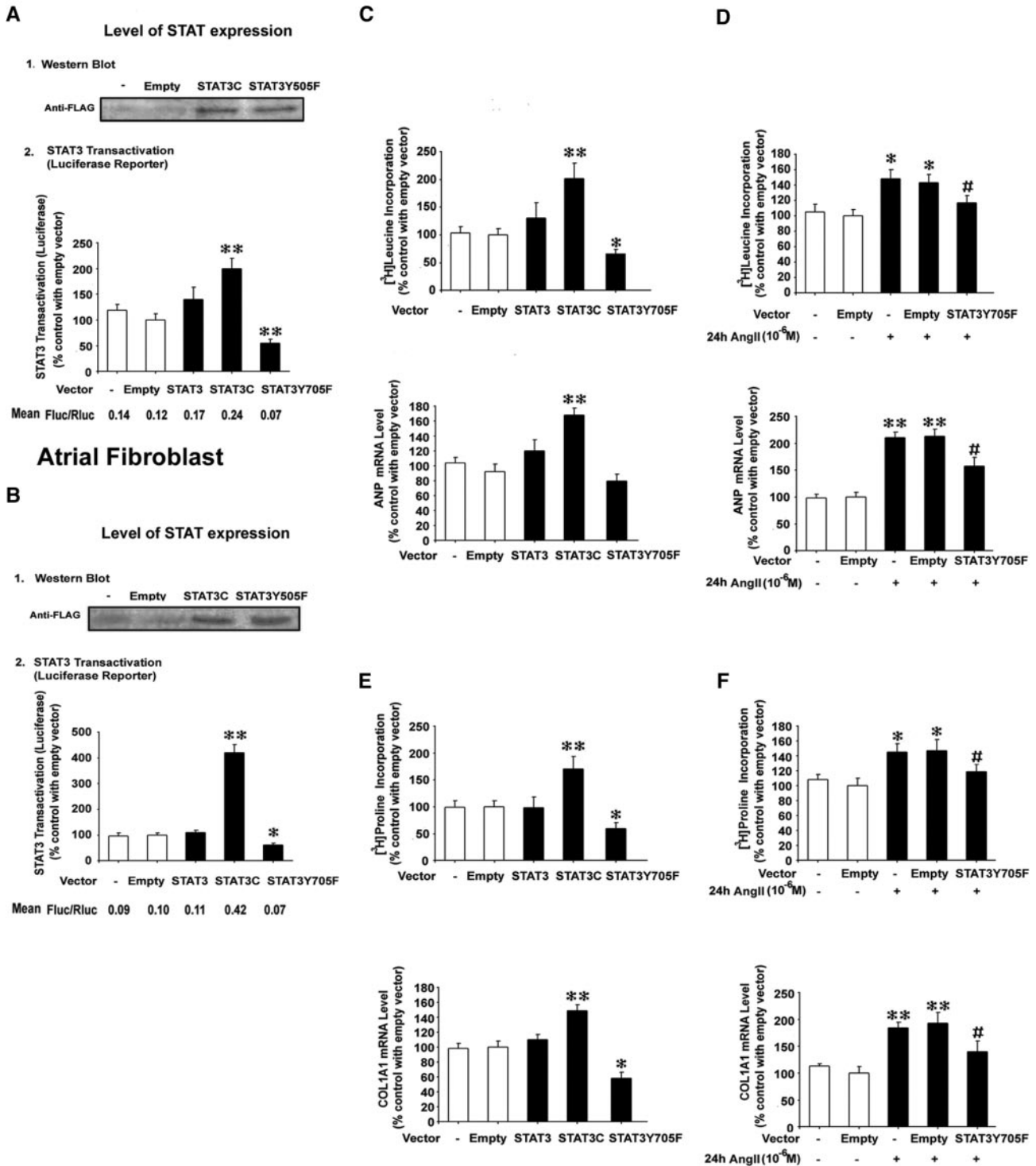


Figure 5. Constitutively active STAT3 (STAT3C) increased protein synthesis and expression of hypertrophy marker (ANP) in HL-1 atrial myocytes, as well as protein synthesis and expression of fibrosis marker (COL1A1) in atrial fibroblasts. Dominant-negative STAT3 (STAT3Y705F) attenuated Ang II-induced hypertrophy and fibrosis in HL-1 atrial myocytes and atrial fibroblasts, respectively. A and B, HL-1 atrial myocytes and atrial fibroblasts were transfected with either empty vector, wild-type STAT3, STAT3C, or dominant-negative STAT3 (STAT3Y705F). Level of overexpression was measured by immunoblotting of FLAG-tagged STAT3. STAT3 transactivation activity was evaluated by measuring luciferase expression driven by a promoter that contained the STAT3 binding sequence (STAT3 reporter). C, HL-1 cells transfected with STAT3C had significantly increased [³H]leucine incorporation and ANP expression, whereas those transfected with STAT3Y705F showed a trend toward the opposite effect. D, STAT3Y705F showed a trend to attenuate Ang II-induced [³H]-leucine uptake and ANP expression in HL-1 cells (column 4 versus column 5, uncorrected $P < 0.05$). E, Atrial fibroblasts transfected with STAT3C had increased [³H]proline incorporation and COL1A1 expression, whereas those transfected with STAT3Y705F showed the opposite effect in [³H]proline incorporation and a trend toward the opposite effect in COL1A1 expression. F,

