Aldosterone stimulates proliferation of cardiac fibroblasts by activating Ki-RasA and MAPK1/2 signaling

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Stockand, James D., and J. Gary Meszaros. Aldosterone stimulates proliferation of cardiac fibroblasts by activating Ki-RasA and MAPK1/2 signaling. Am J Physiol Heart Circ Physiol 284: H176–H184, 2003. First published September 5, 2002; 10.1152/ajpheart.00421.2002.—Aldosterone plays a pathological role in cardiac fibrosis by directly affecting cardiac fibroblasts. Understanding of the cellular mechanisms of aldosterone action in cardiac fibroblasts, however, is rudimentary. One possibility is that aldosterone promotes proliferation of cardiac fibroblasts by activating specific cellular signaling cascades. The current study tests whether aldosterone stimulates proliferation of isolated adult rat cardiac myofibroblasts (RCF) by activating Kirsten Ras (Ki-RasA) and its effector, the MAPK1/2 cascade. Aldosterone (10 nM) significantly increased RCF proliferation. This action was sensitive to the mineralocorticoid receptor (MR) antagonist spironolactone. Expression of MR in RCF and the whole rat heart was confirmed by immunoblotting. Aldosterone significantly increased absolute and active (GTP bound) Ki-RasA levels in RCF. Aldosterone, in addition, significantly increased phospho-c-Raf and phospho-MAPK1/2. The effects of aldosterone on Ki-RasA and phospho-c-Raf proteins were inhibited by spironolactone but not RU-486, suggesting that aldosterone acts via MR. Inhibitors of MEK1/2 and c-Raf prevented aldosterone-induced activation of MAPK1/2 and proliferation. These results show that aldosterone directly increases RCF proliferation through MR-dependent activation of Ki-RasA and its effector, the MAPK1/2 cascade. Activation of cardiac fibroblasts through such a cascade may play a role in the pathological actions exerted by aldosterone on the heart.

mitogen-activated protein kinase; Kirsten Ras; mineralocorticoid receptor; myofibroblast; fibrosis

CARDIAC FIBROBLASTS are the major nonmyocyte cell constituent in the myocardium and play important roles in cardiac hypertrophy and failure. Proliferation of activated cardiac fibroblasts, followed by excess matrix deposition, are the two key events that contribute to the development of these pathological states and subsequent cardiac fibrosis. The phenotypically distinctive cardiac myofibroblast is an example of an activated cardiac fibroblast that is thought to play a role in heart pathology. Myofibroblasts are normally present in areas of myocardial damage and play an active role in wound healing and repair (21, 25, 40). A number of endocrine and paracrine factors activate cardiac fibroblasts through the action of diverse signal transduction cascades (18). However, the full compliment of hormones and the mechanisms by which they influence proliferation are not fully appreciated. It is accepted that, as in most other cell types, MAPK1/2 [also known as extracellular signal-regulated kinases 1/2 (ERK1/2) and p42/p44-MAPK] signaling mediates proliferation of cardiac fibroblasts through the activation of a number of tyrosine kinase-associated receptors and G protein-coupled receptors (7). Most notably, angiotensin II (ANG II) activates MAPK1/2 in cardiac fibroblasts to initiate, in part, its mitogenic and fibrotic effects (1, 7, 13, 29, 42).

Investigators of the Randomized Aldactone Evaluation Study (RALES) recently demonstrated that aldosterone plays a pathological role in heart disease (26). Several additional in vitro and in vivo studies support RALES results by demonstrating that aldosterone alters cardiac remodeling and promotes fibrosis (3–5, 9, 14). Indeed, aldosterone in some in vitro models promotes collagen production in cardiac fibroblasts (4, 9, 42) and in vivo increases collagen deposition, fibrosis, necrosis, and fibroblast proliferation in rat hearts in a spironolactone-sensitive manner (3, 5). Thus it has been suggested that aldosterone mediates some and possibly all of its pathological actions on the heart by direct effects on cardiac cells, including cardiac fibroblasts. These and other studies have sparked considerable interest in understanding the cellular and molecular mechanisms of aldosterone action in myocardial cells, where little is known about the signal transduction pathways utilized by this steroid hormone. In comparison, more is known about aldosterone action in its classic target tissues, colonic and renal epithelia (23, 35, 38, 39), and much may be gained with

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respect to study of aldosterone action in the heart by considering its action on these cells.

