Cardiac fibroblasts (CFs) are the major non-contractile cells present in the myocardium. The primary responsibility of CFs is to regulate the synthesis and secretion of extracellular matrix (ECM) proteins. Excessive proliferation and differentiation of CFs during cardiovascular pathologies directly leads to the development of cardiac fibrosis, a condition characterized by a stiffening of the myocardium, which has significant effects on cardiac function. Resveratrol (RES), a component of red wine, has been identified as an agent having considerable cardioprotective properties. The first Specific Aim of my dissertation research was designed to investigate the effects of RES on CF function. I concluded that 25 μM RES significantly inhibited CF proliferation and myofibroblast differentiation, thus identifying RES as a potential anti-fibrotic agent.

A common characteristic of cardiovascular diseases is the activation of the renin-angiotensin system, resulting in increased circulating angiotensin II (ANG II). ANG II directly stimulates CF proliferation, myofibroblast differentiation, and de novo synthesis of collagens and other ECM proteins and therefore, an elevation in systemic ANG II is a major contributing factor in the progression of cardiac fibrosis. The intracellular signaling pathways mediating the effects ANG II signaling are most well characterized in vascular smooth muscle cells (VSMCs). Proliferation of CFs by ANG II is dependent on the ERK 1/2 signaling pathway, but the exact pathway from the ANG II type 1 receptor to ERK...
1/2 has not yet been elucidated in adult CFs. Through studies outlined in the second Aim, I determined that ANG II-induced ERK 1/2 activation is dependent on both intracellular calcium and protein kinase C (PKC). I also concluded that ANG II stimulation did not induce transactivation of the epidermal growth factor receptor, a finding which distinguishes ANG II signaling in adult CFs from that in neonatal CFs and VSMCs. In addition, I determined that ANG II did not signal to ERK 1/2 through a phosphatidylinositol 3-kinase (PI3-K) and atypical PKCζ, another distinguishing feature of ANG II signaling in adult CFs. I did, however, conclude that cellular proliferation was dependent on active PI3-K and PKCζ, and that prolonged PKCζ inhibition resulted in DNA fragmentation and cytoskeletal degradation, indicating that the cells were undergoing apoptosis.

The third Aim was designed to explore whether or not α-adrenergic receptor (α-AR) signaling had a role in myofibroblast differentiation. I first found that the expected effects of selective α1-AR stimulation with phenylephrine (PHE) were lacking in adult CFs, including inositol phosphate production and intracellular calcium release. Second, there was no increase in myofibroblast differentiation in response to PHE. Lastly, PHE appeared to increase ERK 1/2 activation, but this effect was not blocked by a specific antagonist of the α1-AR. Therefore, I concluded that α-ARs are not involved in myofibroblast differentiation.

The information gained from this project was that resveratrol has potential anti-fibrotic properties and that angiotensin II signaling pathways mediating proliferation of adult cardiac fibroblasts differ considerably from those identified in neonatal CFs and VSMCs.
SIGNALING MECHANISMS CONTROLLING THE PROLIFERATION AND DIFFERENTIATION OF CARDIAC FIBROBLASTS

A dissertation submitted to Kent State University in cooperation with Northeastern Ohio Universities College of Medicine in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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DEDICATION

This dissertation is dedicated to my son Jacob, who has sacrificed so much without ever realizing it and who has helped me realize what truly matters in life.
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And last, but certainly not least, I am eternally thankful for my amazing wife Jennifer. She has been through every bit of this with me and her energy and enthusiasm has carried me through the highs and lows of the last five years. She always had confidence in me even when I wasn’t sure about myself and her love and support is unending. I am truly lucky to have her by my side and am excited about beginning the next big step in our lives. Thank you for being an incredible wife and a wonderful mother to Jacob and know that this degree is every bit as much yours as it is mine.
# TABLE OF CONTENTS

List of Figures ....................................................................................................................................... viii

List of abbreviations ................................................................................................................................. ix

Chapters

I. Introduction
   Role of cardiac fibroblasts .................................................................................................................. 1
   Activation of fibroblasts during normal and pathological wound healing .................................... 2
   G protein coupled receptor signaling ............................................................................................... 3
   Physiologic and pathologic functions of angiotensin II ................................................................. 7
   MAPK activation and downstream signaling ...................................................................................... 9
   Mitogenic signaling through ERK 1/2 .............................................................................................. 13
   Activation of ERK 1/2 by GPCRs ...................................................................................................... 16
   PI3-K activation and downstream signaling through PKCζ .......................................................... 20
   The role of PKCζ in mitogenesis, focal adhesion/cytoskeletal signaling and apoptosis ................. 27
   Transforming growth factor-β signaling and role in cardiac fibroblast function ......................... 30
   Signaling mechanisms of α-adrenergic receptors .......................................................................... 33
   The “French Paradox” and the biological functions of resveratrol .................................................... 34
   Specific aims and hypotheses ............................................................................................................ 39

II. Inhibitory effects of resveratrol on cardiac fibroblast proliferation and differentiation
   Introduction ........................................................................................................................................... 41
   Methods .............................................................................................................................................. 43
   Results ............................................................................................................................................... 48
   Discussion ........................................................................................................................................ 65

III. Mechanism of angiotensin II-induced proliferation and ERK 1/2 activation in adult rat cardiac fibroblasts
   Introduction ........................................................................................................................................ 70
   Methods ............................................................................................................................................ 73
   Results ............................................................................................................................................... 77
   Discussion ........................................................................................................................................ 96
IV. The role of PKCζ in modulating the cytoskeleton and attachment properties of cardiac fibroblasts
   Introduction .................................................................................................. 108
   Methods ........................................................................................................ 110
   Results .......................................................................................................... 113
   Discussion .................................................................................................... 121

V. The effects of α-adrenergic stimulation on cardiac myofibroblast differentiation
   Introduction .................................................................................................. 125
   Methods ........................................................................................................ 127
   Results .......................................................................................................... 129
   Discussion .................................................................................................... 132

VI. Overall conclusions and discussion ............................................................ 137
   Perspectives ................................................................................................. 149

VII. Bibliography ............................................................................................ 150
LIST OF FIGURES

1. Angiotensin II type 1 receptor-induced $G_q$ activation and classical downstream signaling events ................................................................. 5
2. Epidermal growth factor receptor-induced MAPK activation ...................... 11
3. AT$_1$R-induced ERK 1/2 activation ................................................................ 19
4. GPCR-induced PI3-K activation ................................................................... 22
5. Domains of different PKC isozymes ............................................................... 26
6. Integrin and focal adhesion complex-mediated signaling events ..................... 29
7. Structure and biological actions of resveratrol .................................................... 37
8. Pretreatment with resveratrol inhibits MEK 1/2 and ERK 1/2 activation in cardiac fibroblasts .................................................................................. 50
10. RES inhibits epidermal growth factor-induced ERK activation in CFs .......... 56
11. RES does not affect protein kinase B (Akt) or p70S6K phosphorylation in CFs .... 59
12. Expression of $\alpha$-smooth muscle actin increased as passage of cultured CFs increased (P2-P5) .............................................................................. 62
13. RES prevents ANG II- and TGF-β-induced cardiac myofibroblast differentiation . 64
14. Angiotensin II-induced cardiac fibroblast proliferation is not EGFR-dependent .... 79
15. ANG II activates ERK 1/2 via an EGFR-independent mechanism ....................... 82
16. c-Src does not play a role in ANG II-induced proliferation in cardiac fibroblasts .. 84
17. Inhibition of PI3-K blocks ANG II-induced [$^3$H]-thymidine incorporation but not ERK 1/2 activation ................................................................. 87

18. Inhibition of PKCζ blocks ANG II-induced [$^3$H]-thymidine incorporation but not ERK 1/2 activation ................................................................. 90

19. ANG II does not induce membrane translocation of PKCζ ............................................ 92

20. ERK 1/2 is activated by direct stimulation of classic and novel PKCs,
   but inhibition of these isozymes did not block ANG II-induced ERK 1/2 phosphorylation ........................................................................................................ 95

21. Chelation of intracellular calcium with BAPTA/AM does not prevent ANG II-induced ERK 1/2 activation ........................................................................................................ 98

22. ANG II-induced ERK 1/2 activation is inhibited by PMA-induced PKC
downregulation and intracellular calcium chelation simultaneously ..................... 100

23. Proposed model for ANG II-induced ERK 1/2 activation in adult cardiac fibroblasts ........................................................................................................ 106

24. CFs treated with ζ-PS display reduced cellular detachment and sensitivity to trypsinization ........................................................................................................ 115

25. CFs treated with ζ-PS for 48 hrs have reduced polymerized f-actin and focal adhesion kinase expression ................................................................. 117

26. Treatment with ζ-PS causes positive TUNEL staining in CFs, indicating that cells are apoptotic ................................................................. 120

27. Effects of subcutaneous ISO and EPI infusion on cardiac α-SMA expression .... 131
28. Selective stimulation of α-ARs with phenylephrine does not induce differentiation or ERK 1/2 activation in isolated CFs .............................................. 134

29. Proposed signaling mechanism of ANG II-induced ERK 1/2 activation in adult cardiac fibroblasts and summary of data ................................................. 148
LIST OF ABBREVIATIONS

AG; AG 1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline

α-AR; alpha-adrenergic receptor

α-SMA; alpha-smooth muscle actin

ANG II; angiotensin II

β-AR; beta-adrenergic receptor

BAPTA/AM; 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester

Calcium

CF; cardiac fibroblast

DAG; diacylglycerol

ECM; extracellular matrix

EGFR; epidermal growth factor receptor

EPI; epinephrine

FURA-2/AM; 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N'N'-tetraacetic acid penta(acetoxymethyl) ester

IP₃; inositol-1,4,5-trisphosphate

ISO; isoproterenol

LY; LY 294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one

PHE; phenylephrine

PI3-K; phosphatidylinositol 3-kinase

PIP₂; phosphatidylinositol-4,5-bisphosphate
PIP<sub>3</sub>; phosphatidylinositol-3,4,5-trisphosphate

PKC; protein kinase C

PLC; phospholipase C

PP2; 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine

RES; resveratrol, trans-3,4’,5-trihydroxystilbene

TGF-β; transforming growth factor-beta

TUNEL; terminal deoxynucleotidyl transferase mediated-dUTP nick-end labeling

ζ-PS; protein kinase C zeta pseudosubstrate inhibitor peptide
CHAPTER ONE

INTRODUCTION

Role of cardiac fibroblasts

Cardiac fibroblasts (CFs) are the major non-muscle cell type present in the myocardium, comprising approximately 20% of the myocardial mass and accounting for over half the total cell number in the heart. The primary function of these cells under normal physiological conditions is to regulate the balance of myocardial extracellular matrix (ECM) deposition and degradation (Camelliti et al. 2005). These cells also act as important mediators of paracrine activity due to their ability to secrete cytokines and growth factors (Lee et al. 1995; Katwa et al. 1997; Booz et al. 1999). CFs actively synthesize and secrete ECM proteins, predominantly type I and type III collagen which collectively comprise approximately 80-90% of the myocardial collagen content (Bashey et al. 1992; Pelouch et al. 1993). In addition, CFs secrete matrix metalloproteinases, enzymes with significant collagenase activity which break down the ECM (Soini et al. 2001). This ongoing process of cardiac remodeling is normally in a state of dynamic equilibrium, resulting in a zero net deposition of myocardial ECM. However, under pathologic cardiovascular conditions, significant changes to both the cellular and extracellular components of the myocardium occur. The precise changes to the myocardium are dependent on the specific pathology, e.g. myocardial infarction vs. hypertension. However, the systemic neurohumoral changes that occur during these pathologies are often conserved, and several of these circulating hormones have a profound impact on CF activity. The consequence of elevated CF activity during these
pathologies is cardiac fibrosis, a condition characterized by a significant increase in ECM deposition leading to a loss of cardiac function (Pearlman et al. 1982; Huysman et al. 1989; Silver et al. 1990; Beltrami et al. 1994). Cardiac fibrosis impairs the heart by limiting myocyte contraction and relaxation, predisposes the heart to ventricular arrhythmias by disrupting the electrical circuitry, and can ultimately result in heart failure.

**Activation of fibroblasts during normal and pathological wound healing**

The formation of a scar resulting from injury or trauma depends on the activation of fibroblasts. Ischemia caused by coronary artery blockage leads to cardiac myocyte death and tissue remodeling (Weisman et al. 1985; Swynghedauw 1999; Sun et al. 2000). Cardiac fibroblasts undergo several activation steps during the wound healing process. Following myocardial infarction, significant neurohumoral changes occur resulting in CF activation. As a result, CFs migrate to the sites of cardiac damage and proliferate. The dead myocytes are removed from the myocardium and the existing ECM is degraded. The next step in CF activation is differentiation to myofibroblasts, a specialized type of fibroblast with hypersecretory capacity and contractile properties due to the expression of $\alpha$-smooth muscle actin ($\alpha$-SMA) in highly organized stress fibers (Hirschel et al. 1971; Hinz et al. 2001; Tomasek et al. 2002). Differentiation is a critical step in the wound healing process because contraction of myofibroblasts is required for wound closure. The secretion of new ECM proteins stabilizes the closed wound and, under normal conditions, the myofibroblasts then undergo apoptosis, leaving behind an acellular scar with resident
fibroblasts maintaining the ECM. However, in some cases myofibroblast presence in the scar tissue can be detected for weeks (or even years) after repair of the wound, indicating that under certain conditions either these cells are resistant to apoptosis or they reappear following initial wound healing (Desmouliere et al. 1997). Cardiac fibrosis, which occurs as a result of extended presence and elevated activity of myofibroblasts in the heart, results from a shift from dynamic equilibrium between collagen production and degradation to net deposition. The excess collagen compromises the ability of the heart to contract, thus reducing cardiac output. In addition, excess collagen interrupts electrical conductivity and predisposes the heart to ventricular arrhythmias. As a result, cardiac fibrosis is a major factor in the progression to heart failure and sudden cardiac death (Burlew and Weber 2002).

**G protein coupled receptor signaling**

G protein coupled receptors (GPCRs) constitute the largest class of plasma membrane receptors in eukaryotic cells and several of these receptors are pharmacological targets for the treatment of cardiovascular diseases. These proteins are characterized by seven transmembrane spanning domains, an extracellular N-terminal domain and an intracellular C-terminal domain. The intracellular loop domains and the C-terminal tail can associate with heterotrimeric G proteins consisting of α-, β-, and γ-subunits (Kim and Iwao 2000). The α-subunit of an inactive G protein is bound to guanine diphosphate (GDP) and is associated with the β- and γ-subunits. Agonist binding to a GPCR (i.e. the angiotensin II type 1 receptor, or AT_1R) causes a conformational
FIGURE 1. Angiotensin II type 1 receptor-induced $G_q$ activation and classical downstream signaling events. 

$A$: Upon ligand binding, conformational changes in the $\text{AT}_1\text{R}$ promote exchange of GDP for GTP on the $\alpha$ subunit of heterotrimeric $G_q$ proteins and subsequent dissociation of $\alpha$ and $\beta\gamma$ subunits. $B$: (1) A major target of $G_q\alpha$ subunits is phospholipase C$\beta$ (PLC$\beta$), a protein which cleaves (2) phosphatidylinositol-(4, 5)-bisphosphate (PIP$_2$) into insoluble diacylglycerol (DAG) and soluble inositol-(1,4,5)-trisphosphate (IP$_3$). (3) IP$_3$ binds receptors on the endoplasmic reticulum to liberate calcium from intracellular stores. $C$: (1) $G_{\beta\gamma}$ subunits recruit G protein coupled receptor kinase 2/3 to the plasma membrane. (2) GRK 2/3 phosphorylates the $\text{AT}_1\text{R}$ which serves to desensitize the receptor to further agonist stimulation and prevents further G protein activation. (3) $\beta$- Arrestin binds to the phosphorylated receptor. (4) The receptor internalizes to endosomes and is dephosphorylated by GPCR phosphatases or undergoes lysosomal degradation.
A. 

AT_{1R} 

\( \alpha_q \) GTP \( \alpha_q \beta_\gamma \) GTP GDP \( \beta_\gamma \) 

B. 

\( \alpha_q \) GTP \( \alpha_q \) GTP \( \alpha_q \) GTP \( \beta_\gamma \) PIP\textsubscript{2} DAG IP\textsubscript{3} \( \uparrow [\text{Ca}^{2+}]_i \) PLC\textsubscript{β} 

C. 

\( \beta_\gamma \) GRK 2/3 P P P β-Arrestin 1 2 3 4 GRK 2/3 Internalization
change in the receptor that enables it to act as a guanine nucleotide exchange factor for
the G protein, resulting in exchange of guanine triphosphate (GTP) for GDP. GTP-bound
Gα subunits dissociate from the βγ-subunits, and both the α- and βγ-subunits then interact
with downstream effectors (Figure 1A). Heterotrimeric G proteins are classified into four major families named Gs, Gi/o, Gq/11 and G12/13 according to the function of the Gα subunits (Hur and Kim 2002). Gαs subunits stimulate the production of cyclic AMP by activating membrane bound adenylcyclase (AC). Cyclic AMP binds to the regulatory subunits of cyclic AMP-dependent protein kinase (PKA) which activates the kinase by causing release of the catalytic subunits. Gαi subunits are capable of inhibiting the production of cyclic AMP by inhibiting AC. This inhibition occurs even when AC is directly stimulated by exogenous forskolin. G proteins in the Gi/o family are also sensitive to pertussis toxin, which ADP-ribosylates Gαi/o subunits. This inhibits the α-subunits from reassociating with βγ-subunits and effectively prevents subsequent activation of the Gi/o proteins by GPCR activation. Gαq subunits stimulate the β isotype of phospholipase C, which catalyzes the cleavage of phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 then interacts with IP3 receptors on the endoplasmic reticulum to induce release of Ca2+ into the cytosol. Thus, a major effect of Gq activation is a rapid and transient increase in cytosolic Ca2+ (Figure 1B). Lastly, G12/13 regulates the activation of small monomeric GTP-binding proteins.

Gβγ-subunits also activate downstream effectors once released from the α-subunit and are responsible for initiating the desensitization of GPCRs (Ferguson 2001). One
important function of the release of βγ-subunits is that they act as a signal of GPCR activation. GPCR kinases (GRKs) are recruited to the plasma membrane by Gβγ subunits, specifically GRK2 and GRK3 (Koch et al. 1993; Touhara et al. 1994). Gβγ subunits bind to the C-terminal domains of GRK 2 and GRK 3, which then bind to the GPCR and phosphorylate residues in the serine and threonine rich regions of the C-terminal tail and intracellular loop domains. Initially, this event prevents reassociation of G proteins and serves to desensitize the receptor to further agonist stimulation. Subsequently, β-arrestin is recruited to the phosphorylated GPCR and induces internalization via a clathrin and dynamin dependent mechanism. GPCRs in internalized endosomes are then dephosphorylated by GPCR phosphatases and recycled to the membrane or the endosomes fuse with lysosomes resulting in receptor degradation (Figure 1C).

**Physiologic and pathologic functions of angiotensin II**

The angiotensin type 1 receptor (AT1R) is a GPCR which mediates the majority of the cardiovascular effects of angiotensin II (ANG II) and which is centrally important to the development of cardiovascular diseases. Systemic ANG II is produced through the renin-angiotensin-aldosterone system (RAAS) and plays a critical role in blood pressure regulation (Matsusaka and Ichikawa 1997; Touyz and Berry 2002). An important physiologic sensor of hypotension is renal perfusion pressure, and when this pressure drops, the juxtaglomerular cells of the kidneys release renin into the circulation. Renin catalyses the cleavage of inactive circulating angiotensinogen to form the inactive
decapeptide angiotensin I (ANG I). The vascular endothelium (primarily in the lungs) contains angiotensin converting enzyme (ACE) which cleaves two residues from ANG I to form the active octapeptide ANG II. ANG II binds to and activates G_{q}-coupled AT_{1}Rs on vascular smooth muscle cells (VSMCs) to induce intracellular calcium release and constriction of the vascular bed. AT_{1}Rs are also expressed in the adrenal cortex and stimulate the production of aldosterone, which promotes sodium and water reabsorption in the distal tubule. This effectively raises blood pressure in an effort to maintain cardiac function. ANG II type 2 receptors (AT_{2}Rs) typically antagonize the effects of AT_{1}Rs, and are ubiquitously expressed during fetal development, but display reduced expression in the adult (Kim and Iwao 2000). Therefore most of the physiologically relevant effects of ANG II are mediated by the AT_{1}R and can be inhibited by the use of AT_{1}R selective antagonists such as losartan or valsartan.

The RAAS is often hyperactivated in cardiovascular pathologies such as chronic hypertension or during cardiac remodeling following myocardial infarction. This hyperactivation results in elevated circulating levels of ANG II, an effect that is often clinically manipulated by the use of ACE inhibitors or AT_{1}R antagonists. During these pathological states, there is considerable hypertrophy of vascular smooth muscle cells, an effect largely due to the actions of ANG II. The AT_{1}R is expressed by different cells in a multitude of tissues, including cardiac fibroblasts and myocytes, and so the effects of systemic ANG II elevation are not limited to the vasculature and the adrenal glands. Chronic infusion of ANG II results in the development of cardiac hypertrophy through direct effects on cardiac myocytes in a similar manner to VSMCs. Cardiac fibroblasts,
whether in cell culture or in vivo, undergo proliferation in response to ANG II (Booz et al. 1994). In addition, ANG II stimulates transcription of procollagen types I and III mRNA in CFs and promotes α-SMA expression, indicating that ANG II induces cardiac myofibroblast differentiation (Brilla et al. 1994; Swaney et al. 2005). These findings identify ANG II as an important mediator of CF activity and suggest that ANG II is involved at multiple levels of both physiologic and pathological cardiac remodeling.

