Aldosterone and its Antagonists

Modulate Elastin Deposition in the Heart

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Laboratory Medicine and Pathobiology

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Doctor of Philosophy

Laboratory Medicine and Pathobiology
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ABSTRACT

Myocardial infarction activates the renin-angiotensin system, consequently upregulating aldosterone production that may stimulate pathological cardiac fibrosis via mineralocorticoid receptor (MR) activation.

Results presented in this thesis were derived from an in vitro experimental model using cultures of human cardiac fibroblasts to study the effect of aldosterone on elastin production. They first confirmed that treatment with 1-50 nM of aldosterone leads to a significant increase in collagen type I production via MR activation. Most importantly, we discovered that treatment with 1-50 nM of aldosterone also increases elastin mRNA levels, tropoelastin synthesis, and elastic fiber deposition. Strikingly, pretreatment with MR antagonist spironolactone did not eliminate aldosterone-induced increases in elastin production.

Interestingly, while cultures treated with elevated aldosterone concentrations (100 nM and 1 μM) showed a further increase (~3.5-fold) in collagen and (~3-fold) in elastin mRNA levels, they demonstrated subsequent increases only in the net deposition of collagen but not elastin. In fact, cultures treated with elevated aldosterone concentrations
displayed a striking decrease in the net deposition of insoluble elastin, which could be reversed with spironolactone or with MMP inhibitors doxycycline or GM6001.

Most importantly, we discovered that the pro-elastogenic effect of aldosterone involves a rapid increase in tyrosine phosphorylation of the insulin-like growth factor-I receptor (IGF-IR) and that the IGF-IR kinase inhibitor AG1024 or an anti-IGF-IR neutralizing antibody inhibits both IGF-I- and aldosterone-induced elastogenesis (Bunda et al., Am J Pathol. 171:809-819, 2007). Furthermore, we showed that the PI3 kinase signaling pathway propagates the elastogenic signal following IGF-IR activation and that activation of c-Src is an important prerequisite for aldosterone-dependent facilitation of the IGF-IR/PI3 kinase signaling.

Results of explorative microarray analysis of 1 hour aldosterone-treated cultures revealed that aldosterone treatment upregulated expression of a heterotrimeric G protein, Gα13, that activates the PI3 kinase signaling pathway. We additionally demonstrated that aldosterone treatment transiently increases the interaction between Gα13 and c-Src and that siRNA-dependent elimination of Gα13 inhibited the pro-elastogenic effect of aldosterone.

In summary, aldosterone, which stimulates collagen production in cardiac fibroblasts through the MR-dependent pathway, also increases elastogenesis via a parallel MR-independent pathway involving the activation of Gα13, c-Src, and IGF-IR/PI3 kinase signaling.
ACKNOWLEDGEMENTS

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# TABLE OF CONTENTS

## INTRODUCTION

The Extracellular Matrix

Elastic Fibers

* Tropoelastin Gene Expression
* Tropoelastin Synthesis and Secretion
* Elastic Fiber Formation

Figure A: The mechanisms involved in the process of tropoelastin secretion and assembly

Diseases and Conditions Affecting Elastic Fibers: Elastinopathies

Collagen Fibers

* Collagen Synthesis and Regulation
* Collagen Fiber Assembly

Figure B: Overview of the process of collagen fiber assembly

Components of the Myocardial ECM

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## Cardiac Remodeling After Myocardial Infarction

Aldosterone

* Classic Actions of Aldosterone
* Aldosterone Synthesis
* The Renin-Angiotensin System
* Epithelial Actions of Aldosterone
* Mechanism of Aldosterone Action via the MR
* Aldosterone-Induced Genes/Proteins
* Target Tissue Specificity of the MR

Figure C: Classic epithelial actions of aldosterone

New Concepts in Aldosterone Research: the Presence of Aldosterone in the Heart

* Aldosterone Production in the Heart
* Activation of Cardiac MR by Aldosterone
* MR-Independent or “Non-Genomic” Aldosterone Actions

Figure D: Genomic and non-genomic aldosterone actions

---

## The Effect of Aldosterone on Cardiac Remodeling After Myocardial Infarction

Benefits of MR Antagonists after MI

Experimental Evidence

Evidence from Clinical Studies

MR Activation and Cardiac Fibrosis

Insight into the Role of Elastin in Cardiac Remodeling
Rationale/Hypotheses/Experimental Model and General Objectives…51

MATERIALS AND METHODS…………………………………………………….52

Materials…………………………………………………………………………52
Identification of Elastin in Rat Hearts Following MI and Eplerenone Treatment………………………………………………………………………….54
Cultures of Human Cardiac Fibroblasts…………………………………………54
Immunostaining………………………………………………………………….56
Quantitative assays of Tropoelastin and Insoluble Elastin……………………57
One-Step RT-PCR Analysis…………………………………………………….58
Western Blotting……………………………………………………………….59
Immunoprecipitation…………………………………………………………….60
Silencing Elastin and Ga13 Expression using siRNA Specific Oligonucleotides……………………………………………………………………62
Elastin Specific siRNA Oligonucleotides……………………………………….62
Transfection of Elastin siRNA Oligonucleotides………………………………62
Ga13 Specific siRNA Oligonucleotides…………………………………………62
Transfection of Ga13 siRNA Oligonucleotides…………………………………63
Microarray Analysis using the Affymetrix GeneChips………………………63
RNA Isolation……………………………………………………………………63
Oligonucleotide Arrays (Hybridization and Staining)…………………………63
Affymetrix GeneChip Data Analysis…………………………………………64
Data Analysis…………………………………………………………………….64

RESULTS………………………………………………………………………….65

1- The effect of aldosterone and its antagonist on cardiac matrix remodeling following experimental MI in rats…………………………………………………………………………..65

2- The effect of various aldosterone concentrations on the production of collagen type I and elastin in cultures of cardiac fibroblasts isolated from fetal and adult human hearts……………………………………………………………………66

2A- Moderate aldosterone levels upregulate collagen type I gene expression and the deposition of collagen fibers in an MR-dependent manner……………………………………………………………………..67

2B- Moderate aldosterone levels also upregulate elastin gene expression and the net deposition of elastic fibers, but in an MR-independent manner…………………………………………………………………….67

2C- High levels of aldosterone further induce collagen type I and elastin gene expression but also trigger a parallel MR-dependent mechanism leading to decreased net deposition of elastic fibers……………………………………………………………………..68

2D- High doses of aldosterone promote elastin degradation
via an MR-dependent mechanism ................................................. 69

3- Elastin and elastin degradation products modulate collagen type I deposition in cardiac fibroblast cultures ........................................... 70

4- The aldosterone-induced increase in elastin deposition involves the activation of the IGF-I receptor ......................................................... 71

4A- The PI3 Kinase/Akt signaling pathway propagates the elastogenic signal upon IGF-IR activation ................................................. 73

5- Aldosterone facilitates IGF-IR signaling via e-Src activation ............... 74

6- AT1 and AT2 receptors do not participate in aldosterone-dependent IGF-IR activation ................................................................. 76

7- Microarray analysis identifies upregulated genes following 1 hour of aldosterone treatment ............................................................... 77

7A- Microarray Analysis: The heterotrimeric G protein $\alpha$ subunits, $G_{\alpha}13$ and $G_{\alpha}i$, show increased expression following 1 hour of aldosterone treatment .......................................................... 77

8- The $G_{\alpha}13$ effector Rho/ROCK pathway is not involved in the aldosterone-dependent increase in elastin production ......................... 80

9- Aldosterone increases the interaction between $G_{\alpha}13$ and c-Src ............... 81

10- Silencing $G_{\alpha}13$ in cardiac fibroblast cultures eliminates aldosterone-induced elastogenesis .......................................................... 82

11- The possible involvement of Src family associated phosphoprotein (SCAP2) in aldosterone-mediated Src signaling ........................................... 83

FIGURES ................................................................................. 85

Figure 1 ....................................................................................... 85
Figure 2 ....................................................................................... 87
Figure 3 ....................................................................................... 89
Figure 4 ....................................................................................... 92
Figure 5 ....................................................................................... 94
Figure 6 ....................................................................................... 96
Figure 7 ....................................................................................... 99
Figure 8 ..................................................................................... 101
Figure 9 ..................................................................................... 103
Figure 10 ................................................................................... 105
Figure 11 ................................................................................... 107
Figure 12 ................................................................................... 109
Figure 13 ................................................................................... 110
Figure 14 ................................................................................... 112
Figure 15 ................................................................................... 114
Figure 16 ................................................................................... 115
Figure 17 ................................................................................... 117
Figure 18 ................................................................................... 119
Figure 19 ................................................................................... 121
DISCUSSION .......................................................................................... 148

Figure E: Parallel mechanisms in which aldosterone modulates cardiac ECM remodeling .................................................. 168

REFERENCES .......................................................................................... 169
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
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<tr>
<td>IGF-IR</td>
<td>Insulin-like growth factor receptor-I</td>
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<tr>
<td>IGF-I</td>
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</tr>
<tr>
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<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>SCAP2</td>
<td>Src family associated phosphoprotein 2</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
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<td>Microfibril-associated glycoprotein-1</td>
</tr>
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<td>Transforming growth factor-β</td>
</tr>
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<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NF-1</td>
<td>Nuclear factor-1</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>EBP</td>
<td>Elastin binding protein</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>SVAS</td>
<td>Supravalvular aortic stenosis</td>
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<tr>
<td>WBS</td>
<td>Williams-Breuren Syndrome</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal–regulated kinases 1 and 2</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin-angiotensin-aldosterone system</td>
</tr>
<tr>
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<td>Angiotensin converting enzyme</td>
</tr>
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<td>G-protein-coupled receptors</td>
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<td>Protein kinase C</td>
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<td>ENaC</td>
<td>Epithelial sodium channel</td>
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<td>Serum-and glucocorticoid-inducible kinase 1</td>
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<td>11β-HSD2</td>
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<tr>
<td>CHIF</td>
<td>Corticosteroid hormone-induced factor</td>
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<tr>
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<td>Angiotensin II type 1</td>
</tr>
<tr>
<td>AT₂</td>
<td>Angiotensin II type 2</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH₂-terminal kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPK/ERK</td>
<td>MAPK/extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>PIINP</td>
<td>Procollagen type III N-terminal amino peptide</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet derived growth factor receptor</td>
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INTRODUCTION

The Extracellular Matrix (ECM)

The ECM not only provides the framework and physical support for cellular structures but also plays a critical role in modulating the development, growth, and biochemical properties of virtually every tissue and organ.\(^1\) While the ECM is ubiquitous, its composition varies significantly from organ to organ and is carefully regulated.\(^2\) The fibroblast, which communicates with many cell types and responds to various cytokines, growth factors, and hormones, is the main cell type responsible for the production of ECM macromolecules.\(^3,4\) The two main classes of ECM macromolecules are:

1. fibrous proteins that perform mainly structural functions, such as providing support and resisting deformation (e.g., collagen and elastin) or serving as an adhesive (e.g., fibronectin and laminin); and

2. polysaccharide chains of glycosaminoglycans (GAGs) that are usually covalently linked to proteins to form proteoglycans.\(^3\)

The ECM is not a static structure, but rather a complex system involving dynamic interactions between matrix molecules, signaling proteins, and transmembrane proteins.\(^5\) Elastic fibers are major components in the ECM that endow tissue with resilience, extensibility, and elastic recoil. Collagen fibers provide tissues with mechanical strength. GAG and proteoglycans associate with collagen and elastin to form complex polymeric structures. Adhesive glycoproteins such as fibronectin promote the attachment of fibroblasts and other cells to the ECM via integrin interactions.\(^6\) The polysaccharide gel
resists compression and facilitates the diffusion of nutrients, metabolites, hormones, growth factors, and pharmacological agents, as well as affecting the activity and stability of proteins and signaling molecules secreted within the matrix.\textsuperscript{3,7}

The ECM undergoes continuous remodeling, and thus the net accumulation of matrix components in tissues is a result of a balance between the production and degradation of these components.\textsuperscript{6,8} The factors that contribute to the synthesis of matrix components are widespread and include cytokines, neurohormones, and growth factors, while the main protease responsible for the degradation of the ECM belongs to the family of matrix metalloproteinases (MMPs), which in turn are regulated by a family of tissue inhibitors of metalloproteinases (TIMPs).\textsuperscript{6,8} Abnormalities in any step of the synthesis and/or degradation of ECM components may result in various pathological states,\textsuperscript{5} such as excessive ECM accumulation as seen in fibrotic diseases (hearts).\textsuperscript{3}

**Elastic Fibers**

Elastic fibers are major insoluble ECM components that have the ability to stretch repetitively and reversibly and therefore provide tissues with elasticity and resilience.\textsuperscript{9} These properties are critical for maintaining repeated cycles of extension and recoil in the cardiovascular system, as well as for the proper functioning of the lungs, skin, and cartilage.\textsuperscript{10} The functional properties of elastic fibers reflect their tissue-specific architectures.\textsuperscript{11} They consist of two morphologically distinct components: an amorphous core made of cross-linked elastin (90% of the fiber) and parallel 10-12 nm microfibrils composed of numerous glycoproteins, such as fibrillin-1 and fibrillin-2, microfibril-associated glycoproteins (MAGP-1 and MAGP-2), and the latent transforming growth
factor-β (TGF-β) binding proteins.\textsuperscript{9,10,12,13} The microfibrils serve as a scaffold for the deposition and assembly of elastin. There are also numerous molecules that localize at the microfibril-elastin interface or at the cell surface–elastic fiber interface,\textsuperscript{10} such as elastin microfibril interface-located proteins (EMILINs),\textsuperscript{14} fibulins,\textsuperscript{15,16} and chondroitin sulfate proteoglycans (e.g., versican, biglycan, decorin).\textsuperscript{17} The role of these microfibril-associated molecules in the assembly of elastic fibers is not fully understood.

Numerous cell types, including vascular smooth muscle cells, endothelial cells, chondrocytes, fibroblasts, and keratinocytes, have been found to synthesize tropoelastin.\textsuperscript{18,19} Elastic fiber formation (elastogenesis) is a very complex process that is still not completely elucidated.\textsuperscript{10} An early stage of elastogenesis involves the pericellular accumulation of microfibrillar proteins, upon which secreted tropoelastin and precursor monomer molecules have to be properly assembled and covalently cross-linked with each other to form a resilient polymer, insoluble elastin.\textsuperscript{20}

Production of tropoelastin is developmentally regulated. It reaches its highest levels in the third trimester of the fetal life and steadily decreases during early postnatal development.\textsuperscript{21,22} The net deposition of elastin appears to be controlled on both the transcriptional level (through tropoelastin mRNA message expression) and post-transcriptional level (through tropoelastin message splicing and stability). Deposition is also affected by several other post-transcriptional events, which involve the secretion of tropoelastin protein monomers and their proper extracellular assembly and cross-linking into the polymeric “insoluble elastin”. The insoluble elastin is metabolically inert and remains the most durable element of the ECM. In undisturbed tissues it may last over the entire human lifespan.\textsuperscript{23}
Elastin is a relatively protease-resistant component of the ECM, mostly degraded by specific proteinases: pancreatic and leukocyte elastases and elastase-like enzymes such as MMPs (MMP-2, MMP-9, MMP-7, and MMP-12) and cathepsins G, K and L.\textsuperscript{24}

Altered elastin homeostasis leads to various pathological states that until recently were attributed to a structural imbalance of elastic fibers within the matrix. However, recent evidence has shown that elastic fiber components and their degradation products engage cell surface receptors to elicit intracellular signaling that modulates cellular adhesion and proliferation. This suggests that elastic fibers may also be actively involved in the progression of pathologies such as cardiovascular remodeling.\textsuperscript{20}

**Tropoelastin Gene Expression**

Tropoelastin, the precursor of elastin, is encoded by a single elastin gene that has been localized to the 11.2 region of chromosome 7q.\textsuperscript{25-27} The human elastin gene is 45 kb in size and contains 34 exons separated by large introns, with an intron:exon ratio of about 20:1.\textsuperscript{25,28} This sequence codes for an mRNA of ~3.5 kb that after extensive alternative splicing translates into several tropoelastin isoforms with molecular weights ranging from 68 to 74 kDa.\textsuperscript{25,28,29} Analysis of the structure of the gene in various species shows that the hydrophobic domains and hydrophilic cross-linking domains of all tropoelastin isoforms are encoded by separate, alternating exons.\textsuperscript{30} Exon 36 is conserved (>70%) amongst species and codes for the basic C-terminus, in addition to a large 3'-untranslated region\textsuperscript{28} that has been shown to have a function in regulating the stability of elastin mRNA.\textsuperscript{31}
The proximal promoter of the tropoelastin gene lacks a classical TATA box, and the 5' flanking region is generally GC rich (66%), with a high frequency of CpG dinucleotides and multiple cis-elements that serve as potential transcription start sites. All of this implies that the elastin promoter resembles regulatory sequences of genes that are constitutively expressed.\textsuperscript{30,32} The multiple cis-elements within the 5' flanking region of the elastin promoter associate with potential binding sites for transcriptional regulatory factors, a characteristic indicative of complex regulation.\textsuperscript{30} These binding sites include multiple Sp1, AP-1, and AP-2 binding sites, glucocorticoid response elements (GRE), 12-0-tetradecanoylphorbol-13-acetate (TPA), cyclic adenosine monophosphate (cAMP)-responsive elements (CRE), and nuclear factor-1 (NF-1) binding sites.\textsuperscript{33}

Currently, very little information is available about the factors that control and regulate elastin gene transcription.\textsuperscript{20} The majority of published studies on specific cis-acting elements and their respective trans-acting factors have focused on the downregulation of elastin transcript levels. It has been shown that tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) downregulates elastin gene expression, which is mediated thorough Jun/Fos binding to an AP-1 site located in the elastin promoter.\textsuperscript{34} Kuang \textit{et al}.\textsuperscript{35} have demonstrated that interleukin (IL)-1\(\beta\)-induced down-regulation of elastin mRNA in rat lung fibroblasts is dependent on the activation of nuclear factor-kappa B (NF-\(\kappa\)B) subunit p65 and the expression of CCAAT/enhancer binding protein-\(\beta\) (C/EBP-\(\beta\)). It has also been shown that basic fibroblast growth factor (b-FGF) decreases elastin gene transcription through a hybrid AP-1/cAMP response element in cultured aortic smooth muscle cells.\textsuperscript{36} Expression of B-Myb represses the activity of the elastin promoter in adult bovine smooth muscle cells, and the level of elastin mRNA is significantly reduced
in the aortas and isolated smooth muscle cells of B-Myb transgenic mice compared to wild-type mice.\(^{37}\) In addition, Vitamin D\(^{38}\) and phorbol esters (TPA)\(^{39}\) have been shown to inhibit tropoelastin production.

However, only two cis-acting elements within the elastin promoter have been shown to induce transcription. These include a Sp1/Sp3 sequence in the proximal promoter region\(^ {40}\) and an NF-1 sequence located in the distal promoter region.\(^ {33}\) The former site controls upregulation by insulin-like growth factor-I (IGF-I) in aortic smooth muscle cells through a mechanism of derepression involving the abrogation of Sp1/Sp3 binding. The latter site appears to control the level of elastin gene transcription via the binding of different NF-1 family members, although the specific factors regulating the differential binding of family members in elastogenic cells is unknown. Recently, a positive-acting element was found at the 5' end of the first exon of the elastin gene. It has been suggested that binding of Sp1 to this element stabilizes the transcription complex and thus enhances transcription of the elastin gene.\(^ {41}\)

It has further been established that TGF-β, one of the most potent stimulators of elastin production, exerts its effect by stabilizing elastin mRNA.\(^ {42-44}\) Recently, it has also been shown that TGF-β activates elastin transcription via a phosphatidylinositol 3-kinase (PI3 kinase)/Akt signaling pathway.\(^ {45}\) Metallic ions, such as iron, upregulate elastin gene expression as well.\(^ {46}\) In addition, dexamethasone and retinoids\(^ {47,48}\) and nitric oxide\(^ {49}\) have been shown to increase elastin gene expression through cyclic GMP stimulation.

The results presented in this thesis for the first time indicate that mineralocorticoids also upregulate elastin gene expression.\(^ {50}\)
**Tropoelastin Synthesis and Secretion**

Translation of tropoelastin mRNA takes place in the rough endoplasmic reticulum (RER), and polypeptide chains of tropoelastin are released into the lumen of the RER after cleavage of its signal peptide.\(^{51}\) In contrast to other ECM molecules such as collagen, tropoelastin undergoes very little post-translational modification and there is no evidence for glycosylation. Hydroxylation of some prolyl residues is caused by the enzyme prolyl hydroxylase. Inhibition of prolyl hydroxylase does not affect tropoelastin secretion, and it has been suggested that it is not necessary for elastic fiber formation.\(^{52}\) However, over-hydroxylation caused by the addition of ascorbate, a cofactor of prolyl hydroxylase, causes a decrease in net elastin deposition, and some investigators have suggested that over-hydroxylation also interferes with stabilization of the elastin polymer.\(^{53,54}\)

In normal conditions the newly synthesized tropoelastin is secreted from the cell interior within 20-60 minutes. It has been established that the highly hydrophobic and non-glycosylated tropoelastin has to be chaperoned through the intracellular secretory pathways by the 67-kDa elastin binding protein (EBP) that provides tropoelastin with secretory signals and protects tropoelastin from intracellular degradation and premature self-aggregation.\(^{55-58}\) The 67-kDa EBP is an enzymatically inactive spliced variant of \(\beta\)-galactosidase (S-GAL)\(^{59,60}\) that binds to the repeating hydrophobic VGVAPG-like domains on elastin, but it may also interact with moieties containing \(\beta\)-galactosugars through a separate "lectin-like" domain\(^{61}\) to form a molecular complex\(^{55}\) with neuraminidase-1 (Sialidase, Nase, EC 3.2.1.18) and with Cathepsin A (Cath A, EC 3.4.16.1), which is also called protective protein (PPCA).
The association between tropoelastin and EBP lasts until the complex is secreted into the extracellular space where the lectin domain of EBP can interact with galactosugars protruding from the microfibrillar glycoproteins and thereby release a tropoelastin molecule that, in turn, binds to the microfibrillar scaffold. It has been established that most of the EBP molecules, following the release of their tropoelastin cargo, recycle back to endosomal compartments to pick up another newly synthesized tropoelastin molecule and facilitate its secretion. It has been demonstrated, however, that a high pericellular concentration of free galactosugar-bearing moieties, such as chondroitin sulfate or dermatan sulfate GAGs, may lead to premature release of the tropoelastin from the EBP and consequent disruption of elastic fiber production.

**Elastic Fiber Formation**

Prior to the deposition of newly secreted soluble tropoelastin, microfibrils have to assemble close to the cell surface. Microfibrils are complex structures that appear as repeating globules on filamentous linear arrays. Fibrillins are the principal structural components that provide the framework of the microfibrils. Fibrillins are large (350 kDa), acidic, cysteine-rich glycoproteins that upon secretion undergo N- and C-terminal cleavage by the enzymes of the furin/PACE family. This proteolytic cleavage facilitates the directional linear accumulation and lateral interaction between fibrillin molecules and thus aids subsequent assembly.
MAGP-1 is an acidic, 31 kDa glycoprotein that is possibly the integral microfibril-associated molecule that provides structural integrity to the microfibrils.\textsuperscript{10,65,67} MAGP-1 binds strongly in a calcium-dependent manner to the N-terminal sequence of fibrillin-1, which has been localized to the bead region of the microfibrils.\textsuperscript{68} Heparin sulfate competes with MAGP-1 for this binding, suggesting that heparin sulfate proteoglycans play an important role in microfibril assembly. Assembled microfibrils are stabilized by transglutaminase cross-links.\textsuperscript{10,69}

There are two high-affinity binding sites for tropoelastin on fibrillin-1.\textsuperscript{70} One of these is located near the centre of the molecule, adjacent to the beads of the microfibril structure. By means of a transglutaminase cross-linkage, tropoelastin can bind to a specific fibrillin-1 sequence in this site and thus stabilize the newly deposited tropoelastin. MAGP-1 is thought to facilitate the deposition of tropoelastin onto the microfibril template, since it interacts with tropoelastin through multiple binding sites.\textsuperscript{71,72} In addition to the C-terminus region,\textsuperscript{69} several other binding sites within tropoelastin have been shown to interact with MAGP-1 in a calcium dependent manner.\textsuperscript{68} Transglutaminase-mediated cross-linking of MAGP-1 to tropoelastin covalently locks their association.\textsuperscript{73}

The exact mechanistic involvement of other elastic fiber-associated molecules in the assembly and function of elastic fibers is less clear.\textsuperscript{10} Fibulin-1, -2, -4, and -5, which colocalize with elastic fibers and bind to tropoelastin,\textsuperscript{74-77} likely stabilize their primary structure. Fibulin-5, which also interacts with integrin receptors, may additionally anchor elastin to surrounding cells.\textsuperscript{77}
Once aligned, most of the lysyl residues of the tropoelastin molecule are deaminated and oxidized to allysine following the action of the copper-requiring enzyme lysyl oxidase.\textsuperscript{78} The cross-links are then formed by the reaction of the allysine molecules with themselves or with unmodified lysine to give rise to a variety of inter- and intramolecular structures such as desmosine and isodesmosine which insolubilize the tropoelastin chains as the elastin network grows. When lysyl oxidase is inhibited, cross-linking is greatly reduced and tropoelastin accumulates in tissues, a response that demonstrates the vital importance of this enzyme in elastogenesis.\textsuperscript{79} Nutritional deprivation of copper, which is needed for lysyl oxidase activation, may lead to the occurrence of hemorrhaging and aortic aneurysms.

