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ATP Release and Signaling via P2Y Receptors Regulate Cardiac Fibroblast Phenotype and Activity

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ATP Release and Signaling via P2Y Receptors Regulate Cardiac Fibroblast Phenotype and Activity

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Molecular Pathology

by

David Lu

Committee in charge:
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Professor Lars Eckmann
Professor Hemal Patel
Professor JoAnn Trejo
Professor Francisco Villarreal

2013
The Dissertation of David Lu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2013
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LIST OF ABBREVIATIONS

5'-NT  5’ nucleotidase
ADA    adenosine deaminase
Ado    adenosine
ADP    adenosine diphosphate
Ang II angiotensin II
Apy    apyrase
ATP    adenosine triphosphate
BP     blood pressure
BFA    brefeldin A
cAMP   3’-5’-cyclic adenosine monophosphate
CBX    carbenoxolone
CFTR   cystic fibrosis transmembrane regulator
CHD    coronary heart disease
CFs    cardiac fibroblasts
CREB   cAMP response element-binding protein
CTGF   connective tissue growth factor
CVD    cardiovascular disease
Cx     connexin
DAG    diacyl glycerol
DAPI   4’,6-diamidino-2-phenylindole
DDR-2  discoidin domain receptor-2
ECM    extracellular matrix
EDV    end-diastolic volume
ENTPD  ectonucleoside triphosphate diphosphohydrolase
ERK    extracellular signal-regulated kinase
FSK    forskolin
FSP-1  fibroblast-specific protein-1
FN     fibronectin
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptors</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>Iso</td>
<td>isoproterenol</td>
</tr>
<tr>
<td>LA</td>
<td>left atrium</td>
</tr>
<tr>
<td>LOX</td>
<td>lysyl oxidase</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>NECA</td>
<td>1-(6-Amino-9H-purin-9-yl)-1-deoxy-N-ethyl-β-D-ribofuranuronamide</td>
</tr>
<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>Panx</td>
<td>pannexin</td>
</tr>
<tr>
<td>PBC</td>
<td>probenecid</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>POM-1</td>
<td>polyoxotungstate-1</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated kinase</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoendoplasmic reticulum Ca(^{2+})-ATPase</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>sST2</td>
<td>soluble ST2</td>
</tr>
<tr>
<td>TAC</td>
<td>transverse aortic constriction</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TnC</td>
<td>troponin C</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine diphosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
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PUBLICATIONS


ABSTRACT OF THE DISSERTATION

ATP Release and Signaling via P2Y Receptors Regulate Cardiac Fibroblast Phenotype and Activity

by

David Lu

Doctor of Philosophy in Molecular Pathology

University of California, San Diego, 2013

Professor Paul Insel, Chair

Cardiac remodeling is an essential process that facilitates heart development and wound healing responses post-injury. Cardiac fibroblasts (CFs) are the primary cell type responsible for maintaining myocardial structure and the cardiac extracellular matrix (ECM). CFs contribute to both basal ECM synthesis and its enhanced generation in response to cardiac injury: unregulated, these actions can lead to cardiac fibrosis and
decreased cardiac performance. Soluble factors and mechanical cues can stimulate the
transformation of CFs into myofibroblasts that increase ECM synthesis, proliferation and
migration and that express numerous pro-fibrogenic genes. The work to be described here
investigates a novel pro-fibrotic signaling pathway initiated by the release of cellular
adenosine triphosphate (ATP) from CFs via connexin (Cx) gap junction hemi-channels
following mechanical perturbations (Chapter 3). Importantly, released ATP functions as
an autocrine/paracrine signaling molecule, which activates P2Y₂ nucleotide receptors and
drives myofibroblast transformation, as indicated by increased collagen synthesis, CF
migration and proliferation and the up-regulation of pro-fibrotic genes including α-
smooth muscle actin (α-SMA) and plasminogen activator inhibitor (PAI)-1 (Chapters 3 &
4). Collagen synthesis and PAI-1 expression are sensitive to MAPK/ERK inhibition,
while α-SMA expression and CF contractile responses are sensitive to RhoA/ROCK
inhibition; both pathways are activated upon P2Y₂ stimulation by ATP. However,
attenuating this signaling are ectonucleoside triphosphate diphosphohydrolases
(ENTPDs), extracellular enzymes that hydrolyze ATP and play an essential role in
regulating pro-fibrotic nucleotide signaling. ENTPD inhibition with siRNA or
pharmacological agents increased basal extracellular ATP concentration and was
sufficient to drive CF collagen synthesis and myofibroblast transformation (Chapter 5).
Furthermore, ENTPD activity not only hydrolyzes pro-fibrotic ATP but also facilitates
the generation of adenosine, a bioactive molecule that activates A₂₅ adenosine receptors
with anti-fibrotic effects. Together, these findings identify an autocrine/paracrine
mechanism of CF regulation initiated by cellular ATP release and integrating pro-fibrotic
ATP-P2Y and anti-fibrotic adenosine-P1 signaling, which are mediated by nucleotidase
activity. This regulatory system contributes to both basal CF phenotype and response to acute cellular stress. These findings not only have direct implications in the understanding of cardiac fibrosis, but they may represent a general mechanism underlying the regulation of cellular homeostasis and response to injury.
CHAPTER 1:

CARDIAC PHYSIOLOGY AND REMODELING: THE ROLE OF CARDIAC FIBROBLASTS

1.1 Cardiovascular Disease: An Overview

Cardiovascular disease (CVD) encompasses various pathologies involving the heart or associated blood vessels and has remained the number one cause of death for nearly a century. In 2007, 813,804 deaths in the United States were due to CVD, 33.6% of all deaths recorded that year (1). Coronary heart disease (CHD), including myocardial infarction (MI) caused 1 out of 6 deaths, and an estimated 785,000 Americans experience a first-time coronary attack each year (1). Though diseases and risk factors such as diabetes mellitus, tobacco use and aging contribute to the prevalence of CVD (1), regardless of its initial etiology, CVD represents a critical global health issue where optimal treatment requires the understanding of the myriad underlying pathophysiological mechanisms.
1.2 Myocytes as a Contractile Unit

In the human heart, 75% of the myocardial mass consist of cardiomyocytes, the contractile muscle cells in the heart. Myocytes are cylindrical in shape, contain actin and myosin filaments and are responsible for executing nearly 3 billion heartbeats in an average human lifespan (2, 3). Myocytes are arranged into bundles of myofibers surrounded by an extracellular matrix (ECM) composed primarily of collagen. In addition to cardiomyocytes, cardiac fibroblasts (CFs) are the major non-contractile cell type in the heart and are found interspersed between myocytes (Fig. 1.1). CFs secrete, maintain and remodel the collagen-rich ECM, facilitating proper myocyte shortening for cardiac contraction and aiding in the essential electrical coupling required for concerted cardiac activity (4, 5).

The contractile elements within the cardiomyocyte consist of myofibrils from overlapping myosin and actin filaments (Fig. 1.2). Collectively, overlapping thick myosin and thin actin filaments form the sarcomere, the fundamental contractile unit of the myocyte. Myosin filaments consist of two heavy chains and four light chains. The heavy chains terminate in a myosin head that bind the neighboring actin filaments to initiate a “power stroke,” facilitating myocyte contraction. At rest, preventing the binding of the myosin head to actin is the troponin complex of regulatory proteins that lies between the actin filaments, which inhibit these actin-myosin interactions (3).

Upon depolarization by electrical impulses initiated and paced by the sinoatrial node located in the right atrium, extracellular Ca$^{2+}$ enters the myocyte via L-type voltage sensitive Ca$^{2+}$ channels. T-tubules, invaginations of the myocyte sarcolemma, facilitate the entry of extracellular Ca$^{2+}$ that then trigger the opening of ryanodine receptors on the
sarcoplasmic reticulum (SR), inducing rapid release of Ca$^{2+}$ stores from the SR. Ca$^{2+}$ concentration in the SR is \( \sim 1 \) mM; release of SR Ca$^{2+}$ stores during cardiac contraction raises the concentration of cytosolic Ca$^{2+}$ from \( \sim 0.1 \) \( \mu \)M during diastole to \( \sim 10 \) \( \mu \)M at the peak of systole (3).

Released cytosolic Ca$^{2+}$ binds troponin C (TnC), inducing a conformational change within the troponin complex and relieving the inhibitory association preventing myosin-actin binding. Now free to associate with actin, each myosin head binds and hydrolyzes two adenosine triphosphate (ATP) molecules, extending the head and associating with the once-adjacent actin molecule. Hydrolysis of the terminal phosphate induces a strong binding interaction and a “power stroke” that slides the actin filament \( \sim 10 \) nm in the direction of the power stroke. Collectively, these power strokes shorten the sarcomere and result in myocyte contraction. At the end of the contractile cycle, Ca$^{2+}$ re-uptake is mediated by ATP-dependent sarcoendoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) pumps, allowing for re-association of the inhibitory troponin complex.

1.3 The Cardiac Cycle

The cardiac cycle, as defined by Carl Wiggers, breaks the cardiac contraction/relaxation cycle into three stages (Fig. 1.3). The sinoatrial node initiates cardiac depolarization, which first reaches the atria and then travels through the atrioventricular junction into the ventricles. First, depolarization of the left ventricle (LV) causes release of Ca$^{2+}$ from SR stores in the myocytes, which induces isovolumic contraction and the start of systole; both the aortic and mitral valves that separate the LV
from the aorta and the LV from the left atrium (LA), respectively, are closed. As LV developed pressure exceeds aortic pressure, the aortic valve opens and blood is ejected. Subsequently, cytosolic Ca\textsuperscript{2+} concentration decreases through SERCA pump activity, resulting in myocyte relaxation and the start of diastole. Pressure in the LV decreases, and when LV pressure falls below aortic pressure, the aortic valve closes leading to isovolumic relaxation. Finally, when LV pressure drops below LA pressure, the mitral valve opens resulting in rapid LV filling, culminating with atrial systole, which maximizes LV blood volume and prepares the heart for the next cardiac cycle (3).

1.4 Systolic and Diastolic Dysfunction

The Law of Laplace, named after French mathematician Pierre-Simon Laplace, models the heart as a modified cylindrical shape where wall stress (σ) equals the product of pressure (P) and radius (R), divided by twice the wall thickness (h):

\[
\sigma = \frac{P \times R}{2h}.
\]

The Law of Laplace represents the LV as a simplified geometric shape and can be used to calculate the relationship between ventricular radius, pressure and myocardial wall thickness (6).

A variety of mechanisms can lead to the pathogenesis of cardiac dysfunction, which can be simplified into two general categories: systolic and diastolic dysfunction. Systolic dysfunction is characterized by abnormal ventricular ejection resulting from a decreased rate of developed pressure (+dP/dt) during isovolumic contraction. This reduces the stroke volume, the volume of blood pumped from the LV during each contraction. Causes of systolic dysfunction include MI, volume overload-induced
Myocardial infarction (MI), tissue death via necrosis and apoptosis caused by loss of blood flow to the myocardium, initiates a complex remodeling cascade in the ischemic area as well as in the border zone and distal regions of the heart (10). Shortly after MI, macrophages and neutrophils recruited by the release of pro-inflammatory cytokines and hormones (e.g., interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)-α, angiotensin (Ang)-II and TGF-β) migrate to the site of injury to clear the area of cellular debris, (11-13). Several days post-MI, resident CFs migrate to the site of injury and transform to myofibroblasts, which synthesize collagen (primarily types I and III), fibrin, elastin and
fibronectin in the site of injury to begin the wound healing process and form a fibrous scar, which helps prevent subsequent cardiac rupture (14, 15).

Myocyte death results in impaired contractile activity and typically leads to systolic dysfunction and reduced stroke volume. Eccentric myocyte hypertrophy, the addition of sarcomeres in series, occurs as a compensatory mechanism. This increases myocyte length and ventricular volume and may temporarily restore stroke volume. However, this compensatory mechanism is accompanied by a deleterious increase in wall stress, which further induces cardiac dilation and leads to heart failure (16). Furthermore, the same mechanisms leading to scar formation in the infarct zone can also induce pathological interstitial fibrosis in the non-infarcted areas of the myocardium, leading to reduced myocardial compliance and diastolic dysfunction (17). Notably, after MI, 22% of male and 46% of female patients develop heart failure within 6 years (18).

1.6 Cardiac Hypertrophy

Cardiac afterload is defined as the systolic load on the LV after contraction begins and is the force the LV must overcome to eject blood (7). Increased afterload can occur as a result of aortic stenosis, the narrowing of the aortic valve caused by calcification, or of chronic arterial hypertension, which increases arterial and aortic blood pressures (3). Pressure overload develops in these cases because increased LV pressure is necessary to overcome the increased afterload and maintain normal stroke volume. Concentric cardiac hypertrophy, the parallel addition of sarcomeres that increases myocyte width and wall thickness, is required to normalize wall stresses in a pressure-overloaded heart; the LV
thickens as a result (7). Concentric cardiomyocyte hypertrophy is associated with fibrosis, increased ECM production and collagen cross-linking, which decreases myocardial compliance and can result in diastolic dysfunction, poor LV filling during diastole (19, 20). Numerous hormones and locally produced cytokines and peptides mediate remodeling. TGF-β, the most studied pro-fibrotic mediator, plays a central role in cardiac remodeling during pressure overload: the use of a TGF-β-neutralizing antibody or a TGF-β receptor knockdown mouse substantially reduces fibrosis (21).

In contrast, volume overload hypertrophy can occur in response to valvular regurgitation whereby incomplete closing of the mitral valve during systole or the aortic valve after systole leads to regurgitation of ejected blood (3, 22). The increased end-diastolic volume (EDV) stretches cardiomyocytes. Following the Law of Laplace, an increase in cavity volume associated with an increased EDV requires a higher wall stress to achieve any given pressure (23). To compensate for increased EDV, cardiomyocytes undergo eccentric hypertrophy, increasing LV diameter (24). Although also requiring CF activity and the production of pro-fibrotic factors and matrix metalloproteinases, in contrast to pressure overload hypertrophy, volume overload often may not be accompanied by substantial cardiac fibrosis (22).

1.7 The Cardiac ECM and Cardiac Fibrosis

The cardiac ECM is a highly dynamic environment intimately connected to cardiac function, and investigation of the mechanisms regulating ECM production and turnover is essential to understanding and treating the progression of heart disease. CFs
produce and maintain the fibrillar collagen lattices surrounding individual cardiomyocytes and myofiber bundles; respectively termed endomysial and perimysial collagen, they consist primarily of collagen types I and III (25). Perimysial bundles are covered in yet another layer of ECM dense in collagen I and III, the epimysium (26).

Compensatory myocyte hypertrophy resulting from MI or remodeling secondary to diseases such as hypertension, aortic stenosis and valvular regurgitation is accompanied by substantial changes in the myocardial ECM (27, 28). Whether accompanying the increased wall thickness resulting from pressure overload or the LV dilation due to MI or volume overload, changes in ECM turnover occur, often deleteriously resulting in cardiac fibrosis (Fig. 1.4) (26). In heart failure patients with aortic stenosis, myocardial collagen increases by as much as 3-6 fold (29). Cardiac fibrosis is a result of CF activation and transformation: mechanical and chemical stimuli result in CF recruitment, proliferation and transformation to myofibroblasts (30). CF transformation results in a net increase of collagen synthesis and production of ECM-binding glycoproteins such as fibronectin (31). Overall, the accumulation of interstitial and perivascular collagen increases myocardial stiffness, leading to diastolic dysfunction and a decline in cardiac performance (25, 32). The following sections will focus on the origin and function of CFs, the predominant cardiac cell type responsible for ECM homeostasis and remodeling, and detail the signaling pathways regulating CF phenotype and myofibroblast transformation. These mechanisms underlie cardiac homeostasis and remodeling.
1.8 Origins of Cardiac Fibroblasts

Cardiac fibroblasts play essential roles in the production and maintenance of the collagen-rich ECM, regulation of wound healing, cardiac remodeling and facilitation of electrical conduction (4, 33). CFs are cells of mesenchymal lineage, however their precise origins are varied and can be difficult to define due to the scarcity of CF-specific markers (4). Discoidin domain receptor (DDR)-2, a collagen receptor, and fibroblast-specific protein (FSP)-1, a calcium binding protein, have been used as semi-specific markers for fibroblasts as neither are expressed in cardiomyocytes. However, DDR2 can be found in vascular smooth muscle cells, both DDR2 and FSP1 are found in leukocytes and FSP1 is present in bone marrow derived cells (34-36).