**MAPK activation and downstream signaling**

An important effect of ANG II signaling in several cell types including CFs is activation of the mitogen activated protein kinase (MAPK) signaling cascade. MAPKs are key components of pathways controlling cell proliferation, protein synthesis and hypertrophic growth, cell spreading and motility (Pearson et al. 2001). There are three major families of MAPKs, the extracellular signal-regulated kinase (ERK 1/2), c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK), and p38 families. Of the three families of MAPKs, the ERK 1/2 (also called p44/p42 MAPK) pathway is the best characterized and the most commonly utilized pathway for induction of cell proliferation by extracellular ligands. Both the JNK/SAPK and the p38 MAPK pathways are activated in response to cellular stress. The most well defined signaling pathway from the plasma membrane to MAPK is through activation of receptor tyrosine kinases (RTKs) by growth factors (Pawson and Scott 1997). Binding of RTKs by endogenous ligands results in receptor dimerization and autophosphorylation of tyrosine residues directly adjacent to the cytosolic catalytic domains. Tyrosine phosphorylation acts as a recruitment signal for
FIGURE 2. Epidermal growth factor receptor-induced MAPK activation. A: EGF binding induces receptor dimerization which facilitates autophosphorylation of residues in the catalytic domain. This phosphorylation is required for formation of a multiprotein complex which recruits the guanine nucleotide exchange factor SOS. SOS exchanges GTP for GDP on the small monomeric G protein Ras. B: Ras recruits the first kinase in the classic MAPK cascade Raf-1. Activation of Raf-1 is a highly coordinated event which requires phosphorylation on at least four residues (shown in bold) by several intracellular kinases including p21 activated kinase (PAK) and the soluble tyrosine kinase c-Src. MEK 1/2 is the substrate for activated Raf-1 and full activation of MEK 1/2 is achieved by phosphorylation of two residues S217 and S221. MEK 1/2 is a dual specificity kinase which phosphorylates ERK 1/2 on T202 and Y204 (human) within a conserved TEY motif.
A.

EGF

Ras

GTP

SOS

GDP

B.

EGF

Raf-1

MEK 1/2

ERK 1/2

PAK (S338)

Src (Y341)

(T419/S494/S497/S499)

(S217/S221)

(T202/Y204)

TEY

ERK 1/2
adaptor proteins containing Src homology (SH2) domains and leads to the formation of a multiprotein complex which associates with the guanine nucleotide exchange factor Sos and catalyzes the exchange of GTP for GDP on the small G protein Ras (Songyang et al. 1993). GTP-bound Ras interacts with several effector proteins, and the best characterized effector of Ras is Raf-1. Raf-1 (or c-Raf) is a MAP kinase kinase kinase (MAPKKK) and the first component of the MAPK signaling cascade. Raf-1 is a highly regulated protein and requires phosphorylation of multiple residues (S338/Y341/T491/S494) for full activation (Chong et al. 2001). This is a highly coordinated process which requires the cooperation of several protein kinases, including the soluble tyrosine kinase Src (Y341) and p21 activated kinase (Ser 338). In addition to these activating phosphorylations, Raf-1 can be inhibited by Akt-mediated phosphorylation of Ser 259. Raf-1 associates with the second kinase in the MAPK pathway, MAPK/ERK kinase (MEK) 1/2, and phosphorylates two conserved serine residues (S217/S221) to induce activation (Alessi et al. 1994; Zheng and Guan 1994). This step serves the important function of signal amplification since MEK 1/2 is expressed at much higher levels than Raf-1. Once active, MEK 1/2 associates with and phosphorylates one or both of the two related kinases ERK 1 and ERK 2. These proteins are encoded by two separate genes, but are nearly 85% identical, with the highest conservation in the substrate binding regions. MEK 1 and MEK 2 are dual specificity kinases in that they phosphorylate both serine/threonine residues and tyrosine residues in a conserved TEY motif (T183/Y185 of rat ERK, T202/Y204 of human ERK) in the activation loop of ERK 1/2 (Payne et al. 1991).
The MEK-ERK interaction occurs in the cytoplasm, since MEK contains a nuclear export sequence that prevents accumulation in the nucleus (Zheng and Guan 1994; Fukuda et al. 1997). However, the subsequent distribution of active ERK 1/2 is highly varied and likely depends on the stimulus for activation. The factors determining the distribution of ERK 1/2 have been suggested to depend on cytoplasmic anchoring, nuclear entry by diffusion, dimerization, active transport of monomers or protein complexes across the nuclear membrane, nuclear export of ERK 1/2 complexes, and binding to nuclear retention signals (Fukuda et al. 1997; Khokhlatchev et al. 1998; Lenormand et al. 1998; Adachi et al. 1999; Adachi et al. 2000).

New cellular substrates for ERK 1/2 are continuously being discovered. Currently, there have been about 160 substrates found for ERK 1/2 alone (Yoon and Seger 2006) and include proteins localized to the plasma membrane (i.e. phospholipase A2), cytoplasm (downstream kinases) and nucleus (transcription factors), all of which contain specific substrate recognition motifs. The largest subset of ERK 1/2 targets is protein kinases, including p90 ribosomal S6 kinases (RSKs/MSKs), MAPK activated protein (MAPKAP) kinases, and MAPK-interacting kinases (MNKs). MAPKAP kinases and MNKs are targets of p38 MAPK in addition to ERK 1/2, but the RSKs are selectively activated by ERK 1/2.

**Mitogenic signaling through ERK 1/2**

Stimulation with angiotensin II results in robust cellular proliferation in several cell types, including cardiac fibroblasts, in an ERK 1/2-dependent manner (Schorb et al. 2006).
Proliferation in response to ERK 1/2 requires a high degree of coordination among several proteins which are activated by ERK 1/2 directly or by ERK 1/2 substrates. A select group of cytosolic substrates of ERK 1/2 play critical roles in cellular proliferation. For example, ERK 1/2 has been demonstrated to directly phosphorylate proteins localized in focal adhesions to mediate cell spreading and motility. The cytoskeletal protein paxillin constitutively interacts with MEK 1/2 and will recruit ERK 1/2 to focal adhesions to facilitate the phosphorylation of focal adhesion kinase (FAK). Activation of FAK can result in increased cell motility, which is important for proliferation (Liu et al. 2002). ERK 1/2 also directly influences cell cycle progression by stimulating association of cyclin D1 and cyclin-dependent kinase 4, which leads to the subsequent phosphorylation and inhibition of retinoblastoma protein in VSMCs (Garnovskaya et al. 2004).

Phosphorylation of proteins involved in cellular proliferation is largely mediated by the substrates of ERK 1/2. RSKs phosphorylate and inhibit proteins responsible for prohibiting cell cycle progression, including p27kip1, which normally prevents cell cycle progression from G1 to S (Fujita et al. 2003), and Myt1, which inhibits entry into the M phase (Palmer et al. 1998). A major function of the MNKs is to regulate mRNA translation by interacting with eukaryotic initiation factor 4E (eIF4E) following mitogen stimulation and ERK 1/2 activation, although the exact role of the MNKs is unknown. However, it is well established that eIF4E is an important component of a complex involved in mRNA translation and that protein synthesis from eIF activity is critical for cell growth and proliferation (Sonenberg and Dever 2003).
There are several nuclear responses to ERK 1/2 activation during mitogen stimulation as well. ERK 1/2 modulates gene expression by several distinct mechanisms. First, several targets of the RSKs, MSKs, and MAPKAP kinases are important regulators of transcription, including cyclic AMP response element binding protein (CREB), CREB binding protein, serum response factor (SRF), and several estrogen receptor isotypes (Rivera et al. 1993; Nakajima et al. 1996; Xing et al. 1996; Joel et al. 1998). MSK 1/2 also plays the role of mediating the nucleosomal response to ERK 1/2 activation. Nucleosomal targets of MSKs include histone H3 and high mobility group protein, HMG-14, which are important for DNA packaging into chromatin (Thomson et al. 1999). Second, ERK 1/2 translocates to the nucleus and phosphorylates transcription factors involved in the immediate-early response. AP-1 (activating protein-1), which consists of homo and heterodimers of c-Jun and c-Fos, is a major target of ERK 1/2 and when phosphorylated on N-terminal residues, the dimers are active and stabilized to allow DNA binding (Chen et al. 1993). Ternary complex factors (i.e. Elk-1) are also MAPK substrates and are phosphorylated on several residues within the C-terminal transactivating domain in response to ERK 1/2 activation, which results in increased formation of ternary complexes with SRF and DNA (Janknecht et al. 1993). All of these targets are important mediators of the ERK 1/2 response on cell growth and control of proliferation.
Activation of ERK 1/2 by GPCRs

ERK 1/2 activation by GPCRs is highly diverse and the mechanism varies depending on the type of G protein. Elevation of cyclic AMP by Gαs subunits has been reported to reduce ERK 1/2 activation by PKA-mediated phosphorylation of Raf-1 at inhibitory residues Ser^{43} and Ser^{621} (Hafner et al. 1994). However, some GPCRs that are known to associate with Gs stimulate ERK 1/2 phosphorylation. For example, the β2-adrenergic receptor (β2-AR) couples to Gs to stimulate cyclic AMP production and PKA activation, but this Gs-coupled receptor has also been identified as an activator of ERK 1/2 (Kim et al. 2002). PKA phosphorylates the β2-AR to induce a switch of receptor coupling from Gs to Gi, and it has been suggested that Gi is responsible for the subsequent activation of ERK 1/2 (Daaka et al. 1997). Contrary to Gs, it is the Giβγ-subunits that mediate the signal transduction. In fact, when a peptide corresponding to the C-terminus of GRK 2 (also referred to as β-adrenergic receptor kinase, βARK-ct) is co-expressed with the β2-AR, stimulation with the β-AR agonist isoproterenol does not result in ERK 1/2 activation. This is due to the sequestration of βγ-subunits by βARK-ct. Typically, c-Src can be activated by β-arrestin during GPCR desensitization (Miller et al. 2000). Phosphorylation of proline-rich tyrosine kinase (PYK2) or focal adhesion kinase by c-Src creates SH2 binding domains and allows for the formation of a multiprotein complex in a manner analogous to RTKs (Dikic et al. 1996). Subsequently, this complex associates with Ras to induce activation of Raf-1, MEK 1/2 and ERK 1/2.

Gq-induced ERK 1/2 activation often involves the downstream activation of protein kinase C (PKC). The classic isotypes of PKC contain domains sensitive to both
diacylglycerol and Ca^{2+} and the activation of G_{q} therefore leads to plasma membrane recruitment and subsequent activation of PKC. Direct phosphorylation and activation of Raf-1 by PKC has been suggested to occur and PKC-dependent phosphorylation sites on Raf-1 have been identified, but the mechanism is not fully understood.

Activation of GPCRs can lead to ERK 1/2 phosphorylation via recruitment of multiple tyrosine kinases, whether cytosolic or membrane bound receptor tyrosine kinases. The epidermal growth factor receptor (EGFR) is an important example of a membrane protein with tyrosine kinase activity that can mediate ERK 1/2 activation in response to GPCR stimulation. The G_{s}/G_{i}-coupled β_{2}-AR has been determined to induce ERK 1/2 activation through a mechanism involving c-Src, matrix metalloproteinases, EGFR and PI3-K in adult rat cardiac fibroblasts (Kim et al. 2002). Src activation by the β_{2}-AR was determined to be required for EGFR activation, but the activation mechanism is unclear (Miller et al. 2000). Src has been demonstrated to mediate EGFR phosphorylation on tyrosine residues near the catalytic domain, allowing for the formation of the previously discussed multiprotein complex involved in Ras activation (Bokemeyer et al. 2000). Alternatively, c-Src may be involved in activation of matrix metalloproteinases (MMPs) which serve to shed heparin-binding EGF (HB-EGF), an EGFR ligand, from the extracellular matrix. Once cleaved, HB-EGF can act in a paracrine manner by activating EGFRs on neighboring cells. Both ERK 1/2 activation and subsequent DNA synthesis induced by isoproterenol, a β-AR agonist, can be attenuated by pretreating cardiac fibroblasts with inhibitors of c-Src, MMPs, or EGFR kinase activity (Kim et al. 2002).
FIGURE 3. AT$_1$R-induced ERK 1/2 activation. A major means of ANG II-induced ERK 1/2 activation is through stimulation of classic and novel PKCs. Both types of PKC are recruited by DAG, but the novel PKCs are not dependent on calcium. PKC can interact with Ras to facilitate Raf-1 activation and subsequent activation of the MAPK pathway. Alternatively, ANG II-induced ERK 1/2 activation can proceed through an EGFR-dependent mechanism. The AT$_1$R can induce EGFR activation through Src-dependent activation of matrix metalloproteases (MMPs) which cleave a tethered membrane-associated ligand called heparin binding EGF. Once cleaved, HB-EGF is free to interact with the EGFR to induce activation in a manner identical to direct EGF stimulation.
AT$_1$R-mediated ERK 1/2 activity has been determined to occur through an EGFR-dependent mechanism in several cell types as well, including neonatal cardiac fibroblasts and VSMCs (Eguchi et al. 1998; Murasawa et al. 1998). There are several potential intracellular components of the signaling mechanism which are believed to be required for EGFR transactivation, including calcium, reactive oxygen species, and soluble tyrosine kinases including c-Src, PYK2, and Janus kinase (Saito and Berk 2001). The MMP-dependent EGFR transactivation mechanism has also been described in VSMCs in response to ANG II stimulation, indicating that transactivation is not limited to G$_i$ coupled receptors (Saito et al. 2002). The mechanism of AT$_1$R-induced ERK 1/2 activation is likely cell-specific and depends on localization and availability of intracellular substrates, since both EGFR-dependent and -independent mechanisms and several downstream signaling mediators have been identified.

**PI3-K activation and downstream signaling through PKCζ**

There are three major classes of phosphatidylinositol 3-kinases, class I, II and III. Class I PI3-Ks are agonist sensitive and are further subdivided into class IA and IB based on their structure and mode of activation (Anderson and Jackson 2003). In general, class I PI3-Ks are heterodimeric proteins consisting of a 110 kDa catalytic subunit (p110) and a smaller regulatory subunit that is constitutively associated with an N-terminal domain. There are three class IA PI3-Ks which are encoded by separate genes and every mammalian cell expresses at least one class IA PI3-K. The α and β isotypes are the most widely expressed, but the δ isotype is primarily found in leukocytes. The regulatory
FIGURE 4. GPCR-induced PI3-K activation. A: A major target of GPCR signaling is phosphatidylinositide 3-kinase (PI3-K). Released $G_{\beta\gamma}$ subunits interact with the p110 catalytic subunit of PI3-K and activate the kinase. B: PI3-K phosphorylates phosphatidylinositol phosphates on the position 3 carbon, with the preferred substrate being PI(4,5)P$_2$ to form PI(3,4,5)P$_3$. 
A. 

PKA

\[ \begin{align*} 
&\text{GTP} \quad \text{GDP} \\
&\text{PI}(4,5)P_2 \rightarrow \text{PI}(3,4,5)P_3 \\
&\text{PI3-K} \\
\end{align*} \]

B. 

\[ \begin{align*} 
&\text{PI}(4,5)P_2 \rightarrow \text{PI}(3,4,5)P_3 \\
&\text{PI3-K} \\
\end{align*} \]
adaptor proteins of class IA PI3-Ks are typically 85 kDa and contain no catalytic activity. Their primary responsibility is to facilitate protein-protein interactions through their modular domains, which include an SH3 domain, two SH2 domains and an inter-SH2 domain which binds the p110 subunit. The class IB p110γ isotype shares homology with the α, β and δ isotypes but does not contain a p85 binding site. This isotype instead binds to an unrelated p101 subunit which lacks the protein-protein binding motifs characteristic of p85 subunits. The p110γ isotype was initially very interesting with respect to GPCR signaling because it was determined that this PI3-K was sensitive to activation by Gβγ-subunits. However, the p110γ/p101 complex was later found to only be abundantly expressed in white cells, and the physiologic importance of its activation is therefore not likely relevant in most cells. The widely expressed p110β isotype has also been determined to be sensitive to Gβγ subunits and has been linked to MAPK activation, thus making it a potential mediator of GPCR signaling to ERK 1/2.

Active PI3-K phosphorylates phosphatidylinositol lipids on the 3-position carbon of the inositol ring. The preferred substrate of PI3-K is phosphatidylinositol-4,5-bisphosphate (PIP₂), although other PIPs have been found to be phosphorylated by PI3-K in vitro. Phosphatidylinositol-3,4,5-trisphosphate (PIP₃) is a membrane recruitment signal for several proteins. A well studied effector of PIP₃ is Akt (protein kinase B), which is recruited to the membrane PIP₃ and phosphorylated by phosphoinositide-dependent protein kinase (Alessi et al. 1997). Activation of Akt stimulates mRNA translation, proliferation by cell cycle progression, cell survival through anti-apoptotic mechanisms and several other cell-specific effects (Brazil et al. 2004).
Another important effector of PIP₃ that is of particular interest with respect to MAPK signaling is atypical protein kinase Cζ. Atypical PKCs do not contain diacylglycerol/phorbol ester-sensitive domains unlike the classic and novel isotypes, and they are also lacking domains sensitive to calcium that are found in classic PKCs. PKCζ has been demonstrated to require phosphatidylinositol 3-phosphate for activation and was found to be preferentially recruited to the plasma membrane and activated in response to PIP₃ generation compared to other PIPs (Nakanishi et al. 1993).

PKCζ has been determined to be critical for activation of MAPKs by ANG II in VSMCs and for cellular proliferation of neonatal cardiac fibroblasts induced by transforming growth factor-β (Liao et al. 1997; Braun and Mochly-Rosen 2003). In VSMCs, PKCζ was shown to interact directly with the small G protein Ras in a manner similar to Raf-1 and cause ERK 1/2 activation in response to ANG II. This increase in ERK 1/2 activity was completely attenuated by PKCζ downregulation using antisense oligonucleotides. A similar role of PKCζ was discovered in MCF-7 cells, a breast cancer cell line in which stimulation with ANG II leads to ERK 1/2 activation (Muscella et al. 2003). In this case, PKCζ was inhibited using a myristoylated peptide that corresponded to the pseudosubstrate domain of endogenous PKCζ (amino acids 113-125). The pseudosubstrate resembles PKC substrates except that it contains an alanine residue in place of a predicted serine/threonine residue and normally interacts with the kinase domain until PKCζ is activated. Addition of exogenous pseudosubstrate peptides overwhelms the kinase domain and prevents activation and phosphorylation of downstream targets. In MCF-7 cells, treatment with pseudosubstrate peptide or LY
FIGURE 5. Domains of different PKC isozymes. The three classes of PKC isozymes are classic, novel, and atypical and different isozymes are classified based on the presence or absence of specific domains. The regulatory domain of classic PKCs contain a DAG/phorbol ester-sensitive C1 (further divided into C1A and C1B) domain, calcium sensitive C2 domain and a pseudosubstrate domain which interacts with the catalytic domain to prevent constitutive activity. Novel isozymes contain similar domains with the exception that the C2 domain is insensitive to calcium. Atypical PKCs lack C2 domains altogether and the C1 domain is insensitive to DAG and phorbol esters even though it contains some sequence homology.
Pseudosubstrate – inhibits catalytic domain
C1 domain – DAG/phorbol ester (PE) binding
Atypical C1 domain – does not bind DAG/PE
C2 domain – Ca\(^{2+}\) binding
Novel C2 domain – does not bind Ca\(^{2+}\)
Catalytic domain – kinase activity
29402, a PI3-K inhibitor, prevented ANG II-induced ERK 1/2 phosphorylation. Interestingly, this study also indicated that the ANG II effect on ERK 1/2 was EGFR-independent (Muscella et al. 2003). The AT₁R and other GPCRs have been demonstrated to induce both PI3-K and EGFR activation in several cell types and this finding in MCF-7 cells suggests that there are both cell type specific and GPCR specific signaling mechanisms from the receptor to ERK 1/2.

**The role of PKCζ in mitogenesis, focal adhesion/cytoskeletal signaling and apoptosis**

PKC is involved in cellular processes dependent on remodeling of the cytoskeleton, including cell spreading, adhesion, migration, polarity, and proliferation (Larsson 2006). In mouse fibroblasts, vascular smooth muscle cells and xenopus oocytes, PKCζ is critical for mitogenic signal transduction (Dominguez et al. 1992; Berra et al. 1993; Liao et al. 1997). PKCζ has also been found to associate directly with spindle microtubules in the mitotic apparatus to mediate cellular proliferation (Lehrich and Forrest 1994).

PKC typically does not affect the cell cytoskeleton directly. A major substrate of all PKCs is the myristoylated alanine-rich C kinase substrate (MARCKS), a protein that has been identified to mediate many of the cytoskeletal-dependent effects (Ramsden 2000), including filamentous actin crosslinking (Hartwig et al. 1992). In addition, tubulin has been identified as a PKCζ binding protein (Garcia-Rocha et al. 1997). The interaction occurs at the PKCζ pseudosubstrate domain and is weak compared to other microtubule-
FIGURE 6. Integrin and focal adhesion complex-mediated signaling events. Integrin receptors, composed of $\alpha$ and $\beta$ heterodimers, cluster at focal adhesions. The $\beta$ subunits associate with intracellular proteins important for actin cytoskeleton organization and formation of signaling complexes, including vinculin, talin, and paxillin. PI3-K associates with integrin-linked kinase (ILK) and activates Akt and PKC$\zeta$ to promote cell survival by inhibition of proapoptotic proteins. The myristoylated alanine-rich C kinase substrate (MARCKS) is an important target of PKC$\zeta$ and is responsible for mediating f-actin polymerization. The Rho family GTPase Cdc42 is also a target of PKC$\zeta$ and is key for cellular migration and stress fiber degradation. Focal adhesion kinase (FAK) is another critical focal adhesion protein which can stimulate the ERK 1/2 pathway and induce PI3-K activity through activation of e-Src and growth factor receptors.
associated proteins. However, the interaction was important since disruption of microtubule structures with nocodozal impaired native activity of PKCζ.

Both PI3-K and PKCζ associate with focal adhesion complexes and mediate integrin and growth factor-induced signaling (Delcommenne et al. 1998; Crean et al. 2004). PI3-K mediates attachment-dependent survival through activation of Akt. Interestingly, PKCζ has been identified as a mediator of hyperosmotic stress-induced phosphorylation of caspase-9 at an inhibitory site, and the same study suggests the potential for additional inhibitory sites which are targets for PKCζ (Brady et al. 2005).