The following diagram summarizes elastogenesis:
Figure A: The mechanisms involved in the process of tropoelastin secretion and assembly. EBP binds tropoelastin intracellularly and acts as a molecular chaperone for this hydrophobic and nonglycosylated protein. EBP protects tropoelastin from premature coacervation and proteolytic degradation. All three subunits of the elastin binding complex act in concert in the process of extracellular assembly of elastic fibers. Active Nase (proteolytically processed from an inactive precursor by PP) removes terminal sialic acid residues from carbohydrate chains of microfibrillar glycoproteins. This causes unmasking of penultimate galactosugars. Unmasked galactosugars can interact with galactolectin site of the EBP and induces release of transported tropoelastin from its chaperone.

Diseases and Conditions Affecting Elastic Fibers: Elastinopathies

Elastic fibers produced in fetal life and in the neonatal period are designed to maintain their resilient nature for a lifetime. However, various enzymes such as MMPs and serine proteases are able to cleave elastic fiber components. Loss of elasticity due to degradative changes is a major factor contributing to the aging of connective tissues,
solar elastosis of the skin, and lung emphysema, as well as the development of aortic aneurysms and the progression of other cardiovascular complications.11,81-83

On the other hand, genetic haploinsufficiency of elastin gene expression causes early cardiovascular complications. One such complication is supravalvular aortic stenosis (SVAS), which occurs in isolation or as part of a complex developmental disorder, Williams-Beuren syndrome (WBS).84,85 SVAS is an obstructive vascular disorder associated with heterozygous mutations or deletions in the elastin gene that leave only a single functional allele.86 To better define the pathogenic mechanism of SVAS, Li et al.87 generated mice that were hemizygous for elastin (ELN+/−). In comparison to wild-type mice, ELN+/− mice exhibit a 50% decrease in elastin mRNA, and their arterial walls are significantly thickened, with a 35% increase in the number of lamellar units, which results in increased cardiac stiffness. The pathological features of SVAS include narrowing of the lumen of the ascending aorta (as a result of deregulated hyperproliferation of vascular smooth muscle cells in the subendothelium), thinner elastic lamellae, abnormal arterial mechanical properties, often increased blood pressure, and heart hypertrophy and failure if the condition is not corrected.87-89

Mice deficient in elastin (ELN−/−) die prematurely in early postnatal life from arterial occlusion due to excessive subendothelial proliferation and the accumulation of vascular smooth muscle cells.90 Elastin is therefore unique among the elements of the ECM because disruption of its expression leads to pathological vascular proliferation in both humans and animals.91 Our laboratory has also demonstrated that insoluble elastin is an important regulator of cellular proliferation, since decreased elastin deposition in both
SVAS and WBS was found to coincide with increased cellular proliferation, which could be reversed by the addition of exogenous insoluble elastin.\textsuperscript{92}

Elastin gene mutations resulting in the secretion of mutated elastin and defective elastic fiber assembly have also been reported in patients with cutis laxa,\textsuperscript{93,94} a condition characterized by redundant, loose, sagging, and inelastic skin, cardiovascular complications, and pulmonary emphysema.\textsuperscript{86} Impaired elastic fiber production has also been observed in heritable diseases involving mutations of the genes encoding microfibrillar proteins. Mutations in the gene encoding the microfibrillar protein fibrillin-1, for example, cause Marfan syndrome, an autosomal dominant disorder characterized by widespread connective tissue abnormalities that chiefly affect the cardiovascular, ocular, and skeletal systems.\textsuperscript{1,95,96}

Multiorgan complications, including cardiovascular pathology, can also be observed in several genetic diseases in which the inadequate secretion of normal tropoelastin and the disruption of its extracellular assembly into elastic fibers can be induced by a high pericellular concentration of free galactosugar-bearing moieties (e.g., chondroitin sulfate or dermatan sulfate glycosaminoglycans). These substances disrupt elastic fiber production by inducing the premature release of tropoelastin from EBP (as in Hurler syndrome and Costello syndrome).\textsuperscript{97-100} Interestingly, our laboratory has demonstrated that in patients with Hurler syndrome \textsuperscript{97} and Costello syndrome \textsuperscript{98} inadequate deposition of insoluble elastin and increased cellular proliferation lead to occlusive arterial lesions and progressive cardiomyopathy. Finally, disruption of elastogenesis can be due to a lack of proper elastin cross-linking caused by a deficiency
of lysyl oxidase (as in Ehlers Danlos syndrome), \(^{101}\) which also affects another structural component of the ECM, collagen.

**Collagen Fibers**

Collagen is the major fibrous protein in the ECM that provides extracellular support, maintains tissue integrity, and contributes to the proper function of numerous organs.\(^3\) The collagen molecule consists of three polypeptide chains wound in a triple helical conformation.\(^{102}\) Each individual chain (α-chain) of these molecules is composed of a repeating glycine (Gly)-X-Y triplet, in which X and Y are usually proline and hydroxyproline respectively.\(^{103}\) This triplet motif results in a helix that intertwines with two other helices to form a triple-stranded helical structure, which can be homotrimeric or heterotrimeric, depending on the collagen type.\(^{103}\) The helix is flanked by terminal globular domains, known as the N- and C-propeptides, that do not exhibit the repeated Gly-X-Y motif. Proteolytic removal of the propeptides results in triple-helical collagen molecules that can assemble into highly ordered, string-like aggregates known as fibrils.\(^{103}\)

Each of the approximately 25 α-chains that have been identified is encoded by a separate gene, and different tissues express different combinations of these genes, thus producing the different α-chains that make up the triple-stranded helical structures of different collagens.\(^{3,104}\) Among the various collagen species, collagen type I, II, III, V, and VI are the fibril-forming collagens, with collagen type I representing the principal fibrillar component of most tissues, including those of the heart.\(^{105}\)
Collagen Synthesis and Regulation

Biosynthesis and maturation of collagen is a highly complex, precisely integrated, multistage process. It begins with the transcription of particular pro-α collagen genes, consistent with the well-established mechanism of mammalian gene transcription. Type I collagen, the major component of ECM, is the product of two genes, COL1A1 and COL1A2 (chromosomes 17 and 7 in humans, respectively), composed of two α1(I) chains and one α2(I) chain. The pro-α1(I) and pro-α2(I) polypeptide chains are synthesized in a 2:1 ratio by mesenchymal cells, such as fibroblasts and osteoblasts, and they undergo extensive post-translational modifications before assembling into the characteristic triple helix.

Even though potentially each step of the collagen biosynthesis pathway is regulated, exaggerated tissue deposition of collagen type I, which is evident in fibrotic disease, is largely due to an increase in the rate of transcription of collagen genes.

There are several repressor and enhancer elements within the promoter region of both the COL1A1 and COL1A2 genes. Several transcription factors interact with these upstream regulatory elements and control the basal collagen type I gene expression, which is cell-type specific and cytokine- and growth factor-modulated. These upstream regulatory elements include the binding sites for several well-characterized transcription factors, including CCAAT binding factor (CBF), NF-1, C/EBP, Sp1, AP-1, NF-κB, Smads, Myc, extracellular signal–regulated kinases 1 and 2 (ERK1/2), p53, and others. These transcription factors upregulate or downregulate collagen gene expression, depending on the tissue or cell type and the combination in which particular
transcription factors are present as well as the physiological/pathological condition of the tissue.\textsuperscript{104,107}

Several growth factors, cytokines, and steroid hormones influence the expression of collagen genes. TGF-β is the key regulator of ECM assembly and remodeling.\textsuperscript{8} TGF-β stimulates collagen synthesis at both the transcriptional level and post-transcriptional level and is mediated in part through Smad family signaling molecules.\textsuperscript{8} Interferon-γ inhibits both basal and TGF-β-stimulated collagen type I gene expression.\textsuperscript{104} TNF-α also inhibits collagen type I gene transcription and demonstrates antagonistic activity against TGF-β through the involvement of antagonistic transcription factors such as AP-1 and NF-κB that interfere with the TGF-β-induced Smad signaling pathway.\textsuperscript{8} Interleukin-1β (IL-1β) can both increase and decrease collagen synthesis at the transcriptional and post-transcriptional level, depending on the cell type.\textsuperscript{104} Steroid hormones can also modulate collagen gene expression: glucocorticoids inhibit, while mineralocorticoids enhance collagen synthesis.\textsuperscript{3}

**Collagen Fiber Assembly**

After translation, the pro-α1(I) and pro-α2(I) polypeptides enter into the endoplasmic reticulum, in which a number of molecular chaperones and enzymes assist its folding and trimerization.\textsuperscript{103} For example, the enzyme prolyl-4-hydroxylase is required to convert the proline residues of pro-α chains to hydroxyproline residues, which are essential for the folding of the newly synthesized pro-α chains into triple helix procollagen molecules.\textsuperscript{103,104} If hydroxylation is prevented, unfolded procollagen is retained within the endoplasmic reticulum.\textsuperscript{103} Lysine residues are also hydroxylated by a family
of lysyl hydroxylases, and are subsequently further modified by specific enzymes that add galactose and glucose moieties to hydroxylysine residues in the endoplasmic reticulum.\textsuperscript{103} Folding is also facilitated by other enzymes and by molecular chaperones, all resident within the endoplasmic reticulum.\textsuperscript{103} The modified procollagen molecules are transported through the Golgi complex in which N-linked carbohydrate groups are further processed and then transported within secretory vesicles to the cell membrane where they are secreted by exocytosis to the extracellular space.\textsuperscript{102}

The N-terminal and C-terminal propeptides within the secreted procollagen molecules are cleaved by procollagen N-proteinase and procollagen C-proteinase respectively, yielding triple helical collagen molecules with short non-triple helical terminal telopeptides at each end.\textsuperscript{104} The processed collagen molecules then spontaneously self-associate and assemble into multimeric fibrillar aggregates.\textsuperscript{102} The final post-translational modification in the formation of insoluble fibrous collagen is also catalyzed by lysyl oxidase.\textsuperscript{102} Lysyl oxidase oxidizes selected lysine residues within the N- and C-terminal telopeptides to peptidyl aldehydes, which then undergo spontaneous chemical reactions with other lysyl oxidase–derived aldehyde residues, or with unmodified lysine residues, to insolubilize and stabilize the collagen molecules within the fibers.\textsuperscript{102} The following diagram (adapted from Canty and Kadler, 2005\textsuperscript{103}) summarizes the process of collagen fiber assembly:
Figure B: Overview of the process involved in collagen fiber assembly.

While regulation of the composition of the ECM varies significantly from organ to organ, the abnormal accumulation of predominantly stiff collagenous fibers in the ECM is characteristic of fibrotic diseases. Abnormal accumulation of collagen fibers in the heart following a myocardial infarction (MI) is a leading cause of cardiac dysfunctions.\textsuperscript{2,3}

Components of the Myocardial ECM

The myocardium may be differentiated into compartments occupied by cardiac myocytes, the interstitial fibroblasts, and the extensive network of blood vessels.\textsuperscript{108} The
heart’s primary function as a pump depends mainly on the cardiac myocytes, while its structural and functional integrity depends largely on the non-myocyte fibroblasts. Cardiac myocytes account for 70% to 75% of the myocardium by cell volume but only 25% to 30% by cell number. Cardiac fibroblasts are the most abundant cell type of the myocardium and produce various proteins found in the ECM. Cardiac fibroblasts regulate the levels of cardiac ECM through at least three mechanisms: (1) by regulating the synthesis and deposition of matrix molecules; (2) by mediating matrix degradation and turnover through the production and release of MMPs and TIMPs; and (3) by maintaining mechanical tension on the matrix network.

The myocardial ECM network consists of three major components: the epimysium, the perimysium, and the endomysium. The epimysium contains large bundles of fibers that form a sheath around the myocytes. It is the largest component structure of the cardiac interstitium and envelops whole muscle bundles. The perimysium is composed of large bundles of organized matrix proteins that surround and connect bundles of cardiac myocytes and link the epimysium to the endomysium. The endomysium is a complex weave that is wrapped around individual myocytes and thus forms the most direct point of contact between the ECM and the myocytes. This component of the ECM not only provides a supportive scaffold for the myocytes and blood vessels but also contributes to the function of the heart. The energy produced during systole is stored within the compressed ECM, which expands during diastole and contributes to the relengthening of the cardiac myocytes.

The cardiac ECM proteins include the major fibrillar collagens type I and III, which comprise the bulk of the ECM, as well as elastin, microfibrillar proteins (fibrillin),
fibronectin, glycoproteins, GAG, bioactive signaling molecules, and other components.\textsuperscript{110,113-115} Most studies however, have focused on the myocardial collagen network, and detailed study of the role of other ECM components, particularly elastic fibers in the heart, has largely been neglected.

The presence of elastin in the myocardium has been documented in early studies, which demonstrated that large bundles of elastic fibers are present in the epimysium along with collagen fibers.\textsuperscript{115} Evidence from these studies that elastic fibers provided interconnections between cells and wound helically around myocytes suggested that the presence of elastin may significantly contribute to the function of the myocardium by promoting elongation in tandem with intermyocyte collagen struts.\textsuperscript{115}

The fibrillar collagens (types I, II, III, IV, and V) form the structural backbone of the myocardial ECM network.\textsuperscript{3} Type I collagen, the major fibrillar collagen, comprises about 80\% of the total myocardial collagen content, while type III comprises about 10\%, and the other collagen types make up the remaining 10\%.\textsuperscript{113,116} The tensile strength of cardiac collagen species decreases progressively from type I to type IV – that is, type I is stronger than type II, which is stronger than type III, which is stronger than type IV. Because of these differences, the changes induced by contraction and relaxation can be effectively distributed throughout the heart.\textsuperscript{113,116} Collagen types I and III form aggregate struts that are widely distributed between myocytes and among muscle fibers.\textsuperscript{113} The endomysial intermyocyte collagen struts resists compression and excessive stretch, prevent myocyte slippage, and may also play a role in the mechanical coupling of myocytes.\textsuperscript{3,112,117} Alterations in the content of the collagen network result in changes in left ventricle structure and function and contribute to adverse ventricular remodeling
following MI. Furthermore, loss of myocardial elastin has also been suggested to promote fibrosis.\textsuperscript{118} Thus, myocardial content of these matrix components may determine the extent of ventricular remodeling.

**Cardiac Remodeling After Myocardial Infarction (MI)**

Cardiac remodeling involves two primary responses at the cellular level: (1) hypertrophy, dysfunction, and death of cardiac myocytes, and (2) increased deposition and alteration of the cardiac ECM.\textsuperscript{109} Until recently, the study of the cellular and molecular biology of heart failure centered almost exclusively on the first of these responses, myocyte dysfunction. However, it has now become increasingly clear that the ECM is a dynamic microenvironment and a major contributor to the adverse ventricular remodeling that follows an MI.\textsuperscript{119}

MI is a frequent cause of chronic heart failure, which is a leading cause of morbidity and mortality in developed countries.\textsuperscript{120} The consequent loss of contractile myocardium following an MI results in an abrupt increase in loading conditions that induces a unique pattern of remodeling involving the infarcted border zone and the remote non-infarcted myocardium.\textsuperscript{108} The adjustments that occur to accommodate the increase in workload of the remaining myocardium include reparative changes such as dilatation, hypertrophy, and scar formation. Ventricular remodeling begins immediately and may continue for weeks or months until the distending forces are counterbalanced by the tensile strength of the collagen scar.\textsuperscript{121} While adaptive remodeling is initially beneficial, its continued progression becomes deleterious and leads eventually to congestive heart failure.\textsuperscript{120}
Post-infarction remodeling can be divided into an early phase (within the first three days) and a late phase (beyond the first three days). Early remodeling occurs mainly in the infarct zone and involves infarct expansion, which may result in early ventricular rupture or aneurysm formation. Infarct expansion results from the degradation of the inter-myocyte collagen struts by serine proteases and the activation of MMPs released from inflammatory cells and fibroblasts. The release of pro-inflammatory cytokines such as TNF-α further induces MMP production, all of which shifts the MMP/TIMP balance towards increased proteolytic activity and ECM degradation within the first few days following an MI. The loss of collagen begins within minutes of the onset of ischemia, and within hours infarct expansion occurs, results in myocyte slippage, wall thinning, and ventricular dilatation, and causes the elevation of diastolic and systolic wall stresses. Increased wall stress is a powerful stimulus for hypertrophy. It is mediated by mechanoreceptors and transduced to intracellular signaling partly via angiotensin II release, which initiates the increased synthesis of contractile assembly units.

Chronic inflammatory cells such as macrophages phagocytose the necrotic myocardium and secrete growth factor and cytokines, which in turn stimulate fibroblasts to proliferate within the infarct zone and synthesize collagen. Late phase remodeling involves alterations in ventricular architecture to distribute the increased wall stresses more evenly as the ECM forms a collagen-rich scar to stabilize the distending forces and prevent further deformation. However, the failure to normalize increased wall stresses results in continued ventricular dilation, hypertrophy, and reactive fibrosis of the remote non-infarcted myocardium, which are maladaptive processes that contribute to left
ventricle dysfunction and the progression to heart failure.\textsuperscript{128} Myocardial fibrosis enhances myocardial stiffness, generates arrhythmias, and hampers systolic ejection and diastolic relaxation.\textsuperscript{129} Therefore, agents that act on fibroblasts and the ECM to reduce maladaptive fibrosis without affecting the healing of the infarction may significantly improve left ventricle function and survival after MI\textsuperscript{3,128}.

Post-infarction activation of the renin-angiotensin system (R\textsuperscript{A}S), an important neurohormonal system, leads to increased production of aldosterone.\textsuperscript{130} In addition to expanding the vascular volume, aldosterone also induces maladaptive fibrosis and adverse ventricular remodeling.\textsuperscript{130} Numerous experimental animal models and clinical studies have demonstrated that one of the major cardioprotective effects of blocking aldosterone action (via mineralocorticoid receptor antagonists) is the alleviation of post-MI maladaptive fibrosis.

**Aldosterone**

Aldosterone, the major mineralocorticoid hormone secreted by the adrenal cortex, was isolated 50 years ago.\textsuperscript{131} Aldosterone is a neurohormonal mediator of the renin-angiotensin-aldosterone system (R\textsuperscript{A}A\textsuperscript{S}) and plays an important role in the regulation of fluid and potassium balance. The effects of aldosterone were traditionally thought to be produced through a genomic mechanism involving the activation of intracellular mineralocorticoid receptors (MR). The activated aldosterone-MR complex translocates to the nucleus where it modulates the transcription and translation of “aldosterone-induced” proteins that regulate electrolyte and fluid balance and subsequent blood pressure homeostasis. More recently, it has been shown that aldosterone also has marked
effects in a wide range of non-epithelial tissues (such as the heart), that it can be synthesized and regulated in a number of extra-adrenal tissues, and that it may act through membrane receptors other than the traditional MR (alternative receptors) in epithelial and nonepithelial tissue in a non-genomic manner.

**Classic Actions of Aldosterone**

**Aldosterone Synthesis**

Aldosterone is synthesized from cholesterol in the zona glomerulosa of the adrenal cortex by a series of specific enzymatic reactions.\(^{132}\) First, cholesterol must cross the outer mitochondrial membrane to the inner membrane where the first enzyme in the steroidogenic pathway is located. This process, now considered to be the rate-limiting step in steroidogenesis, is mediated by steroidogenic acute regulatory protein (StAR), which is present in all steroidogenic tissue.\(^{133,134}\) Subsequent aldosterone synthesis is regulated by two critical enzymatic steps: (1) the formation of pregnenolone from cholesterol by the mitochondrial enzyme P450scc (side chain cleavage), and (2) the conversion of corticosterone to aldosterone by cytochrome P450 11β-hydrolase 2 (CYP11B2, aldosterone synthase).

A number of factors have been shown to stimulate or inhibit aldosterone production. However, the principal regulators of aldosterone synthesis and secretion are angiotensin II and the concentration of extracellular potassium, while sodium and adrenocorticotropic hormone (ACTH) are weaker regulators.\(^{132}\)
The Renin-Angiotensin System (RAS)

Aldosterone biosynthesis is regulated principally by the RAS. Renin is synthesized and released by the juxtaglomerular cells in the afferent arteriole of the kidney in response to a decrease in intravascular volume, which is detected by baroreceptors (mediated by β-adrenoreceptor activation) and by a reduced sodium concentration at the macula densa. Renin catalyses the hydrolysis of angiotensinogen to angiotensin I (Ang I), which is then converted to angiotensin II by angiotensin converting enzyme (ACE), which is present in the lungs and vascular tissue. Complete RAS have also been described in other tissues, including those of the brain, heart, vasculature, and adrenal cortex.

Angiotensin II acts on vascular smooth muscle to cause vasoconstriction, and on the adrenal zona glomerulosa to stimulate aldosterone production. The adrenal response to angiotensin II occurs within minutes, a time course that implies that no new protein synthesis is required. This acute, angiotensin II-mediated release of aldosterone may involve rapid synthesis from intermediate compounds in the steroidogenic pathway or de novo synthesis from cholesterol, leading to increased transport of cholesterol to the inner mitochondrial membrane. Chronic stimulation by angiotensin II results in zona glomerulosa hypertrophy and hyperplasia, increased CYP11B2 expression, and subsequent aldosterone secretion.

The mechanism by which angiotensin II stimulates aldosterone production is not yet fully understood. However, it is known to act on specific G-protein-coupled receptors (GPCR) (angiotensin II type 1 receptors), which cause phospholipase C to stimulate intracellular production of inositol 1,4,5-trisphosphate (IP3) and 1,2-
diacylglycerol (DAG), which then activate protein kinase C (PKC).\textsuperscript{132} 1,4,5-Trisphosphate (IP\textsubscript{3}) also increases the concentration of intracellular free calcium, causing several Ca\textsuperscript{2+}/calmodulin-dependent protein kinases (CaM kinases) to phosphorylate and activate such transcription factors as activating transcription factor (ATF)-1, ATF-2, and cAMP-response-element (CRE)-binding protein (CREB).\textsuperscript{137,138} These bind CRE and other cis-acting elements (e.g. Ad-5 and NBRE-1), which are unique to the 5'-untranslated region of the \textit{CYP11B2} gene.\textsuperscript{138}

Extracellular K\textsuperscript{+} concentration is the other key determinant of aldosterone secretion, which in turn helps to maintain K\textsuperscript{+} homeostasis.\textsuperscript{137} Increased K\textsuperscript{+} concentration causes depolarization of cell membranes in zona glomerulosa, leading to a rapid rise in intracellular Ca\textsuperscript{2+} and subsequent activation of calmodulin and Ca\textsuperscript{2+}/calmodulin-dependent protein (CaM) kinases, which phosphorylate transcription factors to stimulate \textit{CYP11B2} gene transcription, as mentioned for angiotensin II.\textsuperscript{137,139}

**Epithelial Actions of Aldosterone**

Classically, aldosterone acts on epithelial cells, particularly in the renal collecting duct, but also in the colon, salivary glands, and sweat glands, where it regulates the transport of Na\textsuperscript{+}, K\textsuperscript{+}, and water.\textsuperscript{140} Aldosterone-responsive epithelial cell monolayers act as barriers separating the internal and external environment; they also permit the reabsorption of Na\textsuperscript{+} and water. These functions are facilitated by the lipid composition of the apical membrane and by the formation of high-resistance tight junctions. Transport through these cells is facilitated by an electrochemical potential across the apical membrane from urine to cell and by an active-transport mechanism across the basolateral
membrane from cell to interstitium. Sodium reabsorption across the apical membrane is mediated by the luminal amiloride-sensitive epithelial sodium channel (ENaC). Transport across the basolateral membrane is driven by the ouabain-sensitive serosal Na+/K+-ATPase which drives the entry of sodium and the excretion of potassium from the cell to the lumen through the luminal K+ channel. Water follows the movement of Na+ across the monolayer. ENaC is removed from the cell membrane and targeted for proteosomal degradation by the Nedd4 family of ubiquitin protein ligases. The activity of ENaC is susceptible to modulation by serine proteases, phosphorylation, and methylation. Similarly the activation of Na+/K+-ATPase is influenced by the intracellular sodium concentration and the availability of ATP and by membrane-spanning modulator proteins, which may decrease the affinity of the pump to sodium ions.

ENaC and Na+/K+/ATPase are considered to be the principal mediators of aldosterone action in epithelial cells. However, other protein targets in the apical membrane have also been identified – e.g. the luminal Na+/H+ exchanger (NHE3) in the colon and the luminal thiazide-sensitive Na+/Cl- cotransporter in the distal renal tubule – which appear to mediate sodium reabsorption in response to volume depletion.

Mechanism of Aldosterone Action via the Mineralocorticoid Receptor (MR)

Aldosterone mediates its effects through the activation of cytosolic MR. The central role of MR in sodium homeostasis has been established in studies using MR-null transgenic mice, which are characterized by severe salt-wasting.