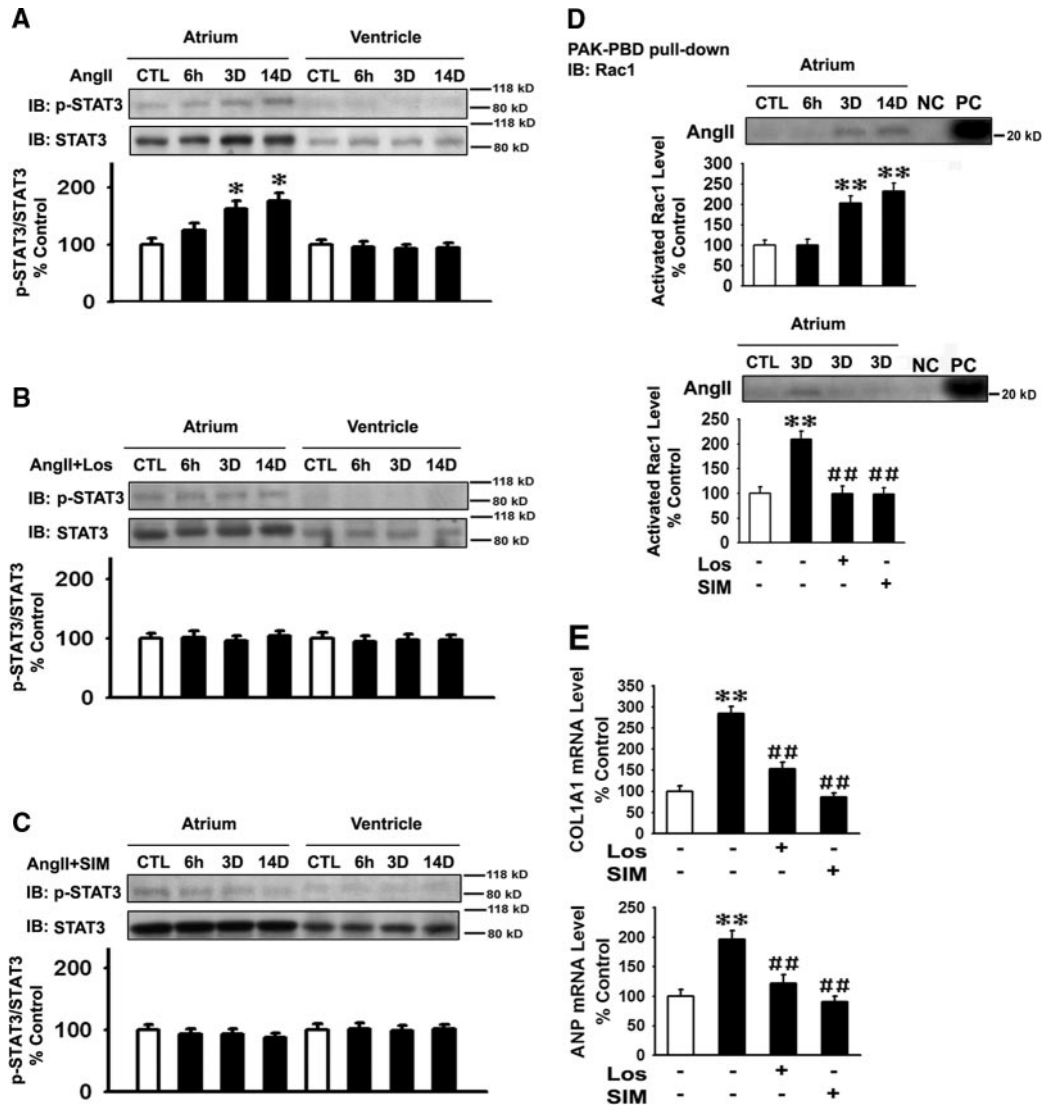


Figure 6. In vivo rat model of Ang II infusion. Ang II activates Rac1 and induces tyrosine phosphorylation of STAT3 in rat atria. A, B, and C, Wistar rats were subcutaneously infused with either vehicle (control) or Ang II for the indicated times. Ang II infusion induced tyrosine phosphorylation of STAT3 in rat atria (A), which was attenuated by oral losartan (B) or simvastatin (C). No change in tyrosine phosphorylation of STAT3 was noted in the rat left ventricle. D, Ang II infusion activated Rac1 in rat atria (upper panel), which was attenuated by oral losartan or simvastatin (lower panel). E, Ang II infusion increased expression of COL1A1 (upper panel) and ANP (lower panel) in rat atria, both of which were attenuated by oral losartan or simvastatin. n=3 Animals per group; data are mean±SD. *P<0.05 (trend toward increase after Bonferroni correction), **P<0.01 vs control; ##P<0.01 vs Ang II infusion. IB indicates immunoblot; CTL, control; p-, phosphorylated; NC, negative control; PC, positive control; Los, losartan; and SIM, simvastatin.

leucine uptake and ANP expression, which was attenuated by overexpressing STAT3Y705F (Figure 5D).

Accumulation of extracellular matrix and fibrosis are also important structural changes in AF.^{33,34} We used [³H]-proline uptake and COL1A1 expression to represent collagen and extracellular matrix synthesis in atrial fibro-