In epithelia, aldosterone modulates cellular activity by directly affecting gene expression (33). Some genes regulated by aldosterone encode signaling factors associated with control of cellular proliferation, growth, and differentiation. The aldosterone-induced genes encoding the A splice variant of the small, monomeric G protein Kirsten Ras (Ki-RasA) and the serine and threonine kinase serum and glucocorticoid-inducible kinase are two examples of such genes (33). Ki-RasA is a well-known activator of the MAPK1/2 cascade. Indeed, we (35) and the laboratory of Verrey (31, 32) have shown that aldosterone activates this cascade in epithelia via induction of Ki-RasA.

Because activation of the MAPK1/2 cascade is known to play a prominent role in promoting growth of myocardiial cells in response to various endocrine signals, including ANG II (22, 29), we asked in the current study whether aldosterone affects cardiac fibroblast proliferation through induction of Ki-RasA and its effector, the MAPK1/2 cascade. The current results demonstrate, for the first time, that aldosterone, via the mineralocorticoid receptor (MR), induces Ki-RasA expression and subsequently activates the MAPK1/2 cascade in isolated rat cardiac myofibroblasts (RCF), with one functional consequence of this being increased proliferation.

**MATERIALS AND METHODS**

**Materials.** Aldosterone, mifepristone (RU-486), and spironolactone were purchased from Sigma and stored frozen as 1.5, 10, and 10 mM (in DMSO) stocks, respectively. PD-98059 and ZM-336372 were from Calbiochem and Tocris, respectively. PD-98059 (0.005% bromophenol blue, 10% glycerol, 3% SDS, 1 mM Tris·HCl, and 20 mM dithiothreitol), lysates were loaded with identical amounts of total protein. Typically Bioreagents. The mouse monoclonal anti-c-Raf-1 antibody was from Transduction Laboratories. The rabbit polyclonal anti-Fra-2 and anti-Ki-RasA antibodies were from Santa Cruz Biotechnology. All phospho-specific antibodies under a ×60 objective.

**Western blot analysis.** All reagents used for Western blot analysis were from Bio-Rad and Pierce unless otherwise noted. For each lysate, the protein concentration was determined with the BCA protein assay. Kodak BioMax Light-1 film was exposed to Kodak Biomax films (Racl) was used to develop Western blots. Whole RCF lysates were extracted on ice after three washes with Tri-s-buffered saline (TBS) using standard procedures (35). Cells were scraped and then maintained for 1–2 h at 4°C in gentle lysis buffer (GLB) containing 76 mM NaCl, 50 mM Tris-HCl, and 2 mM EGTA plus 1% Nonidet P-40, 10% glycerol (pH 7.4), and protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, tosylphenylalanil chloride methyl ketone, and 1-chloro-3-tosylamido-7-amino-2-heptanone). For Western blot analysis of phosphorylated proteins, GLB was supplemented with (in mM) 0.1 NaP, 0.5 NaF, 0.1 ZnCl2, and 0.04 Na3VO4 prepared fresh from ×1,000 stocks. After the clearing of cellular debris, standardization of total protein concentration, and addition of Laemmli sample buffer (0.005% bromophenol blue, 10% glycerol, 3% SDS, 1 mM EDTA, 77 mM Tris-HCl, and 20 mM dithiothreitol), lysates were heated to 85°C for 10 min. Proteins were then separated by standard SDS-PAGE and then transferred electrophoretically to nitrocellulose (0.2 µM). Western blot analysis was performed using standard techniques and appropriate antibodies (34, 36), and primary and secondary antibodies were used at 1/1,000 and 1/20,000, respectively. Tween 20 (0.1%) and 5% dried milk (Carnation) were used as blocking reagents. Band intensity was quantified with densitometric scanning using SigmaGel (Jandel Scientific). When possible, the flood configuration with the highest practical threshold was used to measure band density. All lanes within a blot were loaded with identical amounts of total protein. Typically, blots were loaded with 50–80 µg total protein/lane. Ras-GTP assay. c-Raf-1 RBD agarose was from Upstate Biotech. This immobilized protein corresponds to the human c-Raf-1 Ras binding domain (residues 1–149) and binds Ras complexed with GTP. Pulldown experiments were performed in 100 µl (0.4 mg total protein) of whole cell lysate isolated with GLB. Lysates were incubated with 30 µl c-Raf-1 RBD agarose overnight (at 4°C and with constant agitation); pellets were washed five times with 2 vol of fresh GLB each time for a total wash time of 2 h. After being resuspended in sample buffer and heated, c-Raf-1 RBD agarose-precipitated proteins were separated by SDS-PAGE, and Ki-RasA-GTP was identified by immunoblotting.

