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Aldosterone Induces Elastin Production in Cardiac Fibroblasts through Activation of Insulin-Like Growth Factor-I Receptors in a Mineralocorticoid Receptor-Independent Manner

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Aldosterone is known to regulate electrolyte homeostasis, but it may also contribute to other processes, including the maladaptive remodeling of postinfarct hearts. Because aldosterone has been implicated in the stimulation of collagen production in the heart, we investigated whether it would also affect elastin deposition in cultures of human cardiac fibroblasts. We first demonstrated that treatment with 1 to 50 nmol/L aldosterone leads to a significant increase in collagen type I mRNA levels and in subsequent collagen fiber deposition. Pretreatment of cells with the mineralocorticoid receptor antagonist spironolactone, but not with the glucocorticoid receptor antagonist RU 486, inhibited collagen synthesis in aldosterone-treated cultures. Most importantly, we demonstrated that aldosterone also increases elastin mRNA levels, tropoelastin synthesis, and elastic fiber deposition in a dose-dependent manner. Strikingly, neither spironolactone nor RU 486 eliminated aldosterone-induced increases in elastin production. We further discovered that the proelastogenic effect of aldosterone involves a rapid increase in tyrosine phosphorylation of the insulin-like growth factor-I receptor and that the insulin-like growth factor-I receptor kinase inhibitor AG1024 or an anti-insulin-like growth factor-I receptor-neutralizing antibody inhibits both insulin-like growth factor-I and aldosterone-induced elastogenesis. Thus, we have demonstrated for the first time that aldosterone, which stimulates collagen production through the mineralocorticoid receptor-dependent

pathway, also increases elastogenesis via a parallel mineralocorticoid receptor-independent pathway involving I insulin-like growth factor-I receptor signaling. (Am J Pathol 2007; 171:809–819; DOI: 10.2353/ajpath.2007.070101)

The proper mechanical performance of the myocardium depends on the contractile properties of cardiac myocytes that are supported by the mechanical strength and resiliency of the extracellular matrix (ECM).¹ Following myocardial injury, the cardiac ECM undergoes dynamic local remodeling, which leads to the production of scar tissue.² However, overzealous ECM production in postinfarct hearts may lead to maladaptive fibrosis and contribute to heart failure.³

Aldosterone is one of the major mediators involved in cardiac remodeling following cardiac stress and injury.⁴ Its classic effect is attributed to mineralocorticoid receptor (MR)-mediated salt and fluid retention related to the regulation of blood pressure homeostasis.⁴ 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.^{5–7} Additional evidence also suggests that aldosterone mediates many cellular effects independently of its classic MR transcriptional gene activation,^{8–11} which may also be involved in the remodeling process of cardiac tissues.

Two clinical studies, the Randomized Aldactone Evaluation Study¹² and the Eplerenone Postacute Myocardial Infarction Heart failure Efficacy and Survival Study,¹³ demonstrated that low doses of MR antagonists led to a dramatic reduction of the mortality rate. However, the

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molecular mechanisms by which MR antagonists improve heart function are not fully resolved. There are data that suggest that these antagonists may alleviate the maladaptive remodeling of the cardiac matrix.^{14,15}

Because cardiac fibrosis substantially contributes to cardiac dysfunction and arrhythmogenicity associated with sudden death,³ preventing excessive collagen deposition is an obvious target of therapeutic intervention. The possible role of other ECM components, including elastic fibers that provide resilience and elasticity to many tissues, including stroma of the heart, has not been adequately addressed. It has been found that treatment with selective serine elastase inhibitors following myocardial infarction suppresses inflammatory infiltration and inhibits cardiac dilation. This probably results from inhibiting the proteolytic destruction of existing elastic fibers in the heart.^{16,17} Recently it has been shown that transplanting cells that are overexpressing elastin gene fragments into a myocardial scar modifies the scar content, increases cardiac elasticity, and facilitates ventricular function.^{18,19} Increasing elastogenesis in a postinfarct heart thus introduces a novel therapeutic concept, but it needs further mechanistic evaluation.

Our present study focused on the effects of aldosterone and its antagonist on the production of elastin in cultures of human cardiac fibroblasts. Our results indicate that aldosterone might also stimulate elastogenesis in an MR-independent manner and that blocking MR coincides with elastic fiber production. Our data support the hypothesis that stimulation of elastogenesis in a post-myocardial infarction heart may counteract pathological fibrosis and consequent heart stiffness and failure.

