Aldosterone Stimulates Elastogenesis in Cardiac Fibroblasts via Mineralocorticoid Receptor-independent Action Involving the Consecutive Activation of Ga13, c-Src, the Insulin-like Growth Factor-I Receptor, and Phosphatidylinositol 3-Kinase/Akt*

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We previously demonstrated that aldosterone, which stimulates collagen production through the mineralocorticoid receptor (MR)-dependent pathway, also induces elastogenesis via a parallel MR-independent mechanism involving insulin-like growth factor-I receptor (IGF-IR) signaling. The present study provides a more detailed explanation of this signaling pathway. Our data demonstrate that small interfering RNA-driven elimination of MR in cardiac fibroblasts does not inhibit aldosterone-induced IGF-IR phosphorylation and subsequent increase in elastin production. These results exclude the involvement of the MR in aldosterone-induced increases in elastin production. Results of further experiments aimed at identifying the upstream signaling component(s) that might be activated by aldosterone also eliminate the putative involvement of pertussis toxin-sensitive Ga proteins, which have previously been shown to be responsible for some MR-independent effects of aldosterone. Instead, we found that small interfering RNA-dependent elimination of another heterotrimeric G protein, Ga13, eliminates aldosterone-induced elastogenesis. We further demonstrate that aldosterone first engages Ga13, and then promotes its transient interaction with c-Src, which constitutes a prerequisite step for aldosterone-dependent activation of the IGF-IR and propagation of consecutive downstream elastogenic signaling involving phosphatidylinositol 3-kinase/Akt. In summary, the data we present reveal new details of an MR-independent cellular signaling pathway through which aldosterone stimulates elastogenesis in human cardiac fibroblasts.

Aldosterone is a major component of the renin-angiotensin-aldosterone system, which plays an important role in the regulation of electrolyte and fluid balance (1, 2). The majority of aldosterone-induced effects occur after it binds to the intracellular MR. The activated aldosterone-MR complex translocates to the nucleus, where it modulates the transcription and translation of “aldosterone-induced” proteins involved in blood pressure homeostasis.

Aldosterone has also been implicated in the stimulation of collagen synthesis and myocardial fibrosis through a process that is independent of its effect on blood pressure (3–5). Two clinical studies, the Randomized Aldactone Evaluation Study (6) and the Eplerenone Post-acute Myocardial Infarction Heart Failure Efficacy and Survival Study (7), demonstrated that low doses of MR antagonists lead to a dramatic reduction in the mortality rate of patients who suffered acute myocardial infarctions. Despite the suggestion that these MR antagonists may alleviate maladaptive remodeling of the extracellular matrix (ECM) of post-infarct hearts (8, 9), the molecular mechanisms by which they improve overall heart function in those patients have not been fully resolved.

It has been also shown that aldosterone can induce numerous effects in a wide range of nonepithelial tissues, including heart, and that it may act through membrane receptors other than the traditional MR (alternative receptors) in epithelial and nonepithelial tissue in a nongenomic manner (10–13).

Although the classical genomic model of aldosterone action has long been accepted, the rapid, nongenomic mechanism of aldosterone action is not yet fully elucidated (2). However, it has been proposed that some of these nongenomic effects of aldosterone also require the presence of MR or a closely related protein (14). In contrast, other studies have shown that the nongenomic aldosterone effects still occur in cell lines lacking the classical MR and in yeast devoid of MR or in normal cells treated with MR antagonists (2, 11, 15). Such results strongly suggest the involvement of other receptor(s), distinct from the classic MR, that may interact with aldosterone and trigger the nongenomic effects of this hormone. Although full structural characterization of this putative receptor (or receptors) has not been completed yet (16), data suggest that some MR-independ-

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2 The abbreviations used are: MR, mineralocorticoid receptor; ECM, extracellular matrix; IGF-I, insulin-like growth factor-I; IGF-IR, IGF-I receptor; BSA, bovine serum albumin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; JNK, c-Jun N-terminal kinase; Rb, retinoblastoma protein; siRNA, small interfering RNA; PI, phosphatidylinositol 3-kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; MEK, MAPK/ERK kinase.
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ent effects of aldosterone occur after activation of the pertussis toxin-sensitive heterotrimeric G proteins (13, 17).

Results of our previous studies have revealed a novel mechanism in which aldosterone and its antagonists modulate the production of elastin, an important ECM component that provides resilience to many tissues, including stroma of the heart. We discovered that aldosterone can stimulate elastogenesis in cultures of human cardiac fibroblasts via an MR-independent mechanism involving IGF-IR activation (18). We have therefore uncovered another level of complexity in which aldosterone in conjunction with MR antagonists may modulate the remodeling of the injured heart.

In the present study we provide compelling evidence demonstrating that cultured cardiac fibroblasts, in which the production of MR has been inhibited by siRNA, still exhibit the aldosterone-induced increase in elastin production. We also present the first evidence that this MR-independent elastogenic effect of aldosterone can be triggered by a signaling pathway that involves initial activation of the heterotrimeric G protein Go13 and consecutive activation of c-Src, IGF-IR, and PI 3-kinase/Akt signaling.