blasts.^{35,36} We found an increase and decrease in [³H]-proline uptakes and COL1A1 expression by overexpressing STAT3C and STAT3Y705F, respectively (Figure 5E). Ang II also induced [³H]-proline uptake and COL1A1 synthesis, which were attenuated by overexpressing STAT3Y705F (Figure 5F).

Ang II Infusion Activated STAT3 and Induced Atrial Fibrosis in Rats, Which Was Inhibited by Losartan and Simvastatin

To validate the results obtained from cellular studies, we infused Ang II and investigated the activation of STAT3 and structural changes in the atrium using an in vivo rat model. Infusion of Ang II increased activated Rac1 (GTP-bound Rac1; Figure 6D) and phospho-STAT3 (Figure 6A) levels in

Figure 5 (Continued). STAT3Y705F showed a trend to attenuate Ang II-induced [³H]-leucine uptake and ANP expression in atrial fibroblasts (column 4 versus column 5, uncorrected P<0.05). Cells transfected with empty vector were used for the standard (100%). Data are mean±SD. n=6 for [³H] incorporation studies; n=3 for other studies. Fluc/Rluc indicates the ratio of firefly luciferase level (STAT3 reporter) to Renilla luciferase level (internal control). *P<0.05 (trend toward increase after Bonferroni correction), **P<0.01 vs empty vector transfection; #P<0.05 vs empty vector transfection plus Ang II.