Selective inhibition of PKCζ has been determined to prevent growth factor-induced dissolution of focal adhesion complexes, a critical step in cell migration or chemotaxis (Crean et al. 2004; Sun et al. 2005). Cdc42 is a Rho-like GTPase which associates with PKCζ and is important for the development of filapodia, long finger-like cytoplasmic extensions important for sensing the extracellular environment and for cellular migration (Coghlan et al. 2000; Wennerberg and Der 2004). Collectively, these findings identify PKCζ as a key mediator of focal adhesion-cytoskeletal-dependent cellular events.

**Transforming growth factor-β signaling and role in cardiac fibroblast function**

The transforming growth factor-β (TGF-β) proteins are critical for a multitude of processes (Javelaud and Mauviel 2004; ten Dijke and Hill 2004). The three known isoforms of TGF-β that exist in mammals, TGF-β1, -β2, and -β3, share 60-80% homology and have overlapping functions. They play important roles in cell growth, differentiation,
extracellular matrix production and apoptosis. Cardiac fibroblasts are known to produce and secrete TGF-β₁ and this is likely the dominant isoform that exists in the heart (Rosenkranz 2004).

TGF-β is synthesized as a 391 amino acid precursor protein that is cleaved proteolytically into a 25 kDa dimeric protein consisting of two identical 112 amino acid chains linked by a disulfide bridge. This form of TGF-β is secreted bound to latent-associated peptide (LAP) as a high molecular weight complex which lacks activity. The complex is stored associated with the ECM and can be activated by acidification or proteolytic processes which separate LAP from active TGF-β. Once active, TGF-β acts in an autocrine or paracrine manner by binding to locally expressed TGF-β receptors. There are three types of receptor which associate to form one large receptor complex. Types I and II TGF-β receptors (TβRI and TβRII) are single transmembrane domain serine/threonine kinases. TGF-β binds directly to the TβRII which then phosphorylates the TβRI to induce activation. TβRI then mediates the intracellular effects. TβRIII, also known as betaglycan, is a membrane-anchored proteoglycan which serves the purpose of presenting TGF-β to other receptors. The short cytoplasmic domain of TβRIII is important for forming the TβRII-TβRI complex and for the initiation of intracellular signaling (Blobe et al. 2001).

The most studied and best understood effectors of the TβR complex are a family of transcription factors named Smads (Moustakas et al. 2001). In addition to being activated by the TβR, the Smad proteins are activated by the bone morphogenic proteins
(BMPs), a member of the TGF-β superfamily. Smads are classified into three groups, the receptor-associated Smads (R-Smads) consisting of Smad 2/3 (activated by TβR) and Smad 1/5/8 (activated by the BMP receptor), co-Smads consisting only of Smad 4, and the inhibitory Smads (I-Smads) consisting of Smad 6/7. Activation of TβR leads to direct phosphorylation of Smad 2/3 by TβRI on a conserved SSXS motif on their C-terminus (Abdollah et al. 1997). This process is facilitated by Smad 2/3 binding to SARA (Smad anchor for receptor activation, (Tsukazaki et al. 1998)). Phosphorylation reduces Smad 2/3 affinity for SARA and increases affinity for Smad 4. The Smad 2/3-Smad 4 complex then translocates to the nucleus where it associates with co-activators and binds to target genes or it associates with co-repressors which serve to provide a threshold for transcriptional activation or limit the TGF-β signal (Lagna et al. 1996; Moren et al. 2000).

TGF-β plays a critical role in the wound healing process for several tissues, including heart, skin, liver, kidney and eye (Nagy et al. 1991; Zhang et al. 1995; Elder et al. 1997; Li et al. 1997; Phillips et al. 1997; Schmid et al. 1998). TGF-β is known to be a potent inducer of myofibroblast differentiation and collagen production by stimulating mRNA and protein synthesis of α-smooth muscle actin and collagens I, III, IV, V and VI, all of which are found in the adult myocardium (Rosenkranz 2004). In addition, TGF-β function is particularly relevant to ANG II signaling in CFs since ANG II has been demonstrated to induce TGF-β synthesis and release. In fact, the profibrotic effect of ANG II can be attenuated in vivo by application of TGF-β neutralizing antibodies.
Augmented ANG II and TGF-β signaling leads to aberrant scarring and tissue fibrosis by direct induction of myofibroblast differentiation and collagen production, and therefore, compounds that are effective in preventing ANG II and TGF-β signaling might be effective anti-fibrotic agents.

**Signaling mechanisms of α-adrenergic receptors**

The α- and β-adrenergic receptor systems are of the most well-characterized and highly studied hormone receptor systems in mammals, particularly with respect to normal physiologic and pathophysiologic cardiovascular and pulmonary function. In the heart and lungs, the actions of epinephrine and norepinephrine are primarily mediated by the β-ARs, with the β₁-AR mediating positive inotropic and chronotropic effects on cardiac muscle, and the β₂-AR promoting airway dilation. Cardiac fibroblasts also express β₂-ARs and stimulation of these receptors has been shown to induce CF proliferation through MAPK signaling (Kim et al. 2002). The role of the α-AR system is primarily in the vasculature. The α₁-AR subtype is highly expressed by vascular smooth muscle and is classified as a Gq-coupled receptor. Stimulation of these receptors causes VSMC constriction by Gq-dependent calcium release from intracellular stores, and the α₁-AR system is an important pharmacological target for the management of chronic hypertension. Previous reports suggest that α-ARs are not involved in cardiac fibroblast signaling (Stewart et al. 1994; Meszaros et al. 2000), but our lab has collected some pilot data that suggests that α-ARs might be involved in the process of myofibroblast
differentiation. When adult rats are given isoproterenol a β-AR agonist, there is a reduction in α-SMA expression in whole heart tissue after 48 hours of treatment. Stimulation of the β2-ARs expressed by CFs has been shown to prevent differentiation of myofibroblasts through a cyclic AMP-dependent inhibition of Smad signaling (Schiller et al. 2003). CREB binding protein (CBP) acts as a co-activator for Smad 2/3 and stimulates activation of target genes. The current model is that when CREB is activated by cyclic AMP dependent PKA, then CREB binds CBP and prevents the interaction with Smad 2/3, thus acting as an off switch for Smad signaling. However, in rats treated with epinephrine, which activates both α- and β-ARs, the amount of α-SMA expressed in whole heart tissue is increased compared to the hearts of rats receiving vehicle alone (Chapter 5). There are several potential causes of this increase, one being the induction of myofibroblast differentiation. One of the goals of our laboratory then was to determine the effects, if any, of α-AR stimulation on isolated primary cardiac fibroblasts.

The “French Paradox” and the biological functions of resveratrol

From the mid 1980s to the mid 1990s, the World Health Organization conducted the MONICA (MONItoring system for CArdiovascular disease) study (Tunstall-Pedoe et al. 1994). The study was designed to determine mortality rates due to coronary artery disease (CAD) among people of different parts of the world. A major finding of the MONICA study was that the people of France and some parts of Italy had significantly lower CAD mortality rates compared to people of the United States, Canada and the UK, despite having comparable cholesterol levels (~210-230 mg/dl), systolic blood pressures
(BP, 120-140 mm Hg) and body mass indexes (24.5-27.3). It is well established that consuming foods high in saturated fats will directly lead to elevated cholesterol and contribute to CAD. In general, the diet of the French people included in the MONICA study was not significantly different than then diets of others. This phenomenon later became known as the “French Paradox” and it was hypothesized that the cause may be due to moderate consumption of red wine, since annual per capita consumption rates are 5 and 10 fold higher in France compared to the UK or US, respectively.

Analysis of the components of red and white wines revealed one major difference among the classes of compounds found in each. On average, red table wines contain approximately 20-fold higher concentrations of phenols as compared to white wines. There are no significant differences among any of the other major components including ethanol, sugars, fats and vitamins. Further investigation into the phenol content of red and white wines demonstrated several differences, between both flavonoids and non-flavonoid components. The flavonoids (i.e. quercetin) are known to be potent antioxidants and are likely partially responsible for the beneficial effects of red wine consumption on cardiovascular health. However, these compounds are found in several grape products and are not specific to red wine. Within the non-flavonoid components, one class of compounds is found at higher concentrations in red wine as compared to white and was found to be dependent on both grape skins and the microbes and yeast present during the fermentation process, thus indicating a greater specificity for red wine versus other grape products. The stilbenes are a class of polyphenolic compounds in which two phenol groups are separated by a carbon-carbon double bond and each phenol
FIGURE 7. Structure and biological actions of resveratrol. Resveratrol is a member of the stilbene family, a class of polyphenolic compounds containing a double bond between phenol groups and various hydroxyl groups on the phenol rings. Resveratrol exists in both cis and trans forms, but only the trans form has biologic activity.
Resveratrol
*(trans-3,4’,5-trihydroxystilbene)*

<table>
<thead>
<tr>
<th>Biological actions of resveratrol</th>
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<tbody>
<tr>
<td>• Antibacterial/antifungal</td>
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<tr>
<td>• Antioxidant/free radical scavenging</td>
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<tr>
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<td>• Anti-inflammatory</td>
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<td>• Vasorelaxant/inhibits VSMC hypertrophy</td>
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<td>• Inhibits tyrosine kinase activity and PKC</td>
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ring is variably hydroxylated. Trans-resveratrol (RES; trans-3,4',5-trihydroxystilbene) has been identified as a red wine component with important biological effects.

It is likely that the findings of the MONICA study can partially be explained by the direct activity of RES on the vasculature and on cholesterol levels. Since the identification of RES, several studies have been undertaken with the goal of determining the site or sites of action. In VSMCs, RES was determined to inhibit proliferation and hypertrophy induced by ANG II by blocking activation of PI3-K and ERK 1/2 (Haider et al. 2002). In addition, RES was determined to promote relaxation of vascular smooth muscle by stimulating production of nitric oxide which activates soluble guanylyl cyclase in VSMCs and by directly activating membrane-bound guanylyl cyclase (El-Mowafy 2002). Both of these findings indicate that RES might be beneficial in preventing hypertension and maintaining reduced arterial blood pressure through direct actions on the vasculature. There is also evidence that RES might be useful in preventing a myocardial infarction (MI) by several distinct mechanisms. An MI is often the result of atherosclerotic plaque formation and platelet aggregation in the coronary arteries, and both can be effectively prevented by treatment with RES due to the blockade of polyunsaturated fatty acid oxidation which normally impairs low-density lipoprotein catabolism and the blockade of store-operated calcium channels in platelets (Dobrydneva et al. 1999; Fremont et al. 1999).

In addition to these cardioprotective properties, RES is a potential anti-neoplastic agent due to its ability to inhibit proliferation and tumor growth (Delmas et al. 2006). This anti-proliferative property is of interest to CF function as well, since CF
proliferation is an important aspect of activation and contributes to their collective fibrotic potential. RES can block agonist-stimulated proliferation by direct inhibition of the MAPK cascade in several cell types, but the effects of this compound have not been studied in CFs and little is known about RES as a potential anti-fibrotic agent.

**Specific Aims and Hypotheses**

The overall goals of the studies contained within this dissertation were to determine the intracellular signaling mechanism of ANG II-induced proliferation in adult cardiac fibroblasts, the role, if any of the α-adrenergic system in isolated cardiac fibroblasts, and to determine if resveratrol can inhibit proliferation and differentiation of cardiac fibroblasts. The goals were met by testing the following Specific Aims:

1. To determine whether resveratrol inhibits CF proliferation and cardiac myofibroblast differentiation and to identify specific intracellular signaling targets affected by RES.

   *I hypothesized that RES would prevent both cellular proliferation and myofibroblast differentiation and that this inhibition would not be specific to ANG II signaling, but be the result of ERK 1/2 signaling inhibition.*

2. (A) To determine whether ANG II-induced proliferation of adult rat cardiac fibroblasts is dependent on EGFR transactivation and PKCζ activation.

   *I hypothesized that ANG II induces cellular proliferation through multiple intracellular signaling mechanisms. I proposed that ANG II induced EGFR transactivation and*
activated atypical PKCζ through PI3-K in parallel pathways that both lead to ERK 1/2 activation.

2. (B) To determine how inhibition of PKCζ by application of a selective myristoylated pseudosubstrate caused growth arrest and affected adult cardiac fibroblast morphology.

I hypothesized that the inhibition of PKCζ caused cytoskeletal reorganization and induced apoptosis in CFs.

3. To determine whether chronic α-adrenergic stimulation could alter the myofibroblast content of the myocardium.

I hypothesized that α-adrenergic stimulation of adult cardiac fibroblasts would result in myofibroblast differentiation.
CHAPTER TWO
INHIBITORY EFFECTS OF RESVERATROL ON CARDIAC FIBROBLAST
PROLIFERATION AND DIFFERENTIATION

INTRODUCTION

Cardiac fibroblasts are the predominant secretory cells of extracellular matrix proteins in the heart and the key mediators of normal and pathological cardiac remodeling. Prolonged activation of CFs, defined by increased proliferation and subsequent ECM secretion, is a direct consequence of hypertension and heart failure and leads to cardiac fibrosis, a condition characterized by excess ECM deposition and a stiff myocardium. The impaired compliance of the fibrotic heart ultimately compromises contractile performance (Burlew and Weber 2002). In addition to proliferation, CFs differentiate into myofibroblasts, a cell type with an increased capacity to secrete ECM proteins (Weber et al. 1997). The proliferation and differentiation of new ECM-producing cells enhance the deposition of ECM proteins, and thus, limiting these parameters represents a potential therapeutic avenue to reduce pathological myocardial fibrosis.

ANG II levels are elevated during hypertension and heart failure. This peptide hormone has direct and potent effects on CF function, including activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase cascade and cellular proliferation via direct stimulation of the ANG II type 1 receptor (AT_1R; see (Schorb et al. 1993; Villarreal et al. 1993; Booz et al. 1994; Schorb et al. 1995; Dostal et al. 1996; Brilla et al. 1997). Our lab has previously demonstrated that ANG II-induced
proliferation of CFs is dependent on ERK kinase (MEK) and ERK 1/2 activation via pharmacological inhibition of MEK (Stockan and Meszaros 2003). In addition to activating ERK, ANG II regulates collagen type I expression, at least in part, by inducing CFs to produce and secrete transforming growth factor-β (Lee et al. 1995; Ford et al. 1999). TGF-β is a potent paracrine mediator of differentiation and contributes to the development of cardiac fibrosis by increasing the number of myofibroblasts in the heart (Tharaux et al. 2000; Petrov et al. 2002).

Resveratrol (RES; trans-3,4′,5-trihydroxystilbene), a phytoalexin found in the skins of grapes, has been identified as a key biologically active ingredient in red wine. RES has been credited with mediating a number of beneficial effects in the cardiovascular system that accompany moderate red wine consumption. In rat aortic smooth muscle cells, RES suppresses ANG II-induced Akt/PKB and, to a lesser extent, ERK 1/2 activation, both of which are required for ANG II-induced hypertrophy (Haider et al. 2002). RES reduces ERK and JNK phosphorylation in coronary artery smooth muscle cells (El-Mowafy and White 1999) and induces vasorelaxant responses by activating membrane bound guanylyl cyclase (El-Mowafy 2002) and nitric oxide release (Orallo et al. 2002). Together, these findings depict RES as a potential therapeutic agent in a multitude of cardiovascular diseases.

The studies focusing on the cardiovascular effects of RES have primarily examined the coronary artery and vascular smooth muscle cells, whereas the effects of RES on CFs have not yet been examined. Our initial hypothesis was that RES would inhibit both ANG II-induced proliferation and differentiation of CFs. The proliferation of
CFs via ANG II and the ERK 1/2 cascade (Schorb et al. 1995) plays a key role in the development of cardiac fibrosis, and inhibiting prolonged CF activity represents a viable method for preventing the long-term loss of cardiac function that accompanies this condition. We report that RES does inhibit ANG II-induced proliferation and growth of CFs, an effect mechanistically controlled through direct blockade of the ERK 1/2 cascade. The study also demonstrated that RES pretreatment inhibited ANG II-induced CF differentiation to the hypersecretory myofibroblast phenotype. In addition, we have determined that RES attenuated basal CF proliferation, EGF-induced ERK 1/2 activation, and TGF-β-induced myofibroblast differentiation. These findings suggest that RES has anti-fibrotic properties in the myocardium by limiting CF proliferation and myofibroblast differentiation.

MATERIALS AND METHODS

**Materials**

DMEM, penicillin/streptomycin, fungizone, and FBS were all purchased from Invitrogen/GIBCO (Grand Island, NY). Hybond nitrocellulose membrane and [3H]-leucine were purchased from Amersham Biosciences (Piscataway, NJ). [3H]-Thymidine was from ICN Biomedicals (Irvine, CA). Anti-phospho-Akt, anti-phospho-p70S6K, anti-phospho-MEK, and anti-MEK antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-ERK 1/2 and anti-ERK 1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p70S6K antibodies were from Calbiochem (La Jolla, CA). Anti-α-smooth muscle actin (α-SMA) antibodies was from Sigma-Aldrich (St. Louis, MO). Rat-adsorbed horseradish peroxidase-conjugated anti-
mouse secondary antibody was obtained from Serotec (Raleigh, NC). Alexa Fluor 488 goat anti-mouse secondary antibody was purchased from Molecular Probes (Eugene, OR). All other reagents and chemicals were reagent grade and obtained from Fisher Scientific (Pittsburgh, PA).

**Cell culture**

CFs were prepared from the left ventricles of one to two adult male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN). The ventricles were minced, pooled, and digested in a collagenase-pancreatin solution, as previously described (Meszaros et al. 2000; Meszaros et al. 2000). Cardiac myocytes were pelleted by centrifugation at 200 rpm for 2 min, and supernatant containing the fibroblasts was removed. The CFs were then pelleted at 1,000 rpm for 10 min and resuspended in DMEM supplemented with penicillin, streptomycin, fungizone, and 10% FBS. After a 30-min period of attachment to tissue culture plates, cells that were weakly attached or unattached (myocytes, endothelial cells, smooth muscle cells, and red blood cells) were rinsed free and discarded. After 2–3 days, confluent cultures were passaged by trypsinization and replated at a split ratio of 1:3. Passage 2 or 3 cells were used in all experiments. The purity of these cultures at passages 1 through 3 was > 95% CFs, as measured by vimentin and collagen (types I and III) expression as previously described (Meszaros et al. 2000). In all experiments, DMEM containing 10% FBS was washed out, and the cells were equilibrated in serum-free DMEM (SFM) before hormonal stimulation.
**Cell harvest/protein isolation**

CFs were equilibrated in SFM overnight, and on the following day CFs were preincubated with RES for 30 min before agonist stimulation. After the indicated hormonal treatments, the growth medium was removed, and the cells were washed two times with ice-cold PBS. Whole cell lysates were collected in lysis buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 0.02 mg/ml leupeptin, and 0.02 mg/ml aprotinin, pH 7.4). Cells were quickly scraped from the plates, and the protein lysates were sonicated by three 5-s bursts and centrifuged at 20,000 \( g \) for 15 min at 4°C. Supernatants were collected, and protein levels were determined by the bicinchoninic acid method (Pierce, Rockford, IL).

**Western blotting**

Protein samples were diluted in modified Laemmli sample loading buffer and heated at 95°C for 5 min. Equal amounts of protein (10–20 μg) were loaded on a 10% SDS-polyacrylamide gel and electrophoresed at 180 volts for 1 h using the Mini-Protean III system (Bio-Rad, Hercules, CA). After electrophoresis, proteins were transferred to a nitrocellulose membrane at 100 volts for 1 h. The membrane was then blocked for 1 h at room temperature in a Tris-buffered saline (TBS, pH 7.4) solution containing 0.4% gelatin. After being blocked, membranes were incubated overnight at 4°C with appropriate antibodies in TBS containing 0.1% Tween 20 (TTBS). We have employed several phospho-specific antibodies that recognize only the phosphorylated, active form
of the MAPK and Akt proteins. Antibodies used to detect phosphorylated ERK (P-ERK) levels recognize both ERK 1 and ERK 2, which are represented on Western blots by two distinct bands at 42 and 44 kDa. For Akt activation, we used two antibodies that recognize distinct phosphorylation sites (Ser473 and Thr308). Membranes were washed extensively the following day with TTBS and incubated for 1–2 h with appropriate secondary antibodies conjugated with horseradish peroxidase. Signals were detected by chemiluminescence, and membranes were exposed to Kodak X-OMAT AR film for an appropriate length of time and developed according to the manufacturer’s recommendations. To ensure equal protein loading between samples, antibodies bound to nitrocellulose membranes were removed by incubation in stripping buffer (62.5 mM Tris-HCl, 2% (wt/vol) SDS, and 0.7% (vol/vol) 2-mercaptoethanol, pH 6.7) at 50°C for 30 min and reprobed with antibodies that recognize both phosphorylated and nonphosphorylated forms of the proteins. Densitometric data from Western blots were obtained and quantified using a flatbed scanner interfaced with a computer and imaging software (Scion Image, Frederick, MD).

[^H]-Leucine/[^H]-thymidine incorporation assay

Equal numbers of CFs were plated on 12-well tissue culture plates in DMEM supplemented with 10% FBS. Cells were washed with PBS (pH 7.4), placed in SFM overnight, and then treated in triplicate for a period of 48 h. In the leucine incorporation assay, hormone additions were made in the presence of 1 μCi/ml[^H]-leucine. In the thymidine incorporation assay, 0.5 μCi/ml[^H]-thymidine was added to the media only
during the final 4 h of the 48-h stimulation period. At the end of the treatments, the medium containing the label was removed, and the cells were washed two times with PBS, one time with 5% TCA, and two times with 95% ethanol. The cells were solubilized in 1 ml of 0.5 M sodium hydroxide for 30 min at room temperature. The suspension was then trituated several times and then placed in scintillation vials with 5 ml EcoLume scintillation fluid. Samples were counted on a Beckman LS 6500 liquid scintillation counter. Data are expressed as a percentage of vehicle-treated controls (set at 100%) after calculating the ratio of counts per minute per well of hormone-stimulated to vehicle-treated cells.