EXPERIMENTAL PROCEDURES

Materials—All chemical grade reagents, aldosterone, protease inhibitors, agarose-linked protein A, pertussis toxin, recombinant human IGF-I, PD 98059, PD123319, AlCl3, and NaF, as well as secondary antibodies fluorescein-conjugated goat anti-rabbit, fluorescein-conjugated goat anti-mouse, and fluorescein-conjugated rabbit anti-goat were obtained from Sigma. Wortmannin, PP2, SP600125, and Y-27632 were purchased from Calbiochem. Losartan was purchased from Cayman Chemicals Co. (Ann Arbor, MI). A cell-permeable Rho inhibitor (exoenzyme C3 transferase, CT04) was purchased from Cytoskeleton, Inc. (Denver, CO). Iscove’s modified Dulbecco’s medium, fetal bovine serum, 0.2% trypsin with 0.02% EDTA, and other cell culture products were acquired from Invitrogen. Polyclonal antibody to tropoelastin was purchased from Elastin Products (Owensville, MI). Polyclonal antibody to tropoelastin was obtained from Fitzgerald Industries Intl. (Concord, MA). As specified by the manufacturer, 25 aldosterone molecules are covalently linked to each BSA molecule through a carboxymethyl oxyime residue on the C-3 of the hormone, forming a stable conjugate (19).

Cultures of Human Cardiac Fibroblasts—We used cardiac fibroblasts isolated from human fetal hearts (which are responsible for the production of cardiac ECM) to make our studies clinically relevant. Human fetal cardiac fibroblasts of 20–22 weeks gestation, a generous gift from Dr. John Coles, were prepared in accordance with an institutional review board-approved protocol (20). Confluent cultures were passaged by trypsinization and maintained in Iscove’s modified Dulbecco’s medium supplemented with 1% antibiotics/antimycotics and 10% fetal bovine serum. Passage 1–3 cells were used in all experiments. The purity of these cultures at passage 1 was 95%. Cardiac fibroblasts were determined by positive staining for vimentin and negative for von Willebrand factor and α-smooth muscle cell actin, as previously described (21).

In experiments aimed at assessing ECM production, fibroblasts were initially plated (100,000 cells/dish) and maintained in a normal medium until confluency, the point at which they produce abundant ECM. Confluent cultures were then treated for 72 h with or without 50 nM of aldosterone (22).

In separate experiments we also tested the influence of an equimolar concentration of aldosterone that was coupled to BSA, which prevents it from penetrating into the cell interior, as demonstrated in previous studies (23, 24). The G protein inhibitor pertussis toxin (13), MAPK kinase inhibitor PD98059 (25, 26), JNK inhibitor SP600125 (27, 28), PI 3-kinase inhibitor wortmannin (29, 30), c-Src tyrosine kinase inhibitor PP2 (31), and Rho-associated kinase inhibitor Y-27632 (32, 33), as well as the AT1 receptor antagonist losartan (34, 35) and the AT2 receptor antagonist PD123319 (36, 37), were added 1 h prior to aldosterone treatment. Cell-permeable Rho inhibitor (CT04) was added 2 h prior to aldosterone treatment, as specified by the manufacturer. The cells were also treated for 3 h with aluminum fluoride solution (AlCl3 and NaF) prepared immediately before use, as previously described (38). All of the control cell cultures received an equal amount of the solvent vehicle.

Immunostaining—At the end of the 72-h incubation period with the indicated treatment, confluent cultures were fixed in cold 100% methanol at −20 °C (for elastin and MR staining) or in 4% paraformaldehyde at room temperature (for collagen staining) for 30 min and blocked with 1% normal goat serum for 1 h at room temperature. The cultures were then incubated for 1 h with either 10 μg/ml of polyclonal antibody to tropoelastin, 10 μg/ml of monoclonal antibody to MR, or 10 μg/ml of polyclonal antibody to collagen type I. All of the cultures were then
incubated for an additional hour with either fluorescein-conjugated goat anti-rabbit, fluorescein-conjugated goat anti-mouse, or fluorescein-conjugated rabbit anti-goat secondary antibodies to detect elastin, MR, and collagen type I staining, respectively. The nuclei were counterstained with propidium iodide. Secondary antibody alone was used as a control. All of the cultures were then mounted in elvanol and examined with a Nikon Eclipse E1000 microscope attached to a cooled CCD camera (QImaging, Retiga EX) and a computer-generated video analysis system (Image-Pro Plus software, Media Cybernetics, Silver Spring, MD). Initial magnification for all images was 600×.

Quantitative Assay of Insoluble Elastin—Fetal human cardiac fibroblast cultures were grown to confluency in 35-mm culture dishes (100,000 cells/dish) in quadruplicate. Then 2 μCi of [3H]valine/ml of fresh medium were added to each dish and treated as specified in the figure legends. Following a 72-h incubation, the cells were extensively washed with phosphate-buffered saline, and the cells including deposited insoluble ECM were scraped and boiled in 500 μl of 0.1 N NaOH for 30 min to solubilize all matrix components except elastin (39). The resulting pellets containing the insoluble elastin were then solubilized by boiling in 200 μl of 5.7 N HCl for 1 h, and the aliquots were mixed in scintillation fluid and counted (40). The aliquots taken from each culture were also used for DNA determination according to the manufacturer’s instructions, 1 μg of total RNA was added to each one-step RT-PCR kit, and the reactions were set up according to the manufacturer’s instructions in a total volume of 25 μl. The reverse transcription step was performed for elastin and GAPDH reactions at 50 °C for 30 min, followed by 15 min at 95 °C. The elastin PCR (sense primer: 5’-GGTGCGGTGGTTCCCTAGCCCTTG-3’; antisense primer: 5’-GGGCCTTGGAGATACCCCCAGTG-3’; designed to produce a 255-bp product) was performed under the following conditions: 25 cycles of 94 °C denaturation for 30 s, 60 °C annealing for 30 s, and 72 °C extension for 1 min and one cycle of 72 °C final extension for 10 min. 5-μl samples of the elastin, Go13, collagen type I, and GAPDH PCR products from each reaction were run on a 2% agarose gel and post-stained with ethidium bromide. The amount of elastin, Go13, and collagen type I mRNA was standardized relative to the amount of GAPDH mRNA.