Materials and Methods

All chemical-grade reagents, aldosterone, spironolactone, doxycycline, RU 486 (mifepristone), proteinase inhibitors, agarose-linked protein A, pertussis toxin, recombinant human insulin-like growth factor-I (IGF-I), insulin-like growth factor receptor-I (IGF-IR) inhibitor AG 1024, epidermal growth factor receptor (EGFR) inhibitor AG 1478, platelet-derived growth factor receptor inhibitor AG 1295, and transforming growth factor β receptor inhibitor SB 431542 were obtained from Sigma (St. Louis, MO). Iscove's modified Dulbecco's medium, fetal bovine serum, 0.2% trypsin-0.02% ethylenediamine tetraacetic acid, and other cell culture products were acquired from Gibco Life Technologies (Burlington, ON, Canada). Polyclonal antibody to tropoelastin was purchased from Elastin Products (Owensville, MI). Polyclonal collagen type I antibody was purchased from Chemicon (Temecula, CA). Monoclonal antibody against phosphotyrosine (PY99) and polyclonal antibody against IGF-IR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). IGF-IR-blocking monoclonal antibody was purchased from EMD Biosciences (San Diego, CA). Fluorescein-conjugated goat anti-rabbit and fluorescein-conjugated rabbit anti-goat secondary antibodies were purchased from Sigma and Chemicon, respectively. Species- and type-specific secondary antibodies conjugated to horseradish peroxidase, an enhanced chemiluminescence

kit, and the radiolabeled reagent [³H]valine were purchased from Amersham Biosciences Canada, Ltd. (Oakville, ON, Canada). A DNeasy tissue system for DNA assay and an RNeasy Mini Kit for isolating total RNA were purchased from Qiagen (Mississauga, ON, Canada), as well as a One-Step RT-PCR Kit. Bovine serum albumin-conjugated aldosterone (aldo-BSA) was purchased from Fitzgerald Industries Int. (Concord, MA); as specified by the manufacturer, 25 aldosterone molecules are covalently linked to each BSA molecule through a carboxymethyl oxime residue on the C3 of the hormone, forming a stable conjugate.²⁰

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 to 22 weeks of gestation, a generous gift from Dr. John Coles (Cardiovascular Research, The Hospital for Sick Children, Toronto, ON, Canada), were prepared in accordance with an Institutional Review Board-approved protocol.²¹ 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 to 3 cells were used in all experiments. The purity of these cultures at passage 1 was 95%. Cardiac fibroblasts were identified by positive staining for vimentin and negative for von Willebrand factor and α -smooth muscle cell actin, as previously described.²²

In experiments aimed at assessing ECM production, fibroblasts were initially plated (100,000 cells/dish) and maintained in a normal medium until confluence, the point at which they produce abundant ECM. Confluent cultures were then treated for 72 hours with or without 1 to 50 nmol/L aldosterone.²³

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.^{24,25} The aldosterone receptor antagonist spironolactone,⁶ the glucocorticoid receptor antagonist RU 486, and the following IGF-IR, EGFR, platelet-derived growth factor receptor, and transforming growth factor β receptor inhibitors AG 1024,²⁶ AG 1478,²⁷ AG 1295,²⁸ and SB 431542,²⁹ respectively, as well as the G-protein inhibitor pertussis toxin¹¹ and the protein kinase C inhibitor staurosporine,³⁰ and IGF-IR-neutralizing antibody,³¹ were added 1 hour before aldosterone treatment. Control cell cultures received an equal amount of the solvent vehicle. To eliminate the possibility that the observed effects were restricted to the fetal cardiac fibroblasts, we also tested the influence of aldosterone on elastogenesis in cultures of commercially available adult human cardiac fibroblasts ScienCell (San Diego, CA).³²

Immunostaining

At the end of the 72-hour incubation period with the indicated treatment, confluent cultures were either fixed in ice-cold 100% methanol at -20°C (for elastin staining)

or in 4% paraformaldehyde at room temperature (for collagen staining) for 30 minutes and blocked with 1% normal goat serum for 1 hour at room temperature. The cultures were then incubated for 1 hour with 10 μ g/ml polyclonal antibody to tropoelastin or with 10 μ g/ml polyclonal antibody to collagen type I. All cultures were then incubated for an additional hour with fluorescein-conjugated goat anti-rabbit or with fluorescein-conjugated rabbit anti-goat secondary antibodies to detect elastin and collagen type I staining, respectively. 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 charge-coupled device camera (Retiga EX; QImaging, Surrey, BC, Canada) and a computer-generated video analysis system (Image-Pro Plus software; Media Cybernetics, Silver Spring, MD).