**Immunocytochemistry/α-SMA staining**

CFs were plated on 12-well glass slides in DMEM supplemented with 10% FBS and allowed to attach for 4 h. Cells were then washed with PBS and equilibrated in SFM for 2 h. TGF-β was administered for 24 h in the presence and absence of RES, after which the CFs were washed with PBS and fixed with 2% paraformaldehyde in PBS for 30 min. CFs were permeablized with 1% Triton X-100/PBS for 30 min, blocked in 2% goat serum for 1 h, and incubated with mouse anti-α-SMA primary antibodies for 1 h. CFs were then washed and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody. Excess antibody was washed five times, and cells were mounted in Vectashield mounting media containing DAPI, a nuclear stain (Vector Laboratories, Burlingame, CA).
**Data analysis/statistics**

Statistical significance across treatment groups was determined by either a one-way ANOVA with Tukey’s multiple-comparison test or a t-test using GraphPad Prism (GraphPad Software, San Diego, CA) statistical analysis software. Statistical significance is indicated by an asterisk for significance vs. basal or a dagger for significance vs. hormone-stimulated cells. A $P$ value $< 0.05$ indicates a statistically significant difference, and $P < 0.05$, $P < 0.01$, and $P < 0.001$ are represented by one, two, or three symbols, respectively.

**RESULTS**

**RES attenuates basal and ANG II-induced ERK 1/2 activity in CFs**

RES inhibits the MAPK cascade in a number of cell types, and so we asked whether ERK 1/2 was a target of RES in CFs. As shown in [Figure 8](#), RES inhibited basal phosphorylated MEK (P-MEK) levels (A, $34.3 \pm 4.5$, $70.5 \pm 3.1$, and $90.4 \pm 1.7\%$ reduction with 5, 10, and 25 μM RES, respectively) and P-ERK 1/2 levels (B, $50.9 \pm 1.5$ and $71.0 \pm 2.3\%$ reduction with 10 and 25 μM RES, respectively) in a concentration-dependent manner. CFs were stimulated with 100 nM ANG II for 20 min in the presence and absence of 5–25 μM RES (preincubated 30 min before ANG II stimulation). As expected, ANG II increased P-MEK (Fig. 8A, $33.6 \pm 11.3\%$) and P-ERK (Fig. 8B, $102.8 \pm 4.2\%$) levels over controls. A reduction in ANG II-stimulated MEK and ERK activation by RES pretreatment was evident, with statistical significance being achieved at 25 μM RES for P-MEK ($96.1 \pm 0.5\%$ reduction) and at 10 and 25 μM RES for P-ERK
FIGURE 8. Pretreatment with resveratrol (RES) inhibits mitogen/extracellular signal-regulated kinase (MEK) and extracellular signal-regulated kinase (ERK) activation in cardiac fibroblasts (CFs). A: Representative Western blot demonstrating the effects of 30-min pretreatments with 5, 10, and 25 μM RES on MEK phosphorylation (P-MEK). RES reduced basal P-MEK levels in a concentration-dependent manner, and MEK phosphorylation was inhibited by 25 μM RES in CFs stimulated for 20 min with 100 nM ANG II. B: Representative Western blot demonstrating the effects of 30-min pretreatments with RES on ERK 1/2 phosphorylation (P-ERK). RES reduced both basal and ANG II-stimulated P-ERK levels in a concentration-dependent manner. C: Total ERK was unchanged across all conditions, indicating equal protein loading. [RES], RES concentration. D-E: Densitometric analysis of P-MEK and P-ERK levels expressed as % untreated controls. For ERK 1/2, densitometric graphs represent only the 42-kDa band, although similar trends were found for both bands. Data are pooled from 3 experiments incells isolated from separate animals (n = 3) and are expressed as % basal phosphorylation. **P < 0.01, and ***P < 0.001, statistically significant vs. basal levels. †††P < 0.001, significant reduction vs. 100 nM ANG II alone. Significant differences between conditions were determined by a one-way ANOVA and Tukey’s multiple-comparison test.
(26.5 ± 1.5 and 54.6 ± 0.9% reduction in P-ERK levels, respectively). Total ERK levels were unchanged in all conditions, indicating equal protein loading (Fig. 8C). Summary graphs displaying the effects of RES on MEK and ERK activation are shown in Fig. 8, D and E, respectively. We conclude that the inhibition of basal and hormone-stimulated MEK and ERK phosphorylation is a major mechanism by which RES affects mitogenic signaling in CFs.

**RES inhibits growth and proliferation of isolated adult rat CFs**

Our next set of experiments was designed to determine whether the blockade of MAPK activation by RES inhibited growth and proliferation of CFs. To achieve this end, we pretreated cultured CFs with 5, 10, and 25 μM RES in the presence and absence ANG II (100 nM), a known potent mitogen for CFs. Figure 9 demonstrates that 48 h of stimulation of CFs with ANG II increased [3H]-leucine and [3H]-thymidine incorporation by 59.8 ± 5.1 and 34.7 ± 5.6%, respectively (Fig. 9, A and B). RES significantly inhibited ANG II-induced [3H]-leucine incorporation at 10 μM (13.9 ± 5.0% reduction) and 25 μM (37.7 ± 4.5% reduction, Fig. 9A), whereas 25 μM RES significantly lowered [3H]-thymidine incorporation (49.4 ± 3.8% reduction, Fig. 9B). In addition, we observed reductions in basal, unstimulated [3H]-thymidine incorporation in CFs treated with 10 μM (36.9 ± 8.6% reduction) and 25 μM (56.4 ± 12.9% reduction, Fig. 9B) RES, but reductions in basal [3H]-leucine incorporation were not evident. We also determined that RES pretreatment did not alter CF morphology, cause cell detachment, or cause DNA laddering throughout the entire treatment period at any of the concentrations used (data
FIGURE 9. RES attenuates ANG II-stimulated $[^3]H$-leucine and $[^3]H$-thymidine uptake in cultured rat CFs in a concentration-dependent manner. A: $[^3]H$-Leucine uptake in response to 48 h treatment with 5, 10, or 25 μM RES in combination with 100 nM ANG II normalized to unstimulated controls (basal = 100%). RES reduced ANG II-stimulated $[^3]H$-leucine uptake in a concentration-dependent manner, but treatment with RES alone had no effect on basal $[^3]H$-leucine uptake. B: $[^3]H$-Thymidine uptake in response to 48 h treatment with RES and 100 nM ANG II. RES reduced ANG II-stimulated $[^3]H$-thymidine uptake in a concentration-dependent manner, reaching statistical significance at 25 μM. A concentration-dependent reduction in basal $[^3]H$-thymidine uptake was also evident. Data are pooled from 3 experiments, each done in triplicate in cells isolated from separate animals, and are expressed as % vehicle-treated controls (calculated by pooling cpm/well). *$P < 0.05$ and ***$P < 0.001$, statistically significant vs. basal levels. †*$P < 0.05$ and †††*$P < 0.001$, significant reduction vs. 100 nM ANG II alone. Significant differences between conditions were determined by Tukey’s multiple-comparison test.
not shown). The effects of RES on \(^{3}H\)-leucine and \(^{3}H\)-thymidine incorporation are therefore not the result of toxicity or cell death. Given our previous findings that ANG II-induced proliferation is completely inhibited by pharmacological inhibition of ERK 1/2 signaling (Stockand and Meszaros 2003) and the data presented in Figs. 1 and 2, we conclude that the blockade of the ERK 1/2 signaling cascade is a major target for RES to achieve its inhibitory effect on CF proliferation.

**RES attenuates epidermal growth factor-induced ERK 1/2 activation in CFs**

We next sought to test whether the inhibitory actions of RES on ERK 1/2 signaling were the result of a specific blockade of the AT\(_1\)R or inhibition of downstream intracellular signaling. Epidermal growth factor (EGF) is a more robust stimulus for proliferation and activation of ERK 1/2 in isolated CFs, because of direct receptor tyrosine kinase-mediated activation of the MAPK cascade. Initially, we found that 25 \(\mu\)M RES was ineffective in reducing ERK 1/2 phosphorylation induced by 10 nM EGF (data not shown). Treatment with either 100 nM ANG II or 10 nM EGF was sufficient to induce a maximal ERK phosphorylation for the given hormones, but 10 nM EGF induced nearly a 20-fold greater phosphorylation of ERK 1/2 over basal compared with a 2-fold increase induced by 100 nM ANG II (Figure 10, A and B). As previously demonstrated in Fig. 8B, pretreatment with 25 \(\mu\)M RES inhibited the 100 nM ANG II-induced activation of ERK. It is conceivable that treatment with 10 nM EGF is sufficient to overcome the inhibition by 25 \(\mu\)M RES, since EGF is a more efficacious inducer of ERK 1/2 activation than ANG II. Figure 10B indicates that 0.5 and 0.2 nM EGF do not
FIGURE 10. RES inhibits epidermal growth factor (EGF)-induced ERK activation in CFs.  

A: Representative Western blot of P-ERK and total ERK levels in CFs after stimulation with 0.2–10 nM EGF for 10 min.  

B: Densitometric analysis of Western blots comparing ANG II- and EGF-induced ERK phosphorylation. Densitometric data for ANG II were obtained previously and carried over from Fig. 1 for comparison. The amount of ERK phosphorylation in CFs treated with 10, 5, 2, and 1 nM EGF was significantly elevated over 100 nM ANG II. ERK activation induced by 0.5 and 0.2 nM EGF was not significantly different from 100 nM ANG II. Data are pooled from 3 experiments in cells isolated from separate animals (n = 3) and are expressed as % basal phosphorylation. †P < 0.05, ††P < 0.01, and †††P < 0.001, statistical significance vs. 100 nM ANG II determined by Tukey’s multiple-comparison test.  

C: Representative Western blot of P-ERK and total ERK levels in CFs after 30 min pretreatment with 25 μM RES and stimulation with 0.5 and 0.2 nM EGF (the concentrations that did not show a significant elevation in ERK phosphorylation over 100 nM ANG II) for 10 min. The Western blot in C was taken from A and is representative of 3 experiments examining the effects of RES pretreatment on 0.5 and 0.2 nM EGF-induced ERK activation.  

D: Densitometric analysis of experiments represented in C. Data are pooled from 3 experiments in cells isolated from separate animals (n = 3) and are expressed as %basal phosphorylation. †P < 0.05, statistical significance between means determined by a t-test.
significantly induce ERK 1/2 phosphorylation greater than that induced by treatment with 100 nM ANG II. Pretreatment with 25 μM RES was indeed effective in reducing ERK 1/2 activation in CFs stimulated with 0.2 nM EGF (60.6 ± 11.1% reduction, Fig. 10, C and D). Given the data for EGF-induced ERK 1/2 activation, we conclude that the effects of RES are not specific for ANG II signaling and that RES acts as a nonspecific, general inhibitor of ERK 1/2 signaling in CFs.

**RES does not affect Akt/p70S6K signaling in CFs**

Akt has previously been determined to be an important mediator of ANG II-induced proliferation in VSMCs (Dugourd 2003), and RES has been shown to inhibit both Akt and p70 ribosomal S6 kinase in VSMCs, both of which are activated by 100 nM ANG II in this cell type (Haider 2002). **Figure 11A** demonstrates that treatment with 10 or 25 μM RES does not reduce phosphorylated levels of Akt by examining two distinct sites at which phosphorylation is required for activation. Figure 11A also indicates that RES has no effect on phosphorylation of p70S6K, a downstream effector of Akt. In addition, we determined that treatment with 100 nM ANG II did not induce Akt or p70S6K phosphorylation over basal levels in CFs. Figure 11B confirms that 100 nM ANG II is sufficient to activate Akt in VSMCs, and, compared with Fig. 11A, it is clear that this pathway is not activated by 100 nM ANG II in CFs. Thus we conclude that 100 nM ANG II and 10 or 25 μM RES do not affect Akt signaling in CFs, indicating that this pathway is not playing a major role in proliferation of CFs at the concentrations of ANG and RES used in the experiments. These results indicate cell type-specific differences in
FIGURE 11. RES does not affect protein kinase B (Akt) or p70S6K phosphorylation in CFs. A: Representative Western blot demonstrating the effects of 10 or 25 μM RES followed by stimulation with 100 nM ANG II for 5 min on phosphorylated Akt (P-Akt) in CFs at Ser\textsuperscript{473} and Thr\textsuperscript{308} and phosphorylated p70S6K (P-p70S6K). Total p70S6K levels are unchanged, indicating equal protein loading. RES had no effect on P-Akt at either Ser\textsuperscript{473} or Thr\textsuperscript{308} or P-p70S6K. B: Representative Western blot demonstrating phosphorylation of Akt at Ser\textsuperscript{473} and Thr\textsuperscript{308} by 100 nM ANG II in vascular smooth muscle cells (VSMCs). Western blots are representative of 3 experiments in cells isolated from separate animals (n = 3). The appearance of multiple bands is the result of some nonspecific binding of the primary antibody.
A  

Cardiac Fibroblasts

P-Akt (Ser 473)  
P-Akt (Thr 308)  
P-p70S6K  
p70S6K

100 nM Ang II  
-  + +  +  +  + 
+ 10 uM RES  + 25 uM RES

B  

VSMCs

P-Akt (Ser 473)  
P-Akt (Thr 308)

100 nM Ang II  
-  +
the effects of both ANG II and RES, specifically highlighting an important contrast between VSMCs and CFs.

**Passage of cultured cardiac fibroblasts increases myofibroblast differentiation**

Early passage adult cardiac fibroblasts typically express low levels of \( \alpha \)-smooth muscle actin and collectively maintain the properties of undifferentiated fibroblasts. Cultured CFs typically respond to angiotensin II by proliferating and differentiating to myofibroblasts. However, as cultures are amplified by trypsinization and replating, the growth properties markedly change. Higher passage cells (P4 and P5) do not proliferate in response to ANG II stimulation. **Figure 12** indicates that as cultured CFs are split and replated, the expression of \( \alpha \)-SMA increases dramatically. This finding indicates that CFs will differentiate in culture to the myofibroblast phenotype and in order to avoid complicating factors, all cells used in subsequent proliferation and differentiation studies are low passage cells (P2).

**RES reduces ANG II- and TGF-β-induced cardiac myofibroblast differentiation**

Because of the recent appreciation that myofibroblasts contribute to the development of cardiac fibrosis, we next investigated the effects of RES on cardiac myofibroblast differentiation. The expression and organization of \( \alpha \)-SMA are hallmarks of myofibroblast differentiation, and we have assessed the expression levels of \( \alpha \)-SMA after 48- to 72-h treatments with either 200 pM TGF-β or 100 nM ANG II in the presence and absence of 25 \( \mu \)M RES. **Figure 13A** displays immunocytochemical \( \alpha \)-SMA staining
FIGURE 12. Expression of α-smooth muscle actin increased as passage of cultured CFs increased (P2-P5). This indicates that adult CFs are capable of differentiating in culture and therefore, to avoid this phenomenon, all differentiation studies are performed on passage 2 cells.
FIGURE 13. RES prevents ANG II- and transforming growth factor (TGF)-β-induced cardiac myofibroblast differentiation. A: Immunocytochemical staining of CFs using specific α-smooth muscle (α-SMA) actin primary antibodies. Treatment of CFs with 200 pM TGF-β for 48 h caused a marked increase in α-SMA expression and organization, an effect that was attenuated by pretreating CFs with 25 μM RES for 30 min. There were no observable differences in α-SMA expression and organization between untreated CFs and cells treated with 25 μM RES alone. Immunocytochemical data representative of 3 experiments in cells isolated from separate animals (n = 3). B: Representative Western blot showing the effects of 100 nM ANG II or 200 pM TGF-β on α-SMA expression in CFs with and without 25 μM RES pretreatment. C: Summary data displaying % inhibition by 25 μM RES of ANG II- and TGF-β-induced α-SMA expression. RES was effective in attenuating the increase in α-SMA levels induced by ANG II or TGF-β. Data are pooled from 3 independent experiments in cells isolated from separate animals (n = 3) and are expressed as % inhibition of α-SMA by RES. †Statistical significance vs. ANG II or TGF-β alone was determined by a t-test.
in CFs treated with TGF-β with and without RES pretreatment for 30 min. We selected TGF-β as an agonist, since it has been shown repeatedly to be a potent inducer of in vitro cardiac myofibroblast differentiation (Petrov et al. 2002; Lijnen et al. 2003). Pretreatment of CFs with RES was effective in limiting the staining intensity and organization of α-SMA induced by TGF-β. Differences in cell number are apparent between treatment conditions, which would be expected, since the agonists used affect both proliferation and differentiation. Figure 13B contains the representative Western blot demonstrating the expected increase in α-SMA levels induced by TGF-β and ANG II over vehicle-treated controls. Figure 13C shows an inhibition in ANG II- and TGF-β-induced α-SMA expression when CFs are pretreated with 25 μM RES (44.7 ± 5.2% reduction vs. ANG II alone, and 27.4 ± 8.4% reduction vs. TGF-β alone). Both the immunocytochemistry and Western blot data indicate that 25 μM RES was effective in limiting CF differentiation to the highly active myofibroblast phenotype, which identifies another beneficial effect of RES in limiting the myofibroblast contribution to cardiac fibrosis.

**DISCUSSION**

The current study was designed to determine whether RES has direct effects on CF activation and function. We have examined key early events that occur during CF activation, proliferation, and myofibroblast differentiation. CFs have been reported to respond to a variety of hormonal stimuli via proliferation, which predisposes the myocardium to excessive ECM production and ultimately cardiac fibrosis. Differentiation of these cells to a myofibroblast phenotype can contribute to hypersecretion of ECM
proteins as well, and we hypothesize that the myofibroblasts secrete the majority of the myocardial collagen and other ECM proteins. Therefore, interventions that target CF proliferation and differentiation to myofibroblasts represent a viable therapeutic avenue in the prevention of cardiac fibrosis.

We report here that RES, when administered to cultured CFs, inhibits proliferation by attenuation of ERK 1/2 signaling. The MAPK family has been shown to be involved in a variety of chronic disease states and to play a major role in cardiac hypertrophy. Agents that are effective in attenuating MAPK signaling cascades, particularly those that are naturally occurring compounds, may prove to be viable therapeutic tools in the prevention of many hyperplastic and/or hypertrophic diseases. The anti-proliferative effects of RES have been examined intensively, and several studies in various cancer cell lines and smooth muscle cells have determined that the ERK 1/2 cascade is a key pathway targeted by RES (El-Mowafy and White 1999; Yu et al. 2001; Haider et al. 2002). Results from the present study provide further support that RES targets the ERK 1/2 pathway in CFs by directly inhibiting MEK activation. However, the precise mechanism by which RES acts is poorly understood and likely involves multiple intracellular targets.

CF proliferation is vital for ventricular remodeling, and several studies have identified the hormones and growth factors that mediate this process. Growth factor-induced proliferation of CFs is mediated by the classic MAPK signaling pathway through phosphorylation of Raf, MEK, and ERK. G protein coupled receptors, including the AT1R, have been postulated to activate MAPKs by transactivation of the EGF receptor.
(EGFR) in both CFs and VSMCs, and this transactivation is necessary for ERK activation, DNA synthesis, and protein production (Eguchi et al. 2001; Kim et al. 2002; Voisin et al. 2002). Data obtained in the current study indicate that RES is effective in inhibiting ERK phosphorylation regardless whether the initial stimulus is via the AT$_1$R or the EGFR. However, the mitogenic response resulting from ANG II stimulation may only be partially attributed to transactivation of the EGFR, since ANG II activates multiple signaling pathways to induce mitogenesis, and there likely exist other targets of RES upstream of MEK and distinct from the classic MAPK cascade that still remain to be identified.

One target of RES and the mitogenic pathway activated by ANG II in VSMCs is the phosphatidylinositide 3-kinase (PI3-kinase)/Akt pathway. Akt has a multitude of effector proteins and is involved in a number of cellular processes, including proliferation and survival via inhibition of proapoptotic proteins. Akt induces hypertrophy of VSMCs by activating p70S6K. Treatment of VSMCs with RES was found to be an effective method in preventing ANG II-induced hypertrophy by inhibiting phosphorylation of PI3-kinase, the kinase directly upstream of Akt/PKB (Haider et al. 2002). These data suggest that RES is likely exerting beneficial effects on the cardiovascular system by interacting with multiple signaling pathways and is not limited strictly to the MAPK cascade. Our findings indicate that levels of phosphorylated Akt are unaffected by 100 nM ANG II in CFs, which agrees with a previous study in which the investigators had determined that the concentration of ANG II that would activate Akt over basal in CFs was in the micromolar range, far above the concentration of interest in the current study (Tian et al.
We found that neither 10 nor 25 μM RES had any effect on Akt/p70S6K signaling in CFs, results that highlight important cell-specific differences in the inhibitory actions of RES.

We and other investigators postulate that myofibroblast differentiation is stimulated by a number of hormonal and non-hormonal factors, and, even in the normal myocardium, these hypersecretory cells are important players in the wound healing and remodeling processes. CFs and myofibroblasts produce and secrete TGF-β, a potent inducer of differentiation (Campbell and Katwa 1997). TGF-β acts in a paracrine fashion to stimulate myofibroblast differentiation and a concurrent production of collagen (Lijnen et al. 2000). Under normal circumstances, the myofibroblasts are removed from the wound site by apoptosis (Desmouliere et al. 1995). However, a lack of apoptosis of myofibroblasts leads to an overproduction of ECM proteins because of their prolonged presence (Gabbiani 2003). In a cardiac pressure-overload hypertrophy model, increased fibrosis, myofibroblast number, and TGF-β mRNA were observed from 3 to 28 days after suprarenal aortic constriction (Kuwahara et al. 2002). Inhibition of TGF-β function by the injection of anti-TGF-β antibodies resulted in a reduction in both fibrosis and myofibroblast number as well as a reduction in type I and III collagen mRNA. Prevention of cardiac myofibroblast differentiation may therefore represent a potential target for therapies aimed at limiting fibrosis in the heart. In the present study, we found that pretreatment of cultured CFs with RES was effective in preventing myofibroblast differentiation induced by ANG II and TGF-β.
In conclusion, we have determined that RES directly inhibits two critical stages of CF activation, proliferation, and differentiation to myofibroblasts. Both of these physiological parameters are key determinants of cardiac fibrosis, and limiting these activation steps can greatly reduce the overall production of ECM components within the myocardium. This study therefore contributes additional evidence identifying RES as a cardioprotective agent by due to its anti-fibrotic activity. We cannot conclude that consumption of red wine will result in similar favorable effects on CF activity *in vivo*, since there are compounding factors which may limit the amount of RES that reaches the myocardium. Not only do metabolism and distribution affect the amount of RES in the heart, but the actual concentration of the RES in the wine can be highly variable and might not be high enough to achieve the micromolar range in the myocardium. However, we have determined that direct treatment of cardiac fibroblasts with resveratrol does prevent excessive CF activity, which has implications for limiting aberrant remodeling and fibrosis in the myocardium.
CHAPTER THREE
MECHANISM OF ANGIOTENSIN II-INDUCED PROLIFERATION AND ERK 1/2 ACTIVATION IN ADULT RAT CARDIAC FIBROBLASTS

INTRODUCTION

Cardiac remodeling involves changes in both the cellular and extracellular composition of the myocardium. The regulation of extracellular matrix (ECM) remodeling is primarily mediated by cardiac fibroblasts (CFs), the major non-muscle cell type of the heart (Brilla et al. 1995). CFs control matrix turnover in non-pathological states, and their activity is greatly enhanced following an acute cardiac event or during chronic cardiovascular disease states (i.e. myocardial infarction or hypertension, respectively, see (Capasso et al. 1990; Cleutjens et al. 1995; Funck et al. 1997; Sun and Weber 2000)). Active CFs migrate to sites of damage, proliferate, differentiate to myofibroblasts, and secrete large amounts of ECM proteins to repair the myocardium (Lorena et al. 2002). Aberrant remodeling will cause cardiac fibrosis, a condition characterized by reduced contractility and disruption of the electrical circuitry by ECM proteins, which ultimately contributes to heart failure (reviewed in Burlew and Weber 2002). Proliferation is a key early step in CF activation and a major contributing factor to the collective fibrotic potential of CFs.