Western Blotting—Confluent fetal human cardiac fibroblast cultures were exposed with or without the treatment specified in the figure legends for the indicated time points. At the end of each experiment, the cells were lysed using an radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) containing a mixture of antiproteases (20 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) and antiphosphatases (200 μg/ml aprotinin, 2 μg/ml pepstatin). Then 40–60 μg of protein extract was resuspended in sample buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 10% glycerol, 4% 2-mercaptoethanol, and 0.05% bromphenol blue), and the mixture was boiled for 5 min. The protein lysates were resolved by precast SDS-PAGE gel (4–12% gradient), transferred to nitrocellulose membranes, blocked for an hour, and then immunoblotted with polyclonal anti-MR antibody, anti-phospho-c-Src (Tyr416) antibody, anti-phospho-Akt (Ser473) antibody, anti-Go13 (goat) antibody, anti-SCAP2 antibody, or with buffer (TBS-T) at 4 °C overnight. All of the blots were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for an hour and examined using the enhanced chemiluminescence detection system. As indicated in the figure legends, the blots were stripped and reprobed using specified antibodies. For all Western blot experiments human whole cell lysates were also electrophoresed and immunoblotted with the mentioned antibodies that served as a positive control and accordingly produced the appropriate molecular weight band (data not shown).

The degree of expression or phosphorylation of immunodetected signaling molecules was measured by densitometry.

Immunoprecipitation—To evaluate the level of IGF-IR-B phosphorylation, confluent fetal human cardiac fibroblast cultures were incubated for the indicated time in the presence or absence of 50 nm aldosterone or for 10 min with 100 ng/ml of IGF-I, as specified in the figure legends. For co-immunoprecipitation experiments, confluent cultures were incubated with the treatment indicated in the figure legends. At the end of each experiment the cells were lysed as specified above, and 300 μg of protein extract were then precleared for 1 h with normal rabbit agarose-conjugated IgG at 4 °C and incubated with rabbit polyclonal antibodies against IGF-IR-B, c-Src, or with Go13 for 1 h at 4 °C, followed by the addition of 4% protein A-beaded agarose and left overnight, as previously described (42). The resulting protein-antibody conjugate was centrifuged at 4 °C and washed four times with phosphate-buffered saline. The final pellet was resuspended in sample buffer, and the proteins were resolved as specified above. Following immunoprecipitation of IGF-IR-B, the membrane was immunoblotted using monoclonal anti-p-Tyr antibody, stripped, and reprobed using...
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A

anti-MR

anti-GAPDH

% of control

scrambled (96 hours) MR siRNA (96 hours) scrambled (144 hours) MR siRNA (144 hours)

**/***p<0.05

B

Immunostaining with anti-MR

Scrambled siRNA

MR siRNA

C

Deposition of Insoluble Elastin

Incorporation of [H]proline into RNA

CPM / 1 µg DNA

ctr Aldo 50 nM ctr Aldo 50 nM

scrambled siRNA MR siRNA

*p<0.05

D

Immunostaining with anti-Elastin

Scrambled siRNA

MR siRNA

E

anti-phospho-Tyr

anti-IGF-IR

F

anti- phospho-Tyr

anti-IGF-IR

% of control

ctr Aldo 50 nM 10 min ctr Aldo 50 nM 10 min

scrambled siRNA MR siRNA

*p<0.05

ctr Aldo 50 nM Aldo-BSA 50 nM 100 ng/ml

*p<0.05
anti-IGF-IR-β. Following immunoprecipitation of c-Src, the membranes were immunoblotting using polyclonal goat antibodies against anti-Gα13, whereas those immunoprecipitated with anti-Gα13 were developed with monoclonal anti-c-Src antibody. As indicated in the figure legends, the blots were stripped and reprobed for equal loading.

For all of the immunoprecipitation experiments, rabbit IgG was also immunoprecipitated and used as a negative control and accordingly did not produce a band (data not shown). The degree of expression or phosphorylation of immunodetected signaling molecules was measured by densitometry.