Severa Bunda
Page 27
The MR is a member of the steroid-thyroid-retinoid superfamily of ligand-dependent transcription factors. The MR compromises three major domains, an N-terminal domain, a highly conserved DNA-binding domain, and a ligand-binding domain (LBD). The DNA-binding domain contains sites for the consensus sequence on the promoter/enhancer region of the target genes (hormone-responsive element). In the absence of the ligand, MR associates with heat-shock proteins, which tether MR predominantly in the cytoplasm. Hormone binding produces a conformational change, which results in dissociation of the associated proteins, dimerization, and translocation to the cell nucleus. The activated receptor/hormone complex binds to steroid responsive elements in the 5'-untranslated region of aldosterone-responsive genes that activate or repress gene transcription.

The receptor–steroid complex may also act through a process known as transcriptional interference or synergy, whereby it interacts with other transcription factors that themselves bind DNA to activate or repress transcriptional activity. This enables transcriptional modulation without direct interaction between the receptor–steroid complex and DNA. The N-terminal domain is thought to influence receptor activity in transcriptional activation via co-activator binding sites and in interdomain receptor interactions by interacting with ligand-activated LBD.

**Aldosterone-Induced Genes/Proteins**

The epithelial action of aldosterone consists of early (1–6 h) and late (>6 h) phases. The early phase is mediated by changes in gene expression that activate ion channels and signaling proteins, which modulate the activity of electrolyte-transport
proteins. The late phase results from both primary and secondary effects on gene expression.

Of the early aldosterone-induced genes and proteins, including the obvious α-, β- and γ-ENaC subunit genes, serum- and glucocorticoid-inducible kinase 1 (Sgk1) is most firmly established as a mediator of aldosterone action, although others exist.¹⁴¹

Sgk1 is a serin-threonine kinase that regulates a variety of ion transporters, including the ENaC.¹⁵⁵ Sgk1 stimulates sodium reabsorption, partly by phosphorylation of ubiquitin ligase Nedd4, which compromises the interaction with ENaC and, as a result, prolongs the functional half-life of ENaC.¹⁵⁶ Activation of Sgk1 requires phosphorylation via a PI3 kinase-mediated pathway, which suggests that Sgk1 may integrate the signaling of nuclear receptors and membrane-associated tyrosine kinase receptors, such as the insulin receptor.¹⁵⁷

The expression of the small, monomeric Kirsten Ras GTP-binding protein-2A (Ki-RasA) is induced during the early phase of aldosterone action. The effects of aldosterone on sodium transport in renal epithelial cells also appear to depend on this protein and its regulation of the activity and abundance of ENaC. Another important protein involved in mediating aldosterone responses is the glucocorticoid-induced leucine zipper (GILZ) protein.¹⁵⁸ GILZ increases cell surface ENaC expression and subsequently sodium reabsorption.¹⁵⁹

Corticosteroid hormone-induced factor (CHIF) is expressed in the basolateral membranes of epithelial cells in the distal colon and nephron.¹³² It belongs to the FXYD transmembrane family of seven proteins that contain both this motif and three other conserved amino acids that serve to regulate ion channels and transport proteins. The γ-
subunit of Na+/K+-ATPase is part of this family.\textsuperscript{160,161} The role of CHIF in regulating electrolyte balance is highlighted in studies of CHIF knockout mice, where potassium loading plus treatment with furosemide leads to lethality in the knockout group but not in the wild-type controls.\textsuperscript{162} CHIF has been shown to increase the affinity of Na+/K+/ATPases for sodium and also likely mediates the early aldosterone-induced increase in Na+/K+/ATPase activity.\textsuperscript{160}

**Target Tissue Specificity of the MR**

Although the MR is the principal effecter of the cellular responses to aldosterone, glucocorticoids (cortisol) and mineralocorticoids (aldosterone) bind to it with almost equally high affinity.\textsuperscript{163} Even though cortisol circulates at much higher concentrations than aldosterone and the majority of glucocorticoids are bound to the proteins in the plasma, the concentration of glucocorticoids that do not preferentially occupy the MR is still 100-fold higher than expected.\textsuperscript{132} In the epithelial target tissue and in some other tissues, aldosterone specificity is maintained by the action of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2).\textsuperscript{164} 11β-HSD2 metabolizes biologically active cortisol to receptor-inactive cortisone and stoichiometrically generates NAPH.\textsuperscript{140} Inactivation of this enzyme by the active components of licorice or congenital loss of function (the syndrome of apparent mineralocorticoid excess) produces salt retention, hypertension, and potassium wasting, all of which reflect MR activation by endogenous glucocorticoids.\textsuperscript{165}

Although this mechanism accounts for aldosterone specificity in epithelia, it fails to explain how aldosterone can act in cells and tissues, such as cardiac myocytes (see
below), that possess abundant MRs but no or little 11β-HSD2. In addition, the efficiency of 11β-HSD2 alone may not be sufficient to account for the complete lack of MR activation by glucocorticoids. Kinetics experiments, which show that the off-rate of aldosterone from MR is 5 times lower than that of glucocorticoids, point to an intrinsic discriminating property of the receptor. However, molecular studies of the cloned MR show that even though mineralocorticoids and glucocorticoids bind with equivalent affinity, aldosterone achieves greater transactivation. This suggests that ligands can have differential effects on gene transcription at the same receptor. These may be due to conformational differences in the MR-ligand complex, which lead to variable degrees of stability. The interaction with co-activators after binding to response elements may also be dependent on the ligand. Finally, another potential mechanism for maintaining MR specificity comes from very recent studies, which suggest that MR-glucocorticoid complexes are inactivated by high local concentrations of NADH, which is produced by 11β-HSD2.

The following diagram summarizes the classic epithelial actions of aldosterone, adapted from Connell and Davies, 2005.
Figure C: Classic epithelial actions of aldosterone.

Kidney tissue is the classic target of aldosterone action, but the MR is also expressed at high abundance in specific regions of the central nervous system and in the heart, where its role in the pathogenesis of cardiac fibrosis has attracted considerable attention.\textsuperscript{168}

**New Concepts in Aldosterone Research: the Presence of Aldosterone in the Heart**

The renin-angiotensin-aldosterone system (\textit{R A A S}) has been viewed conventionally as a circulating system, involved in the regulation of salt, fluid
homeostasis, and blood pressure.\textsuperscript{169} This traditional concept has been updated in the past two decades. It is now believed that most components of the \textit{R A A S} are synthesized locally in tissues such as the heart and vessel wall.\textsuperscript{170} For instance, in the heart, angiotensin II is synthesized locally by cardiac ACE following the uptake of renin and angiotensinogen from the circulation.\textsuperscript{135,136} This angiotensin II subsequently stimulates cardiac angiotensin II type 1 (AT\textsubscript{1}) and type 2 (AT\textsubscript{2}) receptors.\textsuperscript{171} Local synthesis of angiotensin II at cardiac tissue sites is consistent with the observation that the beneficial effects of ACE inhibitors in heart failure are, at least in part, independent of their effect on blood pressure.\textsuperscript{172}

Traditionally, treatment of heart failure and hypertension has focused on the role of the \textit{R A A S} rather than the \textit{R A A S}, with the assumption that aldosterone will be suppressed once angiotensin II formation is blocked.\textsuperscript{140,169} However, because of “angiotensin II reactivation” or “aldosterone escape,” aldosterone formation does not stay suppressed during prolonged \textit{R A A S} blocking therapy.\textsuperscript{173,174}

Two clinical trials, the Randomized ALdosterone Evaluation Study (RALES)\textsuperscript{175} and the EPlerenone HEart failure and SUrvival Study (EPHESUS),\textsuperscript{176} have convincingly indicated that treatment with MR antagonist (such as spironolactone), and ACE inhibitors, reduce mortality in patients with heart failure and systolic left ventricular dysfunction post-MI. These results and the pioneering experimental work of Weber and colleagues\textsuperscript{177} on aldosterone and dietary sodium in cardiac fibrosis drew attention to the importance of aldosterone as an independent risk factor in the pathophysiology of cardiovascular disease. The benefit of MR antagonists during \textit{R A A S} blockade is not yet fully understood. Their effects cannot be attributed solely to blockade of the renal MR-
mediated effects on blood pressure, and it is now generally assumed that aldosterone also acts directly on the heart, in agreement with the concept of local R A A S synthesis.\textsuperscript{169}

The presence of the MR has been demonstrated in the heart, both at the mRNA and protein level.\textsuperscript{178} Importantly, the enzymes required for the synthesis of aldosterone (\textit{CYP11B2}) appear to be expressed in the human heart as well.\textsuperscript{179} Additional evidence suggests that aldosterone mediates many cellular effects independently of classical MR-mediated transcriptional gene activation,\textsuperscript{180-183} which may also be involved in the remodeling process of cardiac tissues.\textsuperscript{182,184} Together with the fact that angiotensin II is capable of increasing the aldosterone levels in isolated rat hearts and blood vessels,\textsuperscript{185,186} these data suggest that aldosterone, like angiotensin II, may be formed and may act locally in the heart.

**Aldosterone Production in the Heart**

In the last few years it has become apparent that many of the genes encoding enzymes and cofactors involved in aldosterone biosynthesis are also expressed in the cardiovascular system.\textsuperscript{132} As early as 1969, Knox and Lockett\textsuperscript{187} demonstrated that isolated hearts produce a substance whose physiochemical properties are consistent with those of aldosterone. However, the first direct evidence of a local cardiac aldosterone system in the normal rat heart has only recently been provided. Silvestre \textit{et al.}\textsuperscript{185} first described aldosterone synthase (\textit{CYP11B2}) expression in the four cardiac chambers of the adult rat, which was upregulated in response to angiotensin II or sodium restriction. Aldosterone synthase expression has also been proposed in the human heart.\textsuperscript{179,188} Cardiac levels of \textit{CYP11B2} transcripts and aldosterone secretion are reported to be raised
during heart failure, and a direct correlation has been identified between myocardial $CYP11B2$ mRNA expression and collagen volume in the failing human heart.\textsuperscript{179,189,190}

After a MI, rats had increased cardiac levels of $CYP11B2$ transcripts and aldosterone in the non-infarcted areas of their left ventricles, although corticosterone levels actually fell in these areas.\textsuperscript{191} The rat heart also expresses steroidogenic acute regulatory protein (StAR) protein, a crucial factor in the rate-limiting step of aldosterone biosynthesis,\textsuperscript{192} and the gene for P450 scc that catalyzes the production of pregnenolone from cholesterol is transcribed in all chambers of the normal rat heart and adult and fetal human hearts.\textsuperscript{193,194}

While these studies suggested that the whole pathway of aldosterone synthesis is present in the heart, other studies have failed to support the idea of local synthesis of aldosterone in the rat heart.\textsuperscript{195,196} In these studies, the cardiac aldosterone levels were 14-28 times lower than the previously reported estimate of 16 nM (which is 17 times higher than in the plasma).\textsuperscript{185} Subsequent perfusion studies with aldosterone in the isolated Langendorff rat heart\textsuperscript{197} showed that most cardiac aldosterone is derived from the circulation and that the large capacity of cardiac tissue to rapidly accumulate aldosterone explains why the levels of cardiac aldosterone in the rat heart can be up to 10-fold higher than serum levels.\textsuperscript{195}

While the non-epithelial actions of aldosterone, particularly in the heart, are clear, there is no direct evidence that these are achieved through local rather than adrenal production.\textsuperscript{132} The rat may differ from humans in this regard, or heart failure may create conditions that enable cardiac aldosterone expression.\textsuperscript{198,199}
Activation of Cardiac MR by Aldosterone

MR has been shown to be expressed not only in sodium-transporting epithelial cells (e.g., kidney, colon) but also in non-epithelial cells in the brain, blood vessels, and heart. Cardiac MR are localized both in cardiac myocytes and cardiac fibroblasts. The expression of 11β-HSD2 has been demonstrated in the heart by some, but not all, researchers. However, the levels of expression are generally much lower than those found in epithelial tissue and may be limited to specific cell types. Cardiac fibroblasts have been shown to contain 11β-HSD2, while negligible levels are present in cardiac myocytes. Therefore, it has been proposed that cardiac MR are occupied by cortisol rather than aldosterone. Recent experimental evidence has shed further light on the activation of cardiac MR.

In those cells in which MR are not co-expressed with 11β-HSD2, it appears that glucocorticoids do not activate the MR but instead block MR activation by aldosterone. This has been demonstrated in the heart both in vivo and in vitro, as well as in blood vessel walls. In single-cell preparations of cardiac myocytes, aldosterone can increase Na+/K+/2Cl− cotransporter activity, whereas cortisol cannot. Cortisol will, however, block the action of aldosterone when co-infused. When the intracellular redox state is altered using oxidized glutathione (GSSG) to decrease NADH levels and mimic the production of reactive oxygen species (ROS), cortisol then acts in the same manner as aldosterone alone. These findings support the hypothesis that the MR may be activated by normal circulating levels of glucocorticoids in the presence of ROS. However, further studies are necessary to prove whether redox-sensitive
conformational changes in MR are involved in the regulation of MR activation in the heart.\textsuperscript{200}

**MR-Independent or “Non-Genomic” Aldosterone Actions**

The classical genomic model of aldosterone action in target epithelial cells has long been accepted. However, a number of recent studies have generated data that cannot be explained by this model and which suggest that aldosterone can also act by a rapid, non-genomic mechanism in a variety of cell types.\textsuperscript{132} The non-genomic steroid actions are characterized by a rapid time course and insensitivity to inhibitors of transcription and translation and to steroid receptor antagonists. However, this description does not apply to all non-genomic effects of aldosterone.\textsuperscript{211} Evidence exists that some non-genomic effects are mediated by the classical MR-dependent mechanism or by a closely related protein (as has been shown for estrogen and progesterone receptors).\textsuperscript{211} Some rapid-non genomic aldosterone effects have been demonstrated in isolated cardiac myocytes \textsuperscript{212,213} and in human MR-transfected cells.\textsuperscript{214} Furthermore, evidence of cross talk or integration between non-genomic and classical MR-mediated aldosterone action has also been provided.\textsuperscript{215}

However, the great majority of studies have shown that MR antagonists are ineffective in blocking non-genomic aldosterone effects.\textsuperscript{132} Research into non-genomic effects has focused on identifying a specific receptor, distinct from the MR, and elucidating its cellular effects and mechanism of action. The action of such receptors has been demonstrated for other steroid hormones.\textsuperscript{216,217} In the case of rapid, non-genomic actions involving aldosterone, the studies by Wehling and colleagues have made a
significant contribution to our understanding. They have proposed that rapid non-genomic effects of mineralocorticoids are mediated by the activation of a high-affinity membrane receptor. This mechanism is distinct from classical intracellular MR activation, since it is not blocked by spironolactone or canrenone and is not mimicked by cortisol. They further isolated an approximately 50 kDa candidate membrane protein from human lymphocytes. However, full structural characterization of this protein has yet to be completed.

Nonetheless, aldosterone has been shown to have rapid effects on calcium efflux in cell lines lacking the classical MR, and effects on calcium and cAMP have been demonstrated in skin fibroblasts from MR-knockout mice. Chai et al. recently demonstrated that an MR-antagonist (eplerenone) had no blocking effect on the inotropic and vasoconstrictor effects of aldosterone in the Langendorff rat heart, thereby confirming that these effects are exerted in a non-genomic manner involving cell-surface membrane binding.

Non-genomic actions may be important to the responses mediated by local aldosterone production in the vascular system. A recent clinical study showed that administration of aldosterone significantly reduced forearm blood flow within 4 minutes, a time course far too rapid to be accounted for by classical genomic actions. Other studies have shown a small but significant increase in systemic vascular resistance within 3 minutes of aldosterone administration. Similar rapid aldosterone actions have been reported on heart rate, peripheral vascular resistance, and baroreflex sensitivity. Although most studies have focused on the rapid actions of aldosterone, it has now been demonstrated that non-genomic effects of aldosterone on Na+/K+/2Cl-activation in rabbit
cardiac myocytes\(^{211}\) persist for a week in vivo,\(^{213}\) evidence that such rapid actions can be long-lasting.

The non-genomic actions of aldosterone are varied and are mediated by a multitude of second messenger pathways, including changes in intracellular calcium concentration and 1,4,5-trisphosphate (IP\(_3\)) and stimulation of the activity of phospholipase C-PKC, ERK1/2, c-Jun NH\(_2\)-terminal kinase (JNK) 1/2, and cAMP in a variety of tissues.\(^{211}\) Other second messenger pathways that have been linked to the rapid effects of aldosterone include c-Src tyrosine kinase, mitogen-activated protein kinases (MAPK), ROS, AT\(_1\) receptor, the epidermal growth factor receptor (EGFR).\(^{50,224-228}\) Our studies, that consist a major part of this thesis, for the first time demonstrate that the IGF-IR is also involved in the MR-independent/non-genomic action of aldosterone.\(^{50}\)

Although, a complete understanding of the physiological/pathophysiological significance of non-genomic aldosterone action is lacking, it is clear that aldosterone via MR-independent and/or MR-dependent action elicits an array of complex intracellular signaling pathways leading to various cellular responses.

The following diagram summarizes genomic and non-genomic aldosterone actions, modified from Zollner et al., 2008\(^{229}\):
The Effects of Aldosterone on Cardiac Remodeling After Myocardial Infarction

The contribution of angiotensin II, an important component of the RAAS, to the pathophysiology of post-MI remodeling has been well documented. Clinical studies of ACE inhibitors (the Acute Infarction Ramipril Efficacy [AIRE] study, the Trandolapril Cardiac Evaluation [TRACE] Study, and the Survival and Ventricular Enlargement
and of ARBs (the Valsartan in Acute Myocardial Infarction Trial [VALIANT]) have demonstrated the life-saving benefits of these agents in patients after MI.

Aldosterone, another important component of the R A A S, also has been implicated in left ventricular remodeling after MI and is correlated with increased post-MI mortality and morbidity. Activation of the cardiac aldosterone synthase pathway has been shown in rat models to occur during the immediate post-MI process, with corresponding almost 4-fold increases in aldosterone in the myocardium. After MI, myocardial aldosterone stimulates transcription of collagen types I and III mRNA. Further, both aldosterone blockade and angiotensin receptor blockade have been found to prevent increases in ventricular collagen types I and III messenger RNA and fibrosis in rats, and experimental evidence strongly suggests that many of the pathophysiologic effects previously attributed to angiotensin II may, in fact, be caused by aldosterone. In addition, MI, as well as other increased cardiovascular events, has been shown to be more common in patients with primary aldosteronism than in hypertensive control subjects with similar blood pressure. (Primary aldosteronism is a condition characterized by an elevated aldosterone/renin ration and autonomous over-production of aldosterone independent of its normal trophin, e.g., angiotensin II).

Hayashi and colleagues have demonstrated that plasma aldosterone may be extracted by the heart during an MI. The results from this study indicate that in patients with MI the plasma aldosterone extracted by the heart at the acute phase correlates with plasma levels of procollagen type III N-terminal amino peptide (PIIINP) (a biomarker of vascular collagen turnover and fibrosis) and left-ventricle end-diastolic
volume index. These findings are supported by the results of a recent study, which demonstrated that high aldosterone levels in patients presenting with ST-elevation MI (STEMI) within 24 hours of symptom onset are associated with early and late adverse clinical outcomes, including mortality.

**Benefits of MR antagonists after MI**

*Experimental Evidence*- An abundance of experimental studies has demonstrated the beneficial effects of MR antagonists on the cardiovascular system and specifically on post-MI remodeling, including limitation of fibrosis and left ventricular dysfunction. In rat studies, where post-MI activation of aldosterone synthase has been associated with ventricular fibrosis, treatment with spironolactone has been shown to decrease collagen deposition in the non-infarcted left ventricle and interstitial fibrosis. Long-term eplerenone treatment has been shown to attenuate left ventricular remodeling and prevent progressive left ventricular dysfunction in a dog chronic heart failure model as well. The beneficial effects of aldosterone blockade, in comparison with a placebo, were also studied on infarct healing and remodeling at days 3, 7, and 28 post-MI in eplerenone-treated rats. Reparative fibrosis, measured by collagen volume fraction in the infarcted region of the myocardium, was similar in eplerenone-treated and placebo-treated animals. However, evidence of reduced reactive fibrosis was found in viable myocardium at 28 days post-MI in eplerenone-treated animals.

Thus, these data indicate that aldosterone receptor antagonism did not retard infarct healing but rather protected the myocardium against maladaptive responses after MI. Given that the incidence of cardiovascular diseases increases rapidly with age,
another study evaluated the role of aldosterone in aged myocardium following MI by studying the post-MI effect of eplerenone in aged rats. The results of this study demonstrate that eplerenone normalizes diastolic relaxation as well as myocardial interstitial collagen and aortic fibrosis in aged rats with MI, thus demonstrating improvement in cardiac structure and function.

Combination therapy with an ACE inhibitor has been shown to potentiate the beneficial effects of aldosterone blockade on left ventricular remodeling in rats with left ventricular dysfunction after an extensive MI. In a study by Fraccarollo and colleagues, monotherapy with either eplerenone or the ACE inhibitor trandolapril, starting 10 days after MI and continuing for 9 weeks, decreased left ventricle end-diastolic volume and pressure and plasma norepinephrine levels. It also attenuated the increase in collagen type I gene expression and collagen content in the non-infarcted left ventricular myocardium, compared with placebo. Combination eplerenone/trandolapril therapy further improved many of these markers, including left ventricle end-diastolic pressure and volume, and completely prevented increased collagen type I gene expression and collagen content in the non-infarcted left ventricle myocardium. On the other hand, combination treatment offered limited additional benefit beyond monotherapy with either eplerenone or enalapril (ACE inhibitor) in mice following MI. Further research is necessary to better determine the role of combination therapy in this setting.

MR antagonists also appear to be beneficial for the prevention of sudden cardiac death after MI. In addition to hypertrophy and fibrosis, aldosterone plays an important role in other pathophysiologic processes associated with cardiac arrhythmias and sudden cardiac death, such as changes in myocyte electrical properties, abnormal repolarization,
and ion channel abnormalities. Electrical remodeling of the myocardium precedes myocyte hypertrophy following experimental MI, occurring within the first week after infarction.\textsuperscript{247} In this setting, action potential duration is increased due to an increase in myocardial calcium current and a decrease in transient outward potassium current. These changes are important and consistent precursors to cardiac arrhythmias and sudden cardiac death.\textsuperscript{247} It has recently been shown in an experimental model that these early changes in electrical remodeling of the myocardium post-MI are prevented by mineralocorticoid receptor blockade.\textsuperscript{247}

\textit{Evidence from Clinical Studies-} The vast number of experimental studies mentioned support the clinical evidence of the beneficial effects of MR antagonists on post-MI cardiovascular pathophysiology.\textsuperscript{242} Results from the landmark RALES and EPHESUS studies provide convincing evidence that this is the case.\textsuperscript{175,176} In the RALES trial, 3400 patients with severe heart failure were randomized to spironolactone in addition to existing conventional therapy. These patients showed a 30\% reduction in mortality and a 35\% reduction in morbidity.\textsuperscript{175} To examine the beneficial effects of MR antagonists on cardiac remodeling, 261 patients from the RALES trials were randomized to spironolactone or placebo, and serum markers of cardiac fibrosis were assessed at baseline and at 6 months.\textsuperscript{248} Patients with high baseline serum levels of markers of cardiac fibrosis synthesis were significantly associated with poor outcome. Spironolactone therapy significantly decreased these markers of cardiac fibrosis, suggesting that the limitation of aldosterone-stimulated collagen synthesis may be one of the various extra-renal mechanisms contributing to the clinical benefit of spironolactone
in the RALES trial.\textsuperscript{248} Similarly in another study, Hayashi \textit{et al}.\textsuperscript{249} examined the effect of spironolactone on left ventricle remodeling in 134 patients with first anterior acute MI. The results of this study demonstrate that MR antagonist combined with ACE inhibitor can prevent post-infarct left ventricle remodeling better than ACE inhibitor alone, as shown by the suppression of a marker of collagen synthesis (PIIINP).\textsuperscript{249}

The benefits of another MR antagonist, potassium canrenoate, have also been demonstrated on post-MI cardiac remodeling.\textsuperscript{250,251} Modena and colleagues\textsuperscript{250} evaluated the effect of potassium canrenonate on serum levels of PIIINP, which they used as a measure of the collagen synthesis rate in patients treated with an ACE inhibitor after a recent MI. Results of this study demonstrate that the addition of MR antagonists to ACE inhibitor therapy further reduce post-infarction collagen synthesis and progressive left ventricular dilation.\textsuperscript{250} A larger, randomized, double-blind study that evaluated the effects of adding potassium canrenoate to ACE inhibitor therapy further supports the beneficial effects of aldosterone blockade on left ventricle remodeling and function following MI.\textsuperscript{251} The beneficial effects of MR antagonists on the progression of left ventricular remodeling are being currently investigated in a large population (463 mild heart failure patients) in a new clinical trial, Antiremodelling Effect of Aldosterone Receptors Blockade with Canrenone in Mild Chronic Heart Failure (AREA IN-CHF).\textsuperscript{252}

The EPHESUS trial to date is the only long-term clinical outcomes trial of MR antagonists in the setting of post-MI heart failure. The results of this trial showed that eplerenone treatment was associated with significant improvements in early and long-term mortality and morbidity in patients with MI complicated by left ventricle systolic
Aldosterone and Elastogenesis

Severa Bunda

Page 46

dysfunction and clinical heart failure. The ability of eplerenone to add benefit to treatment with conventional therapy (ACE inhibitors and β-blockers) suggests an important synergistic or additive effect of aldosterone blockade in the post-MI setting and provides important scientific evidence supporting the role of aldosterone in post-MI pathophysiology.