**Assessment of cellular proliferation.** Cardiac fibroblasts were seeded onto 35-mm tissue culture dishes at a split ratio of 1:3, or 1.2 × 105 cells/well. These cells were incubated in DMEM containing 10% FBS for 6 h, after which they were
rinced twice with PBS and maintained in serum-free DMEM for 48 h before the addition of pharmacological inhibitors and/or hormones. ANG II (100 nM) and aldosterone (1–10 nM) were added from 1,000 stocks. The inhibitors were added from frozen stocks 30 min before the hormones at the following concentrations: 0.1 μM spironolactone, 10 μM PD-98059, and 1 μM ZM-241385. [3H]thymidine (ICN; Irvine, CA) was added for the duration of the hormone treatments (48 h) at 2 μCi/ml. The growth assays were terminated by three washes in cold PBS followed by 1-h incubation in 10% trichloroacetic acid, solubilization in 0.5 M NaOH, and counting by liquid scintillation spectrometry.

Statistics. All data are presented as means ± SE. Comparisons were made using t-tests and one-way ANOVA with P < 0.05 considered significant.

RESULTS

Cardiac myofibroblast characterization. Isolated cardiac fibroblasts at passage 3 and vascular smooth muscle cells (positive controls) were seeded onto glass microscope slides for immunocytochemical analysis. RCF (Fig. 1, A and B) and vascular smooth muscle cells (Fig. 1, C and D) were double stained with fluorescent antibodies against vimentin (Fig. 1, A and C) and smooth muscle α-actin (Fig. 1, B and D). Both cell types stained positive for vimentin and smooth muscle α-actin in double-stained cultures as well as single-stained ones (data not shown). These latter experiments excluded bleedthrough between the FITC and rhodamine filters. Manual cell counts were conducted for 200 RCF cells at passage 3. These results, where 82% (164/200) of passage 3 cells stained positive for smooth muscle α-actin, indicate that the majority of the cells used in the current study were of the myofibroblast subtype.

Aldosterone increases proliferation of isolated adult RCF. Administration of 10 nM aldosterone to subconfluent cultures of cardiac fibroblasts, as shown in Fig. 2A, significantly increased [3H]thymidine incorporation after 48 h (135.6 ± 4.3% of controls, n = 7). Aldosterone at 1 nM similarly caused marked cell proliferation, although this increase did not reach statistical significance. ANG II, well known for its mitogenic effects on RCF (7, 29), increased [3H]thymidine incorporation (145.8 ± 11.6%, n = 4) as expected. Interestingly, 10 nM aldosterone was as nearly effective as 100 nM ANG II in promoting growth. The [3H]thymidine incorporation results were confirmed by assessment of hormone-stimulated changes in cell number by nuclear staining with crystal violet and manual cell counting (data not shown). The mitogenic effects of 10 nM aldosterone were significantly inhibited by 0.1 μM spironolactone (Fig. 2B), suggesting that aldosterone at this dose affects RCF through MR.

The presence of MR in cardiac cells and RCF in particular is controversial (14, 15). The results shown in Fig. 2B are consistent with MR being present in the cells used in the current study (see also Fig. 5). The results in Fig. 2C are also consistent with this possibility. The representative Western blot (n = 3) in Fig. 2C contained whole cell lysate from RCF, the whole adult rat heart, and A6 cells and was probed with anti-MR antibody. Positive reactivity for a protein of appropriate size (∼120 kDa) was observed in both RCF and the whole rat heart but not A6 cells, a cell line known to lack MR (6).