Silencing MR and Gα13 Expression Using siRNA-specific Oligonucleotides and MR- and Gα13-specific siRNA Oligonucleotides—ON-TARGET plus SMART pool MR siRNA (gene ID 4306) containing a mixture of four SMART-selection predesigned siRNAs exclusively targeting MR (MR siRNA) was purchased from Dharmacon. Two different Silencer® predesigned siRNA duplexes against human Gα13 (standard purity, siRNA identification numbers 119735 and 119733) were obtained from (Ambion). The custom designed oligonucleotide duplex (Dharmacon) was synthesized to correspond to target sequences on the full-length human Gα13 protein. The custom designed oligonucleotide target sequence was as follows: 5′-GAA GAU CGA CUG ACC CAA UC-3′, which was previously shown to completely eliminate Gα13 in HeLa cells (43). A nonsilencing control and GAPDH siRNA duplex sequences (Panomics) were used as controls for the transfections.

Transfection of MR and Gα13 siRNA Oligonucleotides—Cardiac fibroblasts were seeded in 6-well plates, maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). 80–90% confluent cardiac fibroblast cultures were washed in phosphate-buffered saline, and 30 nM of Gα13, GAPDH, or nonsilencing siRNA or 90 nM of MR or nonsilencing siRNA were transfected into cells using DeliverX plus siRNA transfection reagent (Panomics), according to the manufacturer’s instructions. MR production was monitored by Western blotting, whereas Gα13 expression was monitored by one-step RT-PCR and Western blotting post-transfection, as specified in the figure text. The Gα13 siRNA 1 oligonucleotide (Ambion) provided the greatest knockdown of Gα13 and was used in all siRNA experiments to silence Gα13 expression.

Data Analysis—In all of the biochemical studies, quadruplicate samples in each experimental group were assayed in three separate experiments. The means and standard deviations were calculated for each experimental group, and statistical analyses were carried out by analysis of variance. A p value of less than 0.05 was considered significant.

RESULTS

Absence of MR Does Not Prevent an Aldosterone-induced Increase in IGF-IR Phosphorylation and Subsequent Elastin Production in Cultures of Cardiac Fibroblasts—In our previous study we demonstrated that treatment with 1–50 nM of aldosterone increases elastin mRNA levels, tropoelastin synthesis, and elastic fiber deposition in a dose-dependent manner. Strikingly, neither spironolactone (an MR antagonist) nor RU 486 (a glucocorticoid receptor antagonist) eliminated aldosterone-induced increases in elastin production, which were induced after aldosterone-dependent phosphorylation of IGF-IR (18).

The present study was intended to produce a detailed characterization of the signaling pathway through which aldosterone up-regulates elastin production. We first used MR-specific siRNA oligonucleotides to eliminate the production of MR in cardiac fibroblast cultures to exclude the conventional involvement of MR in aldosterone-induced elastogenesis. We first demonstrated using MR-specific siRNA oligonucleotides that MR protein levels decreased to ~11% of the scrambled control levels 96 h after transfection and to ~6% of scrambled control levels 144 h after transfection (Fig. 1A). Importantly, results of the consecutive experiments demonstrated that this effective siRNA-dependent inhibition of MR synthesis in cultures of cardiac fibroblasts (Fig. 1, A and B) did not diminish their elastogenic response to 50 nM of aldosterone (Fig. 1, C and D). Furthermore, we also showed that a 10-min exposure to 50 nM of aldosterone, which produced a transient increase in tyrosine phosphorylation of the IGF-IR in control cultures, produced a similar increase in cultures treated with MR siRNA (Fig. 1E). Then we utilized BSA-conjugated aldosterone to determine whether this membrane-impermeable form of aldosterone would trigger IGF-IR phosphorylation by direct stimulation of a cell surface-residing component (or components). Indeed, treatment for 10 min with 50 nM of BSA-conjugated aldosterone produced the same effect on IGF-IR phosphorylation as treatment with equimolar free aldosterone (Fig. 1F).

Search for the Cell Membrane Component(s) Involved in Aldosterone-induced Elastogenesis—The results described above suggested that MR-independent activation of the IGF-IR leading to increased elastin production by aldosterone does not require the entry of this hormone into the cell interior. We therefore speculated that such an effect might be triggered through the direct interaction of aldosterone with certain cell membrane-residing component(s). Consequently, our next experiments were aimed at identifying the initial stages of aldosterone-induced signaling leading to enhanced elastogenesis.

FIGURE 1. Eliminating the production of MR with siRNA-specific oligonucleotides in cultures of human cardiac fibroblast does not affect aldosterone-induced increases in elastin production. A, representative Western blots of cellular lysates from cultures that were transfected for either 96 or 144 h with scrambled and MR siRNA-specific oligonucleotides. β-actin immunochemistry with anti-MR antibody confirmed that production of MR was completely attenuated in cultures that were transfected for 144 h with MR siRNA. C, results of a quantitative assay of newly deposited insoluble elastin metabolically labeled with [3H]valine in cultures that were initially transfected for 72 h with scrambled or Gα13 siRNA and then transfected again for an additional 72 h and kept in the presence or absence of 50 nM of aldosterone. D, representative photomicrographs immunostained with anti-estatin antibody confirm the results presented in C. E and F, the cultures were either transfected for 96 h with scrambled siRNA control and Gα13 siRNA-specific oligonucleotides and treated for 10 min with or without 50 nM of aldosterone (E) or treated for 10 min with or without 50 nM of aldosterone, 50 nM of aldosterone conjugated to BSA (Aldo-BSA), or 100 ng/ml of IGFI (F). The cell lysates were immunoprecipitated with an IGF-IR antibody and probed with an anti-phosphotyrosine (anti-phospho-Tyr) antibody or anti-IGF-IR antibody. The graphs depict the means ± S.D. of data from three individual experiments, expressed as percentages of control phosphorylation values obtained by normalizing the corresponding total level of IGF-IR. ctr, control.
Inspired by reports indicating that some MR-independent effects of aldosterone may be induced through the modulation of angiotensin II-dependent signaling (12, 44, 45), we first examined the possibility that aldosterone-induced elastogenesis might involve the cross-activation of angiotensin II receptor(s). Our results demonstrated that the addition of angiotensin II type I (losartan) and angiotensin II type 2 (PD 123319) receptor antagonists to cultures of cardiac fibroblasts did not abrogate their elastogenic response to aldosterone (data not shown). Thus, the possibility that angiotensin II receptors were involved was eliminated.