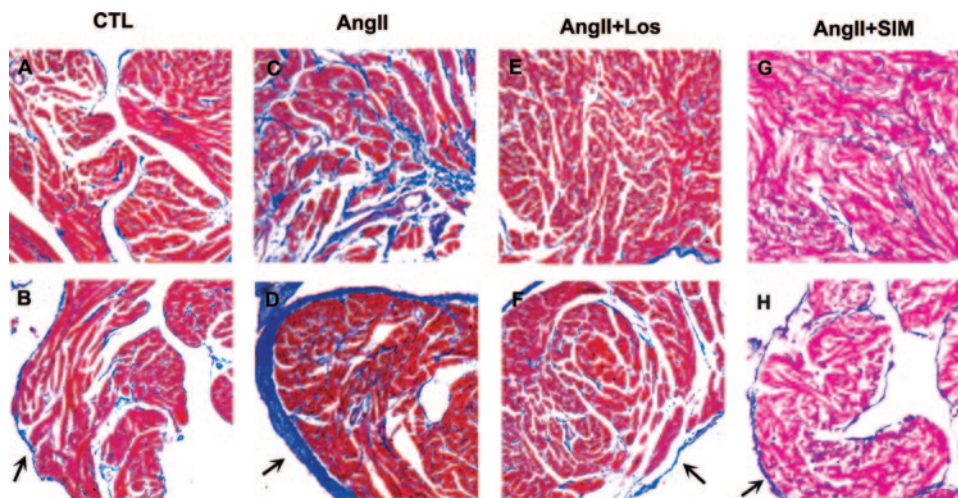


Figure 7. Masson's trichrome-stained light microscopic pictures of left atrial tissues from representative rats in each group. Ang II infusion group showed more extracellular matrix accumulation and fibrosis within the intercellular space (C) and in the pericardium (D; arrows) than the control group (A and B), which were attenuated by cotreatment with oral losartan (E and F) or simvastatin (G and H). Original magnification $\times 200$. $n=3$ animals per group. CTL indicates control; SIM, simvastatin.

the atria in a time-dependent manner, which was blocked or even decreased by oral losartan (Figure 6B and 6D) and simvastatin (Figure 6C and 6D). Ang II infusion for 14 days significantly increased expression of ANP and COL1A1, and this was also attenuated by oral losartan and simvastatin treatment (Figure 6E). Left atrial tissue sections after 14 days' infusion showed atrial fibrosis and pericardial thickening (Figure 7C and 7D), which were attenuated by oral losartan (Figure 7E and 7F) and simvastatin (Figure 7G and 7H).

Human Atrial Samples

The clinical characteristics of the study subjects are shown in the Table. AF patients had greater mean left atrial dimensions than those without AF. No significant differences in other clinical variables were detected between AF and non-AF

patients. Because of the limited amount of protein from each sample, we only investigated the status of STAT3 tyrosine phosphorylation and measured tissue Ang II concentration. We found significantly higher levels of tissue Ang II (39.5 ± 15.5 versus 21.2 ± 7.47 pg/mg protein, $P=0.026$; Figure 8A) and phospho-STAT3 (Figure 8B) in patients with AF than in those without AF, with no significant difference in total STAT3 level.

Discussion

Despite abundant evidencing addressing the detailed signaling mechanisms in the ventricular myocardium, very little information is available about the atrial myocardium. Using in vitro cellular and in vivo rat models, we demonstrated that Ang II/Rac1/STAT3 is an important signaling pathway in the

Table. Patient Characteristics

| | Age, y | Sex | VHD | HTN | DM | LVEF, % | LAD, mm | Drugs |
|--------------------|-----------------|-----|--------|-----|-----|-----------------|-----------------|----------|
| Sinus rhythm (n=6) | 65 | M | MR | 0 | 0 | 42 | 42 | Di |
| | 78 | M | AR | 0 | 0 | 58 | 47 | D |
| | 54 | M | MR | 0 | 0 | 63 | 48 | D, Di, |
| | 80 | M | AR | 1 | 1 | 39 | 51 | C, Di, O |
| | 64 | F | MR | 0 | 0 | 62 | 42 | Di, D |
| | 52 | F | MR | 1 | 0 | 60 | 48 | Di, D |
| Mean \pm SD | 65.5 \pm 11.7 | ... | ... | ... | ... | 54.1 \pm 10.6 | 46.3 \pm 3.6 | ... |
| AF (n=6) | 73 | F | MR | 1 | 0 | 65 | 49 | Di |
| | 55 | M | MR, AR | 1 | 1 | 61 | 57 | O, Di, D |
| | 74 | M | AR | 1 | 0 | 35 | 54 | Di, D |
| | 65 | M | MR | 0 | 1 | 43 | 48 | C, Di, O |
| | 68 | F | AR | 0 | 0 | 60 | 53 | Di, D |
| | 73 | M | MR | 1 | 0 | 56 | 54 | Di, D |
| Mean \pm SD | 68.2 \pm 7.3 | ... | ... | ... | ... | 53.3 \pm 11.7 | 52.5 \pm 4.0* | ... |

VHD indicates valve disease requiring valve replacement; HTN, hypertension; DM, diabetes mellitus; LVEF, left ventricular ejection fraction; LAD, left atrial dimension; M, male; MR, mitral regurgitation; Di, diuretics; AR, aortic regurgitation; D, digoxin; C, calcium-channel blockers; O, oral hypoglycemic agent; F, female; 1, yes; and 0, no.