Aldosterone increases Ki-RasA levels in RCF. Aldosterone increases expression of Ki-RasA in epithelia (32, 35). We examined whether aldosterone also increases absolute and active Ki-RasA levels in RCF. The representative Western blot shown in Fig. 3A is of whole cell lysate from RCF treated with 50 nM aldosterone for various times ranging from 0 to 48 h. This blot was probed with an anti-Ki-RasA antibody, which specifically reacts with Ki-RasA but not Ki-RasB, Ha-Ras, and N-Ras. Often Ras proteins, including Ki-RasA, appear as doublets, as shown here, upon SDS-PAGE. The faster migrating species (∼19 kDa) is mature Ras protein fully processed by posttranslation modifications. As shown in the summary graph of Fig. 3B, aldosterone increased Ki-RasA levels as early as 1–2 h, reaching statistical significance at 4 and 6 h. The Ki-RasA levels (measured as the sum of both bands) at 4 and 6 h were 2.4 ± 0.4-fold (n = 7) and 3.1 ± 0.4-fold (n = 3) over control values, respectively. By 24 h, Ki-RasA levels were 0.9 ± 0.2-fold (n = 3) of pretreatment values.

Aldosterone also increased, as shown by the representative Western blot shown in Fig. 3C, the amount of active, GTP-bound Ki-RasA (2.5 ± 0.7, n = 3). For these experiments, cells were treated with vehicle and aldosterone for 4 h, and the GTP-bound form of Ras was isolated from lysates with 400 μg (in 400 μl) total protein using Raf-RBD (see MATERIALS AND METHODS). Ki-RasA:GTP was identified by subsequently probing Western blots containing Ras:GTP from each group with anti-Ki-RasA antibody.

Aldosterone stimulates the MAPK1/2 cascade in RCF in a sustained manner. The MAPK1/2 cascade is activated by Ki-RasA in many cells; thus we tested, as shown in Fig. 4, whether aldosterone stimulates MAPK1/2 signaling in RCF. The results shown in Fig. 4A (and summarized in Fig. 4B) demonstrate that in RCF, aldosterone increases the activated (phosphorylated) protein levels of several members of the MAPK1/2 cascade. Phospho(Ser259)-c-Raf levels were significantly increased 2.8 ± 0.3-fold (n = 7) after 4 h of aldosterone treatment. In contrast, total cellular c-Raf expression was unaffected by steroid with the relative increase 3.0-fold by 4 h (n = 3). At this time point, total MAPK1/2 levels of 0.8 ± 0.1 were unchanged (n = 4). By 24 h, the relative levels of phosho-MAPK1/2 and total MAPK1/2 did not differ from basal levels at time 0.
Fig. 1. Smooth muscle α-actin expression in cardiac myofibroblasts. Passage 3 adult rat cardiac myofibroblasts (RCF; A and B) and vascular smooth muscle cells (C and D) were fixed, permeabized, and double stained with conjugated primary antibodies for vimentin (rhodamine conjugate; A and C) and smooth muscle α-actin (fluorescein conjugate; C and D) before fluorescent imaging. Positive staining of RCF with smooth muscle α-actin was detected in >80% of the cells.
Aldosterone increases Ki-RasA levels and stimulates MAPK1/2 signaling in RCF through MR. The experiments shown in Fig. 5 tested the possibility that aldosterone activates Ki-RasA and its downstream effectors via MR in RCF. The representative Western blot (n = 3) shown in Fig. 5A shows the dose-dependent effects of aldosterone in the absence and presence of MR (spironolactone) and glucocorticoid receptor (RU-486) antagonists on the levels of Ki-RasA, phospho-c-Raf, total c-Raf, and the activator protein-1 transcription factor Fra-2, which has been shown previously to be a possible downstream target of MAPK1/2 signaling (17). For these experiments, cells were treated for 4 h with aldosterone with and without antagonist (0.1 μM). As shown in the summary graph of Fig. 5B, aldosterone at 1 and 10 nM significantly increased the relative levels of Ki-RasA and phospho-c-Raf, whereas the total c-Raf levels were not affected. Spironolactone but not RU-486 markedly attenuated the actions of aldosterone (10 nM) on Ki-RasA and phospho-c-Raf. Similar to Ki-RasA, Fra-2 protein levels increased to 1.7- and 2.1-fold (n = 2) in response to 4-h treatment with 1 and 10 nM aldosterone, respectively. The aldosterone-induced increase in Fra-2 was partially attenuated by spironolactone but not RU-486 (1.5- and 3.7-fold, respectively).