MR Activation and Cardiac Fibrosis

The overall mechanism(s) by which MR activation in the heart contributes to adverse cardiac effects, including cardiac fibrosis, is not fully understood and is currently under intensive investigation. Aldosterone has been shown to stimulate collagen synthesis in cardiac fibroblasts and MR antagonists have been shown to alleviate the maladaptive remodeling of the cardiac matrix. Thus it has been suggested that within the myocardium, aldosterone acts through the MR to enhance collagen deposition.

Weber and colleagues were the first to firmly establish a direct correlation between aldosterone/salt exposure and the development of cardiac fibrosis and left ventricle remodeling. Subsequent study demonstrated that the myocardial fibrosis observed in response to chronic mineralocorticoid elevation and salt loading is a humorally mediated event independent of hypokalemia, hypertension, and cardiac hypertrophy. Since then several reports have confirmed similar findings that aldosterone promotes cardiac fibrosis independently of blood pressure and that MR antagonists oppose this effect. Aldosterone has also been shown to increase collagen I synthesis in cardiac fibroblasts through MR activation. Whether aldosterone (via MR activation) acts directly on collagen gene expression to induce
cardiac fibrosis or indirectly by involving other signaling components is not fully understood.

Aldosterone has been shown to stimulate the expression of several pro-fibrotic molecules that may contribute to the pathogenesis of cardiac remodeling. For example, aldosterone increases the activity of TGF-β in cultured cardiac myocytes. Aldosterone/salt treatment increases and MR antagonism decreases myocardial TGF-β expression. TGF-β promotes fibrosis and tissue remodeling by increasing the synthesis of matrix proteins and decreasing the production of MMPs.

Increased expression of endothelin-1 may also contribute to aldosterone-mediated cardiac and vascular fibrosis. Endothelin-1 has been shown to be a direct gene target of aldosterone-MR activation. Therefore, aldosterone via MR-induced endothelin-1 expression may stimulate collagen synthesis by cardiac fibroblasts directly or, in part, through the effects of TGF-β.

It has also been suggested that aldosterone-induced cardiac fibrosis involves angiotensin II acting through AT₁ receptors. The density of AT₁ receptors has been shown to be upregulated in the heart and blood vessel walls of aldosterone-treated rats via MR activation. ACE expression is also increased with aldosterone/salt treatment, an effect blocked by both spironolactone and AT₁ receptor antagonists, which suggests local tissue regulation of this system. Although these AT₁ receptor effects have been suggested as mechanisms for potentiating angiotensin II-mediated tissue remodeling, Chander et al. have shown that adrenalectomy in angiotensin II/salt-treated rats completely blocks the fibrotic response. This evidence indicates that MR signaling plays a key role in this model.
Collagen accumulation in the myocardium reflects an imbalance between collagen synthesis and degradation. Aldosterone has been shown to increase the activity and expression of MMP-2 and MMP-9 via activation of the MR and PKC.\textsuperscript{270} Interestingly, the activity of MMP-1, a key enzyme of collagen degradation, in cultured cardiac fibroblasts was not influenced by aldosterone.\textsuperscript{253} Furthermore, in chronic heart failure and post-MI patients, the benefit of aldosterone receptor blockade was associated with decreased collagen synthesis (as shown by decreased serum levels of the collagen synthesis marker PIIINP) but not collagen degradation.\textsuperscript{259} Therefore, limitation of aldosterone-stimulated collagen synthesis is most probably a major mechanism explaining the clinical benefit of MR antagonists.\textsuperscript{259}

Studies of genetically modified mice provide conflicting data as to whether aldosterone or MR activation is the critical step in initiating fibrosis. Garnier et al. have shown that cardiac-specific over-expression of aldosterone synthase causes coronary endothelial dysfunction but not fibrosis.\textsuperscript{271} They suggest that the lack of cardiac fibrosis in transgenic mice, compared to wild mice, is likely due to the slightly higher aldosterone concentration (about 1.7 times higher) in the transgenic animals.\textsuperscript{271} The fact that human MR over-expression in cardiac myocytes leads to cardiac arrhythmias but not to cardiac fibrosis further demonstrates the importance of changes in the concentration of the ligand (aldosterone) for the MR rather than the expression of MR itself in the development of cardiac fibrosis.\textsuperscript{272} On the other hand, transgenic mice over-expressing 11β-HSD2 in cardiac myocytes developed cardiac hypertrophy, fibrosis, and heart failure on a normal-salt diet.\textsuperscript{273} Eplerenone treatment reversed these effects. This study confirmed that the effect of aldosterone on cardiac fibrosis is due to the activation of cardiac MR by
aldosterone and highlights the protective role of glucocorticoids in preventing the harmful actions of aldosterone.\textsuperscript{273}

The exact mechanism by which aldosterone induces cardiac fibrosis remains to be elucidated. However, results of both clinical studies and experimental models demonstrate that MR antagonists alleviate aldosterone-induced cardiac fibrosis. This is likely due to the direct effects of aldosterone in the heart, mediated via fibroblasts, the primary cell type responsible for ECM production in the heart.

**Insight into the Role of Elastin in Cardiac Remodeling**

The alteration in cardiac collagen content during cardiac remodeling has been well documented, but the possible role of other ECM components, including elastic fibers that provide resilience and elasticity, has not been adequately addressed. It has been found that treatment with selective serine elastase inhibitors following MI suppresses inflammatory infiltration and inhibits cardiac dilation. This likely results from inhibiting the proteolytic destruction of existing elastic fibers in the heart.\textsuperscript{274,275} Recently, more direct evidence for the degradation of elastic fibers in the left ventricle myocardium in patients with heart failure has been provided.\textsuperscript{276} Elastolytic cathepsin activity was significantly higher in these patients than in normal subjects and resulted in the degradation of interstitial elastic fibers. It also led, however, to an increase in the content of collagen fibers in left ventricle myocardium.\textsuperscript{276} An imbalance between collagen and elastin levels in the left ventricular myocardium of mice with pressure overload has also been demonstrated by Henderson \textit{et al.}\textsuperscript{118} who suggest that the replacement of lost elastin by collagen may promote fibrosis. Another study has shown that transplanting cells that
are over-expressing elastin gene fragments into a myocardial scar modifies the scar content, increases cardiac elasticity, and facilitates ventricular function. Together, these studies suggest that increasing elastogenesis in a post-infarct heart may act to counterbalance collagen fiber stiffness following myocardial injury and improve cardiac function.

The well-established role of aldosterone in altering cardiac collagen content during cardiac remodeling has been documented. The activation of the RA S has been shown to increase elastin content in the left ventricle following cardiac volume overload in rats. In addition, eplerenone treatment has been shown to normalize a decreased aortic elastin–collagen ratio in experimental MI in rats and to significantly reduce vascular collagen content and increase elastin content in resistance arteries of hypertensive patients compared with vessels of patients before treatment.

However, there is an absence in the literature of detailed studies on the effect of aldosterone and its antagonists on cardiac elastin.
RATIONALE

Compelling evidence indicates that MR antagonists alleviate aldosterone-mediated maladaptive remodeling of post-MI hearts by attenuating overzealous collagen production, but a possible influence of aldosterone and MR antagonists on the deposition of elastic fibers that provide resilience and elasticity has not been addressed. Our pilot in vivo study demonstrated that treatment of rats after experimental MI with MR antagonists led to a net increase of elastic fibers in post-MI scars.

HYPOTHESES

1. Aldosterone affects the deposition of both collagen and elastin via different signaling pathways.
2. Blocking MR-mediated signaling enhances aldosterone-induced elastogenesis that is propagated via other membrane-residing receptor(s).

EXPERIMENTAL MODEL AND GENERAL OBJECTIVES

We utilized normal human cardiac fibroblasts that are capable of elastic fiber production in vitro to explore whether the presence and absence of MR antagonists would affect the pro-elastogenic effect of aldosterone. We also explored the possible cellular mechanisms responsible for this effect.
MATERIALS AND METHODS

Materials

All chemical-grade reagents, aldosterone, spironolactone, doxycycline, RU 486 (mifepristone), proteinase inhibitors, agarose-linked protein A, pertussis toxin, VGVAPG, human angiotensin II, 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 (PDGFR) inhibitor AG 1295, transforming growth factor β receptor (TGF-βR) inhibitor SB 431542, PD 98059, PD123319, and aluminum chloride (AlCl₃) and sodium fluoride (NaF) were obtained from Sigma (St. Louis, MO). Wortmannin, PP2, SP600125, and Y-27632 were purchased from Calbiochem (San Diego, CA). Losartan was purchased from Cayman Chemicals Co. (Ann Arbor, MI). Cell permeable Rho inhibitor, exoenzyme C3 transferase, CT04, was purchased from Cytoskeleton, Inc. (Denver, CO). Iscove’s modified Dulbecco’s Medium (IMDM), fetal bovine serum (FBS), 0.2% trypsin-0.02% EDTA, and other cell culture products were acquired from GIBCO Life Technologies (Burlington, ON). Soluble elastin degradation product, κ-elastin, and polyclonal antibody to tropoelastin were purchased from Elastin Products (Owensville, MI). Polyclonal collagen type I antibody was purchased from Chemicon (Temecula, CA). Polyclonal antibodies against phosphorylated c-Src (Tyr-416), total c-Src, phosphorylated Akt (Ser437), total Akt, and monoclonal antibody against β-actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Monoclonal antibody against phosphotyrosine (PY99), polyclonal antibody against IGF-IR-β, rabbit and goat polyclonal antibodies against Ga13, rabbit polyclonal antibody against Ga12, normal
rabbit or goat agarose conjugated-IgGs, rabbit polyclonal and mouse monoclonal antibody against c-Src, as well as goat polyclonal antibody against SCAP2 (SKAP55-R) (C17 and N15) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). IGF-IR blocking monoclonal antibody was purchased from EMD Biosciences (San Diego, CA). Secondary antibody fluorescein-conjugated goat anti-rabbit (GAR-FITC) and fluorescein-conjugated rabbit anti-goat (RAG-FITC) 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). Pre-cast 4-12% tris-glycine gel was purchased from Invitrogen Canada Inc. (Burlington, ON). A DNeasy Tissue system for DNA assay, RNeasy Mini Kit for isolating total RNA, One-Step RT-PCR Kit, RNAiFect transfection reagent as well as elastin specific and non-silencing short interfering RNA (siRNA) oligonucleotide duplexes were purchased from Qiagen (Mississauga, ON). Two different pre-designed Gα13 siRNA oligonucleotide duplexes were purchased from Ambion, Inc. (Austin, TX), and a custom designed Gα13 siRNA oligonucleotide duplexes was synthesized by Dharmacon (Lafayette, CO). DeliverX plus siRNA transfection reagent kit including GAPDH specific siRNA and non-silencing (scrambled) siRNA oligonucleotide duplexes were purchased from Panomics, Inc. (Fremont, CA). BSA-conjugated aldosterone (aldo-BSA) was purchased from Fitzgerald Industries Intl (Concord, MA). As specified by the manufacturer, 25 aldosterone molecules are covalently linked to each BSA molecule through a carboxymethyl oxyme residue on the C3 of the hormone, forming a stable conjugate.281
Identification of Elastin in Rat Hearts Following MI and Eplerenone Treatment

van Geison (VG) stain was performed to determine the distribution and relative quantity of elastin and collagen within myocardial interstitial space of infarcted rat hearts.\textsuperscript{282} MI was created in male Sprague-Dawley rats by the ligation of the proximal left anterior descending (LAD) coronary artery, as previously published.\textsuperscript{283} The MI groups were randomized to oral eplerenone treatment or placebo consisting of the inactive vehicle thus creating 2 distinct groups; MI-Vehicle control and MI-eplerenone groups. Eplerenone or its carrier vehicle was administered orally embedded in the rat chow. The dietary food intake is very consistent from day to day per individual animal, and the equivalent dose of eplerenone was administered at 100 mg/kg/day starting within 24 hours of surgery. Drug treatment continued until day 35 following surgery at which time all animals were sacrificed for analysis. The University Health Network Animal Care Committee approved the study protocol. Separate groups of animals randomized in the same fashion had hearts harvested 35 days post-MI, snap frozen in OCT (Optimum Cutting Temperature) solution, and stored in liquid nitrogen at -80°C until time of analysis. Tissue samples were sectioned and sent to the histology department at the Hospital for Sick Children in Toronto for VG staining. Sections were viewed at 100x, 600x, and 1000x magnification (Leica).

Cultures of Human Cardiac Fibroblasts

In all experiments, unless otherwise specified, 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. Briefly, the left ventricle myocardium was isolated, minced and washed with PBS. Cell isolation was accomplished with 0.2% trypsin and 1.0 mg/mL type II collagenase in a 0.02% glucose PBS solution at 37°C. After dissection, cells were incubated with IMDM containing 1% antibiotics/antimycotics, and supplemented with 10% FBS on 100-mm culture dishes (Starstedt, Inc, Newton, NC) for 1 hour at 37°C to obtain cardiac fibroblasts. Confluent cultures were passaged by trypsinization and maintained in IMDM supplemented with 1% antibiotics/antimycotics, and 10% FBS. 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.

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 hours with or without 1-1000 nM of aldosterone. 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. The aldosterone receptor antagonist spironolactone, the glucocorticoid receptor antagonist RU 486, the AT1 receptor antagonists losartan, the AT2 receptor antagonists PD123319, and the following IGF-IR, EGFR, PDGFR, and TGF-βR inhibitors AG 1024, AG 1478, AG 1295, SB 431542 respectively, as well as the G-protein
inhibitor pertussis toxin,\textsuperscript{183} the PKC inhibitor staurosporine,\textsuperscript{184,299} MAPK kinase inhibitor PD98059,\textsuperscript{300,301} JNK inhibitor SP600125,\textsuperscript{302,303} PI3 kinase inhibitor wortmannin,\textsuperscript{304,305} e-Src tyrosine kinase inhibitor PP2,\textsuperscript{306} IGF-IR neutralizing antibody,\textsuperscript{307} and Rho-associated kinase (ROCK) inhibitor Y-27632,\textsuperscript{308,309} were added 1 hour prior to aldosterone treatment. Cell permeable Rho inhibitor (CTO4) was added 2 hours prior to aldosterone treatment, as specified by the manufacturer. Cells were also treated with aluminum fluoride solution (AlCl$_3$ and NaF) prepared immediately before use for 3 hours, as previously described.\textsuperscript{310} All 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).\textsuperscript{311}

**Immunostaining**

At the end of the 72-hour incubation period with the indicated treatment, confluent cultures were either fixed in 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 µg/ml of polyclonal antibody to tropoelastin or with 10 µg/ml of polyclonal antibody to collagen type I. All cultures were then incubated for an additional hour with fluorescein-conjugated goat anti-rabbit (GAR-FITC) or with rabbit anti-goat (RAG-FITC) 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 CCD camera (QImaging, Retiga EX) and a computer-generated video analysis system (Image-Pro Plus software, Media Cybernetics, Silver Springs, MD).

**Quantitative Assays of Tropoelastin and Insoluble Elastin**

Fetal human cardiac fibroblasts were grown to confluency in 35-mm culture dishes (100,000 cells/dish) in quadruplicate. Then 2 μCi of [3H]-valine/ml of fresh media were added to each dish along with or without 1-50 nM aldosterone in the presence and absence of spironolactone. Cultures were incubated for 72 hours, and the soluble and insoluble elastin were assessed separately in each dish. The cells were extensively washed with PBS, and the soluble proteins present in the intracellular compartments were extracted overnight at 4°C with 0.1 M acetic acid in the presence of proteinase inhibitors. After centrifugation, the supernatants were precleaned by 30 minutes incubation with 50 μl of 4% protein A-beaded agarose, and then 500 μl of the supernatant was incubated with 5 μg of polyclonal antibody to tropoelastin for 2 hours and subsequently with 50 μl of 4% protein A-beaded agarase 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. The remaining cultures containing cell remnants and deposited insoluble extracellular matrix were scraped and boiled in 500 μl 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 µl of 5.7 N HCl for 1 hour, and the aliquots were mixed in scintillation fluid and counted.\textsuperscript{312} Aliquots taken from each culture were also used for DNA determination, according to Rodems \textit{et al.}\textsuperscript{313} 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 CPM/1 µg DNA. In separate experiments the specified treatments in figure legends were added along with 2 µCi of [\textsuperscript{3}H]-valine/ml media to normal human skin fibroblasts grown to confluency 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 [\textsuperscript{3}H]-valine into the insoluble elastin was assessed as described above.

\textbf{One-Step RT-PCR Analysis}

Confluent fetal human cardiac fibroblast cultures were treated with or without the specified treatment in the figure legend for 24 hours, unless otherwise indicated. Total RNA was extracted using the RNeasy Mini Kit according to the manufacturer’s instructions, 1 µg 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 µl. The reverse transcription step was performed for elastin and GAPDH reactions at 50 °C for 30 minutes, followed by 15 minutes at 95 °C. The elastin PCR reaction (sense primer: 5'-GGTGCGGCTCCTCAGCCTGG-3', antisense primer: 5'-GGGCCTTGAGATACCCCAGTG-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 1 cycle at 72 °C final extension for 10 minutes. The collagen type I (pro-α1(I) chain) PCR reaction (sense primer: 5'-CCCACCAATCACCTGCACGTAAGA-3', antisense primer: 5'-TTCTTGGTCGCTGGTAGACTCTGA-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 1 cycle at 72 °C final extension for 10 minutes. The Ga13 PCR reaction (sense primer: 5'-CGTGATCAAAGGTATGAGGG-3', antisense primer: 5'-CAGATTCACCCAGTTGAAAAT-3'; designed to produce a 249 bp product) was performed under the following conditions: 25 cycles at 94 °C denaturation for 30 seconds, 60 °C annealing for 30 seconds, 72 °C extension for 1 minute, and 1 cycle at 72 °C final extension for 10 minutes. The GAPDH PCR reaction (sense primer: 5'-TCCACCACCCTGTTGCTGTAG-3', antisense primer: 5'-GACCACGTCCATGCCCCAGCAT-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 1 cycle at 72 °C final extension for 10 minutes. 5 μl samples of the elastin, collagen type I, Ga13, and GAPDH PCR products from each reaction were run on a 2% agarose gel and post-stained with ethidium bromide. The amount of elastin, collagen type I, and Ga13 mRNA was standardized relative to the amount of GAPDH mRNA.

**Western Blotting**

Confluent fetal human cardiac fibroblast cultures were exposed for different periods of time to 50 nM of aldosterone or to 1 μg/ml IGF-I for 10 minutes in the
presence or absence of indicated inhibitors, or were incubated with \( \alpha 13 \) or scrambled siRNA oligonucleotides, as specified in the figure legends. At the end of each experiment cells were lysed using an RIPA buffer [(in mM: 50 Tris · HCl, pH 7.4, 150 NaCl, 1 EDTA, 10 NaF, 1% Triton X-100, 0.1% SDS, 1% Na deoxycholate) containing a cocktail of antiproteases (20 µg/ml leupeptin, 10 µg/ml aprotinin, 0.1 mM PMSF, 1 mM DTT) and antiphosphates (200 M orthovanadate, 2 µg/ml pepstatin)], and 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-β-mercaptoethanol; and 0.05% bromophenol blue), and the mixture was boiled for 5 minutes. Protein lysates were resolved by pre-cast SDS-PAGE gel (4–12% gradient), transferred to nitrocellulose membranes, blocked for an hour and then immunoblotted with anti-phospho-c-Src (Tyr416) antibody, anti-phospho-Akt (Ser473) antibody, anti-\( \alpha 13 \) (goat) antibody, anti-SCAP2 antibody, or with buffer (TBS-T) at 4 °C overnight. All blots were then incubated with the appropriate HRP-conjugated secondary antibodies for an hour and examined using the enhanced chemiluminescence detection system. As indicated in the figure legends, blots were stripped and re-probed using specified antibodies. The degree of expression or phosphorylation of immunodetected signaling molecules was measured by densitometry.

**Immunoprecipitation**

To evaluate the level of IGF-IR-β 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 minutes with 100 ng/ml of IGF-I, as specified in the figure legends. Parallel cultures were incubated in serum-free conditions in the presence or
absence of 50 nM aldosterone and incubated with or without 10, 25, 50 ng/ml of IGF-I for 10 minutes. For co-immunoprecipitation experiments, confluent cultures were incubated for indicated time in the presence or absence of 50 nM of aldosterone. At the end of each experiment the cells were lysed as specified above and 300 µg of protein extract after 1 hour pre-clearing with normal rabbit or goat agarose conjugated-IgG at 4 °C were then incubated with rabbit polyclonal antibodies against IGF-IR-β, c-Src, Gα13, or with goat polyclonal antibody against SCAP2 (C17) \(^{314}\) for 1 hour at 4 °C, followed by the addition of 4% Protein A-beaded agarose left overnight, as previously described.\(^{59}\)

The resulting protein-antibody conjugate was centrifuged at 4 °C and washed four times with PBS. The final pellet was re-suspended in sample buffer and the proteins were resolved as specified above. Following immunoprecipitation of IGF-IR-β and SCAP2 the membranes were immunoblotted using monoclonal anti-p-Tyr antibody, stripped and re-probed using anti-IGF-IR-β or anti-SCAP2 antibody. Following immunoprecipitation of c-Src the membranes were immunoblotted using polyclonal goat antibodies against anti-Gα13 or anti-SCAP2, while those immunoprecipitated with anti-Gα13 were developed with monoclonal anti-c-Src antibody. As indicated in the figure legends, blots were stripped and re-probed for equal loading.

For all immunoprecipitation experiments negative controls rabbit or goat IgG were also immunoprecipitated that served 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 Elastin and Ga13 Expression using siRNA Specific Oligonucleotides

**Elastin Specific siRNA Oligonucleotides** - Two different siRNA oligonucleotide duplexes were synthesized (QIAGEN), corresponding to target sequences on human full-length elastin. The target sequences were as follows: for elastin 1, 5'-CCG GGA GTA GTT GGT GTC CCA-3'; for elastin 2, 5'-AAG CTT GTG GCC GGA AGA GAA-3'. A non-silencing control siRNA duplex sequence (QIAGEN) was used as a control for the transfections. The same fluorescently labeled siRNA duplex (QIAGEN) was used to monitor transfection efficiency.

**Transfection of Elastin siRNA Oligonucleotides** - Cardiac fibroblasts were seeded in 6-well plates, maintained in IMDM medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). 80-90% confluent cardiac fibroblast cultures were washed in PBS and 2 mL of fresh media was added. 5 µg of elastin siRNA or non-silencing siRNA were transfected into cells using 20 µL RNAiFect transfection reagent (QIAGEN) according to the manufacturer’s protocol. Elastin expression was monitored 24, 48 and 72 hours post-transfection by one-step RT-PCR analysis using elastin and GAPDH specific primers. The greatest knockdown of elastin was obtained with elastin siRNA 1 oligonucleotide, used in all siRNA experiments to silence elastin expression.

**Ga13 Specific siRNA Oligonucleotides** – Two different Silencer® pre-designed siRNA against human Ga13 (standard purity, siRNA ID # 119735 and 119733) were obtained from (Ambion). The custom design oligonucleotide duplex (Dharmacon) was synthesized corresponding to target sequences on human full-length Ga13. The custom design
oligonucleotide target sequence was as follows: 5'-GAA GAU CGA CUG ACC CAA UC-3', which was previously shown to completely eliminate Gα13 protein level in HeLa cells.\(^{315}\) A non-silencing control and GAPDH siRNA duplex sequences (Panomics) were used as controls for the transfections.

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

**Microarray Analysis using the Affymetrix GeneChips**

**RNA Isolation** - Total RNA was isolated from human cardiac fibroblasts that were treated or untreated with 50 nM of aldosterone for 1 hour by using the TRIzol reagent (GIBCO-BRL) following the manufacturer’s protocol. The quality of RNA was assessed by an Agilent 2100 Bioanalyzer (version A.02.01S1232, Agilent Technologies, Palo Alto, CA) and Only RNA with the OD ratio of 1.99-2.0 at 260/280 was used for microarray analysis.
Oligonucleotide Arrays (Hybridization and Staining) - Total of 2 hybridizations were performed on the Human U133Plus2.0 GeneChip Array with total RNA. Samples were prepared for hybridization according to standard Affymetrix instructions and performed at the genomic core center at the Hospital for Sick Children.\textsuperscript{316}

Affymetrix GeneChip Data Analysis - Data analysis was performed using GeneSpring 7.0 software (Agilent). To identify differentially expressed transcripts, genes were isolated based on fold changes in 1 hour aldosterone treated versus untreated control. Network map was generated using Pathway Architect software (Agilent).