Because other reports also suggested that certain MR-independent effects of aldosterone can be mediated by activation of the pertussis toxin-sensitive heterotrimeric G protein Goi (13, 46), we further explored the role of this signaling pathway in aldosterone-induced elastogenesis. As shown in Figure 2, treatment of human cardiac fibroblast cultures with 50 nM of aldosterone for 24 h resulted in a significant increase in elastin mRNA expression (A) and insoluble elastin deposition (B). In contrast, pretreatment with 0.5 μg/ml of pertussis toxin (PTX) did not significantly affect aldosterone-induced elastogenesis. Immunohistochemical analysis of 1-h pretreated cultures with PTX, followed by 72 h of incubation with aldosterone, further confirmed these findings (C).

**FIGURE 2.** The Goi inhibitor pertussis toxin (PTX) does not attenuate aldosterone-induced increases in elastin production in human cardiac fibroblast cultures. A, results of one-step RT-PCR analysis assessing elastin mRNA transcripts (normalized for GAPDH) in cultures treated for 24 h with or without 50 nM of aldosterone prior to 1 h of preincubation with 0.5 mg/ml of PTX. B, results of a quantitative assay of [3H]valine-labeled insoluble elastin. C, immunohistochemistry with anti-elastin antibody of 1-h pretreated cultures with 0.5 mg/ml of PTX, following 72 h of incubation with 50 nM of aldosterone.
17), we then tested its potential involvement in aldosterone-induced elastogenesis. However, the data we obtained demonstrated that pretreatment of cultured cardiac fibroblasts with pertussis toxin does not attenuate the pro-elastogenic effect of aldosterone (Fig. 2). Thus, the putative involvement of Gα proteins in this process was also eliminated.

We therefore concentrated our investigation on another member of the G protein family, Gα13, which has recently been shown to mediate nongenomic actions of estrogen (46).

**Silencing Gα13 in Cardiac Fibroblast Cultures Eliminates Aldosterone-induced Elastogenesis**—To examine whether Gα13 would be involved in the initiation of the cellular signaling leading to an aldosterone-induced increase in elastin production, we specifically silenced Gα13 mRNA expression to about 8% of scrambled siRNA control levels 24 h after transfection (Fig. 3A) and protein production to ~14 and 9% of scrambled control levels 48 and 120 h after transfection, respectively, in cardiac fibroblast cultures without affecting the levels of its related family member, Gα12 (Fig. 3).

Our results indicated that the aldosterone-induced increase in elastin mRNA (observed in cultures transfected with scrambled siRNA) did not occur in cultures in which Gα13 expression was effectively silenced (Fig. 4A). Consequently, cultures of cardiac fibroblasts that were transfected with Gα13 siRNA did not demonstrate any increase in elastin deposition in response to aldosterone treatment (Fig. 4, B and C). Meaningfully, parallel cultures transfected either with Gα13-specific or with scrambled siRNA demonstrated heightened elastin message levels and increased deposition of mature (metabolically labeled and immunodetectable) elastin in response to IGF-1 treatment. Additionally, we found that in contrast to cultures transfected with scrambled siRNA, which demonstrated a significant increase in IGF-1R phosphorylation, cultures transfected with Gα13-specific siRNA did not demonstrate any up-regulation in IGF-1R phosphorylation following aldosterone treatment (Fig. 4D). We also demonstrated that Gα13 is not involved in the collagenogenic effect of aldosterone (Fig. 4, E and F). These results clearly demonstrated that Gα13 is engaged in the initial stage of the aldosterone-induced increase in elastogenesis that occurs prior to IGF-1R activation.

**Aldosterone Also Induces a Transient Interaction between Gα13 and c-Src That Leads to c-Src Phosphorylation**—Results from further experiments suggested that this initial Gα13-dependent effect may also involve the activation of cytosolic tyrosine kinase c-Src. This conclusion was based on the observation that pharmacological inhibition of c-Src (with PP2) abolished an increase in elastin mRNA levels and the consequent up-regulation in elastic fiber production in aldosterone-treated cultures (Fig. 5).

Because the most characterized downstream signaling mediated by Gα13 involves GTPase Rho (28, 47), we examined the possible involvement of Rho and its downstream effector, Rho-associated kinase, in aldosterone-dependent elastogenesis. Because pretreatment of cultured cardiac fibroblasts, either with a cell membrane-permeable Rho inhibitor, CT04, or with a specific Rho-associated kinase inhibitor, Y-27632, did not eliminate the aldosterone-induced increase in elastin mRNA expression and elastin production in our cardiac fibroblast cultures, we concluded that the Rho pathway is not involved in the described elastogenic effect of aldosterone (data not shown).