Quantitative Assays of Tropoelastin and Insoluble Elastin

Fetal human cardiac fibroblasts were grown to confluence in 35-mm culture dishes (100,000 cells/dish) in quadruplicate. Then 2 μ Ci of [^{3} H]valine/ml of fresh media were added to each dish along with or without 1 to 50 nmol/L aldosterone in the presence and absence of spirinolactone. Cultures were incubated for 72 hours, and the soluble and insoluble elastin were assessed separately in each dish. The cells were extensively washed with phosphate-buffered saline, and the soluble proteins present in the intracellular compartments were extracted overnight at 4°C with 0.1 mol/L acetic acid in the presence of proteinase inhibitors. After centrifugation, the supernatants were precleaned by 30-minute incubation with 50 μ l of 4% protein A-beaded agarose, and then 500 μ l of the supernatant was incubated with 5 μ g of polyclonal antibody to tropoelastin for 2 hours and subsequently with 50 μ l of 4% protein A-beaded agarose for 3 hours at 4°C. The protein A-containing beads were sedimented by centrifugation, washed with immunoprecipitation buffer, mixed with scintillation fluid, and counted.³³ The remaining cultures containing cell remnants and deposited insoluble extracellular matrix were scraped and boiled in 500 μ l of 0.1 N NaOH for 30 minutes to solubilize all matrix components except elastin. The resulting pellets containing the insoluble elastin were then solubilized by boiling in 200 μ l of 5.7 N HCl for 1 hour, and the aliquots were mixed in scintillation fluid and counted.³⁴ Aliquots taken from each culture were also used for DNA determination according to Rodems and Spector,³⁵ using the DNeasy Tissue System from Qiagen. Final results reflecting amounts of metabolically labeled insoluble elastin in individual cultures were normalized per their DNA content and expressed as counts per minute per 1 μ g of DNA. In separate experiments the specified treatments as shown in the figure legends for Figures 2, 4, and 5 were added along with 2 μ Ci of [^{3} H]valine/ml media to normal human skin fibroblasts grown to confluence in 35-mm culture dishes (100,000 cells/dish) in quadruplicate

for 72 hours. The conditioned media were then removed, the cell layers were washed, and the incorporation of [^{3} H]valine into the insoluble elastin was assessed as described above.

One-Step Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Confluent fetal human cardiac fibroblast cultures were treated with or without the specified treatment shown in the figure legends for Figures 1 to 5 for 24 hours. Total RNA was extracted using the RNeasy Mini Kit according to the manufacturer's instructions, 1 μ g of total RNA was added to each one-step RT-PCR (Qiagen One-Step RT-PCR Kit), and reactions were set up according to the manufacturer's instructions in a total volume of 25 μ l. The reverse transcription step was performed for elastin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reactions at 50°C for 30 minutes, followed by 15 minutes at 95°C. The elastin PCR reaction (sense primer: 5' GGT-GCGGTGGTTCCTCAGCCTGG-3'; antisense primer: 5'-GGGCCTTGAGATAC-CCCAGTG-3'; designed to produce a 255-bp product) was performed under the following conditions: 25 cycles at 94°C denaturation for 20 seconds, 63°C annealing for 20 seconds, 72°C extension for 1 minute, and one cycle at 72°C final extension for 10 minutes. The collagen type I PCR reaction (sense primer: 5'-CCCACCAATCACCTGCGTACAGA-3'; antisense primer: 5'-TTCTTGGTTCGGTGGGTGACTCTGA-3') was performed under the following conditions: 20 cycles at 94°C denaturation for 30 seconds, 58°C annealing for 30 seconds, 72°C extension for 10 minutes, and one cycle at 72°C final extension for 10 minutes. The GAPDH PCR reaction (sense primer: 5'-TCCACCACCCTGTT-GCTGTAG-3'; antisense primer: 5'-GACCACAGTCCAT-GCCATCACT-3'; designed to produce a 450-bp product) was performed under the following conditions: 21 cycles at 94°C denaturation for 20 seconds, 58°C annealing for 30 seconds, 72°C extension for 1 minute, and one cycle at 72°C final extension for 10 minutes. Five-microliter samples of the elastin and GAPDH PCR products from each reaction were run on a 2% agarose gel and poststained with ethidium bromide. The amount of elastin mRNA was standardized relative to the amount of GAPDH mRNA.