During embryonic development, CFs resident in the myocardium are thought to be derived from the epicardium as a result of epithelial-mesenchymal transformation (EMT) of epicardial cells induced by periostin and TGF-β (37-39). In the adult heart, proliferation of resident CFs, EMT of epicardial cells or endothelial-mesenchymal transformation (EndMT) of endothelial cells in response to TGF-β stimulation can produce CFs (40, 41). CFs can also originate from fibrocytes, bone-marrow derived progenitor cells, and mesoangioblasts, multipotent mesodermal stem cells (4, 17, 42). Studies that have modeled pressure overload and MI in mice estimate that approximately 25% of CFs in a fibrotic lesion are bone marrow-derived (41).

Given the varied origins of CFs, the relative contribution and localization of CF subpopulations have yet to be firmly established (17). Atrial and ventricular CFs may have distinct gene expression profiles and differential responses to stimuli such as platelet-derived growth factor (PDGF) (43). Studies in the kidney and heart have shown
that perivascular and interstitial fibroblasts may have unique origins and responses to local stimuli, suggesting further subdivision in fibroblast phenotype and response dependent on their localization within an organ (44, 45). Most reports thus far and the studies described herein treat primary isolated CFs as a homogenous population of fibroblasts. Treatment of primary CFs in vitro as a homogenous population potentially obscures differential responses of subpopulations of CFs with different origins and phenotypes.

1.9 Cardiac Fibroblast Function

Normal CFs are not functionally quiescent; they play important roles in basal myocardial remodeling, facilitate electrical conduction in the heart (4) and secrete a variety of growth factors that can regulate cell proliferation and myocyte hypertrophy (41). CFs integrate a variety of environmental cues, which include soluble signaling factors (e.g., TGF-ß, Ang II, ATP) and physical stimuli (e.g., cellular swelling, stretch, hypoxia, substrate stiffness), all of which can induce the transformation of CFs to myofibroblasts (4, 30, 33, 46). Myofibroblasts increase myocardial ECM content, display increased migratory and contractile abilities and synthesize a variety of pro-fibrotic and pro-inflammatory factors that can further increase the extent of fibrosis (30). The details of CF function and the major pathways that stimulate myofibroblast transformation will be outlined in the following sections.
1.10 ECM Synthesis and Degradation by Cardiac Fibroblasts

CFs produce and maintain the cardiac ECM, consisting mainly of collagen I and III (85% and 11% of total collagen in the heart, respectively (47)) but also containing collagen IV, V and VI, fibronectin, laminin, elastin, fibrillin and various glycoproteins (30).

Fibrillar collagen is first synthesized as a pro-α chain precursor molecule, which is then directed to the endoplasmic reticulum. There, selected prolines and lysines are hydroxylated to provide additional hydrogen bonding partners, allowing three pro-α chains to combine and form a triple helical procollagen protein (48). The procollagen is then exported from the cell and the propeptides removed, allowing for the self-assembly of collagen proteins into larger collagen fibrils in the extracellular space (48). Finally, the fibrillar collagen helices are selectively cross-linked by lysyl oxidase (LOX), conferring proteolytic resistance and tensile strength to mature collagen fibrils. Pro-fibrotic agonists can greatly accelerate the production of collagen proteins in CFs and by LOX up-regulation, the extent of collagen crosslinking (49). Increased LOX-mediated collagen crosslinking is implicated in adverse remodeling and stiffening of the myocardium (50).

In addition to de novo collagen synthesis, CFs also produce numerous zinc-dependent matrix metalloproteinase (MMP) enzymes that regulate ECM turnover by degrading collagen and other matrix proteins. Numerous soluble MMP isoforms and the membrane-bound MT1-MMP/MMP-14 isoform have been associated in cardiac remodeling (30, 51). The relevant MMP isoforms and their respective ECM substrates are listed in Table 1.1. Of the MMPs that can degrade collagen, studies in human and rodent CFs have identified MMP-1, -2, -3, -9, -13 and -14 as the major isoforms expressed in
CFs with MMP-2 and -9 being the best understood in the context of cardiac remodeling (52).

MMPs are synthesized in their inactive forms consisting of a pro-peptide sequence that blocks the catalytic domain. Removal of the pro-peptide and activation of the enzyme is achieved by the activity of other proteases including thrombin, plasmin, trypsin and neighboring MMPs (15, 24, 53, 54). Additional control of MMP activity following transcriptional regulation and proteolytic activation occurs by tissue inhibitors of metalloproteinases (TIMPs), which bind to the MMP catalytic domain and prevent substrate access (24). TIMP1 and TIMP2 are released from CFs (52, 55).

1.11 ECM Production and Degradation in Pathologic Cardiac Remodeling

MMPs play a complex role in both post-MI and hypertrophic remodeling. Both MMP-2 and -9, elevated in response to MI and pressure overload hypertrophy, are associated with systolic dysfunction and matrix accumulation (24, 56). MMP-2\(^{-/-}\) mice subjected to experimental MI exhibited a higher survival rate due to a decreased likelihood of cardiac rupture. However, the MMP-2\(^{-/-}\) mice had reduced macrophage infiltration, resulting in reduced clearance of necrotic cardiomyocytes (57). A study of MMP-9\(^{-/-}\) mice demonstrated attenuation of LV enlargement and collagen accumulation after MI (58). These studies indicate that MMP activity can contribute to cardiac rupture and heart failure. The regulated action of matrix proteolysis is an important aspect of remodeling as it facilitates normal macrophage and fibroblast infiltration, essential to proper wound healing. Notably, in addition to their roles in matrix degradation, MMPs
also can cleave and activate latent proteins in the ECM such as TGF-β. The cleavage and activation of pro-TGF-β can stimulate additional ECM production and deposition by CFs, representing a feedback loop that regulates pro-fibrotic CF activity (59, 60).

1.12 Fibroblast-ECM interactions

Myocardial collagen is organized in a complex three-dimensional matrix surrounding the myocytes and vasculature and confers tensile strength and elastic properties to the myocardium (61). CFs are thought to adhere to and contract the collagen network, integrating both chemical and mechanical signals (62). The expression of α-SMA in pro-fibrogenic myofibroblasts increases their contractile ability and aids in wound healing. α-SMA is the most clearly defined marker of transformed myofibroblasts, as quiescent CFs have little α-SMA expression (63). However, the mechanical environment of the ECM also influences the phenotype and activity of resident CFs, and many of these mechanical cues signal via the actin cytoskeleton (63).

Integrins on fibroblasts interact with the surrounding matrix within extensive cell-matrix contacts called focal adhesions (FAs), where the intracellular actin cytoskeleton is mechanically linked to the ECM via the concerted action of integrins αvβ3 and α5β1, and the cytoskeletal proteins vinculin and paxillin (63, 64). The up-regulation of α-SMA expression in fibroblasts seems to require the transmission of force through these FA-actin contacts (65, 66). Fibroblasts cultured in stiff substrates rapidly express α-SMA, whereas fibroblasts in a highly deformable environment do not (67). The mechanism by which force sensing results in myofibroblast transformation is likely to involve FA-
dependent activation of RhoA and Rho-associated kinase (ROCK), which induce actin polymerization and α-SMA stress fiber formation (68-70). Furthermore, Hinz et al. have hypothesized that mechanical tension produced by myofibroblast contraction activates latent TGF-β present in the ECM, which may represent a positive feedback loop wherein α-SMA expression further drives myofibroblast transformation (60).

The infarcted or scarred heart is several-fold stiffer than normal myocardium, and has been shown to interfere with cardiomyocyte contraction and stimulate CF migration and spreading (71). Though force transmission via the actin cytoskeleton and the ECM, fibroblasts receive mechanical cues to regulate their set point and activity in a physical, tissue-dependent context. These cues likely integrate with chemical signals to regulate basal remodeling and response to injury.

1.13 The Role of TGF-β and Ang II Signaling in Fibrosis

TGF-β (TGF-β1) is the best-characterized signaling protein that drives the transformation of CFs and other fibroblasts into myofibroblasts. TGF-β synthesis by local cells and tissues, including macrophages, fibroblasts and myocytes, is induced by stress and injury as well as by circulating Ang II (17, 72-74). Once secreted, TGF-β is usually maintained in an inactive form via its interaction with a latent TGF-β binding protein (LTBP) (72, 75). Extracellular proteases such as MMPs and plasmin can cleave LTBP and release active TGF-β, which can subsequently bind to heteromeric TGF-β type I/II receptor complexes to initiate intracellular signaling (72). The canonical TGF-β receptor signaling pathway involves phosphorylation of intracellular mediators Smad-2 and -3.
The activated Smad2/3 complex binds Smad4 and translocates to the nucleus, where it associates with transcriptional cofactors to up-regulate target gene transcription (72). Additionally, TGF-β can activate other signaling pathways, including ERK, p38 and Rho/ROCK (72, 76).

**Abundant in vitro and in vivo** evidence demonstrates the potent pro-fibrotic effects of TGF-β signaling (21, 59, 72, 73, 77). TGF-β treatment increases collagen synthesis and transformation of CFs *in vitro* (78, 79), and its expression is up-regulated locally at sites of myocardial infarction (59). Furthermore, *in vivo* TGF-β overexpression in rodents stimulates cardiac fibrosis, whereas blockade of TGF-β signaling by a neutralizing antibody attenuates cardiac fibrosis and diastolic dysfunction in pressure-overloaded rat hearts (77, 80).

Another well-characterized pro-fibrotic mediator is Ang II. A product of the renin-angiotensin system, Ang II is a circulating peptide hormone strongly associated with heart failure, hypertension and fibrosis (81). Ang II is produced from angiotensinogen by the activity of angiotensin-converting enzyme (ACE), which is found in renal, pulmonary and cardiac endothelia as well as circulating in the blood (82). The effects of Ang II signaling in the heart are mediated primarily through the activation of AT₁ receptors (81). AT₁ receptor stimulation can activate the Rho/ROCK, ERK, p38 and JNK signaling pathways, resulting in α-SMA expression, ECM synthesis and pro-fibrotic gene expression (52, 81). Notably, Ang II also up-regulates TGF-β expression and thus links TGF-β activity with Ang II signaling (77). *In vivo* administration of Ang II into rodents increases inflammatory cell infiltration into the heart and stimulates myocyte hypertrophy and cardiac fibrosis (83, 84).
Generally, TGF-β and Ang II signaling stimulate ECM synthesis, α-SMA expression and the induction of a pro-fibrotic gene expression profile in fibroblasts (Table 1.2). Of the numerous markers in CFs typically assessed, we found that plasminogen activator inhibitor (PAI)-1 is the most highly up-regulated one by pro-fibrotic signaling (46). PAI-1 (SERPINE1) is a serine protease inhibitor that inhibits urokinase- and tissue-plasminogen activators (uPA and tPA, respectively). uPA and tPA activity results in the cleavage activation of plasmin, a protease that cleaves numerous ECM substrates including fibrin, fibronectin and laminin, and that can activate latent MMPs and TGF-β (85, 86). By inhibiting the activity of matrix-degrading proteases, PAI-1 is implicated in fibrosis in a variety of tissues such as skin, kidney, lung, liver and heart (86). In the heart, PAI-1 up-regulation is associated with post-MI fibrosis and TGF-β synthesis, but PAI-1 deficiency is protective against these pro-fibrotic sequelae (87). However, other studies have demonstrated pro-fibrotic responses to PAI-1 deficiency: increased inflammatory cell and fibroblast recruitment, elevated TGF-β levels and activation of pro-fibrotic Smad and MAPK pathways (88, 89). Thus, the role of PAI-1 in mediating cardiac fibrosis is controversial, as different groups have reported conflicting results. Likely, the pro-fibrogenic action of PAI-1 is dependent on cell type and its temporal and spatial localization during remodeling responses, but further studies are necessary to conclusively understand the role of PAI-1 in the development of fibrosis.

In addition to the relatively well-established pathways mediated by Ang II and TGF-β, another important mechanism regulating cellular homeostasis and response involves the release and autocrine/paracrine signaling of extracellular nucleotides, particularly ATP, but also UTP, ADP and UDP (90). Though the roles of such
nucleotides in the vascular system in regulating vascular tone, inflammation and platelet activation are well documented (90), their roles in fibroblast regulation and tissue remodeling are not clear and are a topic of strong interest. Pathways of extracellular nucleotide release and its signaling roles as a mediator of cellular function are described in the following Chapter.
Figure 1.1: Myocyte-fibroblast arrangement in the myocardium. (Top) Cross-section diagram illustrating the arrangement of cardiomyocytes (green) and cardiac fibroblasts (red). (Bottom) Immunofluorescent staining of rat myocardium: CFs stained red for DDR2 are present between cardiomyocytes (green).
Figure 1.2: A sarcomere, the fundamental unit of muscle contraction. The sarcomere consists of overlapping myosin and actin filaments, which form a unit of contraction delineated by the Z lines. Muscle contraction resulting from ATP-dependent power strokes increases the amount of overlap between myosin and actin filaments, shortening the sarcomere.
Figure 1.3: Wiggers diagram illustrating the cardiac cycle. Plotted as a function of the various stages of cardiac contraction are ventricular pressure (blue), ventricular volume (red), and aortic and atrial pressures (black). Reading from left to right: 1) isovolumic contraction begins systole by increasing ventricular pressure until it exceeds aortic pressure, opening the aortic valve and leading to blood ejection; 2) isovolumic relaxation follows when ventricular pressure first drops below aortic pressure, closing the aortic valve. When ventricular pressure drops below atrial pressure, the mitral valve opens and the ventricles fill with blood; 3) atrial systole further fills the ventricles until the mitral valve closes once again, once again leading to systole.
Figure 1.4: Cross-section of normal and fibrotic mouse heart. Mice were subjected to pressure overload hypertrophy (right). Extensive collagen deposition visualized by Sirius red staining as well as ventricular dilation can be seen in hearts subjected to pressure overload vs. sham control (left).

Table 1.1: MMP subtypes in the myocardium and their known substrates (91).

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Collagens I, II, III, VII, X, gelatin</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Collagens I, IV, V, VII, X, fibronectin, laminins</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Collagens III, IV, IX, X, fibronectin, laminins</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Collagen IV, gelatin, fibronectin, laminins, elastin</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Collagens I, II, III</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Collagens IV, V, XIV, elastin</td>
</tr>
<tr>
<td>MMP-10</td>
<td>Collagen IV, fibronectin</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Elastin</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Collagens I, II, III, gelatin, fibronectin, laminins</td>
</tr>
<tr>
<td>MMP-14/MT1-MMP</td>
<td>Collagens I, II, III, fibronectin, laminins, proMMP-2, proMMP-13</td>
</tr>
</tbody>
</table>
Table 1.2: Genes associated with cardiac fibrosis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression (↑ or ↓) with profibrotic stimuli</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-smooth muscle actin (α-SMA)</td>
<td>↑ (63)</td>
</tr>
<tr>
<td>Collagen I</td>
<td>↑ (4, 5)</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>↑ (15)</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor (PAI)-1</td>
<td>↑ (86, 92)</td>
</tr>
<tr>
<td>Connective tissue growth factor (CTGF/CCN2)</td>
<td>↑ (93)</td>
</tr>
<tr>
<td>Transforming growth factor (TGF)-β</td>
<td>↑ (59, 72, 77)</td>
</tr>
<tr>
<td>Cysteine-rich angiogenic inducer 61 (CYR61/CCN1)</td>
<td>↑ (93)</td>
</tr>
<tr>
<td>Lysyl oxidase (LOX)</td>
<td>↑ (50)</td>
</tr>
<tr>
<td>Secreted protein acidic and rich in cysteine</td>
<td>↑ (11, 94)</td>
</tr>
<tr>
<td>Periostin</td>
<td>↑ (95)</td>
</tr>
<tr>
<td>Monocyte chemotactic protein (MCP)-1</td>
<td>↑ (96)</td>
</tr>
<tr>
<td>Interleukin (IL)-6</td>
<td>↑ (97)</td>
</tr>
<tr>
<td>IL-33</td>
<td>↑ (98, 99)</td>
</tr>
<tr>
<td>sST-2</td>
<td>↑ (98, 99)</td>
</tr>
<tr>
<td>Thrombospondin-1</td>
<td>↑ (100)</td>
</tr>
<tr>
<td>Relaxin</td>
<td>↑ (101, 102)</td>
</tr>
<tr>
<td>Epac1</td>
<td>↓ (79)</td>
</tr>
<tr>
<td>Snail</td>
<td>↑ (103)</td>
</tr>
<tr>
<td>Slug</td>
<td>↑ (103)</td>
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</tbody>
</table>
1.15 References


CHAPTER 2:

NUCLEOTIDE RELEASE AND SIGNALING

2.1 Cellular Nucleotide Release

Adenosine triphosphate (ATP), the ubiquitous energy carrier in the cell, is generated by metabolic processes and accumulates intracellularly in the low millimolar range (1). Burnstock coined the term “purinergic” signaling in 1971 when observing the release of ATP from nerves in the autonomic nervous system. In 1977, Forrester and Williams first observed the release of ATP from isolated cardiac cells (2, 3).