A common occurrence during cardiovascular disease is the systemic elevation of angiotensin II (ANG II). ANG II has been shown to be a potent activator of CF activity, and in cultured CFs, activation of the ANG II type 1 receptor (AT\textsubscript{1}R) stimulates both cellular proliferation and de novo collagen synthesis (Schorb et al. 1993; Schorb et al.
1995; Dostal et al. 1996; Brilla et al. 1997; Swaney et al. 2005). Our lab and others have demonstrated the mitogenic properties of ANG II on CFs, but the exact signaling mechanisms by which ANG II induces proliferation of CFs are not fully understood (Schorb et al. 1993; Schorb et al. 1995; Olson et al. 2005). ANG II-induced proliferation of CFs is dependent upon activation of the extracellular signal-regulated kinase (ERK) 1/2 cascade (Schorb et al. 1995; Stockand and Meszaros 2003), but the precise signaling mechanisms utilized by ANG II to induce ERK 1/2 activation are unknown in adult CFs.

Stimulation of the G_q-coupled AT_1R causes activation of protein kinase C through phospholipase Cβ mediated production of diacylglycerol and release of intracellular calcium. PKC translocation to the plasma membrane allows for interaction with Ras and direct phosphorylation of Raf-1 to initiate MAPK activation. In addition, several cellular processes are calcium dependent and calcium has multiple intracellular targets which may be critical in mediating ANG II-induced ERK 1/2 activation. Since the transient release of calcium into the cytosol is a major result of ANG II stimulation in CFs, it is likely that Ca^{2+} is involved in initiating ERK 1/2 signaling.

Transactivation of the epidermal growth factor receptor (EGFR) has been identified as an intermediate step in ERK 1/2 activation for a number of G protein coupled receptors (GPCRs), including the AT_1R in vascular smooth muscle cells (VSMCs) and in neonatal CFs, as well as the G_q-coupled β_2-adrenergic receptor (β_2-AR) in adult CFs (Eguchi et al. 1998; Wang et al. 2000; Kim et al. 2002; Voisin et al. 2002). The mechanism of AT_1R-induced transactivation has been determined to be dependent on the soluble tyrosine kinase c-Src and intracellular Ca^{2+} in VSMCs (Eguchi et al. 1998;
Bokemeyer et al. 2000). In adult CFs, the $\beta_2$-AR stimulates EGFR transactivation via c-Src-dependent activation of matrix metalloproteinases and cleavage of a membrane-associated heparin-binding EGF-like growth factor (Prenzel et al. 1999; Kim et al. 2002). In addition, Kim et al. (2002) identified phosphatidylinositol 3-kinase (PI3-K) as an upstream activator of $\beta_2$-AR-induced ERK 1/2 activation in adult CFs, since inhibition of PI3-K by wortmannin attenuated isoproterenol-induced ERK 1/2 phosphorylation. Phosphorylation of phospholipids resulting from PI3-K activation has been suggested to provide a recruitment signal or membrane docking site for protein kinase C$\zeta$ (PKC$\zeta$), an atypical PKC which is not activated by phorbol esters and is insensitive to Ca$^{2+}$ and diacylglycerol (Nakanishi et al. 1993; Newton 1995; Mellor and Parker 1998). In VSMCs and in MCF-7 cells, protein kinase C$\zeta$ (PKC$\zeta$) is critical in mediating ANG II-induced ERK 1/2 activation and cellular proliferation, since inhibition of PKC$\zeta$ with an inhibitory myristoylated pseudosubstrate and downregulation of PKC$\zeta$ using antisense oligonucleotides both resulted in a loss of ANG II-induced ERK 1/2 phosphorylation (Liao et al. 1997; Muscella et al. 2003). Additionally, PKC$\zeta$ has been identified as a mediator of proliferation in neonatal rat cardiac fibroblasts, since its selective inhibition reduced both basal and transforming growth factor-β-induced $[^3]$H-thymidine incorporation (Braun and Mochly-Rosen 2003).

The goal of the current study was to identify the intracellular mediators of ANG II-induced proliferation and ERK 1/2 activation in adult rat cardiac fibroblasts. Our initial hypothesis was that ANG II activates ERK 1/2 and stimulates proliferation in a manner which is mechanistically dependent upon either EGFR transactivation or an alternative
mitogenic pathway involving PKCζ. Our findings demonstrate that transactivation of the EGFR by ANG II does not occur in adult CFs, whereas functional PKCζ is required for ANG II to induce proliferation. Sequestering the intracellular calcium released by ANG II or downregulating PKCs using PMA did not have any impact on ERK 1/2 activation. However, inhibition of both events concurrently did block ERK 1/2 phosphorylation, indicating parallel pathways induced by activation of Gq were both responsible for mediating the effect of ANG II. These data suggest that diverse signaling pathways exist between cell types for ANG II-induced ERK 1/2 activation, and that the signaling intermediates utilized by ANG II in adult rat cardiac fibroblasts are distinct from those activated by other GPCR agonists.

**MATERIALS AND METHODS**

**Materials**

Dulbecco’s Modified Eagle’s Medium (DMEM), penicillin/streptomycin, fungizone and fetal bovine serum (FBS) were all purchased from Invitrogen/GIBCO (Grand Island, NY). [3H]-Thymidine was from ICN Biomedicals (Irvine, CA). Anti-phospho-tyrosine, anti-phospho-PKCζ, anti-PKCα, and anti-EGFR antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-ERK 1/2 and anti-ERK 1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail I, protein A agarose, AG 1478 and anti-PKCζ antibodies were from Sigma-Aldrich (St. Louis, MO). Rat adsorbed HRP-conjugated goat anti-mouse secondary antibodies were obtained from Serotec
(Raleigh, NC). HRP-conjugated rabbit anti-mouse secondary antibodies and Hybond nitrocellulose membrane were purchased from Amersham Biosciences (Piscataway, NJ). LY 294002, PP2, myristoylated PKCζ pseudosubstrate, Fura-2/AM (1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N′N′-tetraacetic acid penta(acetoxymethyl) ester), and BAPTA/AM (1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester) were from Calbiochem (La Jolla, CA). All other chemicals were reagent grade and obtained from Fisher Scientific (Pittsburgh, PA).

**Isolation of adult rat ventricular cardiac fibroblasts**

Primary cultured cardiac fibroblasts were isolated from the left ventricles of anesthetized adult male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) exactly as described in Chapter 2.

**Measurement of intracellular calcium release**

Fibroblasts were plated on 35-mm glass coverslips. The cells were washed once in phosphate-buffered saline and incubated in 1 ml of DMEM containing 1 μM Fura-2/AM at 37 °C for 30 min. Cells were then washed once with DMEM and placed in a 37°C chamber containing 1.5 ml of HEPES buffered saline (HBS: 130 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM MgCl₂, 1.0 mM CaCl₂, 25 mM HEPES, pH 7.4), such that groups of 5–8 cells could be viewed using an inverted Olympus IX-70 microscope. Spectrofluorometric measurements were collected using Delta Scan System.
spectofluorometer (Photon Technology), where the field was excited at 380 and 340 nm and the emission ratio was collected at 511 nm. Agonists were administered from 1000x stocks to maintain a constant volume of 1.5 ml.

$[^3]$H-Thymidine incorporation assay

CFs were plated on 12-well tissue culture plates and grown to approximately 50% confluency. Cells were treated in triplicate for a period of 48 hrs, with $[^3]$H-thymidine (2 µCi/ml) being added to the media during the final 4 hrs. The media containing the label was removed and the cells were washed twice with ice-cold PBS and incubated in cold 5% TCA for 30 min. Cells were washed quickly with ice-cold 95% ethanol and solubilized in 1 ml 0.5 M sodium hydroxide for 30 min at room temperature. The amount of incorporated label was determined by liquid scintillation counting.

Protein isolation and Western analysis

Whole cell lysates were collected in lysis buffer (62.5 Tris-HCl, 2mM EDTA, 2.3% SDS, 10% glycerol, pH 6.8, Protease Inhibitor Cocktail: contains AEBSF, pepstatin A, E-64, bestatin, leupeptin, aprotinin, and Phosphatase Inhibitor Cocktail 1: contains cantharidin, bromotetramisole, microcystin LR) following indicated treatments and total protein content was determined by the bicinchoninic acid method (BCA, Pierce, Rockford, IL). Equal amounts of protein samples were separated by SDS-PAGE on 10% acrylamide gels and transferred to nitrocellulose using standard techniques. The membranes were then blocked for 1 h at room temperature in blocking buffer (1% BSA
in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20) and incubated overnight at 4°C with primary antibodies diluted in blocking buffer. Membranes were washed extensively the following day and incubated for 1-2 hrs with appropriate secondary antibodies conjugated to horseradish peroxidase. Signals were detected by chemiluminescence and densitometric data from western blots were obtained and quantified using a Kodak 1D Digital Science Imaging System.

**Immunoprecipitation of the EGFR**

CFs were treated as indicated, washed with ice-cold PBS and then scraped on ice in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM Na₃VO₄, and Protease Inhibitor Cocktail, pH 7.5). Cell lysates were sonicated 3 times for 5 sec, centrifuged at 10,000 x g for 10 min at 4°C and supernatants collected. Equal amounts of protein (500 μg) were incubated with anti-phospho-tyrosine antibodies overnight with end-over-end rotation at 4°C. The following day, protein A-agarose beads were added to the lysates and rotated for an additional 2 hrs at 4°C. Samples were then centrifuged for 30 sec at 12,000 x g and pellets were washed on ice 5 times with lysis buffer. Antibody complexes were dissociated by incubating with 3X sample buffer (187.5 Tris-HCl, 6% SDS, 30% glycerol 150 mM DTT, 0.03% bromophenol blue, pH 6.8) and boiling 5 min. Samples were centrifuged and supernatants subjected to SDS-PAGE and Western analysis as described using 7.5% polyacrylamide gels.
**Plasma membrane translocation of PKC**

CFs were treated, washed with ice-cold PBS and collected on ice in Buffer A (20 mM Tris, 250 mM sucrose, 1 mM EDTA, Protease Inhibitor Cocktail, and Phosphatase Inhibitor Cocktail 1). Cells were sonicated 3 x 5 sec followed by centrifugation at 1000 x g for 10 min at 4°C and the supernatants were then transferred to ultracentrifuge tubes. Cell lysates were then spun at 100,000 x g for 1 hr at 4°C. The supernatants containing the cytosolic fraction were transferred to fresh tubes while the pellets containing the membranes were resuspended in Buffer B (Buffer A containing 0.8% Triton-X 100) and kept on ice for 45 min. Cytosolic and membrane fractions were then subjected to SDS-PAGE and Western analysis as described.

**Data Analysis/Statistics**

Data analysis was performed using GraphPad Prism 4.0 statistical analysis software (GraphPad Software, San Diego, CA). Statistical significance between treatments was determined by analysis of variance (ANOVA) with Tukey’s multiple comparison test.

**RESULTS**

**Angiotensin II induces cardiac fibroblast proliferation and ERK 1/2 activation in an EGFR- and Src-independent manner**

EGFR transactivation is a common mechanism for MAPK activation and proliferation by ANG II and a number of other GPCR ligands in several cell types. Stimulation of cardiac fibroblasts with ANG II resulted in a 44.6 ± 2.6% increase in [³H]-
FIGURE 14. Angiotensin II-induced cardiac fibroblast proliferation is not EGFR-dependent. [\(^{3}\text{H}\)]-Thymidine incorporation was used to determine whether treatment with 1 μM AG 1478 30 min prior to hormone stimulation inhibited 100 nM ANG II- or 10 nM EGF-induced proliferation of serum-starved CFs over 48 hrs. AG completely blocked [\(^{3}\text{H}\)]-thymidine incorporation induced by EGF, but had no significant effect on ANG II-induced proliferation. Each independent experiment was performed in triplicate. Data were pooled from 3 separate experiments each using cells from different animals (n=3) and are expressed as percent of basal ± SEM. *p < 0.05, statistically significant vs. Basal; †p < 0.05, statistically significant vs. EGF. Significant differences between conditions were determined by one-way ANOVA and Tukey’s multiple comparison test.
[\textsuperscript{3}H]-Thymidine Incorporation (% Basal)

- Basal
- 1 μM AG 1478
- 100 nM ANG II
- AG + ANG II
- 10 nM EGF
- AG + EGF

*: Statistically significant difference compared to Basal.
thymidine incorporation over basal (Figure 14). In CFs pretreated with AG 1478 (EGFR kinase inhibitor) 30 min prior to ANG II stimulation, the measured increase in [\(^3\)H]-thymidine incorporation was 30.2 ± 6.2% over basal and was not significantly different from ANG II alone. However, EGF-induced proliferation (75.3 ± 19.6% over basal) was completely inhibited by pretreatment with AG 1478, as expected.

Since ERK 1/2 is a common mediator of both ANG II- and EGF-induced CF proliferation, our next goal was to determine whether ERK 1/2 phosphorylation was affected by AG 1478 pretreatment. Figure 15, panel A demonstrates that AG 1478 blocked the EGF-induced increase in phosphorylated ERK 1/2 (107.2% reduction), but had no effect on phosphorylation induced by ANG II (21.2% reduction, not significant vs. ANG II alone). This finding suggests that ANG II activates ERK 1/2 through a pathway that does not involve transactivation of the EGFR. To confirm this hypothesis, immunoprecipitation of phospho-tyrosine containing proteins in cell lysates was performed followed by Western blotting for the EGFR. Phosphorylation of the EGFR was not induced by ANG II, since densitometric analysis indicated that the levels of tyrosine phosphorylation were not significantly different from basal (Figure 15B).

Src is a common intermediate of ANG II-induced mitogenesis and ERK 1/2 activation through transactivation of the EGFR (Bokemeyer et al. 2000). Inhibition of c-Src signaling by pretreating CFs with 1 μM PP2 had no effect on either ANG II-induced [\(^3\)H]-thymidine incorporation or on ERK 1/2 activation (Figure 16A, B), indicating that c-Src is likely not playing a role in ANG II-induced proliferation of CFs. This c-Src inhibitor has been previously used in other studies at concentrations as high as 10-40 μM.
FIGURE 15. ANG II activates ERK 1/2 via an EGFR-independent mechanism. A: Pretreatment of CFs with 1 μM AG 1478 for 30 min was effective in blocking the activation of ERK 1/2 induced by stimulation with 10 nM EGF for 5 min. However, AG pretreatment had no significant effect on 100 nM ANG II-induced ERK 1/2 activation. Western blot is representative of 3 separate experiments each using cells from different animals (n=3). Densitometric data were pooled and expressed as percent of the ANG II response ± SEM. *p < 0.05, statistically significant vs. EGF. B: Stimulation with 10 nM EGF for 5 min induced EGFR phosphorylation as determined by immunoprecipitation. There was no significant tyrosine phosphorylation induced by 100 nM ANG II after 5 min. Western blot is representative of 3 separate experiments each using cells from different animals (n=3). Significant differences between conditions were determined by one-way ANOVA and Tukey’s multiple comparison test.
A. Basal 100 nM ANG II 10 nM EGF 1 μM AG 1478 AG + ANG II AG + EGF

B. IP: P-Tyr IB: EGFR

IP: EGFR IB: EGFR
FIGURE 16. c-Src does not play a role in ANG II-induced proliferation in cardiac fibroblasts. A: Pretreatment of CFs with 1 μM PP2 for 30 min prior to stimulation with 100 nM ANG II for 48 hrs did not block [³H]-thymidine incorporation. Data were pooled from 3 separate experiments each using cells from different animals (n=3) and are expressed as percent of basal ± SEM. B. Pretreatment with 1 μM PP2 for 30 min did not significantly reduce ERK 1/2 phosphorylation by 100 nM ANG II (5 min). Densitometric data were pooled and expressed as percent of the ANG II response ± SEM. C. Treatment of CFs with 20 μM PP2 was effective in partially inhibiting the activation of ERK 1/2 by ANG II. However, inhibition of EGF-induced ERK 1/2 activation was complete, indicating a loss of inhibitor specificity at this concentration. This effect on EGFR induced ERK 1/2 activation was not seen when PP2 was used at a concentration of 1 μM.

- Basal
- 100 nM ANG
- 1 μM PP2
- ANG II + PP2

B. P-ERK 1/2 (ERK 1/2 pERK/ERK % ANG Response)

- Basal
- 100 nM ANG
- 1 μM PP2
- ANG II + PP2

C. P-ERK 1/2

- Basal
- 100 nM ANG II
- 10 nM EGF
- 20 μM PP2
- PP2 + ANG II
- PP2 + EGF
to prevent downstream ERK 1/2 activation, concentrations which are well above the IC$_{50}$ for c-Src inhibition (4 nM). We investigated the effects of 20 μM PP2 to support our conclusion that c-Src did not mediate ANG II-induced ERK 1/2 activation. Although there was apparent reduction of ANG II-induced ERK 1/2 activation by this high concentration of PP2, the specificity of the compound had been lost. Pretreating CFs with 20 μM PP2 also inhibited EGF-induced ERK 1/2 activation, and since it is well established that the EGFR signaling pathway to ERK 1/2 does not require c-Src, this effect was likely due to non-specific inhibition of tyrosine kinase activity (Figure 16C).

**Inhibition of PI3-K and protein kinase Cζ reduces proliferation of adult CFs, but not ANG II-induced ERK 1/2 activation**

PI3-K has previously been identified as a potential upstream signaling mediator of ANG II-induced ERK 1/2 activation in several cell types. Inhibition of PI3-K with the selective inhibitor LY 294002 (LY, 10 μM) significantly reduced basal $[^3]$H-thymidine incorporation by 36.4 ± 4.5% (Figure 17A). CFs pretreated with LY displayed a significant reduction in $[^3]$H-thymidine incorporation in response to ANG II vs. treatment with ANG II alone (59.6% reduction). However, pretreatment with LY did not inhibit phosphorylation of ERK 1/2 by ANG II (Figure 17B), suggesting that the reduction in $[^3]$H-thymidine incorporation did not occur as a result of inhibition of ERK signaling. In addition, the ANG II-induced activation of PI3-K could not be demonstrated in isolated adult rat CFs since there was no appreciable increase in Akt phosphorylation, a signaling event which occurs downstream of PI3-K activation (Chapter 2). This finding is therefore
FIGURE 17. Inhibition of PI3-K blocks ANG II-induced \(^{3}\text{H}\)-thymidine incorporation but not ERK 1/2 activation. A: Pretreatment of CFs with 10 μM LY 294002 for 30 min prior to stimulation with 100 nM ANG II for 48 hrs reduced \(^{3}\text{H}\)-thymidine incorporation. ANG II-stimulated \(^{3}\text{H}\)-thymidine incorporation was significantly reduced, but the reduction in basal proliferation did not reach statistical significance. Data were pooled from 3 separate experiments each using cells from different animals (n=3) and are expressed as percent of basal ± SEM. *p < 0.05, statistically significant vs. Basal; \(^{*}\)p < 0.05, statistically significant vs. ANG II. B. Pretreatment with LY for 30 min did not significantly reduce ERK 1/2 phosphorylation by 100 nM ANG II (5 min). Densitometric data were pooled and expressed as percent of the ANG II response ± SEM. Significant differences between conditions were determined by one-way ANOVA and Tukey’s multiple comparison test.
A. 


- Basal
- 100 nM ANG
- 10 μM LY 294002
- ANG II + LY

B. 

P-ERK/ERK (% ANG Response)

- Basal
- 100 nM ANG
- 10 μM LY 294002
- ANG II + LY
in agreement with our previous data and suggests that the PI3-K likely acts in a signaling pathway outside of the AT₁R signaling cascade to ERK 1/2.

PKCζ has been shown to be activated through a PI3-K-dependent mechanism and to mediate ANG II-induced ERK 1/2 activation (Nakanishi et al. 1993; Liao et al. 1997; Muscella et al. 2003). Our goal was to determine whether PKCζ was critical for ERK 1/2 activation in CFs. We found that when cardiac fibroblasts were treated with a myristoylated inhibitory PKCζ pseudosubstrate (ζ-PS), ANG II-induced proliferation was inhibited (Figure 18, panel A, 50.8 ± 4.4% reduction). ζ-PS alone significantly reduced the basal [³H]-thymidine incorporation by 26.2 ± 3.8%. We determined, however, that ζ-PS had no effect on the ANG II-induced increase in ERK 1/2 phosphorylation (Figure 18B), which suggests that PKCζ is not downstream of the AT₁R in adult rat CFs. It is important to bring attention to the fact that these experiments were performed in DMEM containing 1% FBS. Interestingly, we consistently observed an increase in ERK 1/2 phosphorylation by treating with the ζ-PS alone when the cells were in serum-free DMEM (Figure 18C). A recent study has indicated that this effect may be due to the myristoylation of the peptide and not the peptide itself, since myristoylation of a scrambled ζ-PS had similar effects in endothelial cells (Krotova et al. 2006).