Immunoprecipitation

Confluent fetal human cardiac fibroblast cultures were incubated for the indicated time in the presence or absence of 50 nmol/L aldosterone or for 10 minutes with 100 ng/ml IGF-I, as specified in the figure legend for Figure 6. Parallel cultures were incubated in serum-free conditions in the presence or absence of 50 nmol/L aldosterone and incubated with or without 10, 25, and 50 ng/ml IGF-I for 10 minutes. Cells were lysed using an radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L ethylenediamine tetraacetic acid, 10 mmol/L NaF, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate containing a cocktail of

antiproteases (20 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ aprotinin, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L dithiothreitol) and antiphosphates (200 mol/L orthovanadate and 2 $\mu\text{g/ml}$ pepstatin)], and 300 μg of protein extract was incubated with an antibody against IGF-IR β for 1 hour at 4°C, followed by the addition of 4% protein A-beaded agarose left overnight, as previously described.³⁶ 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 (0.5 mol/L Tris-HCl, pH 6.8, 10% sodium dodecyl sulfate, 10% glycerol, 4% 2- β -mercaptoethanol, and 0.05% bromophenol blue), and the mixture was boiled for 5 minutes. Proteins were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and then immunoblotted with anti-p-Tyr or anti-IGF-IR antibodies. The degree of expression or phosphorylation of immunodetected signaling molecules was measured by densitometry.

Data Analysis

In all biochemical studies, quadruplicate samples in each experimental group were assayed in three separate experiments. Mean and standard deviations were calculated for each experimental group, and statistical analyses were performed by analysis of variance. A *P* value of less than 0.05 was considered significant.

Results

Aldosterone Up-Regulates Collagen Type I Gene Expression and the Deposition of Collagen Fibers in an MR-Dependent Manner in Cultures of Cardiac Fibroblasts

We first demonstrated that treatment of cultured human fetal cardiac fibroblasts with 1 to 50 nmol/L aldosterone leads to a significant increase in the steady-state level of collagen type I mRNA and to the subsequent deposition of collagen fibers (Figure 1). Then we found that pretreating cardiac fibroblasts with the MR-antagonist spironolactone but not with the glucocorticoid receptor (GR) antagonist RU 486 (1 $\mu\text{mol/L}$), abrogated aldosterone-induced collagen production (Figure 1). These results strongly indicate that the stimulatory effect of aldosterone on collagen production is mediated via MR activation.

Aldosterone Up-Regulates Elastin Gene Expression and the Net Deposition of Elastic Fibers in an MR-Independent Manner in Cultures of Cardiac Fibroblasts Isolated From Fetal and Adult Human Hearts

Analysis of parallel cultures revealed that aldosterone also up-regulated the effective expression of the elastin gene, as detected by heightened elastin mRNA levels, in

Figure 1. The effect of aldosterone, the MR antagonist spironolactone, and the GR antagonist RU 486 on collagen type I production in cultures of human fetal cardiac fibroblast. **A:** A one-step RT-PCR analysis was used to assess collagen type I mRNA transcripts in cultures treated for 24 hours with or without 1 to 50 nmol/L aldosterone or pretreated for 1 hour with spironolactone or RU 486 and normalized to the corresponding levels of GAPDH mRNA transcripts. The results indicate that aldosterone treatment significantly increased collagen type I mRNA transcript levels compared with untreated control values (*P < 0.05). Cells pretreated for 1 hour with spironolactone, before aldosterone treatment, returned the aldosterone-induced increase in collagen type I mRNA levels to untreated values, whereas RU 486 pretreatment had no effect on the aldosterone-induced increase in collagen type I mRNA transcript levels. **B:** Representative photomicrographs of confluent cultures immunostained with antibody to collagen I confirm the results presented in **A**. Fibroblasts were initially plated (100,000 cells/dish) and maintained in a normal medium until confluence. The cultures were then maintained for 72 hours with or without 1–50 nmol/L of aldosterone, in the presence or absence of spironolactone (2 $\mu\text{mol/L}$) or the glucocorticoid receptor antagonist RU 486 (1 $\mu\text{mol/L}$).

a dose-dependent manner (Figure 2A). This was translated to a proportional increase in the net levels of newly synthesized metabolically labeled intracellular tropoelastin and in the net deposition of metabolically labeled insoluble elastin, the major component of elastic fibers (Figure 2, B–D). It is also interesting to point out that raising aldosterone far above “physiological” levels (100 nmol/L and 1 $\mu\text{mol/L}$) did not produce any cytotoxic effects but led to a further increase in elastin production (data not shown).