Though the release of cellular nucleotides and their participation in signaling once in the extracellular milieu has been observed for decades, novel mechanisms and roles of nucleotide signaling continue to be discovered. Nucleotide signaling plays roles in virtually all tissues and organ systems (4). ATP can act as a neurotransmitter (5), regulate vascular tone, control cell proliferation, and stimulate platelet activation and inflammation (3). As an inflammatory mediator, release of ATP from apoptotic cells triggers leukocyte and fibroblast recruitment to sites of injury (6, 7). ATP can also stimulate proliferation of liver portal fibroblasts and pro-fibrotic activation of hepatic stellate cells (8, 9), representing a paracrine mechanism regulating cellular homeostasis.
The extracellular nucleotide signaling cascade is initiated by the release of cellular ATP, either from intracellular vesicles or from cytoplasmic stores (4). Studies examining the pericellular space of airway epithelial cells detected ATP in nanomolar concentrations around resting cells, but hypotonic stimulation can increase pericellular ATP concentrations ~1,000-fold (10), concentrations sufficient to activate various nucleotide receptors on the cell surface (11). UTP, another nucleotide that can activate downstream signaling cascades, is typically found pericellularly at nanomolar concentrations (12), far less abundant than ATP but still potentially contributing to purinergic signaling. It is likely that in any given cell, a variety of ATP (and UTP) release mechanisms work in concert to regulate the release of cellular nucleotides and subsequent downstream signaling events, but their precise contributions in any tissue is largely unclear.

Several non-lytic processes can be involved in the direct release of cellular ATP. Vesicular exocytosis of ATP-dense granules is a Ca\(^{2+}\)-dependent processes commonly found in excitatory and secretory cell types such as neurons, platelets and pancreatic and biliary epithelial cells (13). ATP concentration in secretory vesicles can reach a range of 0.1-1 M. The exact mechanism of active ATP transport into vesicles is not well understood but may involve the SLC17 type 1 phosphate family of vesicular transport proteins (14). Exocytosis requires interaction and docking of v-SNARE and t-SNARE proteins localized on the vesicular and target membranes, respectively (15, 16). Though vesicular release of ATP has been observed in non-excitatory cell types, other mechanisms also play prevalent roles in ATP release. Facilitated diffusion of ATP and other small molecules may occur via ATP binding cassette (ABC) transporters and
voltage- or stretch-activated anion channels (4). The cystic fibrosis transmembrane regulator (CFTR) Cl⁻ channel, an ABC transporter, was thought to release ATP, but additional data indicated that CFTR does not directly conduct ATP, and thus its role as an ATP-permeable pore remain unclear (15). Numerous studies have described the release of ATP via ion channels. Cell swelling (e.g., in response to hypotonic challenge), mechanical stretch or fluid shear can activate stretch- and volume-sensitive ion channels (17), which have a variety of other roles, including cell volume regulation, sensory perception, cell differentiation and action potential initiation (18, 19). Notably, ATP conductance though these channels has been observed in numerous cell types and is sensitive to ion channel blockers such as gadolinium and niflumic acid (15).

Gap junction hemi-channels are another potential route of cellular ATP release and, as described in Chapter 4, are important for the initiation of autocrine/paracrine nucleotide signaling in CFs. The structure and function of connexin and pannexin hemi-channels, and their roles in ATP release, are described in the following sections.

### 2.2 Connexin and Pannexin Hemi-Channel Structure

Connexins (Cxs) are a family of plasma membrane-spanning proteins that form hexameric oligomers (connexons) creating a transmembrane pore bridging the cytosol with the extracellular milieu (20). Connexons on neighboring cells can dock and form non-covalent interactions via their extracellular loops, resulting in electrical and chemical coupling between cells (Fig. 2.1) (21). Transport of molecules of <1 kDa in size can occur through these channels (3). Cx43, the most abundant Cx isoform in the heart, forms
the majority of gap junctions between cardiomyocytes (22). Cx40, Cx43 and Cx45, the major myocardial Cx isoforms, form junctions between neighboring myocytes and fibroblasts (22-24). Importantly, in addition to the transport of molecules between adjacent cells, transport can also occur through undocked “hemi-channels”, which form a ~1.4-1.8 nm diameter pore for intracellular molecules to pass into the pericellular and extracellular space (25, 26).

There are about 20 known human Cx isoforms, though the function and pore-forming ability of several isoforms are as of yet unknown (3). The expression of Cx hemi-channel proteins is cell- and tissue-dependent, as are the permeability and mechanisms of connexon gating (20). Connexon channels can be comprised of both homomeric and heteromeric combinations of Cx proteins, and though the possible combinations of Cx oligomerization are based on their structural similarities, not all physiologically relevant combinations are known (25).

Similar to Cxs, the pannexin (Panx) family are membrane-spanning proteins consisting of three isoforms, Panx-1, -2 and -3, which are thought to share functional homology with Cx proteins, though no sequence homology exists (27). Panx1, which is nearly ubiquitously expressed in all tissues, is the best understood of the three isoforms and is capable of forming hexameric structures akin to Cx hemi-channels. Panx2 expression is thought to be mostly restricted to the brain while Panx3 has been found only in some osteoblast, synovial fibroblast and skin cell lines (28, 29). Notably, the ability of Panxs to form gap junctions has not been observed under physiologic conditions (29). Furthermore, only Panx1 has thus far been implicated in the physiologic formation of hemi-channels in tissues. Reports of Panx3 forming hemi-channels in cell
lines have been reported, but the physiologic roles of Panx1 hemi-channels are the best characterized (27).

### 2.3 Regulation of Hemi-Channel Gating

Studies investigating hemi-channel permeability have used a variety of methods to detect the release of small molecules such as ATP into the pericellular space, as well as the internalization of Cx- and Panx-permeable dyes (20, 23, 30). Cx hemi-channels are usually closed in resting cells (20). However, changes in membrane potential, low extracellular Ca\(^{2+}\) as well as mechanical stimulation increase the likelihood of hemi-channel opening (20). Ca\(^{2+}\) binding sites consisting of aspartate residues have been identified on the extracellular loops of Cx32, and Ca\(^{2+}\) binding causes a conformational change leading to pore occlusion (31, 32). Recent studies by Batra et al. discovered that mechanical force could induce Cx43 hemi-channel opening in osteocytes via activation of α5β1 integrin (33). Though their data noted the dependence of PI3K signaling on integrin-mediated Cx43 opening, the exact signaling mechanism is unclear. These findings are novel in that they describe a mechanism linking physical perturbations of the cell surface with hemi-channel gating. Other studies have observed gating mechanisms dependent on PKC- and PKA-mediated phosphorylation of intracellular serine residues leading to hemi-channel closing (20). Thus, the mechanisms regulating Cx hemi-channel gating likely involve a variety of pathways integrating post-translational modifications, protein kinases and mechanical sensors (e.g., α5β1 integrin). We observed the rapid release of ATP through Cx hemi-channels in response to hypotonic stimulation (Chapter
4), indicating that mechanical forces are sufficient to open Cx hemi-channels in ventricular CFs.

Compared to Cxs, less is known about the mechanisms mediating Panx gating. Similar to many Cxs, Panx1 hemi-channels have been shown to open in response to mechanical stimuli, though they are insensitive to extracellular Ca\(^{2+}\) (30). Furthermore, numerous consensus sites for protein kinases have been identified in the cytoplasmic loops, suggesting a role for phosphorylation and post-translational modification in the regulation of Panx gating (30).

The mechanisms underlying hemi-channel permeability have implications in the regulation of electrical conductance, cell survival and the release of intracellular signaling molecules. The findings to be discussed in this dissertation do not address the precise mechanism of Cx/Panx gating but instead focus on the effects of hemi-channel permeability in the initiation of autocrine/paracrine signaling.

2.4 Paracrine Signaling by ATP Released via Hemi-channels

Hemi-channels provide a permeable pore for the release of ATP and other nucleotides that play a key role in the initiation of downstream signaling via the activation of nucleotide receptors. In the heart, Nishida et al. demonstrated that Panx1 hemi-channels are responsible for the release of UDP from murine cardiomyocytes, and the subsequent activation of P2Y\(_6\) nucleotide receptors, leading to myocyte hypertrophy and cardiac fibrosis (34). Importantly, blockade of hemi-channels with the pharmacological inhibitors carbenoxolone and 1-heptanol suppressed UDP release and
downstream signaling. In a separate study, Panx1 was shown to be up-regulated in canine myocytes following ischemia, corresponding with an increase in ATP release, which induces pro-fibrotic CF activation (35). Furthermore, Clarke et al. demonstrated that Cx-specific mimetic peptides are capable of blocking ATP release from cardiomyocytes following simulated ischemia, suggesting that ATP is a signaling molecule indicative of myocardial injury (36). In the lung, Panx1-mediated ATP release in response to cellular injury triggers an inflammatory and pro-fibrotic response and may contribute to pulmonary fibrosis (6). Together these findings exemplify the importance of hemi-channels as conduits of ATP release, and they identify numerous functional roles of released ATP as a signaling mediator of acute responses to injury or ischemia.

2.5 P2X and P2Y Nucleotide Receptors

Cellular responses to extracellular nucleotides typically involve activation of a diverse family of P2 purinergic receptors to initiate intracellular signaling cascades. Two types of nucleotide (purinergic) receptors exist: the P2X and P2Y subfamilies. Expressed in nearly all mammalian tissues, the P2X receptors are ATP-gated ion channels consisting of seven subtypes, P2X<sub>1-7</sub> (37). P2X receptor subunits are composed of two transmembrane domains, a single extracellular loop and intracellular N- and C-termini. A functional P2X receptor requires the association of the subunits to form a trimer (38). ATP binds via a generally conserved binding pocket lined with positively charged amino acid residues (39). P2X receptors have a high affinity for ATP and ATP-derived analogs, but have weak or no binding to other nucleotides and adenosine (39).
ATP binding to P2X receptors opens the non-selective channel, allowing the passage of monovalent and divalent cations. This results in a change of membrane potential and the elevation of intracellular Ca$^{2+}$ levels, which can stimulate an array of Ca$^{2+}$-dependent intracellular signaling pathways (39).

The P2Y receptor subfamily consists of eight 7 transmembrane/G protein-coupled receptors responsive to a variety of extracellular nucleotides with distinct G protein couplings (Table 2.1). The G$\alpha$-coupled P2Y receptors, P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$ and (partially) P2Y$_{11}$, activate phospholipase C (PLC), which generates diacylglycerol (DAG) and inositol triphosphate (IP$_3$), stimulating the release of intracellular Ca$^{2+}$ from the endoplasmic reticulum (ER) (40). P2Y$_{11}$ is the only P2Y receptor also capable of activating G$_i$ and stimulating the generation of cAMP. The remaining P2Y receptors, P2Y$_{12}$ and P2Y$_{13}$ and P2Y$_{14}$ couple to G$_i$ and inhibit adenylyl cyclase activity, decreasing intracellular cAMP production (40).

Functional responses to P2X and P2Y receptor activation are cell- and context-dependent, and represent the integration of numerous signaling pathways. For instance, in the vasculature, Cx43 is implicated in ATP release from endothelial cells (41), which can stimulate both P2X and P2Y receptors with different effects on vascular tone depending on cell type. ATP induces vasoconstriction on vascular smooth muscle cells, while it has a vasodilatory effect on endothelial cells (42). In mouse cardiomyocytes, P2Y$_6$ activation by UDP produces pro-fibrotic transformation of CFs in response to mechanical stretch (34). P2Y receptor stimulation can also activate RhoA and stimulate actin stress fiber formation and smooth muscle contraction, potentially contributing to hypertension and atherosclerosis (43). In vivo studies in animals demonstrate that ischemia causes the rapid
release of ATP, ADP and UTP into the blood (44), which can induce responses that include vasodilation and increased blood flow, ischemic preconditioning and cardiac arrhythmia (44-46).

These studies demonstrate the diverse role of P2 receptor signaling in regulating cellular physiology and organ function. Nevertheless, the role of ATP release pathways and mechanisms of downstream autocrine/paracrine signaling still represent a relatively poorly understood axis of regulation. Data to be presented in subsequent Chapters seeks to investigate the role of ATP release and P2Y receptor signaling in regulating CF phenotype and activity and examines its implications in cardiac remodeling and fibrosis.

2.6 Enzymatic Hydrolysis of Extracellular Nucleotides

An additional regulator of the nucleotide signaling cascade is the hydrolysis of extracellular nucleotides by endogenously expressed NTPases, which terminate ATP/UTP-mediated signaling but can also initiate ADP/UDP and adenosine-promoted signaling pathways (4). Ectonucleoside triphosphate diphosphohydrolases (ENTPDs) are endogenous Ca\(^{2+}/\text{Mg}^{2+}\)-dependent nucleotidases that hydrolyze tri- and di-phosphate nucleotides into their monophosphate forms. Of the eight ENTPD isoforms (ENTPD1-8), four (ENTPD1-3, 8) are cell surface-localized (47). The membrane-anchored subtypes have two transmembrane domains, which also confer substrate specificity, and ENTPDs are known to oligomerize, increasing catalytic activity (48). ENTPD-1 and -2 are the most studied subtypes; their roles in inflammation, platelet activity and vascular tone are well documented (42, 49, 50). Both ENTPD-1 and -2 prefer adenine nucleotides versus
uracil nucleotides, and while ENTPD1 hydrolyzes ATP and ADP with nearly equal affinity, ENTPD2 strongly prefers ATP and can result in the generation of a pool of bioactive ADP (47, 48). Furthermore, although ENTPDs do not hydrolyze nucleotide monophosphates, the concerted action of membrane-localized 5’-nucleotidases, such as CD73, can generate adenosine, another bioactive signaling molecule (47). ENTPD activity is thus an important aspect of P2 signaling that attenuates nucleotide-dependent responses and induces adenosine-promoted pathways (48).

In a well-studied role, ENTPD1 activity in the vascular endothelium attenuates thrombosis by hydrolyzing ADP and regulating platelet activation (51). Platelets release ADP, which activate P2Y1 and P2Y12 receptors and cause platelet shape change and aggregation (52). Hydrolysis of ADP by ENTPD1, the major nucleotidase in the vascular lumen, attenuates platelet aggregation and thrombosis. By contrast, ENTPD2 may promote thrombus formation due to its preferential hydrolysis of ATP to generate extracellular ADP (49, 51). ENTPD activity has also been implicated in mediating cardiac protection post-ischemia, likely through the facilitation of adenosine generation, a cardioprotective molecule (53): ENTPD1-deficient mice or mice treated with an ENTPD inhibitor were more susceptible to ischemic injury due largely to impaired adenosine generation, while hydrolysis of extracellular ATP with the nucleotidase apyrase increased adenosine generation and reduced infarct size after ischemic insult (53).