Data Analysis

In all biochemical studies, quadruplicate samples in each experimental group were assayed in three separate experiments. Mean and standard deviations (SD) were calculated for each experimental group, and statistical analyses were carried out by t test, or by ANOVA, followed by Bonferroni's test comparing selected groups, as appropriate. \( P \) value of less than 0.05 was considered significant.
RESULTS

1- The Effect of Aldosterone and its Antagonist on Cardiac Matrix Remodeling Following Experimental MI in Rats

After an MI there is significant upregulation of the R A A S, which activates cardiac fibroblasts and smooth muscle cells and modulates ECM remodeling and the healing process. The increased activities of aldosterone in this process has been implicated in the formation of hypertrophic and stiff post-infarct scars and in the initiation of cardiac fibrosis.¹³⁰,³¹⁷

To further examine how elevated levels of aldosterone influence cardiac matrix remodeling after MI, histological sections of rat hearts that underwent experimental MI (created by coronary artery ligation) were treated 35-days post-infarction with the aldosterone receptor antagonist eplerenone (100 mg/kg/day) or with vehicle alone.

The deposition of elastin and collagen in the infarcted and remote non-infarcted regions of the left ventricle were assessed by the van Gieson (VG) method.²⁸² Analysis of VG-stained serial sections revealed that post-MI scars created in the hearts of control rats treated with vehicle alone were greater than those detected in the eplerenone-treated animals and contained abundant bundles of collagen fibers. These large scars contained only scarce and short elastic fibers. In contrast, the post-MI scars created in the eplerenone-treated animals contained dense networks of elastic fibers that alternated with collagen fibers. Also, in contrast to those of the vehicle-treated rats, the remote regions
of the left ventricles of the eplerenone-treated animals did not demonstrate disseminated fibrosis (Figure 1).

We conclude that at least part of the net beneficial effect of MR-antagonist(s) may be attributed to the newly deposited elastic fibers, which may attenuate maladaptive heart repair by replacing the stiff collagen fibers in the post-MI scar.

2- The Effect of Various Aldosterone Concentrations on the Production of Collagen type I and Elastin in Cultures of Cardiac Fibroblasts Isolated from Fetal and Adult Human Hearts

In order to elucidate the molecular and cellular mechanism responsible for the increased deposition of elastic fibers in response to MR antagonist treatment and to examine the role of aldosterone, we employed an in vitro model utilizing cultured cardiac fibroblasts that are mostly responsible for the production of cardiac ECM.\textsuperscript{318}

Since various important differences between rodent and human cardiac fibroblast cultures have been described,\textsuperscript{319,320} we utilized cardiac fibroblasts isolated from human fetal and adult hearts to make our studies more relevant to the human disease. In the series of initial experiments, we examined the net production of collagen and elastin in cultured cardiac fibroblasts treated with physiological and sub-physiological aldosterone concentrations (1-50 nM),\textsuperscript{116,185} designated as moderate aldosterone levels, and with concentrations far exceeding physiological levels (100 nM-1 µM),\textsuperscript{321} designated as high aldosterone levels, in the presence and absence of the aldosterone antagonist spironolactone.
2A- Moderate Aldosterone Levels Upregulate Collagen Type I Gene Expression and the Deposition of Collagen Fibers in an MR-dependent Manner

We first demonstrated that the treatment of cultured human fetal cardiac fibroblasts with 1-50 nM of 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 2).

Then we found that pretreatment of cardiac fibroblasts with the MR-antagonist spironolactone but not with the glucocorticoid receptor (GR) antagonist RU 486 (1 µM) abrogated aldosterone-induced increase in the steady-state level of collagen type I mRNA and subsequent deposition of collagen fibers (Figure 2).

These results strongly indicate that the stimulatory effect of aldosterone on collagen production is mediated via MR activation.

2B- Moderate Aldosterone Levels also Upregulate Elastin Gene Expression and the net Deposition of Elastic Fibers, but in an MR-independent Manner

Analysis of parallel cultures revealed that aldosterone also upregulated the effective expression of the elastin gene, as detected by heightened elastin mRNA levels, in a dose-dependent manner (Figure 3 A). 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 3 B, C and D).

Surprisingly, pretreatment of cardiac fibroblasts with spironolactone, which eliminated the aldosterone-induced increase in collagen type I production, failed to
prevent an aldosterone-induced increase in elastin mRNA expression and in the net content of metabolically labeled intracellular tropoelastin and insoluble elastin (Figure 3). These observations suggest that aldosterone likely 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 pre-incubated the cardiac fibroblasts with the GR antagonist RU 486 (1 µM) in the presence of 1-50 nM of aldosterone. Our results demonstrate that RU 486 had no effect on the aldosterone-induced increase in elastin mRNA levels (Figure 3 A).

To eliminate the possibility that the observed effects might be restricted to fetal cardiac fibroblasts, we additionally utilized 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 4).

2C- High Levels of Aldosterone Further Induce Collagen Type I and Elastin Gene Expression but also trigger a Parallel MR-dependent Mechanism Leading to Decreased net Deposition of Elastic Fibers

We next examined the effect of high aldosterone concentrations (100 nM and 1 µM) on collagen and elastin content. We first demonstrated that raising aldosterone far above “physiological” levels also leads to a significant increase in the levels of collagen type I mRNA and in the deposition of collagen fibers via MR activation (Figure 5). While cultures treated with high concentrations of aldosterone also exhibited a further (up to ~3-fold) increase in elastin mRNA levels and in the synthesis of metabolically labeled
tropoelastin (Figure 6 A and B), they did not show the subsequent increase in the net deposition of insoluble elastin and immunodetectable elastic fibers (Figure 6 C and D). In fact, cultures treated with 100 nM or 1 µM of aldosterone displayed a striking decrease in the net deposition of insoluble elastin compared to cultures treated with 50 nM of aldosterone (Figure 6 C). This indicates that higher doses of aldosterone may also trigger other parallel pathway(s) that lead to the degradation of newly produced elastin. Since the decrease in the net deposition of insoluble elastin in cultures treated with high aldosterone levels could be reversed with spironolactone, resulting in an approximately 3-fold increase in the net deposition of elastin, we speculate that an elastolytic pathway must be induced in an MR-dependent manner.

2D- High Doses of Aldosterone Promote Elastin Degradation via an MR-dependent Mechanism

It has been previously reported that the MR-dependent action of aldosterone leads to an increase in the production and activity of a few metalloproteinases (MMPs), thereby inducing ECM remodeling.

Since fluctuations in the net deposition of elastin may be due to an imbalance between its deposition and degradation rates, we decided to test whether an aldosterone-dependent increase in MMP activity may possibly explain the significant decrease in net elastin content in cultures treated with a high dose of aldosterone (1µM). Therefore, an additional group of aldosterone-treated cultures was also exposed to either of two potent broad spectrum MMP inhibitors, doxycycline and GM6001. Immunofluorescence microscopy and a quantitative assay of newly deposited (metabolically-labeled) insoluble
elastin revealed that cultures jointly treated with 1 µM of aldosterone and 10 µg/ml of doxycycline, or 10 µM of GM6001, displayed significantly higher net deposition of elastin than cultures treated with 1 µM of aldosterone alone (Figure 7). On the basis of this data we suggest that elevated levels of aldosterone that stimulate production of tropoelastin in an MR-independent manner at the same time trigger the MR-dependent signaling pathway leading to the production/activation of elastolytic MMPs.

3- Elastin and Elastin Degradation Products Modulate Collagen Type I Deposition in Cardiac Fibroblast Cultures

Our results demonstrate that high aldosterone concentrations increase the production of collagen and promote the degradation of elastin via MR activation. Since elastin degradation products have been shown to induce various cellular responses we speculate that soluble elastin degradation products, which were likely present in the cultures treated with 1µM of aldosterone, may also have influenced the production of collagen in cardiac fibroblast cultures.

In order to study the effect of elastin degradation products on collagen production, we utilized κ-elastin, a soluble elastin degradation product, and the repeat hexapeptide VGVAPG, which is present in tropoelastin and has been identified as the major ligand for high affinity binding to the cell-surface elastin receptor.

Immunofluorescence microscopy using anti-collagen type I antibody revealed that treatment with these compounds mimicking elastin degradation products leads to increased deposition of collagen type I fibers.
These observations may further explain the effect observed in untreated post-MI hearts, in which the activation of the RAAS and elevated aldosterone levels may stimulate the degradation of elastin, which, in turn, promotes collagen production.

Interestingly, additional experiments, in which we silenced elastin gene expression with siRNA, demonstrated that eliminating the production of elastin in cardiac fibroblast cultures also leads to a significant increase in the deposition of collagen type I fibers (Figure 8 B and C).

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

In the next series of our experiments we aimed to elucidate the mechanism by which aldosterone induces elastogenesis in an MR-independent manner. To explore this we first utilized membrane-impermeable BSA-conjugated aldosterone (forming a stable conjugate) to determine whether aldosterone would induce elastogenesis through the stimulation of cell surface receptors without internalization, as demonstrated in previous studies. Indeed, treatment of cardiac fibroblast cultures with 1, 10, or 50 nM of aldosterone conjugated to BSA produced the same effect on elastin mRNA levels and consequent elastin production as treatment with equimolar free aldosterone (Figure 9).

Since previously published reports examining the MR-independent action of aldosterone demonstrated the involvement of cell-surface residing components, 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 growth factor receptors, EGF (AG 1478), TGF-β (SB 431542), and PDGF-BB (AG 1295), did not affect the aldosterone-induced increase in elastin production. Also treatment with Gαi protein inhibitor, pertussis toxin, or staurosporine (to inhibit PKC activity shown to be involved in some of aldosterone-MR independent action) did not abrogate the aldosterone-induced increase in elastin production (see the following table).

Table 1: A summary of results assessing the effect of specified inhibitors on aldosterone-induced increases in elastin production, obtained by one-step RT-PCR analysis and immunofluorescence microscopy using anti-elastin antibody. The IGF-IR tyrosine kinase inhibitor (AG 1024) antagonizes elastin production.

<table>
<thead>
<tr>
<th>Aldosterone 50 nM</th>
<th>No Inhibitor (Aldosterone alone)</th>
<th>Pertussis toxin 0.5 μg/ml</th>
<th>Staurosporine 2 μM</th>
<th>EGF-R Inhibitor (AG1478) 10 μM</th>
<th>TGF-β Inhibitor (SB4315) 10 μM</th>
<th>PDGF-R Inhibitor (AG1296) 25 μM</th>
<th>IGF-R Inhibitor (AG1024) 5 μM</th>
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<tr>
<td>↑ Elastin Production (2-fold)</td>
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On the other hand, blocking tyrosine kinase activity of the IGF-IR with AG 1024 eliminated the stimulatory effect of aldosterone on elastin mRNA expression and insoluble elastin production (Figure 10 A, C, and E). Since AG 1024 specifically inhibits ligand-stimulated autophosphorylation of the IGF-IR, but not of the insulin receptor, we speculated that aldosterone engages IGF-IR signaling to stimulate elastogenesis. Indeed, blocking the IGF-IR with 1 μg/ml of IGF-IR neutralizing antibody prior to aldosterone treatment eliminated the elastogenetic effect (Figure 10 A, C, and E). Furthermore, we demonstrated that treating cardiac fibroblasts with 100 ng/ml of IGF-I led to an approximately 3-fold 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 µM of AG 1024 or with 1 µg/ml of IGF-IR neutralizing antibody (Figure 10 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 nM of aldosterone for 10, 15, 30, and 60 minutes. Our results showed that a 10-minute exposure to 50 nM of aldosterone led to a transient increase in tyrosine phosphorylation of the IGF-IR above basal level. Exposure for 10 minutes to 100 ng/ml of IGF-I produced a very similar effect (Figure 11 A and B).

However, while IGF-I induced phosphorylation of its IGF-IR, both in the presence and in the absence of FBS, 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, results of further experiments demonstrated that the addition of 50 nM of aldosterone to cultures treated with 10, 25, or 50 ng/ml of IGF-I induced higher levels of IGF-IR tyrosine phosphorylation than in their respective counterparts treated with the same doses of IGF-I alone (Figure 11 C).

4A- The PI3 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 went on to determine which downstream IGF-IR signaling pathway, the PI3 kinase/Akt, or the mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) pathway (Figure 12), propagates the
elastogenic signal. To examine this we utilized a PI3 kinase inhibitor, wortmannin, and an MAPK kinase (MEK) inhibitor, PD 98059, that acts by inhibiting the activation of MAPK. Results from one-step RT-PCR analysis, immunofluorescence microscopy, and metabolic labeling studies demonstrate that the addition of wortmannin to aldosterone and IGF-I treated cultures abolishes the increase in elastin production (Figure 13). Interestingly, blocking the activation of the MAPK pathway by PD 98059 treatment does not eliminate the elastogenic effect of aldosterone but leads to a further increase in the production of elastin (Figure 14). The use of another MAPK family member inhibitor SP600125, which inhibits the JNK signaling pathway, had no effect on aldosterone-induced increases in elastin deposition (Figure 14).

These results demonstrate that the IGF-IR-PI3 kinase pathway propagates the elastogenic signal and that inhibition of the MAPK pathway further enhances the net elastogenic effect observed after IGF-IR stimulation.

5- Aldosterone Facilitates IGF-IR Signaling via c-Src Activation

In the next part of our study we were interested in gaining further insight into the mechanism by which aldosterone facilitates IGF-IR activation and its downstream PI3 kinase signaling pathway.

We decided to test whether the transactivation of the IGF-IR by aldosterone would also involve the cytosolic tyrosine kinase c-Src through a mechanism similar to that involved in aldosterone-dependent transactivation of the EGFR 330,16 (Figure 15). Results of the first experiment demonstrate that in aldosterone treated cultures
pharmacological inhibition of c-Src with PP2 attenuates the increase in elastin mRNA levels and elastic fiber production (Figure 16).

Since phosphorylation of c-Src at tyrosine 416 (Tyr416) in the activation loop of the kinase domain upregulates the enzymatic activity of c-Src, we further examined whether aldosterone treatment would increase c-Src phosphorylation at Tyr416. Indeed, Western blot analysis using anti-phospho-c-Src (Tyr416) specific antibody indicated that the cellular lysates treated with aldosterone displayed increased phosphorylation of c-Src as compared to the control, and that PP2 pretreatment abolished this effect (Figure 17 A). We also utilized an additional phospho-specific antibody that detects phosphorylated Akt, which is located downstream of PI3 kinase and, therefore, functions as part of a wortmannin-sensitive signaling mechanism. Western blot analysis using anti-phospho-Akt antibody revealed that aldosterone-increased basal phosphorylation of Akt may be eliminated by PP2 pretreatment (Figure 17 B).

Interestingly, cultures that were maintained in serum-free conditions and treated with aldosterone did not demonstrate increased basal Akt phosphorylation, while those treated with IGF-I showed a significant increase in Akt phosphorylation (Figure 18A). Furthermore, IGF-I treatment did not affect basal c-Src phosphorylation (Figure 18 A). Consistently, pretreatment with wortmannin, but not with PP2, eliminated the IGF-I-induced increase in Akt phosphorylation (Figure 18 B).

These data demonstrate that aldosterone facilitates IGF-IR-PI3 kinase/Akt signaling via c-Src activation.
6- AT$_1$ and AT$_2$ Receptors do not Participate in Aldosterone-dependent IGF-IR Activation

Our next series of experiments was designed to uncover the cell-surface residing component(s) involved in aldosterone-dependent IGF-IR facilitation and the consequent increase in elastin production (Figure 19). It has been shown that some MR-independent effects of aldosterone may be induced through the modulation of angiotensin II-dependent signaling. Since, angiotensin II-deepened signaling utilizes Src tyrosine kinase to transactivate the IGF-IR, we examined the possibility that aldosterone may also involve angiotensin II-dependent signaling to facilitate IGF-IR activation.

We first tested the effect of angiotensin II on elastin production. Our results demonstrate that treatment with 100 nM of angiotensin II leads to a significant increase in elastin mRNA levels and increased elastin production, as detected by metabolic labeling studies and immunofluorescence microscopy (Figure 20). Moreover, we demonstrated that the IGF-IR tyrosine kinase inhibitor, AG 1024, eliminates the angiotensin II-induced increase in elastin production. This suggests that IGF-IR signaling may also be involved in the mechanism propagating the pro-elastogenic effect of angiotensin II.

Since angiotensin II mediates its effect via the AT$_1$ and AT$_2$ receptors (Figure 21), we utilized their respective antagonists losartan and PD 123319 to determine which angiotensin II receptor subtype is responsible for increased elastin production following angiotensin II treatment. Results demonstrated that only the type I receptor antagonist losartan was able to eliminate the elastogenic effect of angiotensin. However, neither
receptor antagonist was able to eliminate the elastogenic effect of aldosterone (Figure 22 and 23).

These results indicate that, while angiotensin II also utilizes downstream IGF-IR signaling via the AT\textsubscript{1} receptor to propagate the elastogenic effect, aldosterone likely utilizes another cell-surface residing component (or components) to facilitate IGF-IR activation and subsequent elastin production.

7- Microarray Analysis Identifies Upregulated Genes Following 1 hour of Aldosterone Treatment

Since previously published data did not provide any further insight into the identity of other cell-surface residing components that might be involved in an aldosterone-induced increase in elastin production, we performed an explorative microarray assay in the hope of uncovering a candidate or candidates that might be involved in this process. Cardiac fibroblast cultures were maintained for 1 hour with or without aldosterone, and total RNA was extracted and submitted for microarray analysis by the Affymetrix method.

7A- Microarray Analysis: The Heterotrimeric G protein \( G \alpha \) Subunits, \( Ga13 \) and \( Gai \), Show Increased Expression Following 1 hour of Aldosterone Treatment

Microarray analysis identified 201 genes that displayed 1.2-fold or higher expression levels relative to untreated controls following 1 hour of aldosterone treatment. We focused on identifying putative upregulated genes that may be involved in the acute
response of aldosterone and its downstream IGF-IR signaling. We identified 19 upregulated genes on the basis of their potential involvement in this process, which can be broken down into the following clusters: heterotrimeric G proteins and their regulators, small GTPases, components of signal transduction pathways, growth factor receptor activators, protein phosphatases, extracellular matrix components, and transcription factors (see the following table).

<table>
<thead>
<tr>
<th>Table 2. Results of microarray analysis depicting transcription of key genes that were upregulated following 1 hour of aldosterone treatment</th>
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<tr>
<td>Fold Increase Relative to Ctr</td>
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<tr>
<td><strong>Heterotrimeric G proteins and their Regulators:</strong></td>
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<td><strong>Small GTPases:</strong></td>
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<td><strong>Components of Signal Transduction Pathways:</strong></td>
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<td><strong>Growth Factor Receptor Activators:</strong></td>
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<td><strong>Protein Phosphatases:</strong></td>
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<td><strong>Transcription Factors:</strong></td>
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G proteins mediate GPCR signaling through the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the $\alpha$ subunit (Figure 24), which can then activate different signaling cascades (or second messenger pathways) and effector proteins.

Our microarray-derived findings identified two genes, $\alpha_{13}$ and $\alpha_i$, that code for the $\alpha$ subunit of the heterotrimeric G proteins. This observation is consistent with previous reports indicating that some aldosterone MR-independent effects can be mediated by G protein signaling, specifically through $\alpha_i$ protein activation.\textsuperscript{183,328} Although our microarray analysis showed an approximately 1.9-fold increase in the expression level of $\alpha_i$ in aldosterone treated cultures, we also determined that pretreatment with pertussis toxin did not attenuate the pro-elastogenic effect of aldosterone (Figure 25), and we thus eliminated the putative involvement of $\alpha_i$ proteins in this process.

We therefore investigated whether another member of the G protein family, $\alpha_{13}$, might be involved in aldosterone-dependent signaling. $\alpha_{13}$, although previously not suspected to be involved in aldosterone-dependent effects, displayed a significantly higher expression level (2.7-fold increase) in fibroblasts exposed to a 1 hour treatment with aldosterone than did $\alpha_i$ (1.9-fold increase). Moreover, $\alpha_{13}$ along with phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA) (2.4-fold increase) has been shown by protein network mapping to be involved in activating the PI3 kinase pathway (Figure 26). This indicated the possibility of cross-talk between GPCR-$\alpha_{13}$ and IGF-IR-PI3 kinase signaling.
Therefore, we first validated our microarray findings by one-step RT-PCR analysis using Gα13 specific primers, which demonstrated that cultures treated for 1 hour with aldosterone displayed significantly increased Gα13 mRNA levels, in comparison with untreated cultures (Figure 27 A). Furthermore, we showed by Western blot analysis using anti-Gα13 specific antibody that the Gα13 protein level also increased following aldosterone treatment (Figure 27 B).

The following experiments were designed to further evaluate the putative involvement of Gα13 in the effect of aldosterone on elastogenesis.

8- The Gα13 Effector Rho/ROCK Pathway is not Involved in the Aldosterone-Dependent Increase in Elastin Production

There are no known inhibitors of Gα13, and the involvement of Gα13 signaling is usually demonstrated by the presence of constitutive active Gα13 (GTPase defective mutant) or by the presence of Gα13 inhibitory polypeptide derived from the regulator of G-protein signaling domain p115-Rho guanine nucleotide exchange factor (RhoGEF). Therefore, it is difficult to determine if aldosterone activates Gα13 in our culture system using pharmacological inhibition. However, we can gain insight into the possible involvement of Gα13 signaling by evaluating the effect of one of its downstream effector molecules, Rho.

It is well established that Gα13 regulates the activity of small GTPase Rho through RhoGEF, which in turn promotes GDP dissociation from the small GTPase Rho, allowing it to be activated by GTP (Figure 28).333 Gα13-dependent Rho activation has
been shown to modulate some, but not all of the cellular effects of Ga13. To examine the involvement of Rho and its downstream effector Rho-associated kinase (ROCK) in mediating the effect of aldosterone on elastogenesis, we utilized a cell permeable Rho inhibitor, exoenzyme C3 transferase, CT04, which ADP ribosylates and thus inactivates Rho, and a specific ROCK kinase inhibitor, Y-27632. Our results demonstrate that pretreatment with either CT04 or Y-27632 did not eliminate the increase in elastin mRNA expression and elastin production in aldosterone treated cultures (Figure 29). Therefore, these results eliminate the involvement of the Rho/ROCK pathway in aldosterone-dependent elastogenesis and suggest that Ga13 may mediate the effect of aldosterone through a different, Rho-independent pathway.

9- Aldosterone Increases the Interaction between Ga13 and c-Src

Recently, Ga13 has been shown to induce Akt phosphorylation via Src activation in platelets and in cardiac fibroblasts through a process that does not involve the Rho pathway. Ga13 has also been shown to interact with various proteins, including cytosolic tyrosine kinases such as proline-rich tyrosine kinase 2 (Pyk2). Since we showed that aldosterone-dependent facilitation of the IGF-IR is mediated by c-Src (Figure 16-18), we examined whether Ga13 and c-Src interact following aldosterone treatment.

To test this possibility, we immunoprecipitated c-Src from cellular lysates that were treated with or without aldosterone for 1, 5, and 10 minutes. Subsequent immunoblotting of this immunoprecipitation product with anti-Ga13 antibody revealed that Ga13 and c-Src do interact, and that the addition of aldosterone significantly
increases this association. The interaction is most evident after 5 minutes of aldosterone treatment (Figure 30 A). This observation suggests that aldosterone transiently increases the interaction between c-Src and Gα13.

Since it is not yet clear which specific GPCR agonists couple to Gα13, we next investigated whether the pharmacological activation of Gα13 would enforce its (transient) association with c-Src. In this experiment we tested a possible effect of aluminum fluoride, a nonspecific activator of Gα proteins, including Gα13. Our results demonstrated that aluminum fluoride treatment increased the interaction between c-Src and Gα13 (Figure 30 B left panel). Specifically, we found that c-Src immunoprecipitated from cellular lysates treated with aluminum fluoride consistently displayed greater interaction with Gα13 than untreated controls (Figure 30 B right panel). These results demonstrate that activated Gα13 interacts with c-Src.

10- **Silencing Gα13 in Cardiac Fibroblast Cultures Eliminates Aldosterone-Induced Elastogenesis**

In order to establish conclusively that Gα13 is a crucial factor in the initiation of the cellular signaling that leads to an aldosterone-induced increase in elastin production, we silenced Gα13 mRNA expression and protein levels in cardiac fibroblast cultures without affecting protein levels of Gα12 (Figure 31).

We found that the aldosterone-induced increase in elastin mRNA levels is attenuated in cultures in which Gα13 expression is silenced (Figure 32 A). Furthermore, we showed that in cultures which were transfected with Gα13 siRNA, aldosterone
treatment did not increase elastin deposition, whereas scrambled siRNA cultures demonstrated a significant increase in elastin production following aldosterone treatment (Figure 32 B and C). On the other hand, the IGF-I-induced increase in elastin mRNA steady-state levels and deposition into elastic fibers remained unaffected in Gα13 siRNA cultures (Figure 32). These results clearly demonstrate that Gα13 mediates the aldosterone-induced increase in elastogenesis.