Surprisingly, pretreatment of cardiac fibroblasts with spironolactone, which eliminated any aldosterone-induced increase in collagen type I production, failed to prevent an aldosterone-induced increase in elastin mRNA expression

Figure 2. The effect of aldosterone, the MR antagonist spironolactone, and the GR antagonist RU 486 on elastin production in cultures of human fetal cardiac fibroblasts. **A:** A one-step RT-PCR analysis was used to assess elastin mRNA transcripts in cultures treated for 24 hours with or without 1 to 50 nmol/L aldosterone or pretreated for 1 hour with spironolactone or RU 486 and normalized to the corresponding levels of GAPDH mRNA transcripts. The results demonstrate that aldosterone dose-dependently increased elastin mRNA transcript levels compared with untreated control values ($P < 0.05$) and that neither spironolactone nor RU 486 eliminated this increase. **B:** Results of a quantitative assay of newly produced, metabolically labeled, and immunoprecipitable soluble tropoelastin demonstrate that cultures treated for 72 hours with 1 to 50 nmol/L aldosterone synthesize up to approximately three times more [^{3} H]valine-labeled tropoelastin than untreated counterparts ($P < 0.05$). The addition of spironolactone to aldosterone-treated cultures did not abrogate the increase in tropoelastin production. **C:** Results of a quantitative assay of insoluble elastin after metabolic labeling with [^{3} H]valine demonstrate that cells treated for 72 hours with 1 to 50 nmol/L aldosterone incorporate significantly more [^{3} H]valine into extracellular insoluble elastin compared with untreated cells ($P < 0.05$). Pretreating the cells with spironolactone for an hour before aldosterone exposure did not eliminate the increase in insoluble elastin production. **D:** Representative photomicrographs of confluent cultures immunostained with anti-elastin antibody confirm the results presented in **C**. Results of biochemical assays are expressed as the mean $\pm$ SD, as derived from three separate experiments in which each experimental group had quadruplicate cultures.

and in the net content of metabolically labeled intracellular tropoelastin and insoluble elastin (Figure 2). These observations suggest that aldosterone probably induces elastogenesis through an MR-independent process. To exclude the possibility that the increase in elastin production following aldosterone treatment may be mediated through GR activation, we also preincubated the cardiac fibroblasts with the GR antagonist RU 486 (1 μ mol/L) in the presence of 1 to 50 nmol/L aldosterone. Our results demonstrate that RU 486 had no effect on the aldosterone-induced increase in elastin mRNA levels (Figure 2A).

To eliminate the possibility that the observed effects might be restricted to fetal cardiac fibroblasts, we additionally used stromal fibroblasts isolated from adult human hearts to test the influence of aldosterone on their elastogenic abilities. We found that the elastogenic response of adult cardiac fibroblasts to aldosterone and spironolactone was similar to that of their fetal counterparts (Figure 3).

The Aldosterone-Induced Increase in Elastin Deposition Involves Activation of the IGF-I Receptor

To explore further the mechanism by which aldosterone induces elastogenesis in an MR-independent manner, we first used membrane-impermeable BSA-conjugated aldosterone to determine whether aldosterone would induce elastogenesis by the stimulation of cell surface receptors without internalization. Indeed, treatment of cardiac fibroblast cultures with 1, 10, or 50 nmol/L aldosterone conjugated to BSA produced the same effect on elastin mRNA levels and consequent elastin production as treatment with equimolar free aldosterone (Figure 4).

To identify the putative cell surface-residing component involved in the MR-independent action of aldosterone, we blocked the activation of selected cell surface receptors to test whether this might eliminate aldosterone-induced elastogenesis. We found that pretreatment of cultured cardiac fibroblasts with inhibitors of selected

Figure 3. The effect of aldosterone and the MR antagonist spironolactone on elastin mRNA levels and elastic fiber deposition in confluent cultures of adult cardiac fibroblasts. **A:** One-step RT-PCR analysis assessing elastin mRNA transcripts in cultures treated for 24 hours in the presence or absence of 1 to 50 nmol/L aldosterone, with or without spironolactone, and normalized to the corresponding levels of GAPDH mRNA. The results indicate that 1 to 50 nmol/L aldosterone treatment dose-dependently increased elastin mRNA transcript levels compared with untreated control values (* $P < 0.05$). Pretreatment of cells for 1 hour with spironolactone before aldosterone treatment had no effect on the aldosterone-induced increase in elastin mRNA transcript levels. **B:** Representative photomicrographs of confluent cultures immunostained with anti-elastin antibody demonstrate that 1 to 50 nmol/L aldosterone treatment for 72 hours significantly increased the number of immunodetectable elastic fibers compared with untreated controls, and that spironolactone pretreatment did not affect aldosterone-induced increase in elastic fiber deposition.

growth factor receptors EGF (AG 1478), transforming growth factor β (SB 431542), and platelet-derived growth factor BB (AG 1295), did not affect the aldosterone-induced increase in elastin production. In addition, treatment with G protein inhibitor, pertussis toxin, or staurosporine (to inhibit protein kinase C activity) did not abrogate the aldosterone-induced increase in elastin production (data not shown).