Jhandier et al. discovered that ENTPD2 activity in liver portal fibroblasts attenuates bile duct epithelial cell proliferation (9). Loss of portal fibroblast ENTPD2 expression increased epithelial cell proliferation, indicating a paracrine mechanism involving nucleotide signaling and hydrolysis in the regulation of epithelial cell
homeostasis. These results also imply involvement of a similar mechanism in hepatic stellate cell and portal fibroblast proliferation and activation, potentially leading to the development of liver fibrosis (48). The data to be presented in Chapter 5 explores a similar role of ENTPD activity in regulating CF transformation by attenuating pro-fibrotic nucleotide signaling.

2.7 Hypothesis and Significance of the Results Presented Herein

Work in this dissertation investigates the signaling mechanisms regulating CF homeostasis and transformation into myofibroblasts. Understanding the signaling pathways underlying CF phenotype and function aids in our understanding of the pathogenic mechanisms behind cardiac fibrosis and diastolic dysfunction. Early in the work, I discovered that ATP and UTP stimulation of isolated rat ventricular CFs was strongly pro-fibrotic: increasing α-SMA expression, collagen synthesis, CF proliferation and migration, and the production of pro-fibrotic markers (Fig. 2.2). Due to their responsiveness to both adenine- and uracil-derived nucleotides, I hypothesized that activation of P2Y (and not P2X) receptors drives CFs towards a myofibroblast state. I discovered that P2Y2 receptors are the most highly expressed P2 receptor in rat ventricular CFs (results independently confirmed by GPCR gene array analysis) and are the primary receptors responsible for ATP- and UTP-induced effects. Those results are presented in Chapter 3.

The remainder of work described in this dissertation builds upon these initial findings. I hypothesized that nucleotides, in particular ATP, may be released from CFs
subjected to mechanical stimulation to initiate an autocrine/paracrine signaling pathway regulating CF phenotype. If correct, this hypothesis would imply that mechanical forces or cellular stress (e.g., those associated with increased myocardial wall stress or infarction) could instigate CF recruitment and activation through nucleotide-P2Y receptor signaling. Chapter 4 presents my findings demonstrating that cellular swelling in response to hypotonic stimulation causes ATP release from CFs via Cx hemi-channels. Released ATP is then sufficient to activate P2Y$_2$ receptors in an autocrine/paracrine manner, resulting in CF transformation and fibrogenic ECM synthesis.

Furthermore, during experimentation examining the effect of ATP stimulation on CFs, I noted that constitutive release of ATP and signaling seemed to play a substantial role in the regulation of basal CF “tone.” I discovered that hydrolysis of extracellular ATP with the nucleotidase apyrase strongly decreases basal $\alpha$-SMA expression and collagen synthesis. This lead me to hypothesize that the previously discovered ATP-P2Y$_2$ signaling pathway may not only represent a mechanism governing the response of CFs to stress or injury but also contributes to the basal maintenance of CF phenotype. Thus, I hypothesized that endogenous nucleotidases, such as ENTPDs, might play an essential role in the hydrolysis of basally released ATP and attenuation of a pro-fibrotic stimulus. Chapter 5 presents my findings showing that inhibition of endogenous ENTPD activity via siRNA-mediated knockdown and pharmacologic inhibition is pro-fibrotic through the enhancement of ATP signaling in CFs. These data also indicate that adenosine, presumably generated by ATP hydrolysis, activate a counterbalancing pathway via $G_s$-coupled A$_2$ receptor activation to attenuate pro-fibrotic nucleotide-P2Y signaling in CFs.
Together, the results presented in this thesis examine the role of ATP release and P2Y receptor signaling in CFs with implications in the development of cardiac fibrosis (Fig. 2.3). This work investigates a novel pathway of nucleotide release in ventricular CFs that initiates an autocrine/paracrine signaling pathway leading to pro-fibrotic CF activation and collagen synthesis. This signaling mechanism may be involved in CF response to myocardial stress or damage, as well as in the homeostatic maintenance of CF phenotype and activity. In addition to their implications in understanding the pathogenesis of cardiac fibrosis, the data presented here may be applicable to other cell types and tissues. Because of the various potential conduits of ATP release and the ubiquitous expression of P2Y receptors in the body, it is likely that similar pathways can regulate both cellular homeostasis and response to acute stress or tissue damage akin to what occurs in CFs.
2.8 Figures

Figure 2.1: Illustration of connexin proteins and the hexameric structures forming a connexon hemi-channel.

Table 2.1: Preferred ligands and coupling of P2Y receptors (11, 54).

<table>
<thead>
<tr>
<th>P2Y receptor</th>
<th>Preferred ligands (EC_{50}, µM)</th>
<th>Coupling</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2Y_{1}</td>
<td>ADP (1) &gt; ATP (4)</td>
<td>G_{q}</td>
</tr>
<tr>
<td>P2Y_{2}</td>
<td>UTP (0.14) ≈ ATP (0.23)</td>
<td>G_{q}</td>
</tr>
<tr>
<td>P2Y_{4}</td>
<td>UTP (2.5) [rodent: UTP (2.6) ≈ ATP (1.8)]</td>
<td>G_{q}</td>
</tr>
<tr>
<td>P2Y_{6}</td>
<td>UDP (0.300) &gt; UTP (6) &gt; ADP (30)</td>
<td>G_{q}</td>
</tr>
<tr>
<td>P2Y_{11}</td>
<td>ATP (17.4)</td>
<td>G_{i}/G_{q}</td>
</tr>
<tr>
<td>P2Y_{12}</td>
<td>ADP (60.7)</td>
<td>G_{i}</td>
</tr>
<tr>
<td>P2Y_{13}</td>
<td>ADP (60) &gt; ATP (261)</td>
<td>G_{i}</td>
</tr>
<tr>
<td>P2Y_{14}</td>
<td>UDP-glucose (80) &gt; UDP-galactose (124)</td>
<td>G_{i}</td>
</tr>
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</table>

Table 2.2: Major ectonucleotidase enzymes (47).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Major substrates</th>
<th>Products</th>
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</thead>
<tbody>
<tr>
<td>ENTPD1</td>
<td>NTP = NDP</td>
<td>NMP, P_i</td>
</tr>
<tr>
<td>ENTPD2</td>
<td>NTP &gt; NDP</td>
<td>NMP, P_i</td>
</tr>
<tr>
<td>ENTPD3</td>
<td>NTP = NDP</td>
<td>NMP, P_i</td>
</tr>
<tr>
<td>ENTPD4</td>
<td>NTP = NDP</td>
<td>NMP, P_i</td>
</tr>
<tr>
<td>ENTPD5</td>
<td>NDP</td>
<td>NMP, P_i</td>
</tr>
<tr>
<td>ENTPD6</td>
<td>NDP</td>
<td>NMP, P_i</td>
</tr>
<tr>
<td>ENTPD7</td>
<td>UTP, GTP, CTP &gt; ATP</td>
<td>NMP, P_i</td>
</tr>
<tr>
<td>ENTPD8</td>
<td>NTP &gt; NDP</td>
<td>NMP, P_i</td>
</tr>
<tr>
<td>Ecto-5’-nucleotidase</td>
<td>NMP</td>
<td>Nucleoside, P_i</td>
</tr>
<tr>
<td>Ecto-nucleotide pyrophosphatase</td>
<td>NTP = NDP</td>
<td>NMP, PP_i, P_i</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>NTP ≈ NDP ≈ NMP</td>
<td>Nucleoside, P_i</td>
</tr>
</tbody>
</table>
Figure 2.2: Diagram of pro-fibrotic signaling in cardiac fibroblasts.

Figure 2.3: Potential autocrine/paracrine signaling pathways activated by release of cellular ATP.
2.9 References


CHAPTER 3:

URIDINE TRIPHOSPHATE (UTP) INDUCES PRO-FIBROTIC RESPONSES IN CARDIAC FIBROBLASTS BY ACTIVATION OF P2Y₂ RECEPTORS

3.1 Abstract

Cardiac fibroblasts (CFs) play a key role in response to injury and remodeling of the heart. Nucleotide (P2) receptors regulate the heart but limited information is available regarding such receptors in CFs. We thus sought to determine if extracellular nucleotides regulate fibrotic responses (e.g., proliferation, migration and expression of pro-fibrotic markers) of CFs in primary culture. UTP increased rat CF migration 3-fold (p < 0.001), proliferation by 30% (p < 0.05) and mRNA expression of pro-fibrotic markers: alpha smooth muscle actin (α-SMA), plasminogen activator inhibitor-1 (PAI-1), transforming growth factor beta, soluble ST2, interleukin-6 and monocyte chemoattractant protein-1 (MCP-1) by 3.0-, 15-, 2.0-, 7.6-, 11-, and 6.1-fold, respectively (p < 0.05). PAI-1 protein expression induced by UTP was dependent on protein kinase C (PKC) and extracellular signal-regulated kinase (ERK), based on blockade by the PKC inhibitor Ro-31-8220 and
the ERK inhibitor U0126, respectively. The rank order for enhanced expression of PAI-1 and α-SMA by nucleotides (UTPγS>>UDPβS>>ATPγS), the expression of P2Y2 receptors as the most abundantly expressed P2Y receptor in rat CFs and a blunted response to UTP in P2Y2−/− mice all implicate P2Y2 as the predominant P2Y receptor that mediates nucleotide-promoted pro-fibrotic responses. Additional results indicate that P2Y2 receptor-promoted pro-fibrotic responses in CFs are transient, perhaps as a consequence of receptor desensitization. We conclude that P2Y2 receptor activation is pro-fibrotic in CFs; thus inhibition of P2Y2 receptors may provide a novel means to diminish fibrotic remodeling and turnover of extracellular matrix in the heart.

3.2 Introduction

Heart failure is a major cause of morbidity and mortality in economically developed countries. Although heart failure is classically characterized by systolic dysfunction, i.e., decreased cardiac contraction, inadequate filling of the heart, diastolic dysfunction, is increasingly recognized as an important contributor to heart failure (1-3).

Increased deposition of extracellular matrix (ECM) in the heart, cardiac fibrosis, increases the stiffness of the myocardium, thereby contributing to impaired diastolic function. Fibrosis accompanies many types of cardiac pathology, including hypertensive heart disease, post myocardial infarction remodeling, diabetic cardiomyopathy and in aging (1, 3-5). Cardiac fibroblasts (CFs), the most numerous cell type in the heart, play a key role in the homeostatic maintenance of the ECM (6).
CFs are regulated by pro-fibrotic and antifibrotic signals. Transforming growth factor-β (TGF-β) and angiotensin II (Ang II) are two pro-fibrotic peptides that induce signaling events in CFs to increase ECM synthesis. TGF-β activates protein kinase receptors while Ang II signals through Gq-coupled AT1 G-protein-coupled receptors (GPCRs) (4, 7, 8). Offsetting such pro-fibrotic effects, signaling through cAMP and its downstream mediators induce antifibrotic responses (9, 10).

Nucleotides (ATP, ADP, UTP and UDP) can be released into the interstitial space in response to stimuli that include mechanical stretch, chemical stress, platelet activation and cell death (11). Extracellular nucleotides can then stimulate plasma membrane-localized nucleotide receptors: P2X receptors, which are ion channels and P2Y receptors, which are GPCRs (12). P2X receptors preferentially interact with ATP while the 8 P2Y receptor subtypes are adenine nucleotide preferring (P2Y1, P2Y2, P2Y11, P2Y12 and P2Y13), uridine nucleotide preferring (P2Y2, P2Y4 and P2Y6) or responsive to sugar nucleotides (P2Y14) (12). Cell type-specific expression of particular P2Y receptors determines the responses to nucleotide stimulation. In cardiomyocytes, at least three subtypes of P2Y receptors can increase inotropy, regulate hypertrophic growth and modulate the response to pressure overload (13-17). Limited data are available regarding P2Y receptors in CFs. We thus sought to determine if extracellular nucleotides regulate pro-fibrotic responses of CFs and if so, the identity of the P2Y receptors that mediate such responses.
3.3 Materials and Methods

Reagents

UTP, UDP, ATPγS and Ro-31-8220 were purchased from Sigma. Antibody to PAI-1 was from BD Biosciences, α-SMA antibody was from Sigma, ERK antibodies were from Stressgen and GAPDH antibody was from Abcam. U0126 was from Tocris. Antibody to P2Y₂ receptors was from Alomone. UTPγS and UDPβS were a kind gift from Prof. D. Erlinge (University of Lund, Sweden).

Isolation and Culture of Adult Rat and Mouse CFs

CFs were isolated from adult Sprague–Dawley rats (250 – 300 g, male) or C57/BL6 wild-type or P2Y₂⁻/⁻ mice (20 – 25 g, male), as previously described (18). Briefly, CFs were separated from cardiac myocytes by gravity separation and grown to confluency on 10-cm cell culture dishes at 37°C with 90% air/10% CO₂ in growth media (DMEM/10% FBS/1% penicillin/1% streptomycin). All animals were cared for in compliance with the guiding principles of the American Physiological Society and as approved by the UCSD Institutional Animal Care and Use Committee.


[^3]H]Thymidine ([^3]HT) incorporation was used to assess DNA synthesis. CFs (1.5 x 10⁵ per well) were seeded into a 12-well culture plate and serum-starved overnight. One μCi (1 Ci = 37 GBq) of[^3]HT/ml was added in combination with UTP or vehicle control, and the cells were incubated for 24 or 48 h at 37°C. The cells were washed with cold PBS and 7.5% TCA and then dissolved in 0.5 M NaOH before liquid scintillation counting.
**CF Migration**

Migration of CFs was assayed by using the Boyden chamber method. The cells were maintained in serum-free conditions for 24 h and then suspended in serum-free DMEM at a density of $1 \times 10^5$ CFs/100 µl in uncoated chamber inserts. Basal migration was assessed by adding serum-free DMEM (600 µl) to the lower chambers. Cells were allowed to migrate for 16 h, then fixed in 10% formalin and stained with Hema 3 (Fisher Scientific). Cells on the upper surface of the membrane were mechanically removed with a cotton swab. Cells that migrated were counted from 3 different fields (0.1 mm$^2$/field).

**Real-time Polymerase Chain Reaction (RT-PCR)**

Total RNA was isolated by using RNeasy (Qiagen) and cDNA was generated using the Superscript III cDNA synthesis system (Invitrogen) according the manufacturers’ instructions. RT-PCR analysis was performed on a DNA Engine Opticon 2 (Biorad) using the qScript™ One-Step qRT-PCR kit (Quanta Biosciences). Primers for PCR amplification (Table 3.1) were designed based on the nucleotide sequences of the respective gene target and for P2Y receptors were from ref. (19). When possible, each forward and reverse primer set was designed between multiple exons. Amplification efficiency of each primer pair was tested prior to analysis. Relative gene expression levels were determined using the $\Delta\Delta$CT method (20) with 18S as the reference gene.

**Immunoblot Analysis**
Whole cell lysates were prepared in NaCO₃ buffer (pH 11) and homogenized by sonication. Equal amounts of protein (assayed using a dye-binding reagent, Bio-Rad) were separated by SDS/PAGE using 10% polyacrylamide precast gels (Invitrogen) and transferred to a poly(vinylidene difluoride) membrane with the iBlot system (Invitrogen). Membranes were blocked in PBS Tween (1%) containing 5% nonfat dry milk and incubated with primary antibody 18 h at 4°C. Bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and ECL reagent (Amersham Pharmacia). Bands were compared to molecular weight standards to confirm migration of proteins at the appropriate size. Quantitation of protein expression densitometry was performed using ImageJ software (NIH).