Instead, we have established that Gα13 transiently interacts with c-Src proteins following aldosterone treatment. This conclusion was based on results of experiments indicating that Gα13 and c-Src can be co-immunoprecipitated from cellular lysates that were maintained in the presence and absence of aldosterone for 1, 5, or 10 min. Interaction between these two
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A

Go13
Elastin
GAPDH

Elastin mRNA levels (arbitrary units)
ctr Aldo 50 nM IGF-I 100 ng/ml ctr Aldo 50 nM IGF-I 100 ng/ml

* p<0.05

B

Deposition of Insoluble Elastin

Incorporation of [3H]-Valine into Elastin
CPM/1 μg DNA
ctr Aldo 50 nM IGF-I 100 ng/ml ctr Aldo 50 nM IGF-I 100 ng/ml

scrambled siRNA Go13 siRNA

* p<0.05

C

Immunostaining with anti-Elastin Antibody

scrambled siRNA ctr
scrambled siRNA Aldo 50 nM
scrambled siRNA IGF-I 100 ng/ml

Go13 siRNA ctr
Go13 siRNA Aldo 50 nM
Go13 siRNA IGF-I 100 ng/ml

* p<0.05

D

anti-phospho-tyr
anti-IGF-IR

% of control
ctr Aldo 50 nM IGF-I 100 ng/ml ctr Aldo 50 nM IGF-I 100 ng/ml

scrambled siRNA Go13 siRNA

* p<0.05

E

Steady-state levels of Collagen Type I mRNA

Collagen Type I
GAPDH

Collagen Type I mRNA levels (arbitrary units)
ctr Aldo 50 nM ctr Aldo 50 nM

* p<0.05

F

scrambled siRNA ctr
scrambled siRNA Aldo 50 nM

Go13 siRNA ctr
Go13 siRNA Aldo 50 nM
proteins was most evident after 5 min of aldosterone exposure (Fig. 6A).

We then investigated whether pharmacological activation of Gα13 enforces its transient association with c-Src. We found that a nonspecific activator of Ga proteins, aluminum fluoride (48, 49), also increased the interaction between c-Src and Gα13 (Fig. 6B). Specifically, we found that c-Src immunoprecipitated from cellular lysates treated with aluminum fluoride consistently displayed greater interaction with Gα13 than untreated controls (Fig. 6B).

Because phosphorylation of c-Src at Tyr416 in the activation loop of the kinase domain up-regulates the enzymatic activity of c-Src (50), we then examined whether aldosterone treatment would increase c-Src phosphorylation at Tyr416. Indeed, Western blotting with a specific anti-phospho-c-Src (Tyr416) antibody indicated that lysates of cells treated with aldosterone displayed increased phosphorylation of c-Src on Tyr416, as compared with the control. We also demonstrated that PP2 pretreatment abolished this effect (Fig. 6C). Importantly, we also found that the Gα13 siRNA-transfected cultures did not demonstrate any increase in c-Src phosphorylation in response to aldosterone treatment. This was in contrast to scrambled siRNA-transfected cultures, which demonstrated a significant increase in c-Src phosphorylation after treatment with aldosterone (Fig. 6D). These results thus further enforced the notion that in cardiac fibroblasts aldosterone engages Gα13 signaling that in turn interacts with c-Src, causing its activation.

PI 3-Kinase/Akt Signaling Pathway Propagates the Elastogenic Signal upon IGF-IR Activation—Having established that the IGF-IR receptor mediates the effect of aldosterone on elastin production, we now attempted to determine which downstream IGF-IR signaling pathway, the PI 3-kinase/Akt or the MAPK/ERK pathway (51), propagates the elastogenic signal. Results from metabolic labeling studies and immunofluorescence microscopy demonstrated that blocking the activation of the MAPK pathway by its specific MEK inhibitor, PD 98059, did not eliminate the elastogenic effect of aldosterone but instead led to a further increase in the production of elastin (Fig. 7, A and B). Also, treatment with an inhibitor (SP600125) that inactivated another MAPK family member, JNK, did not diminish the elastogenic effect of aldosterone (Fig. 7, A and B). On the other hand, results from one-step RT-PCR analysis and metabolic labeling studies demonstrated that the addition of the PI 3-kinase inhibitor wortmannin to cultures treated with aldosterone or IGF-I abolished the elastogenic effects of both stimulators (Fig. 7, C and D). These results indicate that the IGF-IR-PI 3-kinase pathway propagates the elastogenic signal and that inhibition of the parallel MAPK pathway further enhances the net elastogenic effect.

To finally link the early steps of aldosterone-induced signaling (Gα13-dependent c-Src activation) with the downstream elastogenic pathway (PI 3-kinase/Akt signaling transduced through the IGF-IR following its activation), we tested whether this IGF-IR-dependent downstream signaling event would still occur after inhibition of c-Src with PP2 and in cultures lacking Gα13. Western blot analysis using anti-phospho-Akt antibody revealed that the aldosterone-induced increase in the phosphorylation of Akt is indeed eliminated in cultures treated with the c-Src inhibitor PP2 and in cultures transfected with Gα13 siRNA (Fig. 7, E and F). Furthermore we showed that the levels of tropoelastin mRNA began to significantly increase as early as 1 h after exposure to aldosterone, reached a maximum level between 3–6 h, and remained elevated throughout the course of the experiment (Fig. 7G). This endorsed the suggested link between the early aldosterone induced signaling and consequent increase in elastin mRNA steady-state levels. Thus, the data presented reveal the details of an elastogenic signaling pathway that is triggered by aldosterone and involves the consecutive activation of Gα13, c-Src, and IGF-IR and its downstream PI 3-kinase/Akt signaling.