On the other hand, blocking the IGF-IR with a specific inhibitor, AG 1024, eliminated the stimulatory effect of aldosterone on elastin mRNA expression and insoluble elastin production (Figure 5 A, C, and E). Because AG 1024 spe-

cifically inhibits ligand-stimulated autophosphorylation of the IGF-IR but not of the insulin receptor,²⁶ we speculated that aldosterone engages IGF-IR signaling to stimulate elastogenesis. Indeed, blocking the IGF-IR with 1 μ g/ml IGF-IR neutralizing antibody before aldosterone treatment eliminated the elastogenic effect (Figure 5, A, C, and E). Furthermore, we demonstrated that treating cardiac fibroblasts with 100 ng/ml IGF-I led to an approximately threefold increase in elastin mRNA levels and in the net production of insoluble elastin. We also demonstrated that this increase could be prevented by pretreating the fibroblasts with 5 μ mol/L AG 1024 or with 1 μ g/ml IGF-IR neutralizing antibody (Figure 5, B and D).

To determine whether tyrosine phosphorylation of the IGF-IR is affected by aldosterone treatment, we performed IGF-IR immunoprecipitation from cultures incubated in the presence and absence of 50 nmol/L aldosterone for 10, 15, 30, and 60 minutes. Our results showed that a 10-minute exposure to 50 nmol/L aldosterone led to a transient increase in tyrosine phosphorylation of the IGF-IR above basal level. Exposure for 10 minutes to 100 ng/ml IGF-I produced a very similar effect (Figure 6, A and B). However, whereas IGF-I induced phosphorylation of its IGF-IR, both in the presence and in the absence of fetal bovine serum, aldosterone induced a similar effect only in the presence of serum. These results suggest that aldosterone may facilitate but not induce IGF-IR-dependent signaling. Indeed, in further experiments, cultures treated with 50 nmol/L aldosterone and 10, 25, or 50 ng/ml IGF-I showed higher levels of IGF-IR tyrosine phosphorylation than their respective counterparts treated with the same doses of IGF-I alone (Figure 6C).

Figure 4. The influence of cell-impermeable aldosterone conjugated to BSA on elastin mRNA levels and deposition of elastic fibers. Results demonstrate that 1 to 50 nmol/L aldosterone conjugated to BSA [aldo (1 to 50 nmol/L)-BSA] produced the same effect on elastin mRNA levels (**A**) and [³H]valine incorporation (**B**) into insoluble elastin as 1 to 50 nmol/L aldosterone treatment alone. Cells treated with an equimolar concentration of BSA, as aldo (50 nmol/L)-BSA, served as an additional control. *Statistically different from control group ($P < 0.05$).

Discussion

Remodeling of the cardiac ECM can be triggered by various chemical and physical insults, including myocar-

Figure 5. IGF-IR inhibitor (AG 1024) and neutralizing antibody (α IGF-IR) antagonize aldosterone- and IGF-I-induced increases in elastin production in fetal cardiac fibroblast cultures. One-step RT-PCR analysis assessing elastin and GAPDH mRNA transcripts in cultures treated for 24 hours with 50 nmol/L aldosterone (**A**) or with 100 ng/ml IGF-I (**B**) before 1 hour of preincubation with 5 μ mol/L AG 1024 or with 1 μ g/ml α IGF-IR. The results show that inhibiting IGF-IR tyrosine kinase activity or blocking IGF-IR abolished aldosterone- and IGF-I-induced increases in elastin mRNA transcript levels. Incorporation of [^{3} H]valine (quantitative assay of insoluble elastin) demonstrated that cultures treated for 72 hours with 50 nmol/L aldosterone (**C**) or with 100 ng/ml IGF-I (**D**) before 1 hour of preincubation with 5 μ mol/L AG 1024 or with 1 μ g/ml α IGF-IR returned insoluble elastin production to control values. **E:** Representative photomicrographs of confluent cultures immunostained with anti-elastin antibody confirm the results presented in **C**. *Statistically different from control group ($P < 0.05$).

dial infarction. Although the formation of a postinfarct scar is important for the host's survival, excessive collagen deposition and fibrosis in the remote cardiac regions increase the possibility of heart failure.⁴ Aldosterone has been shown to promote pathological cardiac fibrosis via MR activation.^{7,37–39} Results of clinical trials that showed that the MR antagonists spironolactone and eplerenone exerted cardioprotective effects were primarily connected to the mechanisms that prevent collagenous fibrosis.^{12,13,39,40} It has also been shown that MR antagonists do not abolish all aldosterone-induced effects. The existence of MR-independent action, often referred to as the “nongenomic” effects,^{40–42} has been further confirmed by MR knockout studies.⁹