Typically, activation of PKCζ by ANG II involves translocation from the cytosolic compartment to the plasma membrane (PM). Figure 19 demonstrates that ANG II did not stimulate PM translocation of PKCζ. PMA also did not stimulate PKCζ translocation, as expected, since PKCζ lacks the phorbol ester sensitive domain common
FIGURE 18. Inhibition of PKCζ blocks ANG II-induced $[^3\text{H}]$-thymidine incorporation but not ERK 1/2 activation. 

*A:* Pretreatment of CFs with 10 μM ζ-PS for 30 min prior to 48 hr stimulation with 100 nM ANG II reduced $[^3\text{H}]$-thymidine incorporation. ANG II-stimulated $[^3\text{H}]$-thymidine incorporation and basal proliferation were significantly reduced. Data were pooled from 3 separate experiments each using cells from different animals (n=3) and are expressed as percent of basal ± SEM. *p < 0.05, statistically significant vs. Basal; †p < 0.05, statistically significant vs. ANG II.

*B:* Pretreatment with ζ-PS for 30 min had no effect on ERK 1/2 phosphorylation by 100 nM ANG II (5 min). These experiments were performed in the presence of 1% FBS.

*C:* Treatment with ζ-PS alone in serum-free medium induced ERK 1/2 phosphorylation maximally at 10 min. Phosphorylated ERK 1/2 levels remained above basal for at least 4 hrs.
**A.**

**[3H]-Thymidine Incorporation (%% Basal)**

- **Basal**
- **100 nM ANG II**
- **10 μM PKCζ-PS**
- **ζ-PS + ANG II**

**B.**

+ 1% FBS

- **Basal**
- **100 nM ANG II**
- **10 μM PKCζ-PS**
- **ζ-PS + ANG II**

**C.**

Serum-free conditions

- **Basal**
- **100 nM ANG II**
- **10 μM PKCζ-PS**
- **ζ-PS + ANG II**

**Notes:**

- *: P < 0.05
- †: P < 0.01

**Legend:**

- **P-ERK 1/2**
- **ERK 1/2**
FIGURE 19. ANG II does not induce membrane translocation of PKCζ. CFs were stimulated with 100 nM ANG II or 100 nM PMA for 20 min to induce plasma membrane translocation. Cytosolic and membrane fractions were separated as described in Materials and Methods and subjected to Western analysis. PKCζ did not translocate in response to ANG II or PMA. The classic isoform PKCα did translocate in response to PMA as expected, indicating that cytosolic and membrane fractions were successfully separated.
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<th>Cytosolic fraction</th>
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<tr>
<td>PKCζ 78 kDa</td>
<td>![Image of PKCζ]</td>
<td>![Image of PKCζ]</td>
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<tr>
<td>PKCα 80 kDa</td>
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<th>Condition</th>
<th>Basal</th>
<th>100 nM ANG II</th>
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to both the classic and novel isoforms of PKC. However, PMA did stimulate PKCα translocation, thus verifying the successful separation of cytosolic and PM fractions. Antibodies directed against PKCζ detected a higher molecular weight band at approximately 80 kDa, which is likely PKCα since this is the predicted molecular weight of the α isoform and since it translocated to the PM following PMA treatment. Based on PM translocation and the lack of ERK 1/2 inhibition with ζ-PS, we determined that PKCζ is unresponsive to ANG II, a finding that suggests that stimuli unrelated to the AT₁R may regulate the distribution and activation of PKCζ.

**Concurrent inhibition of calcium release and protein kinase C blocks angiotensin II-induced ERK 1/2 activation in cardiac fibroblasts**

The Gq-coupled AT₁R stimulates intracellular calcium release and production of diacylglycerol which then activates protein kinase C. Figure 20A demonstrates that ERK 1/2 activation in CFs can be achieved by stimulating classic and novel isozymes of PKC directly with 100 nM PMA for 5 min. These isozymes of PKC are sensitive to long term exposure to PMA and are downregulated after 18 hr of treatment. Figure 20B demonstrates the effect of long term 100 nM PMA treatment on PKCα expression, as a representative example of all PKCs with phorbol ester-sensitive domains. Expression of PKCζ, an atypical PKC which lacks a phorbol ester-sensitive domain, is unaffected by long term PMA treatment. We determined that following downregulation of classic and novel PKCs, ERK 1/2 is still phosphorylated in response to ANG II (Figure 20C).
FIGURE 20. ERK 1/2 is activated by direct stimulation of classic and novel PKCs, but inhibition of these isozymes did not block ANG II-induced ERK 1/2 phosphorylation. A: Representative Western blot indicating that stimulation of CFs with 100 nM PMA (5 min) induces ERK 1/2 activation over basal, indicating that PKC is upstream of ERK in CFs. B: Representative blot demonstrating that prolonged exposure to 100 nM PMA (18 hrs) downregulates phorbol ester-sensitive PKCα, indicated by a loss of band density in lysates from treated cells. C: Downregulation of classic and novel PKCs with 100 nM PMA (18 hrs) does not inhibit ANG II-induced ERK 1/2 activation.
Next, we employed an intracellular calcium chelator BAPTA/AM to sequester calcium released by ANG II, and found that 30 μM BAPTA/AM was required for complete inhibition (Figure 21A). Pretreatment of CFs with BAPTA/AM prior to stimulation with ANG II did not have any inhibitory effect on ERK 1/2 phosphorylation (Figure 21B). However, when CFs were pretreated with BAPTA/AM after 18 hr treatment with PMA, the ERK 1/2 response to ANG II did not rise significantly over basal (Figure 22). This finding suggests two parallel, yet convergent, pathways that mediate ANG II-induced ERK 1/2 activation, one via PKC and another via Ca^{2+}.

**DISCUSSION**

The current study was designed to identify the signaling mediators of angiotensin II-induced proliferation and ERK 1/2 activation in adult cardiac fibroblasts. Our goal was to determine the signaling intermediates utilized by ANG II to activate ERK 1/2. It is well established that ANG II-induced proliferation of CFs is dependent on ERK 1/2 phosphorylation, since it has been shown that selective inhibition of this pathway will attenuate this process (Stockand and Meszaros 2003). However, several of the intermediates between the AT_{1}R and ERK 1/2 in CFs remain to be elucidated.

PKC is a common intermediate between ERK 1/2 and Gq-coupled receptors like the AT_{1}R. Classic and novel PKCs are activated by diacylglycerol which is produced by Gq-activated phospholipase C. PKC interacts with the small G protein Ras and can directly phosphorylate Raf-1 to stimulate MAPK activation. The diacylglycerol sensitive domains make these isozymes of PKC sensitive to phorbol esters like PMA as well. Short term treatment activates classic and novel PKCs, but after prolonged stimulation they are
FIGURE 21. Chelation of intracellular calcium with BAPTA/AM does not prevent ANG II-induced ERK 1/2 activation. A: Intracellular calcium release in response to ANG II was detected by preloading CFs with 1 μM Fura-2/AM. Stimulation with 100 nM ANG II induced rapid calcium release from intracellular stores. Calcium release in response to ANG II was attenuated when cells were pretreated with the 30 μM of the calcium chelator BAPTA/AM. B: Representative Western blot indicating that treatment with 30 μM BAPTA/AM for 30 min prior to stimulation with 100 nM ANG II (5 min) had no effect on ANG II induced ERK 1/2 activation.
A.

B.

Basal
100 nM ANG II
30 μM BAPTA
30 μM BAPTA + ANG II

P-ERK 1/2
ERK 1/2
FIGURE 22. ANG II-induced ERK 1/2 activation is inhibited by PMA-induced PKC downregulation and intracellular calcium chelation simultaneously. A: Representative Western blot demonstrating that ANG II-induced ERK 1/2 activation is inhibited when intracellular calcium is blocked with 30 μM BAPTA/AM in PKC-downregulated CFs. Densitometric data from three separate experiments were pooled and expressed as percent of the ANG II response ± SEM. *p < 0.05, statistically significant vs. Basal. †p < 0.05, statistically significant vs. ANG II.
A.

B. 

C. 

D.
downregulated. We determined that downregulation of PKC with chronic PMA treatment was insufficient to prevent the ANG II-induced ERK 1/2 activation.

Intracellular calcium is liberated from stores when CFs are stimulated with ANG II. This second messenger has several intracellular targets, including multiple cytosolic tyrosine kinases like the c-Src family and the focal adhesion kinase/PYK2 family, many of which have been implicated in ERK 1/2 activation. Using an intracellular Ca\textsuperscript{2+} chelator BAPTA/AM, we were able to completely block the rise of intracellular calcium, but this treatment had no effect on ANG II-induced ERK 1/2 phosphorylation. Interestingly, when intracellular Ca\textsuperscript{2+} chelation and PKC downregulation were employed simultaneously, ERK 1/2 phosphorylation was significantly blocked. This finding indicates two convergent pathways involved in ERK 1/2 activation; one mediated through classic/novel PKC and another which is mediated by Ca\textsuperscript{2+}.

Activation of MAPK pathways by G protein coupled receptors has been attributed to the transactivation of epidermal growth factor receptors in several cell types, such as the AT\textsubscript{1}R in vascular smooth muscle cells and neonatal cardiac fibroblasts, as well as the β\textsubscript{2}-AR in adult cardiac fibroblasts (Bokemeyer et al. 2000; Kim et al. 2002; Braun and Mochly-Rosen 2003). This mechanism is dependent upon the actions of numerous intracellular signaling intermediates including G\textsubscript{i/o}-βγ subunits, intracellular Ca\textsuperscript{2+}, c-Src, reactive oxygen species, and matrix metalloproteases, which cleave membrane tethered heparin-binding EGF-like ligands to activate EGFR. The collective data all suggest that there is no universal mechanism for ERK 1/2 activation by agonists of GPCRs, but that the signaling pathway varies depending on the particular GPCR activated and on the cell
type studied. Our findings indicate that in adult cardiac fibroblasts, neither the EGFR nor c-Src is involved in signal transduction between the ANG II receptor and ERK 1/2.

The phosphatidylinositol 3-kinase/Akt pathway has also been implicated in ANG II-induced proliferation. In intestinal epithelial cells and in vascular smooth muscle cells, PI3-K mediates ANG II-induced proliferation in an ERK 1/2-independent mechanism (Dugourd et al. 2003; Chiu et al. 2005). Through pharmacological inhibition of PI3-K with 10 μM LY 294002, we demonstrated that ERK 1/2 activation by ANG II is not dependent on this pathway. We did however observe a significant reduction in $[^{3}H]$-thymidine incorporation following 48 hr exposure to LY. A similar effect of LY has also been reported in human CFs, although the mechanism by which LY exhibited its effects on $[^{3}H]$-thymidine incorporation was not determined (Hafizi et al. 1999). Activation of the Akt/PKB pathway promotes cell survival through inhibition of the downstream pro-apoptotic signaling protein BAD (Datta et al. 1997). Since LY is known to inhibit this pathway, it is likely that the reduction in basal $[^{3}H]$-thymidine incorporation is partially due to cell death during the treatment period. We did indeed observe a 20% reduction in the total number of adherent cells as a result of 48 hr exposure to 10 μM LY 294002 and a 2-4 fold increase in cell detachment compared to untreated cells, which suggests that LY is affecting cell survival (unpublished observations). However, any effect of LY on proliferation of CFs occurs in a pathway parallel to the MAPK cascade, since LY did not have any significant effect on ANG II-induced ERK 1/2 activation.

We were previously unable to detect activation of the PI3-K/Akt pathway in adult rat CFs stimulated with ANG II under serum-free conditions (Chapter 2 and (Olson et al.
A separate study demonstrated that ANG II could activate Akt in adult rat CFs, but in this case the investigators used low serum conditions (DMEM + 0.1% FCS) during treatment and 10-fold higher ANG II concentrations (Tian et al. 2003). Complete serum starvation increases the lipid content of phosphatidylinositide-3,4,5-trisphosphate (PI(3,4,5)P₃) and phosphatidylinositide-3,4-bisphosphate (PI(3,4)P₂) in wild type MDCK cells (Khwaja et al. 1997). Phosphorylation of the 3-position carbon indicates an increase in PI3-K activity when serum is removed. In addition, survival of cultured cells is often dependent on surface attachment via inhibition of pro-apoptotic proteins by PI3-K/Akt activation (Re et al. 1994; Delcommenne et al. 1998; Grigoriou et al. 2005). Delcommenne et al. (1998) demonstrated that integrin-linked kinase-1, which interacts with the cytoplasmic domain of β₁, β₂, and β₃ integrin subunits, activates Akt in a PI3-K-dependent manner. It is therefore likely that under the conditions utilized in this study the PI3-K/Akt pathway is being activated by attachment and serum starvation to such an extent that further activation by ANG II cannot be achieved.

The soluble tyrosine kinase c-Src is often involved in the activation of ERK 1/2 by GPCR agonists (Kim et al. 2002; Godeny and Sayeski 2006). ANG II-induced ERK 1/2 phosphorylation and EGFR transactivation have been demonstrated to be dependent on c-Src in several cell types. Our data indicate that inhibition of c-Src with 1 μM PP2, a selective c-Src inhibitor at this concentration, has no effect on the ANG II-induced ERK 1/2 response. Other studies have shown inhibition of the ANG II response with this and much higher concentrations of PP2, one as high as 40 μM (Wang et al. 2000). The reported IC₅₀ for PP2 inhibition of c-Src activity is approximately 4 nM. The compound
begins to lose specificity at concentrations above 1 μM and is known to inhibit other tyrosine kinases, including the membrane bound EGFR. Figure 19C demonstrates that 20 μM PP2 has a slight inhibitory effect on ANG II-induced ERK 1/2 activation, but can completely block activation by 10 nM EGF (with a reported IC$_{50}$ of 480 nM) in adult rat CFs. This finding reinforces the importance of using proper inhibitor concentrations and brings attention to the questionable importance of c-Src in ANG II signaling in other cells, specifically with regard to EGFR transactivation-dependent ERK 1/2 activation.

Protein kinase Cζ is now appreciated as an important mediator of ANG II-induced signaling. ERK 1/2 activation and cellular proliferation caused by ANG II stimulation is dependent on PKCζ in VSMCs, MCF-7 cells and neonatal rat cardiac fibroblasts (Liao et al. 1997; Braun and Mochly-Rosen 2003; Muscella et al. 2003). Initially, we believed PKCζ to be a potential mediator of ANG II induced ERK 1/2 activation in CFs. Since atypical PKCs are not downregulated in response to long term PMA treatment and ANG II induced ERK 1/2 phosphorylation under these conditions, PKCζ was a logical candidate to mediate the ANG II response. Our data indicate that proliferation of adult rat CFs induced by ANG II requires functional PKCζ, since addition of a myristoylated pseudosubstrate peptide blocked [³H]-thymidine incorporation, but that PKCζ is not upstream of ERK 1/2 in the ANG II mitogenic response. Typically, production of PI(3,4,5)P$_3$ from PI(4,5)P$_2$ by PI3-K provides a recruitment signal that induces PKCζ translocation to the plasma membrane (Nakanishi et al. 1993). However, our results demonstrate that this phenomenon does not occur when adult CFs are stimulated with ANG II. This result is consistent with our finding that ANG II did not stimulate Akt
FIGURE 23. Proposed model for ANG II-induced ERK 1/2 activation in adult cardiac fibroblasts. ANG II induces intracellular calcium release and activation of classic and novel PKCs through PLC-induced diacylglycerol production. Both calcium and PKC signal independently to ERK 1/2. PI3-K, c-Src, and the EGFR do not play roles in signal transduction between ANG II and ERK 1/2 in adult cardiac fibroblasts.
phosphorylation since both Akt activation and PKCζ translocation require PI3-K. Western analysis following subcellular fractionation of CFs indicated that PKCζ was distributed evenly between the cytosolic fractions and the membrane fractions, suggesting that PKCζ could be localized to the plasma membrane in unstimulated serum-starved CFs. Conversely, PKCα was predominantly localized in the cytosol and membrane translocation was stimulated by PMA.

Overall, our findings indicate that ANG II-induced ERK 1/2 activation and proliferation in adult cardiac fibroblasts occurs through a two distinct pathways, one mediated by calcium and one mediated by PKCs. Both mechanisms are independent of the EGFR and c-Src. Selective inhibition of PI3-K had no effect on ERK 1/2 activation and the effect on [³H]-thymidine incorporation was in part due to a reduction in cell survival, rather than an inhibition of cellular proliferation. Our results indicate that pretreatment of adult CFs with PKCζ pseudosubstrate peptide prevents both basal and ANG II-induced CF proliferation, but PKCζ is not activated directly by ANG II. In conclusion, this study highlights the complexity of signaling involved in ANG II-induced ERK 1/2 activation and indicates divergent signaling pathways to ERK 1/2 between cardiac fibroblasts and other cell types in which ANG II signaling is prominent.
CHAPTER FOUR

THE ROLE OF PKCζ IN MODULATING THE CYTOSKELETON AND ATTACHMENT PROPERTIES OF CARDIAC FIBROBLASTS

INTRODUCTION

Protein kinase Cζ has been shown to be essential for angiotensin II-induced ERK 1/2 activation in vascular smooth muscle cells (Liao et al. 1997). When this PKC isozyme was downregulated using antisense oligonucleotides, ERK 1/2 phosphorylation in response to ANG II is significantly inhibited. In this same study, PKCζ was found to directly bind to H-Ras, a phenomenon that is believed to be required for downstream activation of MEK 1/2 and subsequently ERK 1/2. The current evidence that suggests that PKCζ acts as a MEK 1/2 kinase in place of Raf-1 in the classic MAPK signaling cascade, or in some instances it may facilitate the activation of Raf-1 in order to activate MEK. In addition, several studies have demonstrated an inhibition of ANG II-stimulated growth and proliferation when PKCζ activity is blocked using a myristoylated pseudosubstrate peptide (Braun and Mochly-Rosen 2003; Muscella et al. 2003). This peptide is identical to the pseudosubstrate domain of endogenous PKCζ and elicits its effects by interacting with the catalytic domain to prevent substrate phosphorylation.

Our investigation into the role of PKCζ as a potential mediator of ANG II-induced cardiac fibroblast proliferation has lead us to discover new and unexpected effects of ζ-PS in cardiac fibroblasts. Our initial hypothesis was that blocking PKCζ by using ζ-PS would inhibit the ERK 1/2 response to ANG II. Our data indicate that over a period of 48 hrs, ANG II-induced CF proliferation is in fact significantly inhibited by
approximately 26% (see Chapter 3, Figure 16). This result was expected and agrees with the findings of Braun and Mochly-Rosen (2003), who determined that TGF-β-induced proliferation of neonatal CFs was significantly inhibited by ζ-PS. However, we also discovered that ζ-PS by itself caused a rapid increase in ERK 1/2 phosphorylation, a seemingly contradictory finding since the ERK 1/2 cascade is a major mitogenic signaling pathway. A very recent study has indicated that myristoylation of the ζ-PS peptide might be the cause of the increased ERK 1/2 activation, since the same modification to a scrambled ζ-PS peptide also induced ERK 1/2 activation (Krotova et al. 2006). The fact that ζ-PS reduced fibroblast proliferation even though it also caused ERK 1/2 phosphorylation prompted us to investigate the role of PKCζ more closely.

The overall goal of this set of experiments was to explore the effects of ζ-PS and PKCζ inhibition in greater detail by first determining whether 10 μM of ζ-PS was toxic to CFs and caused cell death. We were surprised to find that after 48 hr exposure to ζ-PS, cell detachment from tissue culture plates was actually reduced when compared to basal and also found that the cells were less sensitive to trypsinization. Lastly, ζ-PS treatment had significant effects on the cytoskeleton, as determined by assessing f-actin organization, and also caused DNA cleavage. Overall, the data suggest that inhibition of PKCζ causes significant changes in the morphology of CFs and attachment properties, which are likely early steps in apoptosis.
MATERIALS AND METHODS

Materials

Dulbecco’s Modified Eagle’s Medium (DMEM), penicillin/streptomycin, fungizone, fetal bovine serum (FBS), trypsin, and rhodamine-conjugated phalloidin were all purchased from Invitrogen (Grand Island, NY). Anti-FAK was obtained from BD Transduction Laboratories (San Jose, CA). Anti-phospho-FAK (Y397) antibodies were from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 488-conjugated goat anti-mouse and Alexa Fluor 568-conjugated goat anti-rabbit secondary antibodies were purchased from Invitrogen/Molecular Probes (Eugene, OR). Dichlorotriazinylaminofluorescein (DTAF)- and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibodies conjugated secondary antibodies were obtained from Jackson Laboratories (West Grove, PA). DeadEnd™ Fluorometric TUNEL System was purchased from Promega (Madison, WI). All other chemicals were reagent grade and obtained from Fisher Scientific (Pittsburgh, PA).

Isolation of adult rat ventricular cardiac fibroblasts

Primary cultured cardiac fibroblasts were isolated from the left ventricles of anesthetized adult male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) exactly as described in Chapter 2.
**Assessment of cell detachment**

CFs were cultured in 12-well tissue culture dishes and treated in triplicate for a period of 48 hrs. Growth medium from each of the wells was then collected in conical tubes and centrifuged at 1000 rpm for 10 min. The medium was removed and the pellets were resuspended in a total volume of 10 mL of Isotone® II diluent. CFs adhered to 12-well tissue culture dishes were washed twice with PBS and 0.25 ml of trypsin containing 25 mg/L EDTA was added to each well. Cells were then incubated at 37°C for 2 min and then collected in 2.5 mL DMEM containing 10% FBS. Cells were pooled and centrifuged at 1000 rpm for 10 min. The medium was then removed and the pellets resuspended in 10 mL of Isotone® II diluent. Automatic cell counting was performed using a Coulter Particle Counter (Beckman Coulter). Phase pictures were taken on an Olympus IX-70 microscope following trypsinization. Digital pictures were taken using identical aperture and exposure settings, and the images were optimized for brightness and contrast using Adobe Photoshop.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)**

TUNEL assaying was modified for use in cells plated on 12-well dot slides and otherwise performed according to manufacturer’s protocol. Following treatments, slides were washed with PBS and fixed using 2% paraformaldehyde for 30 min at RT. Cells were then permeabilized with 0.3% Triton X-100 for 15 min and then washed extensively with PBS. Cells were then equilibrated with supplied Equilibration Buffer for 5 min and incubated at 37°C in a humidified incubator for 1 hr in TdT/nucleotide reaction buffer.
Slides were then washed with supplied 2X SSC for 15 min. At this point, slides were washed with PBS and then subjected to immunocytochemistry as described below.