Flow Cytometry

FACS analysis was performed on a BD FACScan flow cytometer equipped with an argon laser capable of excitation at 488 nm. FITC fluorescence was detected with a 530/30 bandpass filter (FL-1). Amplifier gains and instrument voltage were not changed for the duration of each experiment. CFs were serum-starved in DMEM overnight and treated for 4 and 24 h with UTP, UTPγS, or Ang II. Cells were then detached with PBS with 5mM EDTA (pH 7.2), fixed in 1.5% formalin and permeabilized in ice-cold methanol.

CFs were washed in PBS containing 1% BSA, 0.05% NaN₃ and incubated for 1 h at room temperature with antibody against α-SMA, PAI-1, or mouse-isotype control antibody diluted in PBS/1% BSA. Cells were washed and incubated for 30 min with Alexa Fluor 488 donkey anti-mouse antibody (Invitrogen) and then washed and
resuspended in PBS for FACScan analysis. Fluorescence was acquired in log-scale via an FL-1 filter and data was analyzed and plotted with CellQuest (BD) and Weasel (WEHI).

**Immunofluorescence Analysis**

Cardiac ventricles were harvested, frozen and mounted on a cryostat to cut 10-µm sections. Sections were fixed in cold acetone, blocked with 4% BSA in 0.1% Tween and PBS, and incubated with primary antibodies (1:100) in 4% BSA/0.1% Tween/PBS. After incubation with Alexa-conjugated secondary antibody (Molecular Probes) (1:250), samples were mounted in Vectashield (Vector Laboratories) mounting media containing DAPI. Specificity of staining was determined by omission of the primary antibody. Images were obtained by using a Zeiss LSM510 Laser Scanning Confocal Microscope and Zeiss Image Examiner software.

**Statistical Analysis**

Calculations and statistics were performed using GraphPad Prism 5.0 software. Values are presented as mean ± S.E.M. ANOVA with Dunnett’s post-test was used to compare quantitative RT-PCR relative expression data with untreated controls. Analysis of experiments with multiple comparisons was by ANOVA with Bonferroni’s correction.
3.4 Results

3.4.1 UTP induces proliferation and migration in rat CFs

Proliferation and migration of CFs are key events in the fibrotic response of cardiac remodeling and extracellular nucleotides are known to have mitogenic effects in other cell types such as vascular smooth muscle cells (21). Stimulation of serum-starved, quiescent rat CFs with 10 µM UTP increased [3H]thymidine incorporation by 24 h (Fig. 3.1A). We observed a similar effect with 10 µM ATP at 24 h (data not shown). UTP also induced a 3-fold-increase (p < 0.001) in cell migration by 24 h, as assessed by the Boyden chamber method (Fig. 3.1B).

3.4.2 UTP induces transcription of pro-fibrotic genes

The fibrotic response in the heart is modified by a large number of genes that regulate ECM homeostasis. Alpha smooth muscle actin (α-SMA) is a marker of the conversion of resting CFs to activated, phenotypically distinct myofibroblasts. Incubation of rat CFs with UTPγS (10 µM, 4 h) increased the expression of α-SMA mRNA 3.0-fold (p < 0.001) (Fig. 3.2). Plasminogen activator inhibitor (PAI-1) plays a key role in tissue fibrosis as a protease inhibitor that regulates matrix metalloproteinases (22). UTPγS (10 µM, 4 h) increased PAI-1 mRNA expression 15-fold (p < 0.001) and mRNA expression of soluble ST2 (sST2), a receptor for interleukin-33, a prognostic marker in heart failure 7.6-fold (p < 0.001) (Fig. 3.2) (23). In addition, 4 h UTPγS increased mRNA expression of the proinflammatory, pro-fibrotic factors TGF-β and monocyte chemoattractant
protein-1 (MCP-1) by 2.0- and 6.1-fold, respectively (p < 0.01 and p < 0.001, respectively) (Fig. 3.2).

UTP transiently stimulated pro-fibrotic and proinflammatory markers in CFs. Even with the use of an ectonucleotidase-resistant analog, UTPγS, most gene expression was only significantly up-regulated at 4 h of treatment, with the exception of interleukin-6 (IL-6) which had a peak 11-fold increase (p < 0.01) in mRNA levels after 8 h (Fig. 3.2). Desensitization of P2Y receptors rather than degradation of extracellular UTP thus likely accounts for the decrease in responses over the 24 h period of treatment, especially because CF treated with TGF-β or Ang II show increased pro-fibrotic gene expression past 24 h (data not shown).

3.4.3 UTP induces PAI-1 protein expression in a concentration- and time-dependent manner, but response to UTP desensitizes

We further characterized PAI-1 expression in UTP-treated CFs since its mRNA increase was the most prominent of all the markers we tested. UTP dose-dependently increased mRNA and protein expression of PAI-1 (Fig. 3.3A-C). PAI-1 protein expression increased within 2 h, peaked after 4 h and gradually decreased to basal levels after 24 h (Fig. 3.3D, E), consistent with the trend in PAI-1 mRNA expression after UTP stimulation. UTPγS produced a similar time-dependent pattern of PAI-1 protein expression (data not shown).

Flow cytometry was used as an additional approach to assess PAI-1 protein expression and also, that of α-SMA in rat CFs. Consistent with the data obtained by RT-
PCR and Western blotting, we found that UTP increases PAI-1 expression at 4 h (Fig. 3.4A). However, by 24 h the enhanced PAI-1 expression reverts nearly to baseline levels even though Ang II produces sustained upregulation of PAI-1 expression (Fig. 3.4B). Furthermore, CFs are less responsive to UTP than to Ang II (Fig. 3.4A, B) and UTP does not seem to enhance α-SMA protein expression at either 4 or 24 h. The initial rise in α-SMA mRNA levels (Fig. 3.2) thus does not lead to a later increase in protein expression (Fig. 3.4C, D). UTPγS elicited the same response as UTP in all time points (data not shown). Interestingly, the enhanced expression of α-SMA in response to Ang II is not observed after 4 h treatment but only at 24 h, suggesting that the increase in α-SMA production requires persistent signaling, which does not occur with stimulation by UTP.

3.4.4 UTP induces PAI-1 expression in an ERK-and PKC-dependent manner

P2Y2, P2Y4 and P2Y6 receptors couple to Gq and increase diacylglycerol and intracellular Ca^{2+} leading to protein kinase C (PKC) activation (12). Treatment of CFs with the PKC inhibitor Ro-31-8220 abolished UTP-induced PAI-1 expression (Fig. 3.5A, B). Consistent with the ability of P2Y receptors to increase extracellular signal-regulated kinase (ERK) signaling (12), the MAPK/ERK kinase (MEK) inhibitor U0126 decreased UTP-induced PAI-1 expression (Fig. 3.5A, B); such an effect may derive from transactivation of the EGF receptor or GPCR activation of ERK by Gβγ subunits (24). UTP stimulated ERK-phosphorylation in CFs (Fig. 3.5C). The calcineurin inhibitor cyclosporine A did not affect UTP-induced PAI-1 response (Fig. 3.5A, B).
3.4.5 UTP-induced effects are predominately mediated by P2Y$_2$ receptors

UTP can stimulate P2Y$_2$ and P2Y$_4$ receptors but in addition, UTP is hydrolyzed to UDP, which acts on P2Y$_6$ receptors (12). To identify the P2Y receptor responsible for the effects of UTP that we observed on CFs we used ATP, UDP and the stable nucleotides ATP$\gamma$S, UDP$\beta$S and UTP$\gamma$S and found that the effects of UTP$\gamma$S (10 $\mu$M) on $\alpha$-SMA and PAI-1 mRNA expression were similar to those observed with UTP (Fig. 3.6A, B). By contrast, incubation of CFs with UDP (10 $\mu$M) or the stable agonist UDP$\beta$S (10 $\mu$M) did not increase $\alpha$-SMA expression and increased PAI-1 to a lesser extent than did UTP (Fig. 3.6A, B). Expression of PAI-1 and $\alpha$-SMA mRNA was not significantly increased by ATP or ATP$\gamma$S (both 10 $\mu$M) (Fig. 3.6A, B). The rank order for nucleotide-promoted increase in PAI-1 and $\alpha$-SMA mRNA expression (UTP$\gamma$S $>$ UDP$\beta$S $>$ ATP$\gamma$S) suggests that either P2Y$_2$ or P2Y$_4$ is the main receptor that mediates UTP-induced effects. P2Y$_2$ receptors are much more highly expressed than P2Y$_4$ receptors in CFs (Fig. 3.8A), thus implicating P2Y$_2$ as the predominant receptor sub-type that mediates response to UTP. ATP and ATP$\gamma$S produce different effects than do UTP and UTP$\gamma$S in terms of expression of $\alpha$-SMA or PAI-1 (Fig. 3.6A, B). Since ATP and UTP are equipotent agonists for P2Y$_2$ receptors, more than one subtype of P2Y receptor may mediate the effects of ATP.
3.4.6 UTP-induced PAI-1 expression and cell proliferation are blunted in P2Y2−/− mice

To help define the role of P2Y2 receptors in the response of CFs, we isolated CFs from wild-type and P2Y2−/− mice and assayed ability of UTP to regulate PAI-1 expression and induce proliferation. Incubation with UTPγS (10 µM) prominently increased PAI-1 protein expression in CFs from wild-type mice, an effect completely blunted in CFs from P2Y2−/− mice (Fig. 3.7A, B). These results indicate that the UTP-induced increase in PAI-1 expression is mediated by P2Y2 receptors in CFs from mice and provide data that are complementary to those shown in the previous section regarding the role of P2Y2 receptors in rat CFs. In addition, the stimulatory effect of UTP on CF proliferation is also blunted in P2Y2−/− cells: UTPγS (10 µM) increased proliferation of CF from WT mice by almost 50% (p < 0.01) at 24 h but had no proliferative effect on CFs from P2Y2−/− mice (Fig. 3.7C, D). Proliferation occurred in WT CF at 24 h but not 48 h (Fig. 3.7C).

3.4.7 The P2Y2 receptor is the predominant P2Y receptor in rat cardiac fibroblasts and is detected on cardiac myocytes and fibroblasts in rat left ventricle

Quantification of mRNA for P2Y1, P2Y2, P2Y4, P2Y6 and P2Y12 revealed that the P2Y2 receptors are the most highly expressed (>50-fold higher than P2Y4 receptors, 3.5-fold more than the P2Y6 and 11-fold more than the P2Y1 receptors [p < 0.001]) in rat CFs (Fig. 3.8A). P2Y12 receptors are expressed at very low levels. Immunoblotting detected a single band (with the appropriate size of P2Y2 receptors) in CFs but not human platelets, a P2Y2-null tissue (Fig. 3.8B). Immunohistochemistry and confocal microscopy revealed
P2Y$_2$ receptor expression on cardiomyocytes in rat left ventricular tissue but also co-localized with fibroblasts, identified with a fibroblast-specific DDR-2 antibody (Fig. 3.8C).

3.5 Discussion

Cardiac remodeling following stress or damage to the myocardium is associated with increased morbidity and mortality (25). Stiffening due to fibrosis is a key event in cardiac remodeling. The most effective pharmacological agents for heart failure, inhibition of the renin-angiotensin-aldosterone system and β-adrenergic receptor blockers, improve cardiac remodeling (25). GPCR expression profiling has revealed that P2Y$_2$ receptors are expressed in the left ventricles at a level comparable to that of Angiotensin-1 and β1-adrenergic receptors, suggesting that the P2Y$_2$ receptors contribute to cardiac regulation and perhaps remodeling (26).

Three P2Y receptors (P2Y$_2$, P2Y$_6$ and a P2Y$_{11}$-like receptor) increase inotropy (13, 17). Moreover, UTP can increase hypertrophic growth of rat neonatal cardiomyocytes (14, 16). Neonatal rat cardiac myofibroblasts were recently shown to have P2Y$_{1}$-, P2Y$_2$-, P2Y$_4$-, P2Y$_6$- and P2Y$_{11}$-induced signaling through Gq, Gi and Gs (27). Nishida et al described a role for P2Y$_6$ in modulating G$_{\alpha_{12/13}}$ signaling and cardiac fibrosis in response to pressure overload in mice (15). Those authors concluded that cardiac myocyte P2Y$_6$ receptors contribute to stretch-induced modulation of the ECM but did not consider receptors on CF. Other studies suggest that UTP has a protective role in ischemia-reperfusion injury; the receptors that mediate this protection may include P2Y$_2$.
receptors (28, 29). ATP inhibits proliferation and modulates adrenergic-promoted growth of rat neonatal CFs but the nucleotide receptor(s) and mechanisms for this response are not known (30). Our data that implicate P2Y₂ receptors in fibrotic response of CFs identify a cellular consequence of the increases in phosphoinositide hydrolysis and cellular Ca²⁺ promoted by cardiac P2Y₂ receptors (31).

Communication between CFs and cardiomyocytes has recently been shown to contribute to cardiac development and homeostasis. Release of nucleotides by cardiomyocytes can act in an autocrine and/or paracrine manner to stimulate P2Y receptors on cardiomyocytes and also potentially receptors on CFs (32). Our finding that P2Y₂ receptors on CFs induce TGF-β, sST2 and IL-6 expression, factors that are known to be involved in CF-cardiomyocyte crosstalk, identifies a receptor that mediates such autocrine/paracrine response and CF-cardiomyocyte crosstalk.

The current results show that UTP induces proliferation, migration and an increase in pro-fibrotic gene expression in CFs. The use of pharmacological approaches and assessment of P2Y gene expression in rats and in P2Y₂-knockout mice strongly implicate a predominant role for P2Y₂ receptors in these pro-fibrotic effects. Thus, our data demonstrate a previously unappreciated role for nucleotide receptors in promoting a pro-fibrotic phenotype in CFs. Though UTP does not increase protein expression of α-SMA, a marker of phenotypic transformation to myofibroblasts, the increase in other fibrotic markers suggests that UTP contributes to the acute-phase response after injury of CF, perhaps by modulating formation and composition of cardiac ECM.

Although UTP and ATP are equipotent in stimulating rat P2Y₂ receptors (33), we find that ATP-stimulated increases in PAI-1 and α-SMA mRNA expression are less than
those in response to UTP, thus implicating more than one receptor in ATP response. In mouse cardiomyocytes, which show a similar discrepancy for ATP and UTP, ATP acts via P2Y$_2$ and P2Y$_{11}$-like receptors (13). Our finding that UTP acts predominantly via P2Y$_2$ receptors to increase PAI-1 mRNA and protein expression in CFs contrasts with data in rat vascular smooth muscle cells in which P2Y$_6$ receptors mediate PAI-1 induction (34).

P2Y receptors regulate the proliferation and ECM production by renal mesangial cells and exaggerated release of nucleotides and increase in P2Y-induced fibrotic responses can occur in a diabetic setting (35, 36). Moreover, decreased degradation of extracellular nucleotides in CD39-knockout mice results in more severe glomerular sclerosis after induction of diabetes mellitus (37). ATP also contributes to hypoxia-induced increase in growth of lung fibroblasts and P2Y receptors are profibrogenic in hepatic stellate cells (38, 39). Our results for CFs and those for fibroblasts from kidney, lung and liver suggest that signaling via P2Y receptors is a general pro-fibrotic mechanism.

PAI-1 was initially characterized as an inhibitor of the tissue-type and urokinase-type plasminogen activators (tPA and uPA) (40). Plasmin formed after cleavage of plasminogen, the main substrate for tPA and uPA, regulates fibrin degradation but also matrix metalloproteinase (MMP) activation and TGF-$eta$ activation. In addition to the cleavage of plasminogen, tPA and uPA can activate several MMPs in renal fibroblasts, namely MMP-1, -2, -3, and -9 (41-43). Moreover, PAI-1 is a critical modulator of kidney and liver fibrosis (37, 44, 45). In the heart, PAI-1 increases in diabetes mellitus, with increasing age and contributes to cardiac fibrosis post myocardial infarction (22, 46, 47).
An acute rise in plasma PAI-1 levels after myocardial infarction is a strong prognostic indicator of mortality and development of heart failure (48, 49). Notably, we found that PAI-1 was dramatically upregulated in CFs in response to P2Y<sub>2</sub> receptor activation. The inhibitory role of PAI-1 on fibrinolysis and MMP activation suggests that the acute, transitory increase of PAI-1 in response to UTP may alter ECM dynamics.