The investigations we have presented in this study were encouraged by data from a parallel study that we conducted on a rat myocardial infarction model. These data indicated that animals treated with eplerenone during the postinfarction period produced scars with abundant elastic fibers that replaced the mostly collagenous scars seen in vehicle-treated animals (S. Bunda, P. Liu, S. Wadhawan, Y. Wang, K. Liu, S. Arab, A. Hinek, manuscript in preparation).

Our results from an *in vitro* model of cultured human cardiac fibroblasts (which are mostly responsible for the production of cardiac ECM) have demonstrated that aldosterone up-regulates collagen type I mRNA steady-

state levels and subsequent deposition of collagen fibers (Figure 1). These data are in agreement with some *in vitro* studies^{23,43,44} but are in contrast to other studies^{45,46} that failed to demonstrate a direct effect of aldosterone on collagen production. This disagreement may be related to cell- and species-specific differences or to variations in culture conditions. Nevertheless, we found that in our *in vitro* experimental model an aldosterone-mediated increase in collagen production occurs via MR but not GR activation. The lack of GR involvement in aldosterone-induced collagen production has been previously reported from studies using *in vivo*⁴⁷ and *in vitro*⁴⁵ models. Thus, these findings further confirm the effect of aldosterone on collagen production via MR activation.^{23,43,48} It should also be pointed out that *in vivo* models of exogenous aldosterone administration that produce an extensive MR-mediated cardiac pathology (fibrosis) also require salt intake.

Our results from *in vitro* experiments endorse the idea that aldosterone is involved in the stimulation of collagen deposition but do not exclude the possibility that the initiation and progression of cardiac fibrosis may also involve subtle changes in intracellular sodium levels. Nor do they eliminate the importance of other processes that occur during cardiac remodeling following cardiac stress *in vivo* (such as the initiation of an inflammatory response, proteolytic

Figure 6. Aldosterone rapidly increases tyrosine phosphorylation of the IGF-IR in fibroblast cultures via facilitation. Cardiac fibroblast cultures were treated with or without 50 nmol/L aldosterone for 0, 10, 15, 30, and 60 minutes or 100 ng/ml IGF-I for 10 minutes in the presence of 10% fetal bovine serum (**A**) or in the absence of serum (**B**) or in the absence of serum for 10 minutes (**C**) in the presence or absence of 10, 25, or 50 ng/ml IGF-I alone or with 50 nmol/L aldosterone. Cell lysates were immunoprecipitated (IP) with an IGF-IR antibody, electrophoresed, and probed with an anti-phosphotyrosine (α p-Tyr) antibody or anti-IGF-IR (α IGF-IR) antibody. Graphs depict the mean $\pm$ SD of data from three individual experiments expressed as a percentage of control phosphorylation values obtained by normalizing to the corresponding total level of IGF-IR. Data in **A** and **B** demonstrate that a 10-minute aldosterone exposure in cultures maintained in 10% fetal bovine serum leads to a significant increase in tyrosine phosphorylation of IGF-IR over basal levels, similar to the effect observed after a 10-minute IGF-I treatment. *Statistically different from control group ($P < 0.05$). Data in **C** demonstrate that cultures treated together with 50 nmol/L aldosterone and 10, 25, or 50 ng/ml IGF-I exhibit higher levels of IGF-IR tyrosine phosphorylation than their respective counterparts treated with the same doses of IGF-I alone. *, **, and ***, statistically different from the 10, 25, and 50 ng/ml IGF-I-treated group ($P < 0.05$), respectively.

enzyme activation, degradation of the existing matrix, and subsequent deposition of the matrix components).

Most importantly, we have demonstrated for the first time that aldosterone also up-regulates elastin mRNA expression (Figure 2). Interestingly, although the MR antagonist abolished the collagenogenic effect of aldosterone, it did not eliminate the elastogenic effect of this hormone. In fact, pretreatment with spironolactone supported the aldosterone-induced increase in the net deposition of elastic fibers (Figure 2). This indicated that the beneficial cardioprotective effect of MR antagonist(s) could also be attributed to the deposition of new elastic fibers that may result in the formation of a resilient scar rather than a stiff collagenous scar that could hinder cardiac muscle contraction and relaxation.