DISCUSSION

We previously reported that aldosterone stimulates elastogenesis via IGF-IR signaling in both fetal and adult and cultures of human cardiac fibroblasts, even in the presence of the MR antagonist spironolactone (18). Results of the experiments presented in this report additionally demonstrate that aldosterone still induces elastogenesis in cardiac fibroblast cultures in which the synthesis of MR protein is inhibited by the use of MR-specific siRNA oligonucleotides. Thus, these data further confirm that the elastogenic effect of aldosterone is executed via an MR-independent mechanism. Moreover, we have established that membrane-impermeable, BSA-conjugated aldosterone produces the same magnitude of IGF-IR phosphorylation as equimolar concentrations of free aldosterone (Fig. 1). This suggests that the signaling pathway leading to the MR-independent elastogenic effect of aldosterone may be initiated after the interaction of this steroid hormone with a certain moiety residing on the cell surface of cardiac fibroblasts. This assumption is further supported by other studies that have demonstrated the existence of high affinity membrane-binding sites for aldosterone in human vascular endothelium (52) human mononuclear leukocytes (53) and in pig kidneys (10) and livers (54). It has also been suggested that a 50-kDa protein may meet.
the criteria for the alternative cell surface receptor for aldosterone (53). However, this putative aldosterone receptor that mediates MR-independent action has not been characterized yet.

Because previous reports have suggested that G protein-coupled receptors (GPCRs) are involved in the propagation of certain steroid receptor-independent effects of other steroid hormones in animals (55, 56) and humans (57–60) and that some MR-independent effects of aldosterone can also be mediated through pertussis toxin-sensitive $G_\alpha$ proteins (13, 17), we first investigated whether $G_\alpha$ would propagate the elastogenic
effect of aldosterone. However, the results of our experiments, as depicted in Fig. 2, excluded the possibility that activation of \( \alpha_{i} \) may be involved in aldosterone-induced elastogenesis. Instead, we demonstrated for the first time that another heterotrimeric \( \alpha_{i} \) protein, a member of the \( G_{12} \) subfamily, \( \alpha_{13} \), participates in a cellular response to aldosterone that involves IGF-IR activation and a consequent enhancement of elastogenesis. This conclusion was based on data indicating that the elimination of \( \alpha_{13} \) in cultured cardiac fibroblasts by MR-specific siRNA oligonucleotides completely attenuated the aldosterone-induced increase in IGF-IR phosphorylation and subsequent elastin production (Figs. 3 and 4). At the same time we
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**A** Deposition of Insoluble Elastin

Incorporation of [3H]-valine into Insoluble Elastin

| Condition  | Incorporation (CPM / μg DNA) |
|------------|-----------------------------|
| Ctr        | 250                         |
| Aldo 50 nM | 500                         |
| PD 98059 10 μM | 750                     |
| SP600125 10 μM | 1000                   |

*p<0.05

**B** Immunostaining with anti-Elastin Antibody

- Control
- PD 98059 10 μM
- SP600125 10 μM

**C** Steady-state levels of Elastin mRNA

Elastin

| Condition  | Elastin mRNA levels (arbitrary units) |
|------------|---------------------------------------|
| Ctr        | 0.5                                   |
| Aldo 50 nM | 1.5                                   |
| IGF-I 100 ng/ml | 2.0                         |
| Wort 1 μM  | 0.5                                   |

*p<0.05

**D** Deposition of Insoluble Elastin

Incorporation of [3H]-valine into Insoluble Elastin

| Condition  | Incorporation (CPM / μg DNA) |
|------------|-----------------------------|
| Ctr        | 250                         |
| Aldo 50 nM | 500                         |
| IGF-I 100 ng/ml | 750                     |

*p<0.05

**E** Phospho-Akt and Total-Akt

% of control

| Condition  | Phospho-Akt | Total-Akt |
|------------|-------------|-----------|
| Ctr        | 100         | 100       |
| Aldo 50 nM | 200         | 200       |
| PP2 10 μM  | 100         | 100       |

*p<0.05

**F** Steady-state levels of Elastin mRNA

Elastin

| Condition  | Elastin mRNA levels (arbitrary units) |
|------------|---------------------------------------|
| Ctr        | 0.5                                   |
| Aldo 50 nM | 1.5                                   |
| IGF-I 100 ng/ml | 2.0                         |

*p<0.05
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**FIGURE 8. Proposed mechanism by which aldosterone increases elastin production in cardiac fibroblast cultures.** Aldosterone interacts with a still unidentified GPCR that causes the activation of \( \text{Ga}_{13} \). Activated \( \text{Ga}_{13} \) in turn, interacts with cytosolic c-Src. This interaction facilitates the activation of IGF-IR-IRS/PI 3-kinase/Akt signaling, which occurs even in the presence of sub-physiological levels of IGF-I, and subsequently induces increased elastin transcription and production. This effect of aldosterone is not dependent on the presence of the MR.