11- The Possible Involvement of Src Family Associated Phosphoprotein (SCAP2) in Aldosterone-Mediated c-Src Signaling

Since our microarray experiment revealed that a 1-hour aldosterone treatment also induced an approximately 1.5-fold increase in the expression level of a recently identified adapter protein, Src family associated phosphoprotein (SCAP2), we decided to explore the interesting possibility that this factor may also be involved in aldosterone-dependent Src signaling.

The adapter protein SCAP2, also called SKAP55R, SKAP-HOM, RA70, and PRAP, possesses an amino terminal region predicted to form a coiled-coil structure, then a pleckstrin homology (PH) domain, followed by a central region containing potential tyrosine phosphorylation sites as well as a carboxy-terminal SH3 domain (Figure 33). Even though the exact function of SCAP2 is not known, it has been suggested that SCAP2 may play an essential role in the Src signaling pathway in various cells. SCAP2 binds to the SH2 domains of Src family tyrosine kinases via its tyrosine phosphorylation site.
We first validated our microarray findings by Western blot analysis using anti-SCAP2 antibody, which demonstrated that the SCAP2 protein level increased in lysates of cells that were treated with aldosterone (Figure 34 A). We then examined the possibility that aldosterone may influence the binding between SCAP2 and c-Src. By means of immunoprecipitation studies using anti-c-Src antibody from cell lysates that had been treated for 10 minutes with or without aldosterone following immunoblotting with anti-SCAP2 antibody, we determined that aldosterone treatment increases the interaction between SCAP2 and c-Src (Figure 34 B). Additional experiments demonstrated that the phosphorylation state of SCAP2 is also increased following 10 minutes of aldosterone treatment (Figure 34 C).

These results demonstrate that aldosterone influences the interaction between c-Src and SCAP2.
**Figure 1:** *Oral administration of eplerenone following experimental myocardial infarction (MI) in rats reduces myocardial collagenous fibrosis and increases production of elastin in post-infarct scars.*

Representative micrographs depicting histological sections of rat hearts that underwent experimental MI (created by the ligation of the proximal left anterior descending coronary artery) and were treated 35-days post-infarction with the aldosterone receptor antagonist eplerenone (100 mg/kg/day) or with vehicle alone. Histochemistry with the van Gieson method demonstrates that treatment with eplerenone (right side panels) induced a significant increase in the deposition of elastic fibers (black) and a decrease in the deposition of collagen fibers (red) in the infarct region (upper and middle panels) and also minimized collagenous fibrosis in the remote region of the left ventricle myocardium (upper and lower panels).

Sections were viewed at initial magnifications; 100x, 600x, and 1000x (top, middle, and bottom panels respectively).
Figure 2: The effect of moderate (1-50 nM) aldosterone levels, the MR antagonist spironolactone, and the GR antagonist RU 486 on collagen type I production in cultures of human fetal cardiac fibroblast.

(A) The results of one-step RT-PCR analysis used to assess collagen type I mRNA transcripts in cultures treated for 24 hours with or without 1-50 nM of aldosterone, or pretreated for 1 hour with 2µM of spironolactone, or 1µM of RU 486. The results, normalized to the corresponding levels of GAPDH mRNA transcripts, indicate that aldosterone treatment significantly increased collagen type I mRNA transcript levels, compared to values detected in untreated control cultures (*P<0.05). Pretreatment of cultures for 1 hour with spironolactone, prior to aldosterone treatment, inhibited the aldosterone-induced increase in collagen type I mRNA levels. Pretreatment with RU 486 had no effect on the aldosterone-induced increase in collagen type I mRNA transcript levels. Results are expressed as the mean ± SD, as derived from three separate experiments in which each experimental group had quadruplicate cultures.

(B) Representative photomicrographs of confluent cultures of human fetal cardiac fibroblast immunostained with anti-collagen type I antibody. Cultures were treated for 72 hours with or without 1-50 nM of aldosterone, with or without 1 hour pretreatment with 2µM of spironolactone or 1µM of RU 486. The results demonstrate that aldosterone treatment increased collagen type I deposition in cardiac fibroblast cultures, compared to untreated controls, and that pretreatment with spironolactone, but not with RU 486, abolished the aldosterone-induced increase in collagen type I deposition. Initial magnification 600x.
Aldosterone and Elastogenesis

A

Steady-state levels of Collagen Type I mRNA

Collagen Type I

GAPDH

![Graph showing steady-state levels of Collagen Type I mRNA](image)

Control, Aldo 1 nM, Aldo 10 nM, Aldo 50 nM, Spironolactone 2 μM, Aldo 1 nM, Aldo 10 nM, Aldo 50 nM, RU 486 1 μM, Aldo 1 nM, Aldo 10 nM, Aldo 50 nM

B

Immunostaining with anti-Collagen Type I Antibody

Control, Spironolactone 2 μM, RU 486 1 μM

![Immunostaining images](image)

Aldosterone 1 nM, 10 nM, 50 nM
**Figure 3:** The effect of moderate (1-50 nM) aldosterone levels, 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 1-50 nM of aldosterone or pretreated for 1 hour with 2µM of spironolactone or 1µM of RU 486. The results (quantified by densitometry and normalized to the corresponding levels of GAPDH mRNA transcripts) demonstrate that aldosterone dose-dependently increased elastin mRNA transcript levels, as compared to 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 1-50 nM of aldosterone synthesize up to ~2.5-times more [³H]-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 [³H]-valine demonstrate that cultures treated for 72 hours with 1-50 nM of aldosterone incorporated significantly more [³H]-valine into extracellular insoluble elastin than untreated cultures (*P<0.05). Pretreatment of cultures with spironolactone for an hour prior to 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). Initial magnification 600x.
**Figure 4:** 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) Results of a one-step RT-PCR analysis assessing elastin mRNA transcripts (normalized to the corresponding levels of GAPDH mRNA) in cultures treated for 24 hours in the presence or absence of 1-50 nM of aldosterone, and with or without 2 µM of spironolactone. The results indicate that treatment with 1-50 nM of aldosterone caused a dose-dependent increase in elastin mRNA transcript levels, compared to untreated control values (*P<0.05). Pretreatment of cells for 1 hour with spironolactone prior to concomitant treatment with aldosterone 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 cultures treated for 72 hours with 1-50 nM of aldosterone had more immunodetectable elastic fibers, compared to untreated controls, and that spironolactone pretreatment did not affect the aldosterone-induced increase in elastic fiber deposition. Initial magnification 600x.
Aldosterone and Elastogenesis

A

Steady-state levels of Elastin mRNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Elastin mRNA Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
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</tr>
<tr>
<td>Aldo 1 nM</td>
<td>1.5</td>
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<tr>
<td>Aldo 10 nM</td>
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<td>3.0</td>
</tr>
<tr>
<td>Spir 2 μM</td>
<td>3.5</td>
</tr>
</tbody>
</table>

B

Immunostaining with anti-Elastin Antibody

- control
- Aldosterone 1 nM
- Aldosterone 10 nM
- Aldosterone 50 nM
- Spironolactone 2 μM Aldosterone 1 nM
- Spironolactone 2 μM Aldosterone 10 nM
- Spironolactone 2 μM Aldosterone 50 nM
**Figure 5:** The effect of high (100 nM and 1 µM) aldosterone levels and the MR antagonist spironolactone on collagen type I production in cultures of human fetal cardiac fibroblast.

(A) Results of a one-step RT-PCR analysis assessing collagen type I mRNA transcripts in cultures treated for 24 hours with or without 100 nM or 1 µM of aldosterone or pretreated for 1 hour with 2µM of spironolactone. The results indicate that aldosterone treatment significantly increased collagen type I mRNA transcript levels, compared to untreated control values (*$P<0.05$). Cells pretreated with spironolactone prior to aldosterone treatment returned the aldosterone-induced increase in collagen type I mRNA levels to untreated values. (B) Representative photomicrographs depicting collagen type I in cultures treated for 72 hours with or without 100 nM or 1 µM of aldosterone, or pretreated for 1 hour with 2µM of spironolactone prior to aldosterone treatment. The results demonstrate that high aldosterone levels increased collagen type I deposition in cardiac fibroblast cultures, compared to untreated controls, and that spironolactone treatment abolished the aldosterone-induced increase in collagen type I deposition. Initial magnification 600x.
A  Steady-state levels of Collagen Type I mRNA

![Bar graph showing steady-state levels of Collagen Type I mRNA](image)

B  Immunostaining with anti-Collagen Type I Antibody

![Images showing immunostaining with anti-Collagen Type I Antibody](image)
**Figure 6:** The effect of high (100 nM and 1µM) aldosterone levels and the MR antagonist spironolactone on elastin production in cultures of human fetal cardiac fibroblasts.

(A) Results of a one-step RT-PCR analysis assessing elastin mRNA transcript levels (normalized to the corresponding levels of GAPDH mRNA transcripts) in cultures treated for 24 hours with or without 100 nM or 1µM of aldosterone or pretreated for 1 hour with 2 µM of spironolactone. The results demonstrate that high aldosterone levels increased elastin mRNA transcript levels, compared to untreated control values (*P<0.05), and that spironolactone failed to eliminate 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 100 nM or 1µM of aldosterone synthesize significantly more [3H]-valine-labeled tropoelastin than their 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 [3H]-valine of cultures treated for 72 hours with 50 nM, 100 nM, or 1 µM of aldosterone or pretreated for 1 hour with 2µM of spironolactone. The results demonstrate that the cultures treated with 50 nM of aldosterone incorporated significantly more [3H]-valine into insoluble elastin than the untreated cultures (*P<0.05), while cultures that were treated with higher doses of aldosterone (100 nM or 1 µM) demonstrated significantly lower levels of [3H]-valine-labeled insoluble elastin than those treated with, 50 nM of aldosterone (**P<0.05). In contrast, pretreating the parallel cultures with spironolactone for an hour prior to exposure to high doses of aldosterone (100 nM or 1 µM) significantly increased the net
deposition of insoluble elastin compared to those treated with high doses of aldosterone alone (***P<0.05). (D) Representative photomicrographs of confluent cultures immunostained with anti-elastin antibody confirmed results depicted in (C) and particularly demonstrated that the cultures treated together with 2 μM of spironolactone and higher doses of aldosterone (100 nM or 1 μM) deposited more elastic fibers than cultures treated with these doses of aldosterone alone. Initial magnification 600x.
**Figure 7:** High concentrations of aldosterone stimulate elastin degradation in cultures of human fetal cardiac fibroblasts.

(A) Results of a quantitative assay of $[^3]$H]-valine-labeled insoluble elastin and (B) representative photomicrographs of cultures immunostained with anti-elastin antibody indicate that the addition of 10 µg/ml of doxycycline or 10 µM of GM6001 to cardiac fibroblast cultures incubated for 72 hours with 100 nM or 1 µM of aldosterone significantly increased net elastin deposition as compared to untreated cultures.

*Statistically different from 1 µM aldosterone treated group (P<0.05).
Aldosterone and Elastogenesis

**A**

**Deposition of Insoluble Elastin**

Incorporation of $[^{3}H]$-Valine into Insoluble Elastin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CPM / μg DNA</th>
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<tr>
<td>Ctrl</td>
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</tr>
<tr>
<td>Aldo 1 μM</td>
<td>250 ± 15</td>
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<tr>
<td>Doxycycline 10 μg/ml</td>
<td>300 ± 20</td>
</tr>
<tr>
<td>GM6001 10 μM</td>
<td>350 ± 25</td>
</tr>
<tr>
<td>Aldo 10 μM</td>
<td>400 ± 30</td>
</tr>
<tr>
<td>Aldo 1 μM</td>
<td>450 ± 35</td>
</tr>
</tbody>
</table>

**B**

**Immunostaining with anti-Elastin Antibody**

- **control**
- **Doxycycline 10 μg/ml**
- **GM6001 10 μM**
- **Aldosterone 1 μM**
- **Doxycycline 10 μg/ml Aldosterone 1 μM**
- **GM6001 10 μM Aldosterone 1 μM**
**Figure 8:** *Elastin degradation products and experimental elimination of elastin production upregulate collagen type I deposition in human fetal cardiac fibroblast cultures.*

(A) Representative photomicrographs of confluent cultures immunostained with anti-elastin antibody or with anti-collagen type I antibody. Cultures were treated for 72 hours with 50 µg/ml of κ-elastin or with 1 µg/ml of VGVAPG peptide. The results demonstrate that the treatment with these soluble elastin degradation product mimetics increased deposition of elastin and collagen type I fibers, compared to untreated controls. (B) Results of a one-step RT-PCR analysis assessing elastin and GAPDH mRNA transcript levels 24, 48, and 72 hours after transfection with negative control, scrambled siRNA control, or elastin-specific siRNA oligonucleotides. The results demonstrate that, 72 hours after transfection with elastin siRNA oligonucleotides, elastin mRNA levels reduced to approximately 5 % of scrambled siRNA levels. *Statistically significant from scrambled siRNA control group (P<0.05). (C) Representative photomicrographs of confluent cultures immunostained with anti-elastin or anti-collagen type I antibody 72 hours after transfection with negative control, scrambled siRNA control, or elastin-specific siRNA oligonucleotides. The results demonstrate that silencing elastin expression in cardiac fibroblast cultures by elastin-specific siRNA oligonucleotides eliminated elastic fiber production and increased the deposition of type I collagen, compared to scrambled siRNA controls. Initial magnification 600x.
Figure 9: The influence of cell-impermeable aldosterone conjugated to BSA on elastin mRNA levels and deposition of elastic fibers in human fetal cardiac fibroblast cultures.

Results of quantitative assays demonstrate that 1-50 nM of aldosterone conjugated to BSA [aldo BSA] produced comparable stimulatory effects on (A) elastin mRNA levels and (B) net deposition of [3H]-valine-labeled insoluble elastin as treatment with 1-50 nM of free aldosterone. Cultures treated with an equimolar concentration of BSA, as aldo (50 nM)-BSA, served as an additional control. *Statistically different from control group ($P<0.05$).
**Figure 10:** *Insulin-like growth factor receptor-I (IGF-IR) tyrosine kinase inhibitor (AG 1024) and neutralizing antibody (α IGF-IR) antagonize aldosterone- and IGF-I-induced increases in elastin production in human fetal cardiac fibroblast cultures.*

Results of a one-step RT-PCR analysis assessing elastin and GAPDH mRNA transcripts in cultures treated for 24 hours with (A) 50 nM of aldosterone or with (B) 100 ng/ml of IGF-I prior to 1 hour pre-incubation with 5 µM of AG 1024 or with 1 µg/ml of anti-IGF-IR neutralizing antibody (α IGF-IR). The results show that inhibiting IGF-IR tyrosine kinase activity or immuno-blocking IGF-IR abolished aldosterone- and IGF-I-induced increases in elastin mRNA transcript levels.

Results of a quantitative assay of [³H]-valine-labeled insoluble elastin demonstrate that 1 hour pre-incubation with 5 µM of AG 1024, or with 1 µg/ml of α IGF-IR, abolished the stimulatory effects of 50 nM of aldosterone (C) and 100 ng/ml of IGF-I (D) on the net deposition of insoluble elastin. (E) Representative photomicrographs of confluent cultures immunostained with anti-elastin antibody confirm the results presented in (C). Initial magnification 600x. *Statistically different from control group (P<0.05).*
Aldosterone and Elastogenesis

Severa Bunda

Page 107

A

Steady-state levels of Elastin mRNA

B

Steady-state levels of Elastin mRNA

C

Deposition of Insoluble Elastin

D

Deposition of Insoluble Elastin

E

Immunostaining with anti-Elastin Antibody

control

AG 1024 5 μM

α IGF-IR 1 μg/ml

Aldosterone 50 nM

Aldosterone 50 nM

AG 1024 5 μM

α IGF-IR 1 μg/ml

Aldosterone 50 nM
**Figure 11:** *Aldosterone increases tyrosine phosphorylation of the IGF-IR in human fetal cardiac fibroblast cultures via facilitation.*

Cardiac fibroblast cultures were treated with 50 nM of aldosterone for 0, 10, 15, 30, and 60 minutes or 100 ng/ml of IGF-I for 10 minutes (A) in the presence of 10% FBS or (B) in the absence of serum or (C) in the absence of serum for 10 minutes in the presence or absence of 10, 25, or 50 ng/ml of IGF-I alone or with 50 nM of 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 ± 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 depicted in (A) and (B) demonstrate that a 10-minute aldosterone exposure in cultures maintained in 10% FBS led to a significant increase in tyrosine phosphorylation of IGF-IR over basal levels. The magnitude of this increase was similar to the effect observed after a 10-minute treatment with IGF-I. *Statistically different from control group (P<0.05).* Data in (C) demonstrate that cultures treated together with 50 nM of aldosterone and 10, 25, or 50 ng/ml of IGF-I exhibit significantly higher levels of IGF-IR tyrosine phosphorylation than their respective counterparts treated with the same doses of IGF-I alone.

*, **, and *** are statistically different from the group treated with 10, 25, and 50 ng/ml of IGF-I (P<0.05), respectively.
Figure 12: Overview of IGF-IR signaling pathways.

The IGF-I signaling pathways (modified from LeRoith, et al., 1995). The binding of IGF-I to the IGF-IR induces autophosphorylation of the receptor β-subunit at multiple tyrosine residues and activation of the receptors’ intrinsic tyrosine kinase. The predominant substrate for IGF-IR is the insulin-receptor substrate-1 (IRS-1). IRS-1 acts as a docking protein, which can bring together and thereby regulate the activity of many SH2 domain-containing proteins, such as the regulatory p85 subunit and catalytic p110 subunit of PI3 kinase and Grb2. Activation of IGF-IR also results in phosphorylation of SHC, which also forms a complex with Grb2, which is associated with the mammalian guanine nucleotide exchange factor Sos, which in turn, activates Ras. This leads to the activation of the Raf-1- MAPK-ERK-pathway and changes in the expression of early response genes. PI3 kinase signaling leads to the activation of Akt signaling. SH2 and SH3 domain-containing adaptor proteins of the CRK family are also substrates for the IGF-IR. When phosphorylated, they interact with Sos and thereby also activate the Ras-Raf-MAPK–pathway. The PI3 kinase and MAPK kinase inhibitors are depicted in red.
Figure 13: The PI3 kinase inhibitor wortmannin eliminates aldosterone- or IGF-I-induced increases in elastin production in human fetal cardiac fibroblast cultures.

(A) Results of a one-step RT-PCR analysis assessing elastin and GAPDH mRNA transcripts in cultures maintained for 24 hours in the presence or absence of 50 nM aldosterone or 100 ng/ml of IGF-I, prior to 1 hour pretreatment with 1 µM of wortmannin. The results demonstrate that wortmannin abolished aldosterone- and IGF-I-induced increases in elastin mRNA transcript levels. (B) Results of a quantitative assay of $[^3]$H-valine-labeled insoluble elastin demonstrate that treatment for 72 hours with 50 nM of aldosterone or with 100 ng/ml of IGF-I prior to 1 hour pretreatment with 1 µM of wortmannin returned insoluble elastin production to control values. (C) Representative photomicrographs of confluent cultures immunostained with anti-elastin antibody confirm the results presented in (B). Initial magnification 600x. *Statistically different from control group ($P<0.05$).
Aldosterone and Elastogenesis

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

<table>
<thead>
<tr>
<th></th>
<th>ctrl</th>
<th>Aldo 50 nM</th>
<th>IGF-I 100 ng/ml</th>
<th>wort 1 μM</th>
<th>Aldo 50 nM wort</th>
<th>IGF-I 100 ng/ml wort</th>
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<tr>
<td>Elastin</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

![Graph showing steady-state levels of Elastin mRNA](image)

**B** Deposition of Insoluble Elastin

<table>
<thead>
<tr>
<th></th>
<th>ctrl</th>
<th>Aldo 50 nM</th>
<th>IGF-I 100 ng/ml</th>
<th>wort 1 μM</th>
<th>Aldo 50 nM wort</th>
<th>IGF-I 100 ng/ml wort</th>
</tr>
</thead>
</table>
|Incorporation of Tril Valine into Elastin CPM/μg DNA | ![Graph showing deposition of insoluble elastin](image)

**C** Immunostaining with Anti-Elastin Antibody

- **Control**
- **Aldosterone 50 nM**
- **IGF-I 100 ng/ml**
- **Wortmanin 1 μM**
- **Aldosterone 50 nM Wortmanin 1 μM**
- **IGF-I 100 ng/ml Wortmanin 1 μM**

![Immunostaining images](image)
**Figure 14:** Inhibition of MAPK kinase (MEK) potentiates aldosterone-induced increases in the net deposition of insoluble elastin, while inhibition of JNK does not eliminate aldosterone-induced increases in elastin production in human fetal cardiac fibroblast cultures.

(A) Results of a quantitative assay of $[^3]$H-valine-labeled insoluble elastin demonstrate that cultures maintained in 10% FBS significantly increased insoluble elastin production when treated for 72 hours with 10 µM of PD 98059, compared to untreated control cultures (*$P<0.05$) and that the addition of 10 µM of PD 98059 prior to treatment with 50 nM of aldosterone further increased insoluble elastin production. In contrast, cultures treated with 10 µM of SP600125 prior to treatment with 50 nM of aldosterone had no effect on aldosterone-induced increase in deposition of insoluble elastin.

(B) Representative photomicrographs of confluent cultures immunostained with anti-elastin antibody confirm the results presented in (A). Initial magnification 600x.
A

Deposition of Insoluble Elastin

Incorporation of [3H]-valine into Insoluble Elastin

<table>
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<tr>
<th>Incorporation (CPM / 1 µg DNA)</th>
<th>ctr</th>
<th>Aldo 50 nM</th>
<th>PD 98059 10 µM</th>
<th>SP600125 10 µM</th>
<th>Aldo 50 nM</th>
<th>Aldo 50 nM</th>
</tr>
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<tr>
<td>trt</td>
<td>250</td>
<td>500</td>
<td>750</td>
<td>1000</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

B

Immunostaining with anti-Elastin Antibody

control | PD 98059 10 µM | SP600125 10 µM | Aldo 50 nM | Aldo 50 nM | PD 98059 10 µM | SP600125 10 µM
Figure 15: A diagram depicting the proposed mechanism by which aldosterone facilitates IGF-IR activation.

Aldosterone may engage the IGF-IR/PI3 kinase signaling cascade, which leads to increased elastin production via c-Src tyrosine kinases. PP2 and wortmannin, depicted in red, are Src kinase and PI3 kinase inhibitors respectively. The figure was modified from Krug, et al., 2002.
Figure 16: Src tyrosine kinase inhibitor PP2 eliminates aldosterone-induced increases in elastin production in human fetal cardiac fibroblast cultures.

(A) Results of a one-step RT-PCR analysis assessing elastin and GAPDH mRNA transcripts in cultures maintained for 24 hours in the presence or absence of 50 nM aldosterone, with or without 1 hour pre-treatment with 10 µM of PP2. The results demonstrate that the addition of PP2 abolished aldosterone-induced increase in elastin mRNA transcript levels. (B) Results of [³H]-valine-labeled insoluble elastin also demonstrate that PP2 treatment eliminated aldosterone-induced increase in the net deposition of insoluble elastin. (C) Representative photomicrographs of confluent cultures immunostained with anti-elastin antibody confirm the results presented in (B). Initial magnification 600x. *Statistically different from control group (P<0.05).
Aldosterone and Elastogenesis
Severa Bunda
Page 117

A  Steady-state levels of Elastin mRNA

B  Deposition of Insoluble Elastin

C  Immunostaining with anti-Elastin Antibody

control  Aldosterone 50 nM

PP2 10 μM  Aldosterone 50 nM

PP2 10 μM
Figure 17: Aldosterone increases basal phosphorylation of c-Src and Akt in human fetal cardiac fibroblast cultures.

Western blot analysis of cellular lysates obtained from human fetal cardiac fibroblast cultures treated for 10 minutes with or without 50 nM of aldosterone after they were preincubated for 1 hour in the presence or absence of 10 µM of PP2. (A) Samples of cell lysates were first immunoblotted with anti-phospho c-Src (Tyr416) and then stripped and re-probed with anti-c-Src antibody. (B) Samples of cell lysates were first immunoblotted with anti-phospho Akt (Ser473) antibody and then stripped and re-probed with anti-Akt antibody. Graphs depict the mean ± SD of data from three individual experiments, expressed as a percentage of control phosphorylation values obtained by normalizing to the corresponding total level of c-Src or Akt.

The results of Western blot analysis demonstrate that exposure of cultures to aldosterone for 10 minutes led to a significant increase in the basal phosphorylation of c-Src (A) and Akt (B) and that PP2 pretreatment eliminated this effect.
**Figure 18:** *In the absence of serum, aldosterone does not, while IGF-I does stimulate Akt phosphorylation in human fetal cardiac fibroblast cultures.*

(A) Representative Western blots of cellular lysates obtained from cardiac fibroblast cultures that were serum starved for 24 hours and maintained for 10 minutes in serum free conditions in the presence or absence 50 nM of aldosterone or 100 ng/ml of IGF-I, with or without 1 hour pretreatment with 10 µM of PP2. Immunoblotting with anti-phospho-Akt (Ser473) antibody demonstrates that in serum free conditions aldosterone did not induce Akt phosphorylation, whereas IGF-I significantly did. The addition of PP2 did not abolish the IGF-I-induced increase in basal phosphorylation of Akt. Consistently, IGF-I did not increase basal phosphorylation of c-Src. β-Actin served as an internal loading control.