In conclusion, the current results show a role for P2Y<sub>2</sub> receptor activation in the function of CFs. Our findings imply that P2Y<sub>2</sub> receptor inhibition is potentially a novel means to diminish fibrotic remodeling in the heart by reducing matrix production and deposition, release of PAI-1 and via MMP-promoted turnover of ECM.
3.6 Acknowledgements

This work was supported by National Institutes of Health Grant 2P01HL066941 (to PAI), Graduate Training in Cellular and Molecular Pharmacology Grant 2T32GM007752-32 (in support of DL) and the Ellison Medical Foundation. OÖB was supported by postdoctoral grants from the Tegger foundation, the Westerström foundation and from the Swedish Heart and Lung foundation. We thank Sandra Peterson and the laboratory of Dr. Martin F. Kagnoff for sharing their FACS facility. The authors have no conflicts of interest to declare.

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Table 3.1: Oligonucleotides used for real-time RT-PCR.

<table>
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<th>Gene</th>
<th>Forward, 5’–3’</th>
<th>Reverse, 5’–3’</th>
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</thead>
<tbody>
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<td>18s</td>
<td>GTA ACC CGT TGA ACC CCA TT</td>
<td>CCA TCC AAT CGG TAG TAG CG</td>
</tr>
<tr>
<td>α-SMA</td>
<td>CAT CAG GAA CCT CGA GAA GC</td>
<td>TCG GAT ACT TCA GGG TCA GG</td>
</tr>
<tr>
<td>PAI-1</td>
<td>GGA GAA GCG AAA CAG GAG GC</td>
<td>TCC AGA AGG GGA TAT GTT GC</td>
</tr>
<tr>
<td>sST2</td>
<td>TTA CCC AGC CAG GAT GTT TC</td>
<td>CTA GGG GCT TGG CTT CTC TT</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>CCT GGA AAG GCC TCA ACA</td>
<td>GTT GGT TGT AGA GGG CAA GG</td>
</tr>
<tr>
<td>IL-6</td>
<td>GCC TTC CCT ACT TCA CAA GTC C</td>
<td>CTG ACC ACA GTG AGG AAT GTC C</td>
</tr>
<tr>
<td>MCP-1</td>
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<td>TTC CTT ATT GGG GTC AGC AC</td>
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<td>TTC AAC TTG TCC GTT CCA CA</td>
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<td>GGA TTC CCT ACA CCC TGA GC</td>
<td>ACC TCC CTG TCC TTT CTT CT</td>
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</tbody>
</table>

Figure 3.1: UTP induces proliferation and migration of rat cardiac fibroblasts. (A) Proliferation of passage 1 rat CFs serum-starved for 24h and stimulated with 10 µM UTP was examined by [³H]thymidine incorporation. UTP increased [³H]thymidine incorporation by 30% after 24 h. (B) Fibroblast migration was assessed by a modified Boyden chamber method in the absence (control) or presence of 10 µM UTP for 24 h. The data are shown as the fold-increase relative to control and are mean ± SEM of at least 3 independent experiments performed in triplicate and compared by using Student’s t test. *, p < 0.05 and ***, p < 0.001 in response to UTP.
Figure 3.2: UTP induces transcription of pro-fibrotic genes in rat cardiac fibroblasts. CFs were serum-starved for 24 h and then incubated with UTP (10 µM) for 4, 8, 16, or 24 h. Real-time RT-PCR was used to quantify PAI-1, α-SMA, sST2, TGF-β1, MCP-1 and IL-6; the data are normalized to 18S RNA. UTP (10 µM) at 4 h stimulated peak upregulation of pro-fibrotic genes: PAI-1, α-SMA, sST2, TGF-β, MCP-1 by 15-, 3.0-, 7.6-, 2.0-, 6.1-fold, respectively. IL-6 expression peaked at 8 h by 11-fold. The data shown represent mean ± SEM of at least 3 independent experiments performed in triplicate and compared by using Student’s t test. *, p<0.05; **, p<0.01 and ***, p<0.001 in response to UTP.
Figure 3.3: UTP induces PAI-1 protein expression in a dose- and time-dependent manner, but response to UTP desensitizes. (A-C) CFs were serum-starved for 24h and then stimulated with 0 – 100 µM UTP for 4h. Cells were assayed by real-time RT-PCR to quantify mRNA expression and immunoblot analysis to quantify protein expression of PAI-1. UTP dose-dependently increased PAI-1 mRNA expression (A) and protein expression (B). (C) Quantification of PAI-1 protein from two independent Western blots is shown as ± SEM compared by using ANOVA with post-hoc multiple comparison tests. *, p<0.05 and **, p<0.01. (D) Time-dependent increase in PAI-1 protein expression was observed after 2 h, peaked after 4 h and decreased to baseline levels after 24 h. (E) Quantification is shown from two independent Western blots as ± SEM compared by using ANOVA with post-hoc multiple comparison tests. **, p<0.01 and ***, p<0.001.
Figure 3.4: UTP has no effect on α-SMA protein expression of rat CFs. Cells were serum-starved for 24 h, treated with UTP (10 µM) or Ang II (1 µM) for 4 or 24 h and protein expression analyzed via flow cytometry. (A) 4 h UTP treatment increased PAI-1 expression in CFs but this increase reverts to basal levels by 24 h (B). Ang II increases PAI-1 at 4 and 24 h (A, B). UTP does not increase α-SMA at 4 h (C) or 24 h (D) but Ang II stimulation for 24 h significantly increases α-SMA protein expression (D).
Figure 3.5: UTP induces PAI-1 expression in rat cardiac fibroblasts in a PKC and ERK-dependent manner. (A) CFs were serum-starved for 24h and then incubated in the presence or absence of: DMSO (vehicle control), Ro-31-8220 (PKC inhibitor), U0126 (MAPK/ERK kinase (MEK) inhibitor), or Cyclosporine A (calcineurin inhibitor) for 30 min. The cells were then stimulated with UTP (10µM, 4 h). Cells were lysed and assayed for PAI-1 protein expression. GAPDH was used to normalize for protein loading. Panel (B) shows quantification of the immunoblots from panel (A). (C) ERK-phosphorylation and total ERK protein were assessed using immunoblots following stimulation with 10 µM UTP for 0, 10, 20, 30, 60 min. The data shown are mean ± SEM of at least 3 independent experiments performed in triplicate and compared by using ANOVA with post-hoc multiple comparison tests. ***, p<0.001.
Figure 3.6: UTP-induced effects in rat cardiac fibroblasts are predominately mediated by P2Y2 receptors. (A-B) CFs were serum-starved for 24 h and then incubated with UTP, UTPγS, UDP, UDPβS, ATP, ATPγS (all 10 µM) or angiotensin II (Ang II) for 4 h. Cells were assayed using real-time RT-PCR to quantify mRNA expression of α-SMA (A) and PAI-1 (B). Incubation of CFs with UTP or the stable agonist UTPγS (10 µM) increased α-SMA and PAI-1 mRNA expression. UDP (10 µM) and the stable agonist UDPβS (10 µM) did not increase α-SMA expression; PAI-1 was increased but to a lesser extent than with UTP. ATP and ATPγS (both 10 µM) did not significantly increase α-SMA or PAI-1. The data shown are mean ± SEM of at least 3 independent experiments performed in triplicate and compared using ANOVA with post-hoc multiple comparison testing.
Figure 3.7: UTP-induced PAI-1 expression and cell proliferation are blunted in P2Y$_2$^{-/-} mice. CFs from wild-type or P2Y$_2$^{-/-} mice were serum-starved for 24 h and then incubated with UTP$_7$S (10 µM) for 4 h. Cells were assayed by immunoblot analysis to quantify protein expression of PAI-1. (A) Immunoblotting shows that UTP$_7$S increases PAI-1 protein expression in CFs from wild-type mice but not from P2Y$_2$^{-/-} mice. Panel (B) shows quantification of the immunoblots from panel (A). The data shown in (B) are the mean ± SEM of at least 3 independent experiments compared by using ANOVA with post-hoc multiple comparison tests. **, p<0.01. (C) CFs from wild-type mice show ~50% increase in proliferation in response to treatment with UTP$_7$S (10 µM) for 24 h but not 48 h. (D) UTP$_7$S is not proliferative in CFs from P2Y$_2$^{-/-} at either time point.
Figure 3.8: The P2Y₂ receptor is the predominant P2Y receptor expressed in cardiac fibroblasts. (A) Quantification of the mRNA for P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₂ reveals that the P2Y₂ receptors are the most highly expressed P2Y receptor subtype in rat CFs. P2Y₂ mRNA expression is 50-, 3.5-, and 11-fold higher than that of P2Y₄, P2Y₆ and P2Y₁ receptors, respectively (p < 0.001). (B) Immunoblotting with a P2Y₂ antibody detects P2Y₂ receptors in CFs but not human platelets. (C) Sections of left ventricles from rats were stained for DDR2 (red), P2Y₂ receptor (green) and nuclei with DAPI (blue). Cross-sectional images of cardiac myocytes and fibroblasts are shown.
3.8 Appendix

3.8.1 P2Y expression profile in WT mouse CFs

Quantitative RT-PCR analysis was used to determine the P2Y receptor expression profile of CFs isolated from wild-type C57BL/6 mice (Fig. S3.1). Similar to rat CFs, only P2Y$_1$, P2Y$_2$, P2Y$_4$ and P2Y$_6$ subtype were detectable. However, unlike rat CFs where P2Y$_2$ receptors were the predominate subtype, P2Y$_6$ receptors are expressed in approximately equally high abundance. Because the pro-fibrotic effects of P2Y$_6$ stimulation have previously been described {Nishida et al., 2008, #89991}, P2Y$_6$ receptor activation may play a greater importance in mouse CFs as compared to CFs from rat. This suggests that mouse CFs may integrate pro-fibrotic signals from both P2Y$_2$ (ATP/UTP) and P2Y$_6$ (UDP) signaling.

3.8.2 The response of P2Y$_2^{-/-}$ CFs to ATP/UTP stimulation

To further assess the effect of P2Y$_2$ deficiency, CFs isolated from P2Y$_2^{-/-}$ mice were treated with ATP, UTP and TGF-β (Fig. S3.2). As expected, the response of P2Y$_2^{-/-}$ CFs to 10 µM ATPγS and UTPγS were generally abolished. UTPγS had a small effect in stimulating α-SMA expression, however this level of up-regulation was relatively small as compared to WT CFs. Nonetheless, this modest effect may be a result of P2Y$_6$ receptor activation. Though predominately an UDP receptor, P2Y$_6$ can be activated by higher concentrations of UTP (the EC50 of UTP on P2Y$_6$ is ~6 µM {Abbracchio et al., 2006, #92774}). TGF-β (0.1-10 ng/mL) strongly up-regulated the expression of all pro-
fibrotic genes assessed, indicating that P2Y$_2$ deficiency does not interfere with pro-
fibrotic responses elicited from other pathways.

**Figure S3.1:** Mouse CF P2Y receptor expression profile, as assessed by qPCR.

![Figure S3.1](image1)

**Figure S3.2:** P2Y$_2^{-/-}$ CFs respond to TGF-β, but their response to ATPγS and UTPγS stimulation are substantially blunted.

![Figure S3.2](image2)
3.9 References


CHAPTER 4:

ATP RELEASED FROM CARDIAC FIBROBLASTS VIA CONNEXIN HEMICHANNELS ACTIVATES PRO-FIBROTIC P2Y₂ RECEPTORS

4.1 Abstract

Cardiac fibroblasts (CFs) play an essential role in remodeling of the cardiac extracellular matrix. Extracellular nucleotide signaling may provoke a pro-fibrotic response in CFs. We tested the hypothesis that physical perturbations release ATP from CFs and that ATP participates in pro-fibrotic signaling. ATP release was abolished by the channel inhibitor carbenoxolone and inhibited by knockdown of either Cx43 or Cx45 (47% and 35%, respectively), implying that hypotonic stimulation induces ATP release via connexin (Cx)-43 and Cx45 hemichannels, although pannexin 1 may also play a role. ATP released by hypotonic stimulation rapidly (<10 min) increased phosphorylated ERK by 5-8 fold, an effect largely eliminated by P2Y₂ receptor knockdown or ATP hydrolysis with apyrase. ATP stimulation of P2Y₂ receptors increased α-smooth muscle actin (α-SMA) production, and in an ERK-dependent manner, ATP increased collagen
accumulation by 60% and mRNA expression of pro-fibrotic markers: plasminogen activator inhibitor-1 and monocyte chemotactic protein-1 by 4.5- and 4.0-fold, respectively. Apyrase treatment substantially reduced the “basal” pro-fibrotic phenotype, decreasing collagen and α-SMA content and increasing matrix metalloproteinase expression. Thus, ATP release activates P2Y2 receptors to mediate pro-fibrotic responses in CFs, implying that nucleotide release under both basal and activated states is likely an important mechanism for fibroblast homeostasis.

4.2 Introduction

Cardiac fibroblasts (CFs) are the predominant non-myocyte cell type and are responsible for the homeostatic maintenance of extracellular matrix (ECM) in the heart (1). CFs generate, maintain and organize the ECM, which is essential for structural organization and correct contraction of the myocardium (1, 2). In addition to producing ECM proteins, including collagen types I and III (3), CFs can couple to adjacent fibroblasts and myocytes to mediate electrical conduction via connexin (Cx) gap junctions (4, 5).

Aging and pathological conditions, such as heart failure and myocardial infarction (MI), can alter function of CFs, which transform into profibrogenic myofibroblasts (6-8). Compared to “resting” fibroblasts, myofibroblasts have increased synthesis of ECM proteins and expression of pro-fibrotic and pro-inflammatory cytokines, including plasminogen activator inhibitor (PAI)-1, monocyte chemotactic protein (MCP)-1, connective tissue growth factor (CTGF) and transforming growth factor-β (TGF-β) (9-
Myofibroblasts also have increased expression of α-SMA, which aids in wound closure at sites of injury (5). Activity of CFs is important in cardiac remodeling and wound healing and, if excessive, can lead to cardiac fibrosis and diastolic dysfunction.

Swelling and increased stretch of cells occurs in the infarcted or pressure-overloaded myocardium and can increase myocyte hypertrophy and activation of CFs (14-17). Physical stretch or damage to cells triggers cellular release of ATP from numerous cell types in the heart, including erythrocytes, cardiac myocytes and endothelial cells (18-21). In addition to their roles in gap-junction coupling, Cx hemichannels have been implicated in the cellular release of ATP (20, 22, 23).

Release of ATP in the heart may be an important response to myocardial damage through its ability to promote the recruitment of phagocytes (24-26) and initiate pro-fibrotic responses (20, 27). Relatively little is known about the mechanisms of ATP release and signaling in CFs. We previously characterized the P2Y subtypes expressed by rat CFs and found that activation of P2Y₂ receptors, the most highly expressed subtype, increases their migration, proliferation and expression of α-SMA and several pro-fibrotic markers (28). In the current study we sought to determine whether physical perturbation of CFs (e.g., hypotonicity-induced cell swelling) causes the release of endogenous ATP and if so, possible mechanisms for this release and whether released ATP participates in pro-fibrotic signaling via activation of P2Y₂ receptors.

4.3 Materials and Methods

Isolation and culture of adult rat cardiac fibroblasts
Ethical approval for the care and use of animals for this study was granted by the UCSD Institutional Animal Care and Use Committee and was in compliance with the guiding principles of the American Physiological Society. CFs were isolated from adult (8-10 wk), male Sprague-Dawley (SD) rats, as previously described (29). Briefly, SD rats were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) via IP injection. The heart was excised and digested with collagenase II (Worthington Biochemical Corp., Lakewood, NJ, USA) via a modified reverse-Langendorff apparatus. CFs were separated from cardiac myocytes by gravity separation and grown to confluency in 10-cm culture dishes at 37˚C, 10% CO₂ in DMEM supplemented with 10% FBS, 1% penicillin, 1% streptomycin. CFs were then split to appropriate-sized culture dishes, allowed to adhere overnight and then serum-starved in DMEM without FBS for 24 h prior to treatment.