It has been previously shown that \( \text{Ga}_{13} \) protein did not eliminate the elastogenic response of IGF-I (Fig. 4). This also reinforced our belief that \( \text{Ga}_{13} \) is located upstream of the IGF-IR in the elastogenic signaling pathway triggered by aldosterone.

Although previous studies have also shown that \( \text{Ga}_{13} \) can stimulate the activation of the cytosolic tyrosine kinase c-Src in various cell types, including cardiac fibroblast cultures (28, 47, 61–65), the results of our co-immunoprecipitation experiments demonstrated that treatment with aldosterone enhances the transient interaction between \( \text{Ga}_{13} \) and c-Src (Fig. 6). Because the inactivation of c-Src (by its specific PP2 inhibitor) eliminated the elastogenic effect of aldosterone, we concluded that the action of this kinase constitutes a prerequisite for the propagation of the aldosterone-dependent elastogenic signal (Figs. 5 and 6).

It has been previously shown that \( \text{Ga}_{13} \) can directly bind and activate various proteins (66), including cytosolic tyrosine kinases such as Pyk2 (67). Currently, we do not know whether the aldosterone-triggered interaction between \( \text{Ga}_{13} \) and c-Src is direct or whether it requires other proteins, such as Pyk2, that might bind and facilitate phosphorylation of c-Src (68, 69). We have established, however, that in aldosterone-treated cardiac fibroblasts, \( \text{Ga}_{13} \) stimulates phosphorylation of c-Src, via the Rho-independent pathway and that the consecutive steps of elastogenic signaling involve increased phosphorylation of the IGF-IR and its downstream PI 3-kinase/Akt signaling pathway (Fig. 7).

It has been previously shown as well that c-Src may not only phosphorylate the IGF-IR on ligand-induced auto-phosphorylation sites but also significantly increase the phosphorylation of this receptor on Tyrs1316 (70), which has been implicated as a potential PI 3-kinase-binding site (71, 72). We may therefore speculate that aldosterone-induced \( \text{Ga}_{13}/c\)-Src activation facilitates IGF-IR signaling by enhancing its Tyrs1316 phosphorylation. This in turn selectively promotes the downstream PI 3-kinase/Akt pathway needed for elastogenesis but not the alternative IGF-IR-propagated mitogenic MAPK/ERK signaling pathway. Our speculation seems to be additionally endorsed by data indicating that the aldosterone-induced elastogenic effect was enhanced in the presence of the MEK inhibitor PD 98059. Also, treatment with an inhibitor (SP600125) inactivating JNK, another MAPK family member, did not diminish the elastogenic effect of aldosterone (Fig. 7, A and B).

Because phosphorylation on Tyr1316 of the insulin receptor, which is closely related to the IGF-IR, has been shown to play an inhibitory role in mitogenic signaling (73), we also speculate that the aldosterone-induced signaling enhancing phosphorylation of Tyrs1316 on the IGF-IR may contribute to the mechanism maintaining the balance between signals stimulating differentiation and mitogenesis. Further studies are needed to confirm this concept.

In this study we did not investigate further the already well disclosed elastogenic mechanism in which IGF-I induces an increase in elastin gene expression. It has been previously documented that in aortic smooth muscle cells (74–76) IGF-I...
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induces an increase in elastin gene expression via a derepressive mechanism involving the abrogation of Sp3, a retinoblastoma protein (Rb)-associated element, that allows for activation of the elastin promoter by Rb on its retinoblastoma control element (75, 76). Because Rb lies downstream of the PI 3-kinase/Akt/mammalian target of rapamycin signaling pathway (77), we may speculate that the aldosterone-dependent activation of this signaling pathway also modulates the interaction between Rb and pro-elastogenic transcription factors (74–76), leading to an increase in elastin gene expression in cardiac fibroblasts. Because we found that inhibition of the promitogenic MAPK/ERK signaling pathway further enhanced the effect of aldosterone on elastin production (Fig. 7, A and B), we may also suggest that the PI 3-kinase/Akt signaling pathway induces elastogenesis by altering the phosphorylation state of Rb, whereas the mitogenic MAPK/ERK pathway antagonizes this effect. Interestingly, a similar pro-elastogenic effect involving the PI 3-kinase/Akt signaling pathway has been reported in lung fibroblasts after exposure to transforming growth factor-β (78).

In summary, the data presented in this study suggest that the elastogenic effect of aldosterone in cardiac fibroblasts is propagated through the MR-independent action of this hormone. This novel mechanism likely involves a still unidentified GPCR (or GPCRs) that couples to Gα13 to stimulate c-Src, which in turn facilitates the activation of tyrosine kinase-dependent phosphorylation of the IGF-IR and its downstream PI 3-kinase signaling pathway (Fig. 8). This signaling pathway ultimately leads to the up-regulation of the elastin gene and the efficient production of elastic fibers by cardiac fibroblasts. We speculate that the heightened production of elastic fibers that results from the MR-independent action of aldosterone may counterbalance MR-mediated maladaptive fibrosis in the post-infarct heart in patients using MR antagonists, thus providing resilience to the cardiac stroma and facilitating normal ventricular function.

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