(B) Representative Western blots of cellular lysates obtained from cardiac fibroblast cultures pretreated for 1 hour with or without 1 µM of wortmannin and then treated under the same culture conditions described in (A). Immunoblotting with anti-phospho-Akt antibody demonstrates that pretreatment with wortmannin eliminated the IGF-I-induced increase in Akt phosphorylation. The total level of Akt was also assessed by re-probing the stripped blot with anti-Akt specific antibody, which served as an internal loading control.

The results presented here are representative of results obtained from three individual experiments.
**Figure 19:** A diagram depicting the proposed mechanism by which aldosterone induces elastin production.

The figure depicts the putative cell-surface residing “receptor”(R) that aldosterone engages to activate c-Src and facilitate the IGF-IR-PI3 kinase signaling pathway, thus inducing increased elastin production.
Figure 20: Angiotensin II also increases elastin production via the IGF-IR signaling pathway in human fetal cardiac fibroblast cultures.

(A) Results of a one-step RT-PCR analysis assessing elastin and GAPDH mRNA transcripts in cultures maintained for 24 hours in the presence or absence of 100 nM angiotensin II, with or without 1 hour pre-incubation with 5 µM of AG 1024. The results show that inhibiting IGF-IR tyrosine kinase activity by AG 1024 pretreatment abolishes the angiotensin II-induced increase in elastin mRNA transcript levels. (B) Results of [3H]-valine-labeled insoluble elastin demonstrate that treatment for 72 hours with 100 nM of angiotensin II prior to 1 hour pre-incubation with 5 µM of AG 1024 returned insoluble elastin production to control values. (C) Representative photomicrographs of confluent cultures immunostained with anti-elastin antibody confirm the results presented in (B). Initial magnification 600x. *Statistically different from control group (P<0.05).
Aldosterone and Elastogenesis

Severa Bunda

Page 124

A

Steady-state levels of Elastin mRNA

Elastin

GAPDH

Elastin mRNA levels (arbitrary units)

- ctr
- Angiotensin 100 nM
- AG 1024
- Angiotensin AG 1024

B

Deposition of Insoluble Elastin

Incorporation of [H]-valine into Insoluble Elastin

CPM / 1 µg DNA

- ctr
- Angiotensin 100 nM
- AG 1024
- Angiotensin AG 1024

C

Immunostaining with anti-Elastin Antibody

control

Angiotensin 100 nM

AG 1024 5 µM

Angiotensin 100 nM

AG 1024 5 µM
**Figure 21**: *Angiotensin II type I (AT$_1$) and type II (AT$_2$) receptor subtypes.*

Angiotensin II stimulates the AT$_1$ and AT$_2$ receptor, resulting in vasoconstriction and sympathetic activation, among other effects, as shown in the figure. The respective receptor inhibitors are depicted in red. The figure was modified from https://secure.pharmacytimes.com/lessons/images/200412-01/20041201f1.gif.
Figure 22: The effect of AT$_1$ (losartan) and AT$_2$ (PD 123319) receptor inhibitors on Angiotensin II- or aldosterone-induced increases in elastin mRNA transcript levels in human fetal cardiac fibroblast cultures.

One-step RT-PCR analysis assessing elastin and GAPDH mRNA transcripts in cultures maintained for 24 hours in the presence or absence of 100 nM angiotensin II or 50 nM of aldosterone, with or without 1 hour pre-incubation with 10 µM of losartan or 10 µM of PD 123319. The results show that inhibiting the AT$_1$ receptor with losartan abolishes angiotensin II-induced increases in elastin mRNA transcript levels. Neither receptor inhibitor attenuated aldosterone-induced increases in elastin mRNA-transcript levels.

*Statistically different from control group ($P<0.05$).
Steady-state levels of Elastin mRNA

Elastin

GAPDH

![Graph showing Elastin mRNA levels with various treatments.](image)
**Figure 23:** The effect of $AT_1$ (losartan) and $AT_2$ (PD 123319) receptor inhibitors on angiotensin II- or aldosterone-induced increase in elastin production in human fetal cardiac fibroblast cultures.

(A) Results of a quantitative assay of $[^3H]$-valine-labeled insoluble elastin demonstrate that 1 hour pre-incubation with 10 µM of losartan, but not with 10 µM of PD 123319 returned insoluble elastin production to control values in cultures treated for 72 hours with 100 nM of angiotensin II. Neither receptor inhibitor was able to eliminate aldosterone-induced increases in insoluble elastin production.

(B) Representative photomicrographs of confluent cultures immunostained with anti-elastin antibody confirm the results presented in (A). Initial magnification 600x.

*Statistically different from control group ($P<0.05$).
### Deposition of Insoluble Elastin

![Graph showing deposition of insoluble elastin](image)

### Immunostaining with anti-Elastin Antibody

- **Control**
- **Losartan 10 µM** (Type I Receptor inhibitor)
- **PD123319 10 µM** (Type II Receptor inhibitor)
- **Angiotensin 100 nM**
- **Losartan Ang 100 nM**
- **Aldo 50 nM**
- **Losartan Aldo 50 nM**
- **PD123319 10 µM Aldo 50 nM**
Figure 24: Activation cycle of G-proteins by G-protein-coupled receptors (GPCR).

When a ligand activates GPCR, it induces a conformation change in the receptor (1) that allows the receptor to function as a guanine nucleotide exchange factor (GEF) that exchanges GTP for GDP on the Ga subunit (2-4). Ga-GTP and Gβγ can then activate different signaling cascades (or second messenger pathways) and effector proteins (5). The Ga subunit will eventually hydrolyze the attached GTP to GDP by its inherent enzymatic activity (6), allowing it to re-associate with Gβγ and start a new cycle (1). The 4 family of Ga subunits are depicted in the middle. Genes that were shown to be upregulated in the microarray study are indicated in blue.

The figure was modified from http://commons.wikimedia.org/wiki/Image:GPCR-Zyklus.png.
**Figure 25:** The *Gai inhibitor pertussis toxin does not attenuate aldosterone-induced increases in elastin production in human fetal cardiac fibroblast cultures.*

(A) One-step RT-PCR analysis assessing elastin and GAPDH mRNA transcripts in cultures treated for 24 hours with or without 50 nM of aldosterone prior to 1 hour pre-incubation with 0.5 mg/ml of pertussis toxin (PTX). The results show that PTX did not attenuate aldosterone-induced increase in elastin mRNA transcript levels. (B) Results of a quantitative assay of $[^{3}H]$-valine-labeled insoluble elastin demonstrate that 1 hour pretreatment of cultures with 0.5 mg/ml of PTX following 72 hours incubation with 50 nM of aldosterone did not inhibit the elastogenic effect of aldosterone. *Statistically different from control group* ($P<0.05$). (C) Representative photomicrographs of confluent cultures immunostained with anti-elastin antibody confirm the results presented in (B). Initial magnification 600x.
A

Steady-State Levels of Elastin mRNA

Elastin

GAPDH

Elastin mRNA levels (arbitrary units)

control Aldo 50 nM PTX 0.5 mg/ml Aldo 50 nM

B

Deposition of Insoluble Elastin

Incorporation of H3-lysine into Elastin

control Aldo 50 nM PTX 0.5 mg/ml PTX 0.5 mg/ml

C

Immunostaining with anti-Elastin Antibody

control

Aldosterone 50 nM

Pertussis Toxin (PTX) 0.5 μg/ml

PTX 0.5 μg/ml Aldosterone 50 nM
**Figure 26:** Network map showing common targets of those genes upregulated following 1 hour of aldosterone treatment leading to PI3 kinase activation.

The network map was constructed using Pathway Assist software. It shows the following common targets of three genes that were upregulated by 1 hour of aldosterone treatment: guanine nucleotide binding protein (G protein), alpha 13 (GNA13), fibroblast growth factor receptor substrate 2 (FRS2), and phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA). Upregulation of these genes leads to the activation of the phosphatidylinositol-3-kinase/PI3 kinase signaling pathway.
**Figure 27:** Aldosterone increases the production of Ga13 in human fetal cardiac fibroblast cultures.

(A) Results of a one-step RT-PCR analysis assessing Ga13 and GAPDH mRNA transcripts in cultures treated for 1 hour with or without 50 nM of aldosterone. Treatment with aldosterone for 1 hour led to approximate 2.5-fold increase in Ga13 mRNA transcript levels, compared to untreated controls (*P<0.05). (B) Western blot analysis of cellular lysates of cardiac fibroblast cultures incubated for 1.5 hour with or without 50 nM of aldosterone and then probed with anti-Ga13 antibody. For normalization purpose blots were stripped and re-probed with anti-β-actin antibody. The results show that 1.5 hour aldosterone treatment increased Ga13 protein levels up to approximately 2.3-fold, compared to untreated controls.
A

Steady-state levels of Gα13 mRNA

B

anti-Gα13

β-actin
**Figure 28:** *The Ga13-Rho/ROCK signaling pathway.*

**Figure 29:** The Rho-associated kinase (ROCK) pathway is not involved in aldosterone-induced elastogenesis in human fetal cardiac fibroblast cultures.

(A) Results of a one-step RT-PCR analysis assessing elastin and GAPDH mRNA transcripts in cultures treated for 24 hours with or without 50 nM of aldosterone prior to pre-incubation with 2 µg/ml of cell permeable Rho inhibitor, exoenzyme C3 transferase (CT04) or 10 µM of Y-27632. The results show that inhibiting Rho GTPase activity with CT04 or its downstream effector ROCK kinase activity with Y-27632 does not attenuate aldosterone-induced increases in elastin mRNA transcript levels. (B) Results of a quantitative assay of [³H]-valine-labeled insoluble elastin demonstrate that treatment of cultures for 72 hours with 50 nM of aldosterone prior to pre-incubation with 2 µg/ml of CT04 or 10 µM of Y-27632 did not eliminate the elastogenic effect of aldosterone. (C) Representative photomicrographs of confluent cultures immunostained with anti-elastin antibody confirm the results presented in (B). Initial magnification 600x.

*Statistically different from control group (P<0.05).
Aldosterone and Elastogenesis

Severa Bunda
Page 138

A
Steady-state levels of Elastin mRNA

Elastin

GAPDH

B
Deposition of Insoluble Elastin

Incorporation of [H]-Valine Into Insoluble Elastin

C
Immunostaining with anti-Elastin Antibody

control

CTO4 2 µg/ml

Y-27632 10 µM

Aldo 50 nM

CTO4 2 µg/ml

Aldo 50 nM

Y-27632 10 µM

Aldo 50 nM
**Figure 30:** Aldosterone treatment increases the interaction between Ga13 and c-Src in human fetal cardiac fibroblast cultures.

(A) Cardiac fibroblast cultures were treated with or without 50 nM of aldosterone for 1, 5, and 10 minutes. Cell lysates were immunoprecipitated (IP) with anti c-Src antibody, electrophoresed, and probed with an anti-Gα13 antibody or anti-c-Src antibody. Graphs depict the mean ± SD of data from three individual experiments, expressed as a percentage of control values obtained by normalizing to the corresponding total level of c-Src. The results demonstrate that aldosterone increased the basal interaction between Gα13 and c-Src. This response was most evident 5 minutes after aldosterone treatment.

(B) Cellular lysates obtained from cultures treated with or without aluminum fluoride for 30 minutes were immunoprecipitated (IP) with anti G-α13 antibody (left panel) or anti-c-Src antibody (right panel), electrophoresed, and Western blotted with an anti-c-Src antibody or anti-Gα13 antibody, respectively. Total levels of Gα13 and c-Src were also assessed after stripping and re-probing the blots with their respective antibodies. Graphs depict the mean ± SD of data from three individual experiments, expressed as a percentage of control values obtained by normalizing to the corresponding total level of Gα13 or c-Src. The results demonstrate that aluminum fluoride treatment led to greater interaction between Gα13 and c-Src.
Aldosterone and Elastogenesis

Severa Bunda
Page 140

A

IP anti-c-Src
Immunoblot anti-\(G_\alpha 13\)

B

IP anti-\(G_\alpha 13\)
IP anti-c-Src
Immunoblot anti-c-Src
Immunoblot anti-\(G_\alpha 13\)
**Figure 31:** Silencing Ga13 expression in human fetal cardiac fibroblast cultures.

(A) One-Step RT-PCR analysis assessing Ga13 and GAPDH mRNA transcript levels in a negative control culture, a scrambled siRNA control culture, Ga13 and GAPDH siRNA specific oligonucleotides cultures, 24 hours after transfection. The results of densitometric evaluation demonstrate that Ga13 siRNA reduced Ga13 mRNA levels to approximately 8% of scrambled siRNA control levels 24 hours after transfection (*P<0.05). The graphs depict the mean ± SD of data from three individual experiments of Ga13 mRNA levels normalized to the corresponding levels of GAPDH mRNA transcripts. GAPDH siRNA, which served as a positive control, completely eliminated GAPDH mRNA levels. (B) Representative Western blot of cellular lysates obtained from cultures that were transfected for either 48 hours or for 48 hours and then transfected again for an additional 72 hours (120 hours) with scrambled and Ga13 siRNA specific oligonucleotides, electrophoresed, and immuno-blotted with anti-Ga13 antibody. The blots were then stripped and re-probed with anti-Ga12 and anti-β-actin antibodies. Graph depicts densitometric evaluation of results obtained from three individual experiments. The mean ± SD of data are expressed as a percentage of scrambled control Ga13 protein levels. Ga13 protein levels decreased to approximately 18% of the scrambled control levels 48 hours after transfection (**P<0.05) and to approximately 10% of scrambled control levels 120 hours after transfection (**P<0.05).
**Figure 32:** Silencing Ga13 expression in cardiac fibroblast cultures attenuates the aldosterone-induced increase in elastin production in human fetal cardiac fibroblast cultures.

(A) Results of a one-step RT-PCR analysis assessing Ga13, elastin, and GAPDH mRNA transcript levels of cultures transfected for 72 hours with scrambled siRNA control and Ga13 siRNA specific oligonucleotides and treated for the last 24 hours with or without 50 nM of aldosterone or 100 ng/ml of IGF-I. The graphs depict the mean ± SD of data from three individual experiments of elastin mRNA levels normalized to the corresponding levels of GAPDH mRNA transcripts. The results demonstrate that the aldosterone-induced increase in elastin mRNA steady-state levels observed in scrambled siRNA control cultures is attenuated in cultures in which Ga13 mRNA expression is silenced. On the other hand, the IGF-I-induced increase in elastin mRNA levels remained unaffected in Ga13 mRNA-silenced cultures as compared to scrambled control cultures. (B) Results of a quantitative assay of newly deposited insoluble elastin metabolically labeled with [3H]-valine of cultures that were initially transfected for 48 hours with scrambled or Ga13 siRNA and then transfected again for an additional 72 hours and kept in the presence or absence of 50 nM of aldosterone or 100 ng/ml of IGF-I. The results demonstrate that the aldosterone-induced increase in insoluble elastin production observed in scrambled siRNA control cultures is attenuated in cultures in which Ga13 mRNA expression is silenced. While the IGF-I-induced increase in insoluble elastin production remained unaffected. (C) Representative photomicrographs of confluent cultures immunostained with anti-elastin antibody confirm the results presented in (B). Initial magnification 600x. *Statistically different from control group (P<0.05).
Aldosterone and Elastogenesis

Severa Bunda
Page 144

A

B

Deposition of Insoluble Elastin

C

Immunostaining with anti-Elastin Antibody

scrambled siRNA

ctr

Aldo 50 nM

IGF-I 100 ng/ml

Gα13 siRNA

ctr

Aldo 50 nM

IGF-I 100 ng/ml
**Figure 33:** Domain structures of SCAP2 and c-Src.

Schematic representation of SCAP2 and c-Src (modified from Parsons and Parsons, 2004). SCAP2 possesses an amino terminal region predicted to form a coiled-coil structure, then a pleckstrin homology (PH) domain, followed by a central region containing potential tyrosine phosphorylation sites, and finally a carboxy-terminal SH3 domain. c-Src exhibits domain organization, which includes a myristoylated N-terminal segment (Myr), followed by SH3, SH2, and tyrosine kinase domains and a short C-terminal tail.
**Figure 34:** The involvement of the adapter protein SCAP2 in aldosterone-dependent c-Src activation in human fetal cardiac fibroblast cultures.

(A) Representative Western blot analysis of cellular lysates obtained from cardiac fibroblast cultures treated for 1.5 hours with or without 50 nM of aldosterone. Samples were first immunoblotted using anti-SCAP2 antibody and then stripped and re-probed with anti-β-actin antibody. Graphs depict the mean ± SD of data from three individual experiments, expressed as a percentage of control SCAP2 protein levels obtained by normalizing to the corresponding levels of β-actin. The results demonstrate that treatment with aldosterone for 1.5 hours leads to an approximately 1.5-fold increase in SCAP2 protein levels compared to untreated cultures. (B) Representative Western blot analysis of cellular lysates obtained from cardiac fibroblast cultures treated with or without 50 nM of aldosterone for 10 minutes, immunoprecipitated (IP) with anti c-Src antibody, electrophoresed, and then immunoblotted with anti-SCAP2 antibody or stripped and re-probed with anti-c-Src antibody. Graphs depict the mean ± SD of data from three individual experiments, expressed as a percentage of control SCAP2 protein levels obtained by normalizing to the corresponding total level of c-Src. The results demonstrate that aldosterone treatment increased basal interaction between SCAP2 and c-Src. (C) Representative Western blot analysis of cellular lysates obtained from cardiac fibroblast cultures treated as specified in (B), immunoprecipitated using anti-SCAP2 antibody and then immunoblotted with anti-phosphorylated tyrosine (phospho-Tyr) antibody. Graphs depict the mean ± SD of data from three individual experiments, expressed as a percentage of control phosphorylation values obtained by normalizing to the corresponding total level of SCAP2. The results demonstrate that aldosterone
treatment increased SCAP2 phosphorylation as compared to untreated controls.

*Statistically different from control group ($P<0.05$).
Aldosterone and Elastogenesis

Severa Bunda

Page 148

A

anti-SCAP2

β-actin

150
200
% of control

ctr  Aldo 50 nM 1.5 hours

B

IP anti-c-Src

Immunoblot anti-SCAP2

anti-SCAP2

Total c-Src

150
% of control

ctr  Aldo 50 nM 10 min

C

IP anti-SCAP

Immunoblot anti-phospho-Tyr

anti-phospho-Tyr

Total SCAP

150
% of control

ctr  Aldo 50 nM 10 min
DISCUSSION

The renin-angiotensin-aldosterone system (R A A S) constitutes an important component of the compensatory mechanism leading to improvement of cardiac output during heart failure. The elevated pressure and increased extracellular fluid volume generated by the renin-angiotensin-aldosterone system increase the hemodynamic load on the heart and lead to compensatory cardiac remodeling. Following an MI, the formation of a post-infarct scar is important for the host’s survival; however, excessive collagen deposition and fibrosis in the remote regions of myocardium increase the possibility of heart failure. Thus, the prolonged activation of this initially beneficial system may be detrimental.

A growing body of evidence links aldosterone excess to the development and progression of several cardiovascular pathologies, including hypertension, congestive heart failure, chronic kidney disease, coronary artery disease, and stroke. Primary aldosteronism in humans is characterized by high plasma aldosterone and low renin production. Interestingly, such patients show more myocardial and vascular fibrosis than essential hypertensive patients. This suggests that aldosterone has direct effects on cardiac remodeling through the activation of the cardiac MR. It has also been proposed that an increase of plasma aldosterone may be a risk factor in cardiovascular diseases. For example, the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS) has shown a relationship between plasma aldosterone concentration and mortality in patients with congestive heart failure, although the underlying mechanism is not known. Beygui and colleagues have provided additional data linking aldosterone to
cardiovascular risk, finding that plasma aldosterone levels drawn soon after admission predict cardiovascular morbidity and mortality in patients presenting with acute ST-segment elevation MI. These observations are relevant to reports showing that elevation of aldosterone in infarcted human hearts and in animal hearts after experimental injury can be linked to pathological remodeling and the development of detrimental cardiac fibrosis. ¹²⁸,¹⁹¹,¹⁹³,²⁰⁶

Results of clinical trials in which the MR antagonists spironolactone and eplerenone exerted cardio-protective effects have shown that these effects are primarily connected to the mechanisms that prevent collaginous fibrosis.¹²⁸,¹⁷⁵,¹⁷⁶,²⁴⁸,²⁴⁹,²⁵⁷,³⁵⁶ 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 “non-genomic” effects,¹⁸¹,³⁵⁶-³⁵⁸ has been further confirmed by MR knockout studies.¹⁸¹

The detailed in vitro investigations describing the MR-independent action of aldosterone that are presented in this thesis were inspired by results obtained from a rat MI model. These observations demonstrated that in eplerenone-treated animals the post-MI collaginous scars were markedly reduced and replaced with tissue containing abundant elastic fibers (Figure 1). Thus, these results not only confirm previously published observations that the cardio-protective effect of selective aldosterone blockade following MI in rats may be attributed to the reduction of pathological fibrosis ¹²⁸ but also introduce an original discovery that the beneficial effect of MR-antagonist(s) can be additionally attributed to the newly deposited elastic fibers. We speculate that this effect
may attenuate maladaptive post-MI processes by forming a resilient scar, which in contrast to the stiff collagenous scar, facilitates cardiac muscle contractility.

Using an *in vitro* model of cultured human cardiac fibroblasts (which are mostly responsible for the production of cardiac ECM), we first demonstrated that aldosterone upregulates collagen type I mRNA steady-state levels and subsequent deposition of collagen fibers (Figure 2). These results are in agreement with some *in vitro* studies but conflict with other studies in which aldosterone failed to demonstrate a direct effect on collagen production. This disagreement may be related to cell- and species-specific differences or to variations in culture conditions. Nevertheless, we consistently demonstrated that in our *in vitro* experimental model an aldosterone-mediated increase in collagen production occurs via MR but not GR activation. This is consistent with previous studies using *in vivo* and *in vitro* models that reported lack of GR involvement in aldosterone-induced collagen production and further confirms the effect of aldosterone on collagen production via MR activation.

Most importantly, we demonstrated for the first time that aldosterone upregulates elastin mRNA expression (Figures 3 and 4). Another crucial finding presented in this thesis indicates that MR antagonists, which abolished the collagenogenic effect of aldosterone, did not eliminate the elastogenic effect of this hormone. In fact, pretreatment with spironolactone reinforced the aldosterone-induced increase in the net deposition of elastic fibers (Figures 3 and 4). This effect was most evident in the presence of elevated levels of this hormone (Figure 6). Thus, the results of our *in vitro* studies also suggest that the net effect of aldosterone on heart tissue remodeling may depend on the local (myocardial) levels of this hormone.
Results from our experiments in which cultured cardiac fibroblasts were exposed to high aldosterone concentrations (100 nM and 1 µM) additionally demonstrated that in this setting, likely mimicking the situation in a post-infarct heart, MR-dependent action prevailed, causing increased elastolysis and increased net deposition of collagen (Figures 5-7).

In addition to elevated levels of aldosterone, the expression level of MR and 11β-HSD2 are also enhanced in the remodeling rat heart post-MI and in diastolic heart failure via MR activation. Therefore, multiple lines of evidence indicate that the use of MR antagonists post-infarction may decrease MR-mediated responses by antagonizing the effect of aldosterone on cardiac collagen production and by decreasing the number of available MRs. This, in turn, likely promotes MR-independent responses, such as increased elastin production as demonstrated in this thesis.

The mechanisms underlying the profibrotic actions of aldosterone are only partially clear. Most previous studies of cardiac injury in which aldosterone infusion produces an extensive MR-mediated cardiac pathology (fibrosis) have been performed on animals with uninephrectomy, excess salt loading, or both. However, Yoshida et al. demonstrated that an excess of aldosterone, even under a normal salt diet, promotes cardiac remodeling via oxidative stress. 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 levels of intracellular sodium or ROS. Our results, however, provide the additional insight that elevated aldosterone levels induce elastolytic MMP activity and degradation of elastic fibers via MR activation (Figures 6 and 7). MMPs and
their natural inhibitors TIMPs are present in the myocardium, and the expression and activation of MMPs have been identified as key events in left ventricle remodeling. In heart failure patients, plasma levels of aldosterone are known to correlate positively with MMP-9 levels. In cardiac myocyte cultures aldosterone has been shown to induce MMP-9 and MMP-2 activity via MR activation. Eplerenone treatment in dogs with heart failure has been shown to reduce MMP-1, MMP-2, and MMP-9 expression, while expression of TIMP-1 and TIMP-2 remained unaffected in left ventricle myocardium. We can therefore speculate that increased cardiac MR activation promotes MMP activity by increasing the expression of MMPs but not of TIMPs, thus causing an imbalance of MMP activity regulation. Although we have not attempted to identify the particular metalloproteinase(s) responsible for the destruction of elastic fibers in our experimental system, MMP-2, MMP-9, and MMP-12 have been previously identified as elastolytic enzymes. Significantly, recent studies from Dr. Peter Liu’s group have demonstrated that post-MI eplerenone treatment in rats caused a significant decrease in the expression level of elastolytic MMP-12 in the infarcted region (unpublished data).