**Immunocytochemistry**

CFs were plated on 12-well dot slides (28.3 mm²/dot) at a density of 2000 cells/dot and treated with 10 μM ζ-PS for 48 hours. Slides were then washed in PBS and the cells then fixed using 2% paraformaldehyde. Cells were then permeabilized with 0.3% Triton X-100/PBS for 15 min, blocked with 2% goat serum for 1 hr, and incubated with appropriate monoclonal primary antibodies overnight at 4°C and secondary antibodies for 1 hr. For f-actin visualization, CFs were incubated with rhodamine-conjugated phalloidin after the blocking step. Slides were then washed extensively, mounted and visualized using an Olympus IX-70 microscope.

**Data analysis/statistics**

Data analysis was performed using GraphPad Prism 4.0 statistical analysis software (GraphPad Software, San Diego, CA). Statistical significance between treatments was determined by Student’s t test or by analysis of variance (ANOVA) with Tukey’s multiple comparison test.
RESULTS

Exposure to PKCζ pseudosubstrate prevents cell detachment

After determining that ζ-PS inhibited proliferation of CFs (see Chapter 3, Figure 16), we were interested in assessing the toxicity of the peptide and the degree of cellular detachment that occurs after exposure for 48 hr. We found that after treatment with 10 μM ζ-PS, the number of non-adherent cells in the culture medium was actually reduced by 79.3 ± 9.7% compared to basal (Figure 24, panel A). Plated CFs were then detached using trypsin containing 0.2% EDTA. We found that CFs that were exposed to ζ-PS were less sensitive to the effects of the trypsin and remained adhered to the culture dishes (62.5 ± 14.6% fewer cells removed vs. basal) whereas the untreated CFs were efficiently detached within 2 min (Figure 24B and C).

Chronic exposure to PKCζ pseudosubstrate alters cardiac fibroblast morphology and induces cytoskeletal degradation

We next investigated the effects of ζ-PS on the formation and maintenance of focal adhesions. Immunocytochemical staining was performed to determine the effect of ζ-PS on the cellular distribution of focal adhesion kinase, a protein known to associate with focal adhesion complexes. We found that untreated CFs expressed phosphorylated focal adhesion kinase predominantly in the nucleus and at focal adhesions, as expected (Figure 25, panels A-E). Following treatment with 10 μM ζ-PS for 48 hrs, the normal punctuate pattern of phospho-FAK staining was completely lost (Figure 25F-I). Staining indicated that focal adhesion complexes were breaking down and there was a general
FIGURE 24. CFs treated with $\zeta$-PS display reduced cellular detachment and sensitivity to trypsinization. 

A: CFs plated in 12-well dishes were treated with 10 $\mu$M $\zeta$-PS in triplicate for 48 hrs and media was collected and pooled. Floating cells in media from untreated wells and from $\zeta$-PS treated wells centrifuged and resuspended in Isotone® II diluent and counted. Data was pooled from 3 separate experiments and expressed as total cells per well detached. 

B: CFs from 12-well dishes were washed and detached using trypsin containing 0.2% EDTA for 2 min. DMEM containing 10% FBS was added to the wells to inactivate the trypsin and the cells were collected, pooled and centrifuged. Cells were then resuspended and counted. Data was pooled from 3 separate experiments and expressed as total cells per well detached by trypsinization. Differences between treatments were assessed by Student’s t test. *p < 0.05. 

C: Phase images following trypsinization demonstrate that $\zeta$-PS treated CFs remained adhered to tissue culture dishes following trypsinization. The untreated controls were completely removed by trypsinization.
Cells detached/well after 48 hr treatment

A. Basal

B. 10 μM ζ-PS

Cells detached/well by trypsinization following treatment

A. Basal

B. 10 μM ζ-PS

C. Basal

Post-trypsinization

10 μM ζ-PS (48 hr)

Post-trypsinization
FIGURE 25. CFs treated with ζ-PS for 48 hrs have reduced polymerized f-actin and focal adhesion kinase expression. A-E: Polymerized f-actin in untreated CFs is clearly visible by rhodamine-phalloidin staining using (A) 10x, (B, D) 40x, and (E) 80x objectives. Nuclei are stained with DAPI. (C) Focal adhesion complexes (white arrows) can be easily identified with P-FAK staining (FITC). Nuclear P-FAK staining is also evident. Image is taken using 40x objective. (D) Overlay of P-FAK (FITC) and f-actin (TRITC) staining at 40x. F-I: CFs treated with 10 μM ζ-PS for 48 hrs have reduced f-actin polymerization. Images were taken using (F) 10x and (G, I) 40x objectives. A general increase in cytoplasmic P-FAK (F, green arrows) and a loss of nuclear P-FAK (H, I) is also evident in ζ-PS treated cells. (I) Overlay of P-FAK (FITC) and f-actin (TRITC) staining at 40x.
F-actin (phalloidin)

P-FAK

Merged
redistribution of FAK to the cytoplasm of CFs treated with ζ-PS. Staining for total FAK showed similar patterns to that of phospho-FAK, except that expression at focal adhesions was not as clearly visible (data not shown). We also determined that exposure to ζ-PS greatly affected the amount of FAK localized in the cell nucleus. Since focal adhesions are important for anchoring of the cytoskeleton to the plasma membrane, and because of the known involvement of PKCζ with MARCKS, a protein responsible for f-actin polymerization, we examined the effect of ζ-PS on f-actin organization. We found that in CFs exposed to 10 μM ζ-PS for 48 hrs, a significant amount of cytoskeletal degradation had occurred, since rhodamine-conjugated phalloidin binding to filamentous actin was greatly reduced following treatment (Figure 25). It was also clear that there was a direct correlation of cells with high levels of cytosolic FAK staining and the lowest amount of phalloidin staining, indicating that these two effects of ζ-PS were not independent.

**Chronic ζ-PS treatment causes DNA cleavage and nuclear condensation**

There was a visible reduction in the size of some of the nuclei of ζ-PS-treated CFs. Nuclear condensation is a step in the apoptotic process and to assess whether ζ-PS treatment was inducing apoptosis in CFs, TUNEL assays were performed. We found that following treatment, there was an elevation in TUNEL staining in CFs treated with 10 μM ζ-PS for 48 hr (Figure 26). Co-staining for nuclei with DAPI also verified that the cells which stained TUNEL positive were those with condensed nuclei. Some cell nuclei were found to be irregularly shaped (Figure 26G), a result that was not found in
FIGURE 26. Treatment with ζ-PS causes positive TUNEL staining in CFs, indicating that cells are apoptotic. A-D: Untreated CFs display prominent f-actin staining with rhodamine-conjugated phalloidin using (A) 10x and (B,D) 40x objectives. No TUNEL staining was seen in untreated CFs (C, D). (D) Overlay of TUNEL (FITC) and f-actin (TRITC) staining at 40x. Nuclei are stained with DAPI. E-H: CFs treated with 10 μM ζ-PS show elevated TUNEL staining using (E) 10x and (G, H) 40x objectives. Some cells have noticeably smaller nuclei (E vs. A) and are irregularly shaped (G).
Basal

A. 10x

B. 40x

C. 40x

D. 40x

10 μM ζ-PS 48 hr

E. 10x

F. 40x

G. 40x

H. 40x

Basal 10 μM ζ-PS 48 hr
untreated cells. There was also a visible negative correlation between TUNEL positive staining and f-actin polymerization (Figure 26).

**DISCUSSION**

Initially, we were interested in the effects of PKCζ inhibition on angiotensin II-induced cellular proliferation. Our findings in adult cardiac fibroblasts agreed with other reports that inhibition of PKCζ with a myristoylated pseudosubstrate prevented neonatal CF proliferation induced by TGF-β (Braun and Mochly-Rosen 2003). We were surprised to discover that inhibition of PKCζ had no effect on ANG II-induced ERK 1/2 activation and that the compound actually increased ERK 1/2 phosphorylation by itself. These seemingly contradictory findings prompted us to investigate alternative potential mechanisms of the prevention of CF proliferation by PKCζ inhibition.

Our first consideration was that ζ-PS was inducing cell death and that this was the cause of reduced [³H]-thymidine incorporation after 48 hrs exposure. Cellular detachment is a simple method in which to assess whether or not a compound is inducing death or is cytotoxic, since dead cells typically lift off cell culture dishes. We had first found that the PI3-K inhibitor LY 294002 (10 μM) induced an approximate 20% increase in CF detachment compared to basal (unpublished results). This finding was not surprising since inhibition of PI3-K, and subsequently Akt, results in increased apoptosis. However, when examining the effect of ζ-PS on cell detachment, we found that cell detachment was actually reduced compared to basal. In addition, fewer cells were sensitive to trypsinization after being treated with ζ-PS for 48 hrs. Both PI3-K and PKCζ have been
reported to interact with components of focal adhesions, including integrin-linked kinases and cytoskeletal proteins (Garcia-Rocha et al. 1997; Delcommenne et al. 1998). The reduced detachment and loss of trypsin sensitivity suggested that inhibition of PKCζ might affect focal adhesion complexes.

The formation of focal adhesions is critical for the normal functioning of fibroblasts during wound healing. Focal adhesions are important for cell migration and for linking the actin skeleton to the plasma membrane to facilitate contraction and wound closure. To assess the effects of PKCζ on focal adhesion formation and on the organization of the cytoskeleton, immunocytochemical staining was performed. We first determined that the ζ-PS was affecting the localization of focal adhesion kinase in CFs. Under basal conditions, FAK is predominantly localized to both the nucleus and to focal adhesions. After exposure to ζ-PS for 48 hrs, there was an apparent reduction in the numbers of focal adhesions and a redistribution of FAK to the cytoplasm. In addition, the FAK found in the nucleus in untreated cells was no longer present after exposure to ζ-PS.

In addition to the effects on focal adhesion formation, we observed a significant restructuring of the actin cytoskeleton. Phalloidin is a toxin from the death cap mushroom that binds irreversibly at the interface of f-actin subunits. When conjugated to a fluorophore, phalloidin is a useful tool to monitor the organization of f-actin. We determined that treatment of CFs with ζ-PS was causing f-actin depolymerization since there was a significant reduction in phalloidin binding. The greatest reduction in phalloidin binding occurred in CFs in which the staining for cytosolic FAK was the highest. Our findings suggest that PKCζ is an important component of focal adhesion
complexes in CFs and that the PKCζ mediates organization of the actin cytoskeleton, possibly through the PKC substrate MARCKS, a protein which is critical for f-actin polymerization (Hartwig et al. 1992). These data also agree with our finding that ζ-PS reduces CF proliferation, since cell motility and f-actin organization are required for cell division. The increase in cellular attachment and a loss of f-actin following ζ-PS treatment therefore may be the main cause for the ζ-PS-induced reduction in proliferation.

Another observation made from immunocytochemical staining was that the CFs with the greatest changes in phalloidin binding and FAK localization also had nuclei that were reduced in size. Cytoskeletal degradation is a preliminary step in the progression of apoptosis, and as cells advance through the apoptotic process condensation and fragmentation of the nucleus occurs. Therefore, a potential cause of the nuclear size reduction was that cells were undergoing apoptosis. Analysis by TUNEL indicates that the DNA of several of the ζ-PS treated cells was fragmenting. Nuclear staining with DAPI also indicated that some of the ζ-PS treated CFs had irregularly shaped nuclei, another characteristic of apoptotic cells. Cells with the highest degree of TUNEL staining were those with the smallest nuclei, the least amount of phalloidin binding and highest cytosolic FAK levels (unpublished observation). This observation suggests that these events are not independent and that the cells are likely progressing through apoptosis in response to PKCζ inhibition.

Our initial aim was to investigate the role of PKCζ in mediating cardiac fibroblast proliferation. We discovered that the inhibition of PKCζ did indeed reduce [$^{3}$H]-
thymidine incorporation compared to untreated controls and that angiotensin did not increase the amount of proliferation in ζ-PS treated cells. However, further examination into the effect of PKCζ inhibition suggested that this was not likely the result of inhibition of signal transduction as is the case in other cell types. Instead, the reduction in CF proliferation caused by PKCζ inhibition was likely an indirect result of the induction of apoptosis and the morphological changes in the cytoskeleton that occur as a result of cell death. In light of the fact that PKCζ has been found to interact with cytoskeletal proteins, the mitotic apparatus, focal adhesion complexes and caspase-9 (Lehrich and Forrest 1994; Garcia-Rocha et al. 1997; Coghlan et al. 2000; Brady et al. 2005), our findings suggest that inhibition of PKCζ causes the reduction in proliferation predominantly through degradation of the cytoskeleton and by induction of apoptosis.
CHAPTER FIVE
THE EFFECTS OF α-ADRENERGIC STIMULATION ON CARDIAC MYOFIBROBLAST DIFFERENTIATION

INTRODUCTION

Adrenergic receptor signaling is one of the most highly studied and well characterized receptor systems and is particularly important to the physiology and pathophysiology of the heart (reviewed in (Lamba and Abraham 2000)). The predominant cardiac adrenergic receptor subtypes are the β-ARs. The β1-AR is the major adrenergic receptor of cardiac myocytes and these receptors mediate the inotropic and chronotropic effects of catecholamine stimulation. The β2-AR subtype is highly expressed in the lungs and skeletal musculature and plays important roles in mediating catecholamine-induced dilation of airway smooth muscle and skeletal muscle vasculature during periods of increased activity. Cardiac fibroblasts also highly express β2-ARs and stimulation of these receptors induces intracellular cyclic AMP production, which negatively regulates myofibroblast differentiation and induces cellular proliferation through activation of the ERK 1/2 signaling pathway (Kim et al. 2002; Swaney et al. 2005). Lastly, there is also a third β-AR subtype, β3-AR, which is primarily localized in adipose tissue.

In contrast to the β-AR system, the majority of systemic effects of α-AR stimulation occur in the vasculature. The α-ARs are classified into two major subtypes α1- and α2-ARs, with the α2-ARs acting mainly as presynaptic feedback receptors on sympathetic neurons. The α1-ARs are highly expressed in vascular smooth muscle cells
and are involved in the regulation of vascular tone and blood pressure. The $\alpha_1$-ARs are typically coupled to $G_{q/11}$ proteins which stimulate the release of calcium from intracellular stores to facilitate VSMC contraction. Physiologically, catecholamine-induced activation of $\alpha_1$-ARs is vital for constriction of the vasculature in capacitance organs (i.e. spleen) to increase venous return to the heart, a critical step in meeting the increased myocardial oxygen demand during periods of elevated activity. However, chronic stimulation of $\alpha_1$-ARs is a major cause of hypertension and can be an important factor in the development of cardiac fibrosis.

The catecholamines epinephrine (EPI) and norepinephrine (NE) both act on $\alpha_1$-, $\alpha_2$-, and $\beta_1$-ARs with similar potency and efficacy. EPI also acts on $\beta_2$-ARs present on cardiac fibroblasts with high affinity, but the affinity for NE is much lower. Isoproterenol (ISO) is a highly potent and selective synthetic agonist of $\beta_1$- and $\beta_2$-ARs and is structurally related to EPI and NE. The affinity of ISO for $\alpha$-ARs is negligible and therefore it does not bind and activate the $\alpha$-AR. The cardiac effects of ISO administration are therefore similar to EPI. However, ISO infusion actually reduces blood pressure since it does not cause vascular constriction in capacitance organs while causing dilation of skeletal muscle vasculature.

We had initially observed interesting differences between the hearts of adult rats following infusion with either isoproterenol or epinephrine for a period of 48 hrs. The hearts of EPI-infused rats were indistinguishable from the hearts of vehicle-infused rats. However, the hearts of ISO-infused rats had visible ischemic damage and were hypertrophied compared to the vehicle-infused rats even after only 48 hrs of exposure.
Examination of whole heart tissue by Western analysis demonstrated that the hearts exposed to 48 hr ISO infusion actually had a reduction in $\alpha$-smooth muscle actin expression compared to the hearts of vehicle-infused rats. This reduction is likely in part due to the direct stimulation of $\beta_2$-AR on cardiac fibroblasts and the prevention of myofibroblast differentiation. Interestingly, although the EPI-hearts looked normal, we determined that the whole heart expression of $\alpha$-smooth muscle actin had increased as a result of EPI-infusion. As previously mentioned, a major difference between the actions of EPI versus ISO is that ISO is selective for $\beta_1$- and $\beta_2$-ARs whereas EPI will bind to all adrenergic receptor subtypes. We were interested in determining whether there was any direct stimulation of $\alpha_1$-ARs on CFs by EPI and whether or not these receptors might be inducing cardiac myofibroblast differentiation. The goal of this study therefore was to determine which, if any, subtypes of $\alpha_1$-ARs were expressed by CFs and whether or not stimulation of these receptors resulted in myofibroblast differentiation in cultured CFs.

**MATERIALS AND METHODS**

**Materials**

Dulbecco’s Modified Eagle’s Medium (DMEM), penicillin/streptomycin, fungizone and fetal bovine serum (FBS) were all purchased from Invitrogen/GIBCO (Grand Island, NY). Anti-$\alpha$-smooth muscle actin ($\alpha$-SMA) antibodies was from Sigma-Aldrich (St. Louis, MO). Phospho-and total ERK 1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rat-adsorbed horseradish peroxidase-conjugated anti-mouse secondary antibody was obtained from Serotec (Raleigh, NC). All
other reagents and chemicals were reagent grade and obtained from Fisher Scientific (Pittsburgh, PA).

**Mini-osmotic pump implantation and tissue collection**

Sprague Dawley (Harlan Sprague Dawley, Indianapolis, IN) rats were first weighed and then mini-osmotic pumps were appropriately filled with epinephrine (200 μg kg⁻¹ hr⁻¹) or isoproterenol (400 μg kg⁻¹ hr⁻¹) in 10⁻⁴ N HCl (pH ~ 3) and then primed overnight by incubating in PBS at 37°C. On the following day, rats were anesthetized with halothane and their backs were then shaved. Pumps were implanted subcutaneously by making a small incision in the skin of the lower back and separating fascia until a large enough opening for the pump was created. Once the pump was inserted, the incision was closed and irrigated. After 48 hr of continuous infusion, rats were anesthetized with sodium pentobarbital i.p. and their hearts excised. Hearts were minced in PBS and washed several times. Heart tissue was then placed in lysis buffer (62.5 Tris-HCl, 2mM EDTA, 2.3% SDS, 10% glycerol, pH 6.8, Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail 1) and homogenized with a tissue tearor. Samples were incubated on ice for 15 min between homogenization cycles and the process was repeated 3-4 times. Finally, samples were centrifuged for 10 min at 1000 RPM. The supernatant was removed and further diluted in lysis buffer and the pellet was discarded. Samples were then frozen and stored at -20°C until analyzed by Western blotting as described below.
**Isolation of adult rat ventricular cardiac fibroblasts**

Primary cultured cardiac fibroblasts were isolated from the left ventricles of anesthetized adult male Sprague Dawley exactly as described in Chapter 2.

**Cellular protein collection and Western Analysis**

Protein lysates were collected exactly as described in Chapter 2.

**Data analysis/Statistics**

Data analysis was performed using GraphPad Prism 4.0 statistical analysis software (GraphPad Software, San Diego, CA). Statistical significance between treatments was determined by analysis of variance (ANOVA) with Tukey’s multiple comparison test.

**RESULTS**

**Infusion of epinephrine or isoproterenol causes differential changes in whole heart α-smooth muscle actin expression**

Adult male Sprague-Dawley rats were infused with either 400 $\mu$g kg$^{-1}$ hr$^{-1}$ isoproterenol, 200 $\mu$g kg$^{-1}$ hr$^{-1}$ epinephrine or vehicle ($10^{-4}$ N HCl) for 48 hrs. Western analysis of the hearts indicated that the ISO-infused rats had reduced expression of total cardiac α-smooth muscle actin expression compared to vehicle-infused rats (Figure 27, panels A, C, 41.3 ± 1.9% reduction). Protein lysates collected from the EPI-infused rats
FIGURE 27. Effects of subcutaneous ISO and EPI infusion on cardiac α-SMA expression. A: Western blot of whole heart protein samples for α-SMA collected from rats infused with 400 μg kg⁻¹ hr⁻¹ s.c. isoproterenol continuously for 48 hrs. Each lane represents proteins from a different rat. B: Western blot of whole heart protein samples for α-SMA collected from rats infused with 200 μg kg⁻¹ hr⁻¹ s.c. epinephrine continuously for 48 hrs. Each lane represents proteins from a different rat. C: Densitometric analysis of α-SMA expression expressed as % vehicle-infused protein samples.
contained a higher α-SMA content compared to the vehicle-infused controls (Figure 27B, C, 17.4 ± 4.4% increase).

**Stimulation of cultured CFs with phenylephrine does not induce α-SMA expression and ERK 1/2 activation**

To assess the importance of α-AR signaling in cultured CFs, cells were stimulated with 10 μM phenylephrine (PHE), a selective α-AR agonist, for a period of 48 hr and α-SMA expression was then assessed by Western analysis. Figure 28, panel A demonstrates that α-SMA expression in CFs treated with PHE are not different compared to untreated controls, suggesting that α-ARs are not mediating differentiation in CFs. Since the α₁B-AR has been demonstrated to activate ERK 1/2 when expressed in rat-1 fibroblasts (Waldrop et al. 2002), we next stimulated CFs with 10 μM PHE in order to investigate whether α-ARs were functionally coupled to ERK 1/2. We first found that there was indeed a slight increase in ERK 1/2 phosphorylation over basal in response to α-AR stimulation (Figure 28B). However, the detected ERK 1/2 activation was not blocked by pretreatment with 1 μM prazosin, a selective α-AR antagonist, indicating that this effect was not likely due to stimulation of α-ARs, but a non-specific effect.