* $P < 0.05$ vs sinus rhythm.

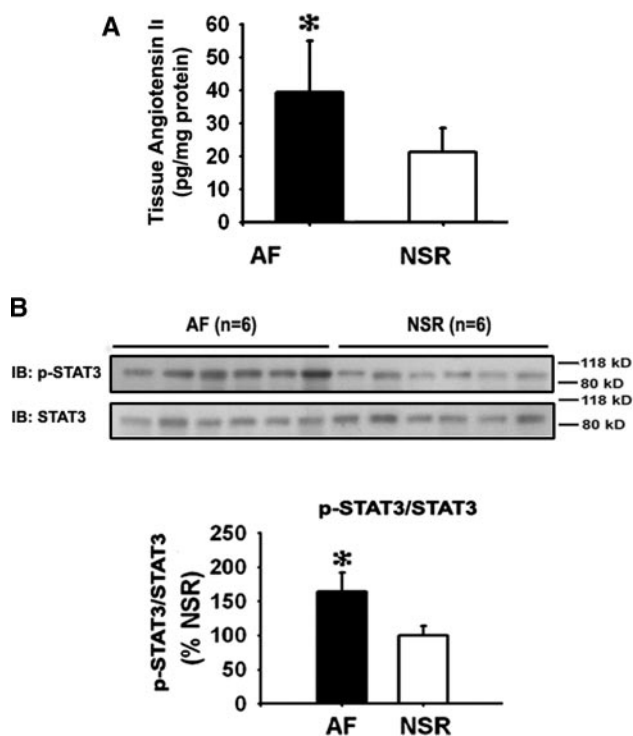


Figure 8. Tissue levels of Ang II and ratios of phosphorylated (p-) to total forms of STAT3 from right atrial appendages were higher in patients with AF than those with normal sinus rhythm (NSR). A, Tissue levels of Ang II were measured by ELISA. B, Tissue levels of phosphorylated and total forms of STAT3 were measured by immunoblotting. n=6 per group; data are mean±SD. * $P<0.05$ vs NSR group.

mediation of structural changes in the atrium that has never been reported before. This pathway may play an important role in the pathogenesis of AF, because we found an increase in tissue Ang II levels and tyrosine phosphorylation of STAT3 in atrial samples from patients with AF.

Recently, several studies have shown the efficacy of statins in the treatment of AF.^{37–39} In atrial myocytes and fibroblasts, Rac1 mediates the Ang II–dependent activation of JAK/STAT and hypertrophic and fibrotic phenotypes, respectively, both of which are inhibited by simvastatin. To the best of our knowledge, this is the first study that demonstrates the molecular mechanism by which statins block Ang II signaling in atrial myocytes and fibroblasts. In this regard, the combination of a statin and an angiotensin receptor blocker or angiotensin-converting enzyme inhibitor may be a good therapeutic option for the treatment of Ang II–induced atrial structural remodeling in AF.

With regard to Ang II–induced STAT3 activation, 2 distinct mechanisms operate in atrial myocytes and fibroblasts, both of which require Rac1 activation. In atrial myocytes, Ang II may induce the direct association of Rac1 with STAT3 and the subsequent activation of STAT3 by a JAK- or TYK2-independent mechanism.^{21,22} This Rac1-mediated STAT3 activation occurred as early as 5 minutes after Ang II stimulation. In atrial fibroblasts, activation of STAT3 by Ang II probably required Rac1-induced autocrine or paracrine factors and the activation of JAKs.^{23,24} Activation of STAT3 was noted >2 hours after stimulation. This

slower rate of tyrosine phosphorylation and inhibition by actinomycin D indicates the possibility of second-phase paracrine factor secretion in the activation of the JAK/STAT pathway.^{23,24}

Recently, it has been shown that Rac1 transgenic mice (racET) develop a significant dilatation of the atria, and downstream activation of PAK by Rac1 is important for the development of structural atrial changes.³⁰ In the present study, we found that Ang II induced PAK activation in atrial fibroblasts but not in atrial myocytes. Therefore, the mechanism of Ang II/Rac1-mediated atrial remodeling may not be through a cytoskeletal change in atrial myocytes, as observed in racET mice, but probably through enhanced atrial fibrosis. However, the significance of PAK activation in atrial fibrosis remains unknown and warrants further study.