Furthermore, following the destruction of elastic fibers, soluble elastin-derived peptides (EDPs) are generated that elicit hyperproliferation and migration of numerous cell types and increase the activity of MMP’s and ECM deposition, including collagen type I deposition (Figure 8A). Degradation of the existing interstitial elastic fibers and an increased content of collagen fibers have also been reported in the left ventricle myocardium of humans and rats with heart failure but not in normal subjects.

Our results demonstrate that in the presence of high aldosterone concentrations the net content of elastic fibers detected in the cultures of cardiac fibroblasts is reduced,
despite the increased amount of elastin mRNA and tropoelastin produced (Figures 5-7). Since the amount of deposited collagen detected in those cultures was actually increased, our observations indicate that high doses of aldosterone induced selective elastolysis.

On the other hand, results of our experiments demonstrated that silencing elastin synthesis in cultured cardiac fibroblasts coincided with a significant increase in collagen Type I deposition (Figures 8 B and C). This indicates that elastin, apart from its major structural role as a resilient ECM component, may also modulate the remodeling process of the cardiac stroma. Since insoluble elastin, the major component of elastic fibers, is able to sequester numerous growth factors, we speculate that the local excess of growth factors in its absence may also contribute to increased collagen deposition in the post-MI hearts. Conversely, factors stimulating the net deposition of elastic fibers may downregulate elevated collagen deposition and thereby reduce post-infarct fibrosis. We therefore postulate that promoting elastin deposition and inhibiting its degradation in the post-infarct heart may provide alternative strategies for the prevention of adverse ventricular remodeling through excessive collagen deposition and subsequent development of congestive heart failure.

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, hormones, and ion channels through a nuclear receptor-independent mechanism. It has been reported that some of aldosterone’s non-genomic effects may be induced through the modulation of angiotensin II-, vasopressin-, and EGF-dependent signaling pathways. These effects may be rapid and
may not involve transcription or translation, or they may be longer lasting and involve
gene expression and protein production. For example, in cardiac myocytes, aldosterone
acutely induces Na\(^+\) uptake via Na\(^+\)/H\(^+\) exchanger-1 in the presence of elevated Na\(^+\)
concentrations via a non-genomic/MR-independent effect, and thus provides an early
protective effect against cellular fluid loss.\(^{378}\) Similarly, aldosterone induces rapid
positive inotropic effects in the heart that, instead of being abolished, are further
enhanced by MR antagonists, a response which, it has been suggested, facilitates the
preservation of cardiac function.\(^{184,197,379}\) On the other hand, Bohmer et al.\(^{380}\) using
*Schizosaccharomyces pombe* as a model (since this yeast does not contain nuclear steroid
receptors), demonstrated that aldosterone is also able to modulate the expression of genes
and the subsequent translation of proteins involved in osmotic regulation and structural
remodeling without the presence of nuclear steroid receptors, including MR.

The results of our study demonstrate, for the first time, that the addition of
aldosterone rapidly enhances the level of IGF-IR phosphorylation, but only in the
presence of serum or IGF-I, leading to the upregulation of elastin mRNA expression in
cardiac fibroblast cultures (Figures 10 and 11). This effect is similar to the effect of
aldosterone on rapid EGFR tyrosine phosphorylation, which requires the presence of
EGF.\(^{330}\) Our data are also consistent to some extent with recently published observations
showing that a hugely elevated level of aldosterone (1.5 µM) transactivates the IGF-IR in
renal epithelial cells by involving MR or GR.\(^{381}\) However, our results suggest that much
lower (physiological) levels of aldosterone facilitate IGF-IR activation in the heart
through an MR-independent pathway. The differences between these two studies could
be due to the different concentrations of aldosterone tested and to the fivefold higher
abundance of MR in renal epithelium (a classic target tissue for aldosterone) as compared to cardiac tissue.\textsuperscript{178} Meaningfully, the direct activation of the IGF-IR by other steroid hormones has been previously described, and it has also been shown that estrogen (17\textbeta-estradiol) induces an increase in tyrosine phosphorylation of the IGF-IR via an estrogen receptor-independent process.\textsuperscript{382}

IGF-I has been previously reported to increase elastin gene expression in aortic smooth muscle cells \textsuperscript{40,383,384} and to exert cardioprotective effects in humans, and in experimental heart failure, by improving cardiac remodeling and function.\textsuperscript{385-388} Moreover, cardiospecific IGF-I overexpression has been shown to attenuate dilated cardiomyopathy in a transgenic mouse model of heart failure \textsuperscript{389} and to reduce collagenous scar formation post-MI.\textsuperscript{390} Overexpression of IGF-I in hepatic stellate cells has also been shown to reduce collagenous fibrosis following acute liver injury.\textsuperscript{391} Interestingly, the benefits of ACE inhibitors in attenuating post-MI cardiac remodeling has been attributed in part to decreased expression of MR \textsuperscript{392} and to increased expression of the IGF-IR, thus sensitizing the myocardium to the positive effects of endogenous IGF-I.\textsuperscript{393}

On the other hand, patients with hypopituitarism, who have diminished growth hormone and IGF-I expression, have an increased risk of congestive heart failure.\textsuperscript{394} Furthermore, in clinical studies of patients with heart failure, IGF-I levels are low and correlate with the severity of ventricular systolic dysfunction.\textsuperscript{395,396} It also has been shown that MI in humans is associated with significant alteration in the IGF-I system, and serum IGF-I concentrations at the time of infarction can be used to predict later development of heart failure.\textsuperscript{386} In the early phase of an MI, IGF-I levels are markedly
decreased, which contributes to a poor outcome afterwards. While IGF-I has biological effects on myocyte survival and angiogenesis, we have focused our studies on its normal matrix modulating effects.

The results from our study demonstrated that in the presence of low IGF-I levels, aldosterone, via an MR-independent pathway, significantly increased IGF-IR activation (Figure 11 C). Thus, we suggest that the use of MR antagonists following MI switches the balance of the cellular response to aldosterone toward the “non-genomic/MR-independent” pathway, which enhances the action of low endogenous IGF-I levels to sufficiently induce IGF-IR signaling resulting in the efficient production of elastic fibers.

Results of further studies aimed at elucidating the detailed cellular mechanism(s) involved of this effect indicated that aldosterone-induces activation of IGF-IR selectively triggers PI3 kinase/Akt signaling, but not the MAPK/ERK signaling pathway, to propagate the elastogenic effect (Figures 13 and 14). A similar pro-elastogenic effect involving the PI3 kinase /Akt pathway has been reported in lung fibroblasts after exposure to TGF-β. Moreover, it has been previously proposed by Conn et al. that IGF-I may induce an increase in elastin gene expression in aortic smooth muscle cells via a mechanism of de-repression, involving the abrogation of a repressor that appears to be Sp3 binding to the retinoblastoma control element located within the elastin promoter. Furthermore, these investigators demonstrated that the retinoblastoma protein (Rb) plays a key role in IGF-I-dependent transcriptional regulation of the elastin gene. Since elastin synthesis is inversely related to cell proliferation, and a major burst of elastogenesis occurs upon confluence, the phosphorylation state of Rb
may establish a boundary between cell growth and matrix formation.\textsuperscript{384} Since Rb lies downstream of PI3 kinase/Akt/molecular target of rapamycin (mTOR) signaling pathway, \textsuperscript{398} we speculate that activation of this signaling may influence the binding between Rb and transcription factors such as Sp1 and/or Sp3 to increase elastin gene transcription.\textsuperscript{40,383,384}

Our results demonstrated consistently that inhibiting the promitogenic MAPK/ERK signaling pathway further enhanced the effect of aldosterone on elastin production (Figure 14). Indeed, studies have shown that the initiation of MAPK/ERK signaling downregulates tropoelastin mRNA levels in response to EGF receptor activation.\textsuperscript{399,400} Thus, the PI3 kinase/Akt signaling pathway mediates the pro-elastogenic signal, likely by altering the phosphorylation state of Rb, while the mitogenic MAPK/ERK pathway antagonizes this effect. On the other hand, results of a recent study have suggested that factors that downregulate collagen deposition may also suppress cardiac myofibroblast proliferation.\textsuperscript{401} The antifibrotic effects of mesenchymal stem cells have also been attributed to both decreased cardiac fibroblast proliferation and collagen production and to increased expression of antiproliferative genes including elastin.\textsuperscript{402} The promitogenic MAPK pathway has been shown consistently to mediate aldosterone-induced increases in collagen production.\textsuperscript{301,403,404}

It is also important to note that activation of PI3 kinase is thought to play a role in the rapid non-genomic actions of classic aldosterone action.\textsuperscript{132,161} In particular, PI3 kinase is an effector that is necessary for aldosterone, insulin, and IGF-I control of the ENaC.\textsuperscript{405-408} Both aldosterone and IGF-I stimulate PI3 kinase signaling in renal epithelial cells.\textsuperscript{406,409} In addition, cardiac-specific overexpression of activated PI3 kinase (p110\alpha)
in transgenic mice has been shown to result in baseline cardiac hypertrophy without
fibrosis,\textsuperscript{410} a response that demonstrates the physiological importance of this kinase and its
downstream target Akt\textsuperscript{411} in the heart. Our results suggest that the IGF-IR may be one of
the key mediators of the non-genomic/MR-independent action of aldosterone that is
responsible for PI3 kinase activation.

A subsequent set of our experiments, which aimed at gaining insight into the early
stages of the mechanism by which aldosterone eventually facilitates IGF-IR signaling,
produced important results. Through these results, we established that aldosterone-
dependent facilitation of IGF-IR/PI3 kinase/Akt signaling is preceded by the activation of
c-Src (Figures 16-18). The involvement of cytosolic tyrosine kinase c-Src has been
previously shown to modulate some aldosterone action\textsuperscript{225} and has been suggested as a
crucial step leading to the transactivation of the EGF receptor in response to aldosterone
treatment.\textsuperscript{56}

It has been shown that c-Src may not only phosphorylate the IGF-IR on the sites
of ligand-induced autophosphorylation but also significantly increase the phosphorylation
of the receptor on Tyr-1316.\textsuperscript{412} The phosphorylation on Tyr-1316 of the insulin receptor,
which is closely related to the IGF-IR, has been shown to play an inhibitory role in
mitogenic signaling.\textsuperscript{413} It has also been shown not to be involved in the transformation
ability of the IGF-IR and has been implicated as a potential PI3 kinase binding site.\textsuperscript{414,415}
This information suggests that aldosterone-induced c-Src activation might facilitate IGF-
IR activity by enhancing the phosphorylation of Tyr-1316, which in turn promotes PI3
kinase signaling and elastogenesis. Further studies are needed to confirm this concept.
Since 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 9), we suggest that aldosterone may exert its MR-independent effect by interaction with certain cell surface-residing moieties. Specific high affinity membrane binding sites for aldosterone have been characterized in human mononuclear leukocytes and in pig kidneys and livers. A 50 kDa putative receptor has also been identified but has not been characterized. Furthermore, Haseroth and colleagues, using dermal fibroblasts from MR knockout mice, demonstrated that the effects of aldosterone on intracellular calcium and cAMP levels are not only present in MR knockout mice but are even greater than in wild-type mice cells. The results of this study not only suggested that the classic MR is dispensable for some of aldosterone’s actions but also indicated that the propagation of MR-induced signals may actually attenuate the MR-independent responses of aldosterone. Our results, which demonstrated that the addition of MR antagonists to cultured cardiac fibroblasts actually enhanced the response of elastin production to aldosterone (Figure 6 C), are consistent with these findings.

Although a specific receptor for aldosterone that mediates MR-independent action has yet to be characterized, GPCRs have been implicated in some non-genomic/steroid receptor-independent action. Recently a GPCR has been identified that elicits rapid responses to ecdysteroids in Drosophila. Like the non-classic ecdysteroid receptor, GPCRs have been shown to mediate some actions of estrogen and the progestins. In addition, a family of non-classic GPCRs, originally identified in fish, has been cloned. These bind progesterone with high affinity and are thought to participate in the
maturation of fish oocytes. In mammalian cells that overexpressed this receptor, progesterone induced ERK phosphorylation and attenuated cAMP production. A homologous family of three related GPCRs, termed mPRα, β, and γ, has also been found in humans. Another GPCR homologue, GPR 30, has been found to mediate estrogen action independently of the estrogen receptor. The response involves a pertussis toxin-sensitive pathway that requires Src-related tyrosine kinase activity and transactivation of the EGF receptor. This finding is consistent with previous reports indicating that some of aldosterone’s MR-independent effects can also be mediated through pertussis toxin-sensitive Ga protein activation. Our microarray analysis of cells exposed for 1 hour with aldosterone also demonstrated upregulation in Ga expression, but the results of further experiments excluded the activation of Ga in aldosterone-induced elastogenesis (Figure 25). Instead, we demonstrated for the first time that another Ga protein subunit, Ga13, which has been shown to mediate some non-genomic actions of estrogen, participates in mediating the cellular response of aldosterone that involves IGF-IR activation and consequent enhanced elastogenesis (Figures 30-32).

Previous studies aimed at elucidating the mechanism by which IGF-I upregulates elastin transcription demonstrated that this effect never occurs before 2-4 hours after exposure to IGF-I. It reaches a maximum level by 8 hours and remains elevated up to 24 hours. While our microarray analysis of 1 hour aldosterone-treated cultures consistently failed to reveal any increase in elastin expression, it revealed an increase in Ga13 expression (Table 2). Furthermore, we demonstrated that the rapid (or non-genomic) aldosterone effect involves the activation of c-Src and subsequent IGF-IR-PI3

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kinase signaling, which eventually increases expression of the elastin gene and enhances the net deposition of insoluble elastin. We thus speculate that aldosterone utilizes a positive feedback loop to upregulate Gα13, which is a crucial factor in the initiation, as well as in the maintenance of the delayed (2-4 hours) pro-elastogenic signal observed upon IGF-IR activation. Similar upregulation of the membrane progestin receptor (mPRα) has been demonstrated to occur in response to progesterone treatment.424,425

The G12 subfamily of heterotrimeric G proteins is comprised of only two members, Gα12 and Gα13.426 However, these α subunit proteins are expressed in virtually every tissue in the body,427 and in the 15 years since their discovery Gα12 and Gα13 have been implicated in a wide variety of cellular events and responses. The first identified and most extensively characterized downstream mediators of signaling through the G12 subfamily are members of the RhoA family of monomeric GTPases, which are activated via direct interaction with RhoGEFs and Gα12 or Gα13.426 Although primarily associated with the regulation of cytoskeletal rearrangements, Rho also conveys signals controlling gene transcription, cell cycle progression, and cytokinesis.428-430 Since then studies have shown that G12 transduces several mutual downstream biological responses via the activation of Rho.426 However, not all Gα12 or Gα13 signals proceed through Rho, and in addition to RhoGEF these Gα subunits also interact with various proteins that may serve as direct effectors or as regulators of G12 signaling.335,426,431

The results of our study exclude the involvement of Rho activation in aldosterone-induced elastogenesis (Figure 29). Many studies have suggested a role for tyrosine kinases in Gα12- and Gα13- mediated signal transduction,336,426,432-434 and in some
systems stimulation of tyrosine kinase activity by members of the G12 subfamily also promoted Rho-independent effects, including the activation of PI3 kinase signaling.\textsuperscript{433} Furthermore, Ga13 binds and stimulates Pyk2, a member of a different family of non-receptor tyrosine kinases.\textsuperscript{336} Although many details of these signaling pathways remain to be elucidated, these studies suggest that members of the G12 subfamily regulate tyrosine kinase activity through direct interaction with kinase proteins.\textsuperscript{426}

Previous studies have also shown that Ga13 stimulates the activation of cytosolic tyrosine kinase Src, which mediates some of the Ga13-mediated responses.\textsuperscript{303,334,435-439} Now, the results of our immunoprecipitation study demonstrate for the first time that activated Ga13 interacts with c-Src and that aldosterone treatment transiently increases this interaction (Figure 30). Recently Ga13 has been shown to induce Akt phosphorylation via Src activation in platelets\textsuperscript{334} and in cardiac fibroblasts\textsuperscript{303} via the Rho-independent pathway. This is also consistent with our results indicating that aldosterone engages Ga13 to stimulate phosphorylation of c-Src via the Rho-independent pathway and thereby facilitate IGF-IR-PI3 kinase/Akt signaling. Whether the interaction between Ga13 and c-Src is direct or requires interaction with other proteins is not clear yet.

Pyk2 links various GPCRs to the activation of receptor tyrosine kinases (such as the activation of the EGF receptor) via c-Src activation.\textsuperscript{440} Studies have shown that Ga13 can directly bind and activate Pyk2,\textsuperscript{336} which links Ga13 activation to PI3 kinase/Akt signaling.\textsuperscript{433} On the other hand, heterotrimeric Ga proteins such as Gai and Gas have also been shown to directly interact with the catalytic domain of c-Src and increase its
activation. Interestingly, activated Pyk2 and Src are not required for linking GPCR with the MAPK signaling cascade.

Since activated Pyk2 directly binds and activates c-Src through the binding of the Src homology 2 domain (SH2) of Src to tyrosine 402 (pY402) of Pyk2, we speculate that Ga13-mediated activation of Pyk2 may be needed for activation of c-Src, which in turn, stimulates IGF-IR-PI3 kinase/Akt signaling and results in the propagation of elastogenesis.

Furthermore, the results of our study demonstrated that elimination of Ga13 by siRNA technique in cultured cardiac fibroblasts completely attenuated the aldosterone-induced increase in elastin production (Figures 31 and 32). Interestingly, we also demonstrated that an increase in elastin production, stimulated by IGF-I, was unaffected in the absence of Ga13 protein (Figure 32). Since Ga12 protein levels in these cultures were unaffected, our result excluded the potential involvement of Ga12 in mediating the elastogenic response of aldosterone.

It has been also shown that physiologically important cell-surface receptors residing on cardiovascular cells, such as the AT1 receptor and endothelin-1 receptor, belong to the GPCR families. Interestingly, both of these receptors have also been shown to couple to Ga13. While, we eliminated the possibility that either AT1 or AT2 receptors may be involved in aldosterone-induced elastogenesis (Figures 22 and 23), we demonstrated that the treatment of cardiac fibroblasts with angiotensin II activates the AT1 receptor, which in turn also engages the IGF-IR-dependent pro-elastogenic pathway
(Figure 20). We assume that this action also involves c-Src tyrosine kinases, as previously demonstrated in smooth muscle cells.\textsuperscript{446}

Jointly, our results indicate that aldosterone activates certain GPCR that couples to $\alpha_{13}$, but not to $\alpha_{12}$, and mediates c-Src-IGF-IR-PI3 kinase downstream signaling. The fact that stimulation of GPCRs can activate signaling from receptor tyrosine kinases, including the IGF-IR, is well established.\textsuperscript{447,448} Although we have not yet identified the particular GPCR that couples to $\alpha_{13}$ and mediates the elastogenic response of aldosterone, we may speculate that it is an unidentified GPCR homologue as previously described to mediate the non-genomic action of estrogen and progestin.

In addition to Rho-dependent cytoskeletal cell shape changes, the activation of $\alpha_{13}$ has been implicated in other cellular processes, such as activation of JNK, stimulation of $\text{Na}^+/\text{H}^+$ exchange, and cell growth.\textsuperscript{426} Although the role of $\alpha_{13}$ in the heart is not clear, it has been shown that overexpression of the $\alpha_{13}$-subunit promotes cardiac hypertrophy\textsuperscript{449} and mediates the hypertrophic response of the $\alpha_1$-adrenergic receptor\textsuperscript{450} and the endothelin-1 receptor\textsuperscript{451} in rat neonatal myocytes. Interestingly, while $\alpha_{13}$ mediates a hypertrophic response in cardiac myocytes it mediates a different, anti-fibrotic response in cardiac fibroblasts.\textsuperscript{303} $\alpha_{13}$-mediated upregulation of canonical transient receptor potential 6 (TRPC6) channels that participate in the resultant activation of nuclear factor of activated T cells (NFAT) through endothelin-1 treatment has been shown to mediate the anti-fibrotic response in cardiac fibroblasts.\textsuperscript{303} Interestingly, this effect also involves Src tyrosine kinase and receptor tyrosine kinase, the EGF receptor.\textsuperscript{303}
Therefore, we suggest that \(G\alpha_{13}\) activation in cardiac remodeling may produce beneficial effects in two ways: (1) by decreasing collagen production via EGF receptor/TRPC6 and NFAT signaling, and (2) by the mechanism presented in this thesis, in which elastin production is increased via IGF-IR-PI3 kinase signaling triggered by aldosterone.

The results of our microarray analysis (Table 2) and Western blot analysis (Figure 34 A) of 1 hour aldosterone-treated cultures also demonstrated increased expression of the adapter protein SCAP2 that binds to the SH2 domains of Src family tyrosine kinases via its tyrosine phosphorylation site.\(^{341,342}\)

Adapter proteins are multifunctional signaling molecules capable of coupling engaged receptors (e.g., GPCRs) to intracellular signaling pathways and effector systems. They do not exhibit enzymatic activity but rather contain a variety of modular domains that mediate constitutive or inducible protein-protein or protein-lipid interactions after the engagement of signal-transducing receptors.\(^{452}\) These proteins represent integration points for different signaling pathways.\(^{314}\) Several cytosolic adapter proteins were implicated in the crosstalk between GPCRs (that utilize Src family kinases) and receptor tyrosine kinases\(^{453,454}\) and in the non-genomic actions of sex steroid hormones.\(^{375}\)

Although the physiological function of the adapter protein SCAP2 has yet to be fully elucidated, it is likely that it plays an essential role in the Src signaling pathway in various cells.\(^{340}\) SCAP2 consistently becomes tyrosine-phosphorylated following c-Src activation.\(^{342}\) In addition to binding to c-Src, SCAP2 (PRAP) has been found to bind to the proline-rich C-terminal domain of Pyk2 via its SH3 domain, and it has been
suggested that Pyk2 recruits c-Src to phosphorylate PRAP (SCAP2).\textsuperscript{342} It has been also suggested that SCAP2 plays a role in maintaining cellular quiescence.\textsuperscript{314,339}

The results of our study demonstrated that aldosterone increased the interaction between c-Src and SCAP2 and SCAP2 phosphorylation (Figures 34 B and C). Since Gα13 directly binds and activates Pyk2,\textsuperscript{336} we can speculate that the activation of Gα13 by aldosterone treatment leads to the activation of Pyk2 and subsequent c-Src-dependent phosphorylation of SCAP2. Although it is not clear why aldosterone treatment increases the association of SCAP2 with c-Src, it is intriguing to speculate that the activation of c-Src in non-proliferative confluent cells recruits this adapter protein to negatively regulate receptor tyrosine kinase-dependent proliferative MAPK signaling and in turn promote PI3 kinase/Akt signaling. Although further studies are required to elucidate the exact function of SCAP2, our results for the first time suggest that SCAP2 is involved in c-Src signaling induced by aldosterone treatment leading to increased elastin production.

In summary, data presented in this thesis uncovered a novel action of aldosterone that can be added to the list of other mechanisms in which this hormone modulates cardiac remodeling. We found that while the collagenogenic effect of aldosterone is mediated through classic MR activation, the elastogenic effect of aldosterone is propagated through the MR-independent action of this hormone. This novel mechanism likely involves certain GPCR(s) that couple to Gα13 to stimulate c-Src, which, in turn, facilitates the activation of tyrosine kinase-dependent phosphorylation of the IGF-IR and its downstream PI3 kinase signaling pathway. The identification of this GPCR(s) will be
the major aim of our future studies. This signaling pathway leads ultimately to the upregulation of the elastin gene, which translates to the efficient production of elastic fibers. Inhibition of MR also overrides the other mechanism, in which overactivation of MR by elevated aldosterone levels promotes elastolysis and destruction of elastic fibers. Therefore, the use of MR antagonists in post-infarct patients may not only attenuate MR-mediated maladaptive fibrosis but also promote the MR-independent action of aldosterone involving stimulation of the IGF-IR, even under low levels of endogenous IGF-I. Aldosterone modulates signaling pathways including IGF-I signaling by interaction with cell surface signaling molecules such as GPCRs.

Our findings endorse the use of these therapeutic strategies for the prevention and management of adverse ventricular remodeling and congestive heart failure.

The following diagram demonstrates aldosterone-dependent mechanisms we believe are operating on cardiac ECM production:
Parallel Mechanisms in which Aldosterone Modulates Cardiac ECM Remodeling

The interaction between aldosterone with its intracellular MR leads to increased synthesis of collagen type I and elastolytic MMPs.

In the parallel (MR-independent) mechanism aldosterone also stimulates elastin mRNA transcription and subsequent increase in elastin deposition.

The data presented in this thesis indicate that aldosterone interacts with a still unidentified GPCR and that this association causes activation of Gα13.

In turn, an activated Gα13 interacts with cytosolic c-Src. This interaction, likely endorsed by the adapter protein SCAP2, facilitates activation of IGF-IR-IRS/PI3 kinase/Akt signaling that occurs even in the presence of sub-physiological levels of IGF-I, which subsequently induces increased elastin transcription.

In response to moderate aldosterone levels elastin and collagen are deposited. However, as aldosterone levels rise there is a shift towards elastolysis and increased collagen production. Elastin degradation products (EDP) may further contribute to increased collagen deposition.
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