Aldosterone promotes RCF proliferation through MAPK1/2 signaling. Because aldosterone promoted RCF proliferation and activated Ki-RasA and MAPK1/2 signaling and because MAPK1/2 signaling is known to impinge upon cardiac cell proliferation, we questioned whether aldosterone stimulates RCF proliferation via the MAPK1/2 cascade. As shown in Fig. 6A, the MEK1/2 inhibitor PD-98059 (10 μM) and the c-Raf kinase inhibitor ZM-336372 (1 μM) significantly attenuated aldosterone-induced RCF proliferation.

The Western blots shown in Fig. 6B were performed to determine whether ZM-336372-dependent inhibition of aldosterone-induced RCF proliferation was due to blockade of MAPK1/2 signaling. The representative Western blot (n = 4) was probed with anti-phospho-MAPK1/2 antibody (Fig. 6B, top) and subsequently stripped and reprobed with anti-MAPK1/2 antibody (Fig. 6B, bottom). The blot contains equal amounts (80 μg) of whole RCF lysate from cells treated with vehicle (control) and 10 nM aldosterone in the absence and presence of ZM-336372 (1 μM) for 4 h. In the presence of aldosterone and ZM-336372, the ratio of phospho-MAPK1/2 to MAPK1/2 was 1.3 ± 0.09-fold of untreated controls (n = 4), which was markedly lower than that for aldosterone alone (2.5 ± 0.7).
DISCUSSION

Our results indicate that aldosterone stimulates proliferation of isolated adult RCF and are consistent with a cellular mechanism of action whereby aldosterone via the MR activates Ki-RasA and the MAPK1/2 cascade to promote this proliferation. These in vitro results are consistent with both the in vitro (2, 9, 29, 42) and in vivo (3, 5, 15, 16, 26, 41) work of others. The data presented here along with those of previous studies suggest that aldosterone-dependent activation of Ki-RasA and the MAPK1/2 cascade in RCF could play a
role in the recently documented pathological actions of this steroid hormone on the heart.

Recent studies demonstrate that aldosterone increases Ki-RasA levels in epithelia (31, 32, 35). This action is dependent on corticosteroid-mediated changes in transcription of the Ki-ras gene at identified cis-

Fig. 5. ALDO increases Ki-RasA levels and activates the MAPK1/2 cascade in RCF via MR. A: Western blots showing the effects of ALDO in the presence and absence of Spiro and RU-486 on Ki-RasA (19 and 21 kDa), phospho-c-Raf (75 kDa), total c-Raf (75 kDa), and Fra-2 (45 kDa) protein levels. Each lane contained equal amounts of total protein extracted from RCF treated for 4 h with vehicle or 1.0 and 10 nM ALDO ± Spiro (0.1 μM) and RU-486 (0.1 μM). C: summary graph of 3 experiments (means ± SE) from Ki-RasA and phospho-c-Raf Western blots (from A) expressed as the degree of change over protein levels of untreated cells at time 0 (assigned a value of 1.0 and denoted by the dotted line). *Statistically significant increase over control levels and **significant reduction vs. 10 nM ALDO alone, P < 0.05 for each by unpaired t-test.

Fig. 6. Inhibition of the MAPK1/2 cascade attenuates ALDO-induced RCF proliferation. A: cells were treated for 48 h with vehicle (control; DMSO), ANG II (100 nM), and ALDO (10 nM) in the absence and presence of the MEK1/2 and c-Raf kinase inhibitors PD-98059 (PD; 10 μM) and ZM-336372 (ZM; 1.0 μM), respectively. Results are expressed as a percentage of control, where control = 100%. *Statistically significant differences between ANG II and ALDO plus PD-98059 and between ALDO plus ZM-336372-treated cells vs. ALDO and ANG II alone by one-way ANOVA (P < 0.05). B: representative Western blot containing whole cell lysates from RCF treated with ALDO (10 nM), vehicle (DMSO), and ALDO plus ZM-336372 (1.0 μM) for 4 h. This blot was probed with anti-phospho-MAPK1/2 antibody (top; ~42 and 44 kDa) and subsequently stripped and reprobed with anti-MAPK1/2 antibody (bottom; ~42 and 44 kDa).
elements (20, 24, 30, 37). The aldosterone-dependent increase in Ki-ras mRNA in both renal and colonic epithelia begins at least 30 min after steroid addition and is a primary action independent of de novo protein synthesis (Ref. 31 and P. Fuller, personal communications). Similarly, elevation of Ki-RasA protein is detectable within 1 h (32, 35). An effect of aldosterone on Ki-RasA levels in tissue other than epithelia, including cardiac cells, to our knowledge has not been investigated. Our data demonstrate that aldosterone markedly increases Ki-RasA protein levels in RCF as early as 1 h, with a significant increase becoming apparent by 2 h. The time course of Ki-RasA induction in RCF, as reported here, is similar to that in epithelia. The MR antagonist spironolactone attenuated induction of Ki-RasA in RCF by aldosterone, suggesting that this effect was mediated by the classic corticosteroid transcriptional paradigm. These data are the first directly linking aldosterone action in rat cardiac fibroblasts to induction of a signaling factor, Ki-RasA, capable of promoting cellular proliferation, as well as other hallmark events of heart disease, such as fibrosis and increased matrix secretion.