In the present study, we demonstrated the important role of STAT3 in atrial structural remodeling. Fibrosis and hypertrophy are important structural changes in AF.^{31–34} Other profibrotic and hypertrophic pathways, such as calcineurin,^{28,32} mitogen-activated protein kinase,^{7,8} and transforming growth factor- β ,³⁹ have also been implicated in atrial remodeling. Therefore, multiple signaling pathways interact with one another to induce atrial remodeling. Most of these pathways are involved as one of the Ang II downstream signaling pathways. This could explain the findings of recent clinical trials that blockade of the renin-angiotensin system is an effective treatment of AF.^{40,41}

In addition to structural remodeling, Ang II may also play a role in electrical remodeling. Recently, we found that Ang II increases expression of the $\alpha 1C$ subunit of the L-type calcium channel (LCC) in atrial myocytes.⁴² Although the STAT binding site is also present in the promoter of the LCC $\alpha 1C$ subunit gene,⁴³ the transcriptional effect of Ang II is through the cAMP response element-binding protein (CREB), not STAT3. Furthermore, the change in expression of the LCC $\alpha 1C$ subunit by Ang II (upregulation) is the reverse of that observed in human AF (downregulation).⁴⁴ The relevance of Ang II–mediated LCC $\alpha 1C$ subunit expression to AF remains unclear and awaits further study.

In the rat model, significant tyrosine phosphorylation of STAT3 was found after 2 weeks' infusion of Ang II in the atrium but not in the ventricle. These results indicate that the atrium is more susceptible to Ang II stimulation than the ventricle, and Ang II–induced JAK/STAT activation is more unique in the atrium than in the ventricle. The mechanism of this finding is unknown and possibly due to the lower Ang II receptor density^{45,46} and the lower basal STAT3 expression (Figure 6) in the ventricle than in the atrium.

The present study included limitations. First, we demonstrated the impact of Ang II/Rac1/STAT3 signaling on atrial fibrosis; however, functional experiments with regard to AF are not provided. The rats also did not develop atrial arrhythmia after Ang II infusion. Second, the number of human atrial samples was limited, although statistical significance had been reached.

In conclusion, Ang II/Rac1/STAT3 is an important signaling pathway in the atrial myocardium, and the combination of a statin and an angiotensin receptor blocker may be a

reasonable approach to prevent or reverse Ang II–induced atrial structural remodeling in AF.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The renin-angiotensin-aldosterone system plays a pivotal role in various cardiovascular diseases. Clinical use of angiotensin-converting enzyme inhibitors or angiotensin blockers is helpful in preventing the progression of many cardiovascular diseases. Basic investigations of the angiotensin II receptor pathways in the heart have focused primarily on ventricular cells. In the present study, we investigated the downstream pathways of the angiotensin II receptor in atrial cells in an attempt to understand the pathogenesis of atrial structural remodeling. An important angiotensin II signaling pathway, the Janus kinase/signal transducers and activators of transcription (STAT) pathway, which has rarely been characterized in the atrium, was found to mediate angiotensin II-induced atrial structural remodeling. Angiotensin II activates signal transducer and activator of transcription 3 through a Rac1-dependent mechanism, which is inhibited by losartan (an angiotensin II type I receptor blocker) and a cholesterol-lowering agent, simvastatin, which inhibits Rac1 translocation. Our investigation not only reappraised the beneficial effects of blockade of the renin-angiotensin-aldosterone system in atrial fibrillation but also further elucidated the downstream pathways of the angiotensin II receptor involved in atrial structural remodeling. We also provide a rationale for the use of statins to prevent angiotensin II-related atrial structural remodeling. The clinical effects of statins in the treatment of atrial fibrillation merit further investigation.

Angiotensin II Activates Signal Transducer and Activators of Transcription 3 via Rac1 in Atrial Myocytes and Fibroblasts: Implication for the Therapeutic Effect of Statin in Atrial Structural Remodeling

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