**Hypotonic stimulation**

Isotonic medium consisted of standard physiological saline (280-290 mOsm) containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES and 100 mM EGTA. In Ca²⁺-containing experiments, EGTA was replaced with the appropriate concentration of CaCl₂. Hypotonic medium (150-160 mOsm) was prepared by reducing [NaCl] and [KCl] to 70 mM and 2.5 mM, respectively.

Isolated CFs were trypsinized and plated onto 6- or 12-well plates (150,000 and 60,000 cells, respectively) and serum-starved. DMEM was then gently aspirated and replaced with hypotonic or isotonic media. Plates were incubated at 37˚C, 10% CO₂ for 5 or 10 min, depending on the experiment. In experiments containing pharmacologic
inhibitors, CFs in 0% DMEM were pretreated with drugs for 30 min. Inhibitors were added to isotonic or hypotonic media used for CF stimulation.

**Extracellular ATP quantification**

ATP-containing isotonic or hypotonic media (100 µl) was carefully removed by placing a pipette as close to the fluid surface as possible to avoid perturbation of the cells. A luciferase-based ATP assay kit (ENLITEN ATP Assay; Promega, Madison, WI, USA) and TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA) were used according to the manufacturers’ instructions. Assays were conducted at room temperature. Separate standard curves of known ATP concentrations were generated with isotonic and hypotonic media and used to quantify the concentration of ATP in the experimental samples.

**siRNA transfection**

Targeted siRNA sequences for rat Cx43 (GJA1; S127779), Cx45 (GJA7; S141483), Panx1 (S163990), P2Y2 (S131806) and negative control siRNA were purchased from Ambion (Grand Island, NY, USA). Cells were transfected with 25 nM siRNA using RNAiMAX (Invitrogen, Grand Island, NY, USA) for 24 h according to the manufacturer’s instructions. The media containing transfection reagent were then replaced with fresh serum-free DMEM.

**Quantitative real-time PCR (qPCR)**
Total RNA was isolated by TRIzol extraction (Invitrogen), and cDNA was generated using the Superscript III cDNA synthesis system (Invitrogen) according the manufacturers’ instructions. qPCR analysis was performed on a DNA Engine Opticon 2 (Bio-Rad, Hercules, CA, USA) using the qScript One-Step qRT-PCR kit (Quanta Biosciences, Gaithersburg, MD, USA). Primers for PCR amplification (Table 4.1) were designed based on the nucleotide sequences of the respective gene target using Primer3Plus software. P2Y receptor primers were obtained from published literature (30). When possible, each forward and reverse primer set was designed between multiple exons. Amplification efficiency of each primer pair was tested prior to analysis. Relative gene expression levels were determined using the ΔΔCT method with 18S as the reference gene (31).

**Immunofluorescent microscopy**

CFs were plated onto 12-mm glass cover slips, allowed to adhere overnight and then serum-starved for 24 h. CFs were treated with either 10 µM ATP or 0.67 U/ml apyrase (or corresponding vehicle control) for 24 h. Cells were washed in PBS, fixed in 10% formalin for 10 min, washed with 100 mM glycine (pH 7.4), permeabilized with 0.3% Triton-X/PBS, washed in 0.1% Tween 20/PBS and blocked in blocking buffer (1% BSA/0.05% Tween 20/PBS). Samples were incubated with primary antibodies to α-SMA (Invitrogen) or Cx43 (Abcam, Cambridge, MA, USA) for 18 h at 4°C. Samples were washed and incubated with fluorescent secondary antibody (Alexa Fluor 488; Invitrogen) for 1 h, then washed and mounted on glass slides with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). Confocal images were acquired on a
Nikon Eclipse laser scanning confocal microscope using Nikon EZ-C1 software (Nikon Instruments Inc., Melville, NY, USA).

**Immunoblot analysis**

Whole cell lysates were prepared in 150 mM Na$_2$CO$_3$ buffer (pH 11) and homogenized by sonication. Equal amounts of protein (assayed using a dye-binding reagent; Bio-Rad) were separated by SDS/PAGE using 10% polyacrylamide precast gels (Invitrogen) and transferred to a poly(vinylidene difluoride) membrane with the iBlot system (Invitrogen). Membranes were blocked in PBS Tween (1%) containing 5% nonfat dry milk and incubated with primary antibody 18 h at 4°C. Bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ECL reagent (Amersham Pharmacia, Pittsburg, PA, USA). Bands were compared to molecular weight standards to confirm migration of proteins at the appropriate size. Quantification of protein expression densitometry was performed using ImageJ software (NIH). Cx43, Cx45 and GAPDH antibodies were purchased from Abcam, α-SMA from Invitrogen, P2Y$_2$ from Alomone Labs (Jerusalem, Israel), and p-ERK and total ERK from Cell Signaling (Danvers, MA, USA).

**Collagenase-sensitive [3H]proline incorporation**

Collagenase-sensitive [³H]proline incorporation assays were used to quantify collagen accumulation and were performed as previously described (29). CFs cultured on 12-well plates were serum-starved for 24 h followed by the addition of 1 μCi/ml
[\textsuperscript{3}H]proline (PerkinElmer, Waltham, MA, USA; 1 Ci = 37 GBq) along with drugs of interest and incubated at 37˚C for 24 h. Cells were lysed in 0.5 M NaOH, and following neutralization with 0.5 M HCl, protein was precipitated overnight with 20% trichloroacetic acid (TCA). Samples were pelleted and washed in 5% TCA, dissolved in 0.2 M NaOH and neutralized with 0.2 M HCl. 2 mg/ml Collagenase II (Worthington) in Tris-CaCl\textsubscript{2}-N-ethylmaleimide buffer was added to each sample and incubated at 37˚C for 1 h. Protein was precipitated in 10% TCA and centrifuged. The radioactive content of the supernatant, corresponding with the amount of [\textsuperscript{3}H]proline incorporated into the digested collagen fraction, was quantified using a liquid scintillation counter.

**Reagents**

ATP, carbenoxolone, probenecid, brefeldin A, suramin and U0126 were purchased from Tocris Bioscience (Minneapolis, MN, USA). Apyrase from potato and TGF-\(\beta\) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Statistical analysis**

Calculations and statistics were performed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Numerical values are presented as mean ± S.E.M. Analysis of numerical data from experiments with multiple comparisons was done using ANOVA with Bonferroni’s correction. \(P<0.05\) was considered significant.
4.4 Results

4.4.1 CFs release ATP in response to hypotonic stimulation, a response that is inhibited by an increase in extracellular calcium

Replacement of growth media with 50% hypotonic media rapidly released ATP from rat CFs: released ATP reached a maximal concentration of 6.2 nM in the bulk media after 2.5 min and decreased over the course of 1 h (Fig. 4.1A). Up to 1 h incubation of CFs in hypotonic media did not increase cell death, as verified by trypan blue staining of cells exposed to each experimental condition (data not shown). Thus, ATP is rapidly released from rat CFs into the extracellular space via non-lytic processes in response to hypotonic stimulation and presumably the resultant cell swelling.

Prior studies document that ATP release is sensitive to extracellular Ca\(^{2+}\) levels (32, 33). We found that the addition of extracellular Ca\(^{2+}\) decreased swelling-activated ATP release from CFs in a concentration-dependent manner (Fig. 4.1B); 1 mM Ca\(^{2+}\) blunted ATP release in response to hypotonic stimulation by 50% (p<0.05) but had no effect on ATP release under isotonic conditions (Fig. 4.1C). Because extracellular Ca\(^{2+}\) can block Cx hemichannel permeability (32, 33), the effect of extracellular Ca\(^{2+}\) on ATP release suggested a role for Cx hemichannels in the release of ATP by CFs.

4.4.2 Cx43 and Cx45 are the most highly expressed subtypes in rat CFs

Using quantitative real-time PCR (qPCR), we found that Cx43 mRNA is the most highly expressed Cx subtype in rat CFs and cardiac myocytes; Cx45 mRNA is expressed at somewhat lower levels and Cx40 mRNA expression is ~10% of that of Cx43 (Fig.
4.2A) in CFs. Immunofluorescence microscopy showed that while Cx43 mediates gap junction coupling between adjacent CFs in culture, a substantial population of Cx43 hemichannels do not form gap junctions and are exposed to the extracellular space (Fig. 4.2B). Pannexin (Panx)-1, another hemichannel protein involved in ATP release (20, 34), is also expressed at low levels in rat CFs (Fig. 4.2A).

4.4.3 Carbenoxolone and probenecid, but not brefeldin A, inhibit ATP release from CFs

To characterize the role of hemichannels in ATP release, we treated CFs with carbenoxolone (CBX) or probenecid (PBC), non-specific gap junction inhibitors: CBX blocks Cx and Panx hemichannels (35-38), while PBC is more specific for Panx than Cx hemichannels (39-41). CBX inhibited ATP release in response to hypotonic stimulation in a concentration-dependent manner (Fig. 4.3A); PBC also blunted ATP release (Fig. 4.3B). By contrast, brefeldin A (BFA), an inhibitor of exocytosis, had no effect on ATP release produced with 5 min stimulation by hypotonic conditions (Fig. 4.3B). These results imply that hemichannels and not exocytosis mediate the release of ATP from rat CFs.

4.4.4 siRNA-mediated knockdown of connexin hemichannels inhibits ATP release by CFs

To more precisely define a role for hemichannels and the hemichannel subtypes involved in ATP release from CFs, we used siRNA to knock down expression of the two
major Cx subtypes in rat CFs, Cx43 and Cx45. Cx43 siRNA decreased Cx43 mRNA and protein expression by >90% (p<0.001, assayed 24 h and 48 h, respectively, after addition of siRNA; Fig. 4.4A, B), and at 48 h it decreased the release of ATP by hypotonic stimulation by 47% (p<0.001, Fig. 4.4C). Cx45 siRNA decreased Cx45 mRNA expression by 79% (p<0.001) after 24 h (Fig. 4.4D) and Cx45 protein expression by 43% (p<0.01) after 48 h (Fig. 4.4E). At 48 h, Cx45 knockdown decreased ATP release in response to hypotonic stimulation by 35% (p<0.05; Fig. 4.4F). The lower abundance of Cx45 expression in rat CFs and the smaller decrease in Cx45 by the siRNA likely explains the more modest effect of this knockdown on ATP release compared to the impact of knockdown of Cx43. Incubation of CFs with siRNAs against both Cx43 and Cx45 decreased ATP release to levels similar to that of isotonic controls (Fig. 4.5A), implying that both Cx43- and Cx45-containing connexons contribute to ATP release.

Panx1 siRNA treatment for 48 h decreased Panx1 mRNA expression by 90% (p<0.001; Fig. 4.5B) but did not significantly alter ATP release from CFs (Fig. 4.5C). These results suggest that Cx43 and Cx45 are the major gap junction hemichannel proteins involved in ATP release induced by hypotonic stimulation and cell swelling of rat CFs.

4.4.5 Released ATP participates in MAPK signaling and is sensitive to apyrase

We next tested whether ATP released from CFs affects downstream signaling, in particular the activation of MAP kinase (assessed as ERK phosphorylation). Addition of ATP (10 µM) increased ERK phosphorylation within 10 min (Fig. 4.6A). Similarly,
treatment with hypotonic media for 10 min increased phosphorylated ERK (p-ERK) levels by 5-fold (p<0.01), but the addition of apyrase, which hydrolyzes nucleotides, substantially blunted this increase (p<0.05; Fig. 4.6B). ATP released from isolated fibroblasts thus induces downstream signaling, an effect that is blunted by hydrolysis of extracellular nucleotides.

Inhibition of ATP release from CFs decreased activation of ERK: inhibition of release by CBX or knockdown of Cx43 or Cx45 expression reduced p-ERK levels after 10 min hypotonic stimulation relative to untreated controls by ~50% (p<0.05; Fig. 4.6C, D). CFs transfected with Cx43 or Cx45 siRNA had ERK phosphorylation in response to added ATP similar to control CFs incubated with scrambled siRNA (data not shown), indicating that the decrease in p-ERK levels in cells treated with siRNA results from decreased ATP-receptor signaling and not off-target effects of Cx43/45 knockdown. Decrease in ATP released in response to hypotonic stimuli thus diminishes ERK activation in response to autocrine/paracrine signaling by ATP.

4.4.6 Released ATP induces ERK phosphorylation through P2Y2 receptor activation

To investigate whether the effects of ATP release occur via P2 receptors, we initially used suramin, a non-specific P2 receptor inhibitor. Treatment of CFs with 100 μM suramin reduced p-ERK levels following hypotonic stimulation by 70% (p<0.001; Fig. 4.6D), consistent with P2 receptor activation by release of endogenous ATP from CFs.
We then sought to define which P2 receptor mediates the activation of ERK by released ATP. Our previous data indicate that P2Y$_2$ receptors are the most highly expressed P2Y receptor in rat CFs (28). Transfection of CFs with P2Y$_2$ targeted siRNA decreased P2Y$_2$ receptor mRNA and protein expression by 84% (p<0.001) and 41% (p<0.01), respectively (Fig. 4.7A, B), without significantly altering P2Y$_6$ expression. Knockdown of P2Y$_2$ receptors decreased ERK phosphorylation in response to exogenous ATP (Fig. 4.7C). Furthermore, ERK activation in response to 10 min hypotonic stimulation decreased 55% in CFs treated with P2Y$_2$ receptor siRNA as compared to scramble-treated controls (p<0.001; Fig. 4.7D, E). The ability of a decrease in P2Y$_2$ receptor expression to prominently decrease ERK activation in response to ATP release implies a key role for those receptors in mediating cellular responses to ATP released from CFs into the extracellular space. In turn, ERK phosphorylation potentially has pro-fibrogenic effects (42), an idea we assessed in subsequent experiments.

4.4.7 ATP stimulation of P2Y$_2$ receptors increases pro-fibrotic marker expression and collagen synthesis via ERK activation

P2Y receptor activation induces pro-fibrotic effects in rat CFs (20, 28). ERK phosphorylation resulting from P2Y activation has been shown to promote proliferation and phenotypic transformation in various cell types (43-46). Treatment of CFs with 10 µM ATP for 24 h increased the expression of α-SMA-positive stress fibers, a response that is a marker of fibroblast-to-myofibroblast transformation (47) (Fig. 4.8A). Moreover, treatment with ATP increased the expression of the pro-fibrotic markers PAI-1 and MCP-
1 by 3.5- and 4-fold, respectively (p<0.05); P2Y2 knockdown eliminated the up-regulation of PAI-1 and MCP-1 in response to ATP stimulation (Fig. 4.8B). Thus, consistent with prior results (28), ATP signals via P2Y2 receptors to promote pro-fibrotic responses in CFs. Treatment with U0126, a MEK/ERK inhibitor, abolished the stimulatory effect of ATP on the expression of PAI-1 and MCP-1, demonstrating that these responses depend on ERK (Fig. 4.8C).

Incubation of CFs with ATP for 24 h increased collagen accumulation by 50-60% (p<0.01, quantified using a collagenase-sensitive [3H]proline ([3H]P) incorporation assay (29)), a response that was eliminated by suramin treatment (Fig. 4.9A). Knockdown of P2Y2 receptor with siRNA (Fig. 4.9B) or inhibition of ERK activation with U0126 (Fig. 4.9C) abolished the stimulation of collagen synthesis by ATP. Together, these data show that release of endogenous ATP by CFs in response to hypotonic media activates the P2Y2-MAPK pathway to stimulate collagen accumulation and expression of the pro-fibrotic markers PAI-1 and MCP-1.