Recently it has been shown that steroid hormones, in addition to their specific nuclear receptor-mediated action, can also modulate intracellular signaling induced by peptide growth factors and hormones through a nuclear receptor-independent mechanism.^{49–52} It has been reported that some of the nongenomic effects of aldosterone may be induced through the modulation of angiotensin II-, vasopressin-, and EGF-dependent signaling pathways.^{51,53–56}

The results of our study demonstrate, also for the first time, that the addition of aldosterone rapidly enhances the level of IGF-IR phosphorylation that triggers intracellular signaling, leading to the up-regulation of elastin mRNA expression in cardiac fibroblast cultures (Figures 5 and 6). These data are to some extent consistent with recently published observations showing that a very highly elevated level of aldosterone (1.5 μ mol/L) transactivates the IGF-IR in renal epithelial cells.⁵⁷ Moreover, the direct activation of the IGF-IR by other steroid hormones has been previously described, and it has also been shown that estrogen (17 β -estradiol) induces an increase in tyrosine phosphorylation of the IGF-IR via an estrogen receptor-independent process.⁵⁸

We have demonstrated that aldosterone-enhanced tyrosine phosphorylation of the IGF-IR occurs only in the presence of serum or IGF-I (Figure 6, A and B). This effect is similar to the effect of aldosterone on rapid EGFR tyrosine phosphorylation, which requires the presence of EGF.⁵⁴ The mechanism by which aldosterone interacts with IGF-IR signaling is not entirely clear. However, our results suggest that aldosterone facilitates IGF-IR activation through an MR-independent pathway. This interpretation is in contrast to that of Holzman et al,⁵⁷ who suggest that MR or GR involvement is a necessary step in the aldosterone-dependent transactivation of the IGF-IR in renal epithelial cells. The differences between these two studies could be due to the fivefold higher abundance of MR in renal epithelium (a classic target tissue for aldosterone) compared with cardiac tissue.⁵⁹

Because we were able to show that membrane-impermeable BSA-conjugated aldosterone produced the same effect on elastin mRNA levels and consequent elastin production as treatment with equimolar free aldosterone (Figure 4), we suggest that aldosterone may exert its MR-independent effect by interaction with certain cell surface-residing moieties. For example, aldosterone may

Figure 7. Diagram depicting parallel mechanisms for proposed MR-dependent and MR-independent modulation of cardiac ECM production by aldosterone. The MR-independent effect stimulates elastin mRNA transcription and subsequent elastin deposition by engaging IGF-IR signaling. Aldosterone also binds to its intracellular MR, which among other genes leads to increased synthesis of collagen.

exert its effect through an angiotensin II type I receptor,^{10,56,60} which, in turn, could transactivate the IGF-IR.⁶¹ The involvement of cytosolic tyrosine kinases of the c-Src family, which have been shown by Krug et al⁵⁴ to transactivate EGFR in response to aldosterone treatment, may also be involved in the transactivation of the IGF-IR.⁶² We also cannot exclude the possibility of a yet unidentified aldosterone membrane receptor, distinct from the classic intracellular MR,⁶³ which may act as a cofactor in facilitating IGF-IR signaling. Future studies are needed for detailed elucidation of the mechanism underlying aldosterone-dependent modulation of IGF-IR signaling.

IGF-I has been previously reported to increase elastin gene expression in aortic smooth muscle cells^{64–66} and to exert cardioprotective effects in humans, and in experimental heart failure, by improving cardiac remodeling and function.^{67–69} Moreover, cardio-specific IGF-I overexpression has been shown to attenuate dilated cardiomyopathy in a transgenic mouse model of heart failure.⁷⁰ Overexpression of IGF-I in hepatic stellate cells has also been shown to reduce collagenous fibrosis following acute liver injury.⁷¹ Our novel observations indicate that aldosterone may further enhance the cellular response to IGF-I that results in an efficient production of elastic fibers.

In summary, our results suggest a novel action of aldosterone that can be added to the list of other mechanisms in which this hormone may modulate cardiac remodeling. We found that this elastogenic effect of aldosterone is not mediated through the classic mode of MR activation but involves the activation of tyrosine kinase-dependent phosphorylation of the IGF-IR and its downstream signaling pathways, thus leading to up-regulation of the elastin gene (Figure 7).

Although in an untreated postinfarct heart, this mechanism may be overwhelmed by an MR-dependent mechanism, leading to up-regulation of numerous genes, including collagen type I, it may prevail in the hearts of patients treated with MR antagonists. We speculate that, heightened deposition of elastic fibers is the beneficial

factor that increases cardiac resilience and facilitates ventricular function in the postinfarct heart.

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