**DISCUSSION**

Our initial observation was that continuous subcutaneous infusion of isoproterenol into adult rats caused the reduction of total cardiac α-smooth muscle actin expression, while epinephrine infusion caused expression to increase. There are several
FIGURE 28. Selective stimulation of α-ARs with phenylephrine does not induce differentiation or ERK 1/2 activation in isolated CFs. A: Representative Western blot indicating that treatment of CFs with 10 μM PHE for 48 hrs did not induce α-SMA expression in CFs. Induction of α-SMA expression by 200 pM TGF-β is clearly visible. B: Representative Western blot indicating first that ERK 1/2 phosphorylation is increased by stimulation with 100 nM ISO, as expected. There was also an apparent increase in phospho-ERK 1/2 in response to 10 μM PHE, but the increase was not blocked by prazosin, indicating that the effect was not likely mediated by α-ARs.
A.

Basal
100 nM ANG II
200 pM TGF-β
10 μM PHE
1 μM PZ
PZ + PHE

α-SMA

B.

Basal
100 nM ISO
10 μM PHE
PZ + PHE

P-ERK 1/2

ERK 1/2
potential causes of this phenomenon. First, EPI is an agonist of both \( \alpha \)- and \( \beta \)-adrenergic receptors while ISO is a selective \( \beta \)-AR agonist. \( \alpha \)-ARs are predominantly localized to vascular smooth muscle cells, a cell type with high levels of \( \alpha \)-SMA expression. Stimulation of \( \alpha \)-ARs will result in VSMC hypertrophy and this could be a major contributing factor in the elevated expression of \( \alpha \)-SMA in the whole heart as a result of EPI infusion. Second, cardiac fibroblasts expressing \( \alpha \)-ARs might be stimulated directly by EPI, which could serve to promote myofibroblast differentiation. Cardiac myofibroblasts express \( \alpha \)-SMA in highly organized stress fibers, a characteristic which is critical for their role in wound healing. Undifferentiated fibroblasts do not express \( \alpha \)-SMA and therefore the increase in total cardiac \( \alpha \)-SMA could potentially be due to direct stimulation of CFs by EPI. Stimulation of \( \alpha_{1A} \)-ARs has been determined to inhibit DNA synthesis and stimulate protein synthesis including \( \alpha \)-SMA in rat-1 fibroblasts, all of which suggests myofibroblast differentiation (Saeed et al. 2004). A similar study also determined that \( \alpha_{1B} \)-AR stimulation induced protein synthesis in rat-1 fibroblasts which was at least partly mediated by ERK 1/2 activation (Waldrop et al. 2002). Our goal therefore was to determine if EPI affected CFs through direct activation of \( \alpha \)-ARs by evaluating the effects of selective \( \alpha \)-AR stimulation in isolated CFs.

The characterization of \( \alpha \)-AR function has predominantly come from studies performed using vascular smooth muscle cells. From these studies, it has been determined that \( \alpha \)-ARs couple preferentially to Gq proteins and, therefore, stimulation results in intracellular calcium release and VSMC constriction. Currently, it is highly
questionable whether or not functional $\alpha$-ARs are expressed in CFs, since mRNA was not previously identified by PCR (Stewart et al. 1994). Several labs, including our own, have found that stimulation of isolated CFs with $\alpha$-AR selective agonists does not result in inositol phosphate production or intracellular calcium release ((Meszaros et al. 2000), E. Olson, unpublished results). Interestingly, it has also been determined that stimulation of $\alpha$-ARs in the ventricular myocardium does not elicit a remarkable increase in inositol phosphate production in both failing and non-failing hearts, where the $\alpha_1$-AR comprised approximately 27% and 15% of the total adrenergic receptor density, respectively (Bristow et al. 1988). Furthermore, adventitial fibroblasts from rat aorta have been found to express very low levels of mRNA for all three $\alpha_1$-AR subtypes, and competition binding experiments with selective $\alpha$-subtype specific ligands indicated that the $\alpha_{1B}$-AR was present in high amounts on the plasma membrane (Faber et al. 2001).

However, our own data indicate that this is not likely the case in isolated CFs. Direct stimulation of isolated CFs with the selective $\alpha$-AR agonist phenylephrine did not result in $\alpha$-SMA expression or $\alpha$-AR-mediated ERK 1/2 activation. Collectively, our data and data from other labs suggest that $\alpha$-ARs in CFs did not mediate the epinephrine-induced increase in whole heart $\alpha$-SMA expression.
CHAPTER SIX

OVERALL CONCLUSIONS AND DISCUSSION

In 2003, the National Health and Nutrition Examination Survey concluded that approximately 71.3 million Americans had at least one type of cardiovascular disease (CVD), with high blood pressure and coronary heart disease being the two most common forms (Thom et al. 2006). There is an obvious direct link between CVD and the incidence of heart failure, defined by the heart having lost the ability to efficiently pump blood in order to provide adequate tissue perfusion. The study estimated that 22% of males and 46% of females who experience myocardial infarction will be disabled with heart failure within 6 years and of the current 5 million cases of heart failure, 75% had antecedent hypertension. Both of these conditions cause aberrant remodeling of the cellular and extracellular myocardial composition. A major contributing factor to the development of heart failure is myocardial fibrosis, a debilitating condition which is characterized by excessive deposition of extracellular matrix proteins. The pathological remodeling that occurs causes a reduction in the pumping capacity due to stiffening of the myocardium, and furthermore, the heart is predisposed to ventricular arrhythmias due to disruption of electrical signaling from the excess ECM. Some of the existing drugs used to treat CVD also reduce ventricular remodeling, but the mechanisms by which this favorable result occurs are not known. Understanding the mechanisms underlying the development of cardiac fibrosis would provide insight into preventing pathological remodeling and would therefore be of great value in the development of new pharmacological agents.
The renin-angiotensin-aldosterone system (RAAS) plays a central role in CVD and is a major factor in the onset and progression of cardiac fibrosis (Brilla et al. 1995; Brilla et al. 1997). The role of angiotensin II in the development of cardiac fibrosis has been studied extensively with the use of several different animal models of hypertension including spontaneously hypertensive rats (Farina et al. 2000), deoxycorticosterone acetate (DOCA)-salt (Brown et al. 1999), and suprarenal aortic banding (Tokuda et al. 2004) as well as in myocardial infarction or ischemia (Yu et al. 2001). Importantly, both the renal RAAS and the intracardiac RAAS have been implicated in the development of fibrosis (Weber et al. 1994; Kyriakidis et al. 1998). An important function of ANG II is stimulation of cardiac fibroblast proliferation early in and during the pathological remodeling process that causes myocardial fibrosis. Excessive proliferation of CFs increases their collective fibrotic potential, and understanding the mechanism of ANG II-induced CF proliferation would therefore be useful in the development of preventative therapies.

One of the goals set in this project was to determine mechanism of ANG II-induced ERK 1/2 activation in adult CFs. The ERK 1/2 signaling cascade is a major mitogenic signaling pathway utilized by growth factors and GPCR agonists in both adult and neonatal CFs, as well as in other cell types where ANG II signaling is prominent (Schorb et al. 1995; Eguchi et al. 1998; Kim et al. 2002; Stockand and Meszaros 2003). Often, activation of ERK 1/2 by ANG II and other GPCR ligands proceeds through an epidermal growth factor receptor-dependent mechanism. Several intermediates involved in EGFR transactivation have been identified and include calcium, matrix
metalloproteinases, c-Src family tyrosine kinases, and reactive oxygen species (Saito and Berk 2001). Our initial hypothesis was that ANG II-induced ERK 1/2 activation was dependent on the EGFR in adult CFs. However, neither proliferation nor ERK 1/2 activation was inhibited by the EGFR kinase inhibitor AG 1478. Further investigation of EGFR phosphorylation of tyrosine residues, a critical step in the activation of EGFR kinase activity, indicated that ANG II was not inducing EGFR phosphorylation and therefore not transactivating the receptor. Another supporting piece of evidence is that inhibition of c-Src had no effect on ERK 1/2 activation. Src-mediated EGFR phosphorylation has been found to occur directly and also indirectly through MMP-induced liberation of heparin-binding EGF, a plasma membrane-bound endogenous ligand which activates the EGFR by direct interaction with the extracellular domain (Kim et al. 2002; Saito et al. 2002). Collectively, our data indicate that ANG II-induced ERK 1/2 activation in CFs does not occur through this mechanism.

Studies performed in vascular smooth muscle have indicated a role of PI3-K in ANG II-induced ERK 1/2 activation. PI3-K has been identified as a signaling intermediate between the AT1R and ERK 1/2. We blocked PI3-K activity with the use of a selective pharmacological inhibitor LY 294002 and determined that ANG II-induced proliferation could in fact be blocked. However, there was no effect on ERK 1/2 activation since ANG II induced ERK 1/2 phosphorylation to an equal extent in the presence of the inhibitor as in the absence. This result suggests that PI3-K is not downstream of the AT1R in adult CFs. Further evidence for this claim comes from the
fact that Akt, a key downstream effector of PI3-K, was not significantly phosphorylated in response to 100 nM ANG II treatment.

The reduction in basal and ANG II-induced proliferation by LY 294002 was intriguing and since PI3-K-induced Akt activation is an important anti-apoptotic survival pathway, we hypothesized that LY was inducing some degree of cell death over the 48 hr treatment period. Analysis of cell detachment from tissue culture plates provides general information about the survivability of cells in culture. We detected approximately 20% more cellular detachment in the presence of LY than in untreated cells. This finding suggests that a portion of the effect of the compound on $[^3\text{H}]-\text{thymidine}$ incorporation could be attributed to cell death.

A third potential signaling intermediate was the atypical isozyme PKCζ. PKCζ mediates ERK 1/2 activation by interaction with Ras and Raf-1 (Liao et al. 1997). PKCζ is recruited to the plasma membrane and activated in response to PI3-K induced generation of PIP3. Interestingly, not only has PKCζ been identified as a mediator of ANG II signaling (Muscella et al. 2003), it was found that PKCζ also played a more direct role in mitogenesis by interacting with tubulin and the mitotic apparatus (Lehrich and Forrest 1994). We utilized a myristoylated peptide which mimicked the pseudosubstrate domain of PKCζ and therefore prevented kinase activity to study the effects of PKCζ inhibition on CF proliferation. Initially we determined that inhibition of PKCζ blocked $[^3\text{H}]-\text{thymidine}$ incorporation in CFs in a manner similar to LY 294002, where both basal and ANG II-induced proliferation were inhibited, but to a lesser degree. However, in the case of the ζ-PS, the effects on ERK 1/2 phosphorylation were
surprising. We found that 10 μM ζ-PS actually induced ERK 1/2 phosphorylation by itself and did so in a time dependent manner, with maximal phosphorylation achieved early (near 10 min) and the signal remaining elevated over basal for at least 4 hrs. A recent study has indicated that modification of the pseudosubstrate peptide by myristoylation is in fact the cause of this effect, since myristoylated scrambled peptides elicit the same effect (Krotova et al. 2006).

We were intrigued by the fact that the ζ-PS inhibited proliferation and yet activated ERK 1/2, which are seemingly contradictory events. This prompted further investigation into the effects of PKCζ inhibition since additional events must have been occurring to offset the activation of ERK 1/2. In a manner identical to LY 294002, we first assayed for cellular detachment as a possible explanation for the reduction in [³H]-thymidine incorporation. We were again surprised to learn that ζ-PS treatment actually reduced cellular detachment. Our next consideration was to confirm equal cell numbers plated by trypsinizing the dishes and counting cells. This assay did not work as expected, but yielded an interesting result. Treatment with ζ-PS desensitized cultured CFs to trypsinization. One possible explanation was that the inhibitor was inducing assembly of focal adhesion complexes to facilitate cellular attachment. We investigated the localization of focal adhesion kinase following ζ-PS treatment and also the organization of filamentous actin, since the cytoskeleton links directly to focal adhesions to facilitate cell migration and polarization. Immunocytochemical staining of ζ-PS treated CFs suggested that the effect of PKCζ inhibition was the dissolution of normal focal adhesions accompanied by relocation of FAK to the cytoplasm, exclusion from the
nucleus, and degradation of the actin cytoskeleton. All of these findings are consistent with the early phase of apoptosis, and since one function of PKCζ is to prevent apoptosis through phosphorylation and inhibition of caspase-9 (Brady et al. 2005), TUNEL was performed to further characterize the effects of PKCζ inhibition. TUNEL indicated that the cells were in fact apoptotic, and the highest amount of TUNEL was seen in CFs with the lowest amount of f-actin staining and the greatest cytosolic FAK. Further evidence is provided by the fact that most cells that stained TUNEL positive had smaller nuclei and some had irregularly shaped nuclei, both of which are indications of nuclear fragmentation.

Cell culture conditions can greatly influence the results of any experiment. An initial observation was that the ζ-PS affected cultured CFs differently depending on the conditions under which the cells were cultured. For example, an important consideration is cell density. Cells plated at high density did not respond to the ζ-PS, as the compound appeared to have no effect on attachment or on f-actin or FAK staining. The two major results of this study were that PKCζ inhibition increased attachment but also caused positive TUNEL staining, which are seemingly contradictory. Considering the fact that culture conditions could affect the outcome of the assay, it is highly likely that the cultured CFs existed in subpopulations dictated by the local conditions, such that the progression through the apoptotic pathway was occurring at different rates for different cell populations. This statement would require further experimentation to be confirmed, but one initial supporting factor is that TUNEL positive cells were even detected at 48 hrs post-treatment, since DNA fragmentation can occur as early as 3-12 hrs. Another
possibility is that the ζ-PS peptide is having multiple cellular effects due to the modification by myristoylation (Krotova et al. 2006). Even though our findings and conclusions are consistent with PKCζ inhibition, this point cannot be ignored and alternative mechanisms of PKCζ inhibition must likely be employed to validate these claims.

Since the mechanism of ANG II-induced ERK 1/2 activation was not found by testing our initial hypotheses, we investigated two other potential signaling mediators, calcium and PKC. We first confirmed that direct activation of ERK 1/2 by PKC could be achieved in CFs by stimulating with PMA, a phorbol ester which activates classic and novel PKC isozymes. Long term treatment with PMA results in classic and novel PKC downregulation, and this effect was confirmed by analyzing PKCα expression. We quickly discovered that c/nPKC downregulation did not block ANG II-induced ERK 1/2 activation. We also investigated the effect of intracellular calcium chelation with BAPTA/AM, since liberation of calcium is an important effect of ANG II in CFs. After determining the concentration required to sequester all of the intracellular calcium released by ANG II, ERK 1/2 activation was assessed. Again we found no inhibition of ERK 1/2 activation when calcium was blocked. Because of the existence of novel, calcium-insensitive PKCs and alternative intracellular mediators of calcium signaling, we measured ERK 1/2 activation in PKC downregulated cells treated with BAPTA/AM. Under these conditions, no significant degree of ERK 1/2 activation by ANG II was detected. This finding indicates that there are two independent, yet convergent, signaling
pathways mediated by PKC and calcium that are responsible for ERK 1/2 activation by ANG II.

Another goal of this project was to investigate the effect of resveratrol on both fibroblast proliferation and differentiation to myofibroblasts. RES is a phytoalexin which is highly produced in grape skins in response to fermentation, and thus it is found in the highest concentrations in red wines. RES is a known inhibitor of proliferation and hypertrophy in response to mitogenic stimulation and can directly inhibit PI3-K and ERK 1/2 activation (El-Mowafy and White 1999; Yu et al. 2001; Haider et al. 2002). RES is also documented as an antioxidant which can prevent ischemia-reperfusion injury in the rat heart (Hung et al. 2002). These properties suggest that it may also be an effective anti-fibrotic agent through inhibition of CF proliferation and myofibroblast differentiation.

We first investigated the effects of RES on ANG II-induced CF growth proliferation and found that both $[^3]$H-leucine and $[^3]$H-thymidine incorporation were reduced in a concentration-dependent manner with 5-25 μM RES. There was no effect on basal protein synthesis, but basal proliferation was also reduced with increasing concentrations of RES. Since ANG II-induced proliferation proceeds through an ERK 1/2 dependent pathway, we next measured the effect of RES on both MEK 1/2 and ERK 1/2 phosphorylation. The result was very similar to the effect on proliferation, in that there was an effect on both ANG II-induced and basal phospho-ERK 1/2 levels. We also detected an effect on MEK 1/2, but only found a complete reduction in ANG II-induced MEK 1/2 phosphorylation with 25 μM RES.
The reduction in basal proliferation and ERK 1/2 phosphorylation suggested that the inhibition by RES was not specific to ANG II, and to test this hypothesis we examined whether RES was effective in blocking EGF signaling. Initially, we found no effect of RES on EGF signaling, but because EGF induces proliferation to a greater degree than ANG, we conducted the experiment in the presence of a range of concentrations for EGF, ranging from 10 nM to 0.2 nM. We found at the lower doses of EGF, which induced ERK 1/2 phosphorylation to a similar degree as 100 nM ANG II, that the increase in ERK 1/2 phosphorylation could in fact be attenuated. This finding provided further evidence that RES is a non-specific inhibitor of proliferation in CFs.

Since ANG II causes CF differentiation to myofibroblasts as well as proliferation, and because of the importance in relation to cardiac fibrosis, we were interested in determining whether RES was an effective inhibitor in this respect as well. We chose to stimulate CF differentiation separately with both ANG II and transforming growth factor-β (TGF-β), since ANG II-induced differentiation and fibrosis occur as a result of TGF-β production (Lee et al. 1995; Kuwahara et al. 2002). We found by Western analysis and immunocytochemistry that 25 μM RES did reduce α-SMA expression induced by both ANG II and TGF-β significantly. This discovery and the finding that RES inhibits proliferation both indicate that RES can have direct effects on CF activity and supply additional evidence that RES is cardioprotective by acting as a potential anti-fibrotic agent.

ANG II has been found to activate ERK 1/2 through a mechanism involving cytosolic phospholipase A₂ (cPLA₂), arachidonic acid (AA) production, and the
subsequent generation of reactive oxygen species resulting from AA metabolism (Gorin et al. 2004). Because cPLA$_2$ is activated by the elevation of cytosolic calcium, this might be the common link between the ERK 1/2 activation mechanism and the inhibition of proliferation by RES, since RES is a known anti-oxidant. Future studies regarding the both the mechanism of ANG II-induced ERK 1/2 activation and inhibition by RES should therefore focus on cPLA$_2$ and arachidonic acid production.

The final aim in this project dealt with the possibility of $\alpha$-adrenergic signaling and cardiac myofibroblast differentiation. Our initial observation was that subcutaneous infusion of rats with the $\beta$-AR agonist isoproterenol reduced total cardiac $\alpha$-SMA expression, but infusion with the non-selective agonist epinephrine actually caused a slight increase over vehicle-infused rats. To examine the possibility of differential signaling through $\alpha$-ARs by EPI, differentiation studies were performed on isolated CFs using an $\alpha$-AR selective agonist phenylephrine. We first found no increase in inositol phosphate production or intracellular Ca$^{2+}$ release, which would normally be expected for an agonist of $\alpha$-ARs since they couple preferentially to G$q$ proteins. We also found no significant elevation in expression of $\alpha$-SMA, indicating that either $\alpha$-AR stimulation did not result in differentiation or $\alpha$-ARs were not expressed by CFs. Although studies have found that some fibroblasts do display high amounts of functional $\alpha_1$-ARs even in the presence of low expression of mRNA (Faber et al. 2001), others have suggested that $\alpha$-AR mRNA for any of the subtypes are not expressed in CFs (Stewart et al. 1994). Based on the data collected, $\alpha$-AR signaling does not appear to be central to the activity of cardiac fibroblasts.
FIGURE 29. Proposed signaling mechanism of ANG II-induced ERK 1/2 activation in adult cardiac fibroblasts and summary of data. ANG II-induced ERK 1/2 activation is not dependent on EGFR transactivation. Two parallel pathways requiring Ca\(^{2+}\) and classic/novel PKCs were identified, and inhibition of either pathway alone was not sufficient to attenuate ERK 1/2 phosphorylation. The unknown mediators between Ca\(^{2+}\) and Raf-1 (?) might include reactive oxygen species, cytosolic phospholipase A\(_2\), and arachidonic acid generated by ANG II signaling. It was also determined that PI3-K and PKC\(\zeta\) did not mediate ANG II signaling to ERK 1/2 in adult cardiac fibroblasts. Instead, it might be that alternative stimuli (attachment and ECM signaling?) activate PI3-K and PKC\(\zeta\) and that these mediate such processes as mitosis, cell polarization and motility. Resveratrol was found to inhibit ANG II-induced proliferation by attenuating ERK 1/2 activation at least at the level of MEK 1/2. Resveratrol is known to have antioxidant properties which might be responsible for this effect if reactive oxygen is found to play a role in ANG II signaling to ERK 1/2 in adult CFs.
PERSPECTIVES

Cardiovascular diseases are routinely treated with a cocktail of pharmacological agents. Two common classes of drugs included in the treatment regimen are AT1R antagonists and ACE inhibitors, both of which are designed to reduce the cardiovascular damage caused by elevated systemic ANG II. The major goal of these treatments is to reduce blood pressure by inhibiting hypertrophy and promoting the relaxation of VSMCs, which therefore alleviates excess strain on the heart. However, an important additional beneficial effect of these agents is the limitation of the progression of myocardial fibrosis, not only in rat models of cardiovascular disease, but in human patients as well (Brilla et al. 1991; Brilla et al. 1996; Brilla et al. 2000). Much of the current understanding of intracellular ANG II signaling is based on studies performed in vascular smooth muscle cells, and my results indicate that several of the pathways known to mediate ANG II-induced proliferation and ERK 1/2 activation are not conserved between cardiac fibroblasts and VSMCs. At this point, I must conclude that regardless of the intracellular mechanisms by which ANG II stimulates VSMC hypertrophy and activation of cardiac fibroblasts, the current line of treatment options is still the best method for treating patients with cardiovascular disease. However, the identification of cell-type specific signaling mechanisms may prove to have great clinical importance. A clearer understanding of ANG II signaling and the identification of intracellular mediators, specifically in cardiac fibroblasts, could lead to the development of new pharmacologic agents that limit the onset and extent of cardiac fibrosis through direct targeting of ANG II-induced signaling mechanisms in cardiac fibroblasts.
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