In addition, our results establish that aldosterone in RCF activates downstream signaling effectors of Ki-RasA in the MAPK1/2 cascade, including phosphorylated forms of c-Raf, MEK1/2, and MAPK1/2. The time course of activation of the MAPK1/2 cascade constituents, which appears maximal at 4 h, is consistent with that of Ki-RasA, suggesting that this drives MAPK1/2 signaling in RCF. Aldosterone activates the MAPK1/2 cascade in epithelia in a similar manner, by first inducing transcription of Ki-Ras (32, 35).

Aldosterone promotes inappropriate remodeling of the heart, fibrosis, increased secretion of collagen from cardiac fibroblasts, and fibroblast proliferation (3–5, 21, 41, 42). The Ras → MAPK1/2 cascade is a well-known mitogenic pathway in many diverse cell types and is activated in some instances during proliferation of cardiac fibroblasts and hypertrophy of cardiac myocytes (7, 8, 10, 12, 22, 29). Thus we tested whether aldosterone-increased Ki-RasA levels and activation of the downstream MAPK1/2 cascade promoted proliferation of isolated male rat cardiac fibroblasts. Inhibition of the MAPK1/2 cascade abrogated the effect of aldosterone to stimulate cardiac fibroblast proliferation in this current study. This finding directly links, for the first time, aldosterone-induced MAPK1/2 signaling to stimulation of RCF proliferation. The fact that the proliferation studies were done in the complete absence of serum suggests that aldosterone alone is a potent mitogenic signal for RCF. Furthermore, pharmacological inhibition of c-Raf and MEK1/2 blocks the mitogenic effects of aldosterone, suggesting that activation of Ki-RasA → MAPK1/2 signaling by aldosterone is causative of RCF proliferation. The importance of MAPK1/2 in causing proliferation of RCF is supported by data showing that ANG II-induced cell growth was also sensitive to MAPK1/2 inhibition, in agreement with previous studies (7, 22, 29). Thus activation of the MAPK1/2 cascade may represent a point of signaling convergence that stimulates cardiac fibroblast growth and contributes to the pathological actions of aldosterone and ANG II in the myocardium.

While it is often difficult and not always appropriate to extend in vitro results to the in vivo situation, the current results are consistent with the findings of others discussed above that suggest that aldosterone has direct effects on cardiac fibroblasts via MR. Further investigation is needed to determine whether aldosterone action on the heart involves other cell types and receptors. The current experimental design tested only whether aldosterone affects Ki-RasA and MAPK1/2 signaling in RCF via MR and did not directly test the parallel hypothesis that this corticosteroid at higher doses is also capable of affecting cardiac cells via glucocorticoid receptors or whether aldosterone exerts non-genomic actions on cardiac tissues. The rationale for such a design is that the pathological action of aldosterone on the heart is abrogated by the specific MR antagonist spironolactone (3, 4, 26, 28, 41).

In conclusion, the current study demonstrates that aldosterone stimulates RCF proliferation via activation of Ki-RasA and the MAPK1/2 cascade in a MR-dependent manner. Activation of the MAPK cascade may represent a key signaling convergence point that stimulates cardiac fibroblast and myofibroblast growth, thus contributing to the pathological actions of aldosterone and ANG II in the myocardium.

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