4.4.8 Nucleotides contribute to basal pro-fibrotic signaling in cardiac fibroblasts

The results shown thus far utilized hypotonic stimuli to release ATP and produce pro-fibrotic effects. “Basal” ATP concentrations in the bulk media of unstimulated CFs are low, but the concentrations of released nucleotides near the cell surface may be sufficient to activate adjacent P2Y receptors and contribute to basal signaling (48, 49). To assess the role of basal extracellular ATP concentrations on pro-fibrotic activity, we added apyrase to CFs in culture for 24 h. We found that incubation with apyrase
substantially reduces the fibrotic phenotype of CFs (Fig. 4.10). Immunofluorescent staining for α-SMA revealed that treatment with apyrase decreases the expression of α-SMA-positive stress fibers (Fig. 4.10A). Treatment with apyrase also decreased α-SMA mRNA expression by >90% (p<0.001; Fig. 4.10B), but importantly, did not prevent the pro-fibrotic response of CFs to TGF-β (Fig. 4.10B, D). The decrease in pro-fibrotic activity by nucleotide hydrolysis thus appears to result from a decrease in basal nucleotide signaling.

Apyrase treatment for 24 h also decreased basal collagen accumulation by 40% (p<0.001; Fig. 4.10C) and increased the expression of matrix metalloproteinases (MMP)-2 and -9 by 4- and 13-fold, respectively (p<0.001). The apyrase-promoted increases in MMP-2 and -9 were blunted if the CFs were incubated with TGF-β (Fig. 4.10D), implying that “basal” levels of those MMPs are regulated by ambient concentrations of extracellular ATP, but retain their ability to respond to TGF-β. Based on the results observed in CFs treated with apyrase, we conclude that ATP signaling represents not only a pro-fibrotic mechanism for cells exposed to physical perturbation (e.g., hypotonic stimulation/cell swelling), but also that ATP signaling helps establish the basal level of fibroblast activation. This conclusion is supported by the ability of suramin treatment to also decrease basal levels of collagen formation by 68% (p<0.001; Fig. 4.10C).

4.5 Discussion

The data presented here demonstrate that ATP is released from rat CFs into the extracellular space in response to hypotonicity (and the presumed swelling of the cells),
and that released ATP induces MAPK signaling and pro-fibrotic cellular responses (Fig. 4.11). Treatment with CBX, a gap junction inhibitor, decreases ATP release; siRNA-mediated knockdown of Cx43 and Cx45 (the two most highly expressed subtypes in rat CFs) also reduces ATP release. Furthermore, because Cx43 and Cx45 can form heteromeric connexons (50, 51), selective knockdown of either Cx subtype conceivably alters the expression and function of these heteromeric channels. Though simultaneous knockdown of both Cx subtypes decreased ATP release to isotonic control levels (Fig. 4.5A), our data do not rule out the potential contribution of heteromeric connexons.

Panx1, the only Panx subtype found in the heart (40, 52, 53) and which is implicated in ATP release from myocytes (20), is not highly expressed in our rat CF cultures. By using siRNA knockdown, we found that Panx1 does not substantially contribute to ATP release from rat CFs; however, since PBC, a Panx-preferring inhibitor (39, 40), partially inhibits ATP release, there may be a role, albeit likely a minor one, of Panx hemichannels in ATP release. Furthermore, because our use of siRNA did not completely eliminate Cx expression, future studies using conditional knockout approaches may be required to precisely define the contributions of each Cx subtype to ATP release from CFs. In addition, because it was necessary to use relatively high concentrations of CBX, its precise site of action cannot be determined in our studies. As such, we cannot unequivocally rule out other pathways for ATP release. Overall, our results indicate that both Cx43 and Cx45 mediate ATP release from CFs.

ATP concentrations near the surface of mechanically stimulated cells are in the micromolar range (49, 54), akin to those that activate P2Y receptors (40). Consistent with the ability of ATP to stimulate P2Y2 receptors and activate MAP kinases (43-45, 55), we
observed a rapid increase in p-ERK following hypotonic stimulation. Inhibiting ATP
release via CBX or Cx43/C45 knockdown, hydrolysis of extracellular ATP or blockade
of P2Y signaling with suramin or P2Y2 siRNA each reduced ERK activation in response
to hypotonic stimulation. Our findings are the first of which we are aware to demonstrate
that physical perturbations of CFs (or fibroblasts from any tissue) can activate
intracellular signaling events via ATP release, P2Y2 receptor activation and pro-fibrotic
responses. The current studies, including via the use of P2Y2 receptor siRNA, show that
ATP stimulation of P2Y2 receptors increases collagen accumulation and PAI-1 and MCP-
1 gene expression in an ERK-dependent manner.

Nucleotide signaling by ATP may represent a mechanism for CFs to respond to
cellular injury/stress. PAI-1 inhibits plasmin activation and can inhibit fibrinolysis and
matrix proteolysis in the heart (11). MCP-1 recruits monocytes and macrophages to sites
of tissue injury and assists in the regulation of wound healing in the myocardium (56).
The ATP-promoted increase in expression of PAI-1 and MCP-1, along with increases in
collagen and α-SMA accumulation in CFs, imply that locally released ATP from stressed
or damaged cardiac cells provides a pro-fibrotic stimulus via P2Y2 receptors to aid in
wound resolution following myocardial injury, such as myocardial infarction. Of note,
ischemia in neurons also induces ATP release, which then signals via P2Y receptors to
induce ischemic tolerance and neuronal survival (57). A similar mechanism may help
protect against ischemic injury in the heart (58, 59).

Our results also implicate a role for ATP activation of P2Y2 receptors in the basal
regulation of the phenotype of CFs. The ability of apyrase to decrease α-SMA and
collagen expression and increase the expression of MMP2 and MMP9 indicates that
signaling by ambient levels of ATP stimulates the basal production and deposition of ECM. Myocardial infarction can increase Cx43 expression and hemichannel opening (60, 61), and Cx43 deficiency has been correlated with reduced fibrosis in infarcted areas (62).

Extracellular ATP can contribute to pro-fibrotic activity in various settings, including in pulmonary fibrosis (27), airway remodeling (63) and hepatic stellate cell activation (64). Our findings thus are likely more broadly applicable: P2Y receptor activation by nucleotides released in response to cellular stress or apoptosis may be a general component of wound healing and fibrotic response. Especially given our data showing that P2Y₂ receptors contribute to both basal and stress-induced regulation of CF phenotype, nucleotide signaling may be a key autocrine/paracrine mechanism that regulates fibroblast activity, which may represent a novel therapeutic target to modulate tissue fibrosis.
4.6 Acknowledgements

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4.7 Figures

**Table 4.1**: Oligonucleotides used for qPCR.

<table>
<thead>
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<th>Gene</th>
<th>Forward, 5'-3'</th>
<th>Reverse, 5'-3'</th>
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<td>ATG CGG AAA ATG AAC AGG AC</td>
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<td>AGT ACC ACC GCA AAG GTC AC</td>
</tr>
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<td>ATC GGA AGG AGT AAT AGA GGG T</td>
</tr>
<tr>
<td>P2Y6</td>
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<td>TGG CAT AGA AGA GGA AGC GT</td>
</tr>
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<td>TCG GAT ACT TCA GGG TCA GG</td>
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<td>TCC AGA AGG GGA TAT GTT GC</td>
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</tr>
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<td>MMP2</td>
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<td>ACA CGG CAT CAA TCT TTT CC</td>
</tr>
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<td>MMP9</td>
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<td>CCA TCC AAT CGG TAG TAG CG</td>
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</table>
Figure 4.1: ATP is released into the extracellular space in response to hypotonic stimulation and is sensitive to extracellular calcium. (A) Rat CFs were exposed to isotonic and hypotonic conditions for the indicated times. Release of ATP after hypotonic stimulation peaked at 6.2 nM (in bulk media) after 2.5 min. ATP concentration in the media of hypotonic-stimulated CFs returned to near-baseline after ~1 h. (B) Addition of extracellular Ca\(^{2+}\) dose-dependently decreased ATP release in response to 5 min hypotonic stimulation. (C) 1 mM extracellular Ca\(^{2+}\) reduced hypotonicity-induced ATP release by 50% (p<0.05) but did not affect basal (isotonic) ATP release. In all panels: \(*p<0.05;\) data are presented as mean ± SEM of 3 independent experiments.
Figure 4.2: mRNA expression of Cx and Panx subtypes in rat CFs. (A) qPCR revealed that the rank order of mRNA abundance of the three most highly expressed Cx isoforms is: Cx43>Cx45>Cx40. Panx1 mRNA expression was detected at one-fifth the relative abundance of Cx43. (B) Immunofluorescence microscopy in rat CFs showed that while Cx43 is expressed between adjacent cells to mediate cellular coupling, a substantial population of Cx43-containing hemichannels are exposed to the extracellular space (arrows; green: Cx43, blue: DAPI).
Figure 4.3: CBX and PBC blunt hypotonicity-induced ATP release in rat CFs; BFA has no such effect. (A) CBX dose-dependently inhibited ATP release in response to hypotonic stimulation, with maximal inhibition at 500 µM CBX. (B) 100 µM PBC inhibited ATP release (~40%, p<0.01); greater concentrations had no additional effect. (C) BFA had no effect on ATP release. In all experiments CFs were incubated with vehicle (veh), CBX, PBC or BFA for 30 min followed by 5 min stimulation with hypotonic media. In all panels: *p<0.05, **p<0.01; data presented are the mean ± SEM of at least 3 independent experiments.
Figure 4.4: Selective siRNA knockdown of Cx43 and Cx45 expression blunts hypotonicity-induced ATP release from CFs. (A) Cx43 siRNA knocked down Cx43 mRNA expression by 91% 24 h after transfection (p<0.001; SCR: scramble siRNA). (B) Cx43 protein levels were decreased >90% 24-48 h after transfection with Cx43 siRNA. (C) CFs transfected with Cx43 siRNA (assayed 48 h post-transfection) released 47% less ATP than controls after 5 min hypotonic stimulation (p<0.001). (D) Cx45 siRNA knocked down Cx45 mRNA expression by 79% (p<0.001) 24 h after transfection. (E) Cx45 protein levels were decreased by 43% (p<0.01) 48 h after transfection with Cx45 siRNA. (F) CFs transfected with Cx45 siRNA (assayed 48 h post-transfection) released 35% less ATP after hypotonic stimulation for 5 min (p<0.05). In all panels: *p<0.05, **p<0.01, ***p<0.001. mRNA and protein expression data are presented as mean ± SEM of 2 independent experiments; ATP release data are presented as mean ± SEM of 6 (Cx43) or 4 (Cx45) independent experiments.
Figure 4.5: Simultaneous Cx43/Cx45 knockdown inhibits ATP release, but Panx1 knockdown does not. (A) Simultaneous transfection of Cx43 and Cx45 siRNA decreased ATP release to the levels of SCR-transfected isotonic controls. (B) Panx1 siRNA decreased Panx1 mRNA expression 90% 24 h post-transfection (p<0.001). (C) Knockdown of Panx1 did not significantly blunt ATP release in response to 5 min hypotonic stimulation 48 after siRNA transfection. In all panels: *p<0.05, **p<0.01, ***p<0.001. mRNA expression data are presented as mean ± SEM of 3 independent experiments; ATP release data are presented as mean ± SEM of 4 independent experiments.
Figure 4.6: ATP released from CFs signals via nucleotide receptors to regulate ERK activation. (A) Addition of 10 µM ATP induced ERK activation by 10 min. (B) p-ERK levels in CFs increased 5-fold after 10 min hypotonic stimulation (p<0.01) and was significantly blunted by addition of 0.67 U/ml apyrase (p<0.05). (C) CFs transfected with Cx43 and Cx45 siRNA, which decrease ATP release, showed diminished ERK activation by ~50% (p<0.05) in response to hypotonic stimulation. (D) Hypotonicity-induced ERK activation was decreased 50% by apyrase (0.67 U/ml; p<0.01), 52% by inhibition of ATP release (100 µM CBX; p<0.01) and 69% by P2 receptor blockade (100 µM suramin; p<0.001). In all experiments, cells were treated with the respective compounds for 30 min and then exposed to hypotonic conditions for 10 min. Densitometric quantification in (B), (C) and (D) of 3 independent experiments are shown ± SEM, *p<0.05, **p<0.01, ***p<0.001.
Figure 4.7: P2Y₂ receptor knockdown decreases extracellular ATP-stimulated ERK activation. P2Y₂ siRNA reduced P2Y₂ gene expression by 84% (p<0.001) (A) and protein expression by 41% (p<0.01) (B). P2Y₂ siRNA transfection (+) reduced ERK activation in response to 10 min ATP stimulation (C) and to 10 min hypotonic stimulation by 55% (p<0.001) (D), compared to scramble-transfected (-) controls. (E) The quantification of 3 independent experiments is shown as the mean ± SEM. In all panels: *p<0.05, **p<0.01, ***p<0.001; +p<0.05 vs. isotonic control, +++p<0.001 vs. isotonic control.
Figure 4.8: ATP increases α-SMA and expression of pro-fibrotic markers PAI-1 and MCP-1. (A) Immunofluorescent microscopy shows that incubation with 10 µM ATP for 24 h increased expression of α-SMA-containing stress fibers. (B) Treatment with 10 µM ATP for 4 h increased mRNA expression of the pro-fibrotic markers PAI-1 and MCP-1 (3.5- and 4-fold, respectively; p<0.05). siRNA knockdown of P2Y2 expression abolished this effect. (C) ERK inhibition with U0126 (U0) eliminated the stimulatory effect of ATP on PAI-1 and MCP-1 expression. In all panels: *p<0.05, **p<0.01, ***p<0.001; data are presented as mean ± SEM of at least 3 independent experiments.
Figure 4.9: P2Y₂ activation by ATP increases collagen synthesis in CFs and is ERK dependent. Collagen synthesis was measured by a collagenase-sensitive [³H]proline incorporation assay. (A) ATP (10 µM for 24 h) increased collagen synthesis by 50% (p<0.01) and was blunted with 50 µM suramin (p<0.001). (B) siRNA knockdown of P2Y₂ receptor expression reduced ATP-stimulated collagen synthesis (at 10 and 100 µM) over 24 h (p<0.001). (C) MEK/ERK inhibition with 10 µM U0126 (U0) prevented ATP-stimulated collagen synthesis (p<0.001). In all panels: **p<0.01, ***p<0.001 vs. untreated control and +++p<0.001 between the groups indicated. Data are presented as mean ± SEM of 3 independent experiments.
Figure 4.10: Hydrolysis of extracellular ATP decreases pro-fibrotic α-SMA and collagen accumulation and increases MMP expression. (A) By immunofluorescent microscopy, apyrase treatment for 24 h decreased expression of α-SMA-containing stress fibers in CFs. (B) Apyrase treatment (24 h) decreased α-SMA mRNA expression by >90% (p<0.001), which was partially reversed by co-treatment with 10 ng/ml TGF-β. (C) Apyrase also decreased collagen accumulation after 24 h by 40% (p<0.001) and (D) increased MMP2 and MMP9 mRNA expression by 4- and 13-fold, respectively (p<0.001). These effects were also partially reversed by TGF-β co-treatment. In all panels: *p<0.05, **p<0.01, ***p<0.001; data are presented as mean ± SEM of at least 3 independent experiments.
Figure 4.11: Model of autocrine/paracrine signaling caused by ATP release. Hypotonic stimulation induces rapid ATP release in CFs. ATP then signals via P2Y$_2$ receptors to up-regulate PAI-1 and MCP-1 expression and collagen accumulation via ERK activation.
4.8 References


CHAPTER 5:

HYDROLYSIS OF EXTRACELLULAR ATP BY ECTONUCLEOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASE ATTENUATES FIBROTIC ACTIVITY IN CARDIAC FIBROBLASTS

5.1 Abstract

The establishment of set points for cellular activities is essential in regulating homeostasis. Here we demonstrate key determinants of the fibrogenic set point of cardiac fibroblasts (CFs) by focusing on the pro-fibrotic activity of ATP, which is released by CFs. We tested the hypothesis that the hydrolysis of extracellular ATP by ectonucleoside triphosphate diphosphohydrolases (ENTPDs) regulates pro-fibrotic nucleotide signaling. We detected two ENTPD isoforms, ENTPD-1 and -2, in adult rat ventricular CFs. Partial knockdown of ENTPD-1 and -2 with siRNA increased basal extracellular ATP concentration and enhanced the pro-fibrotic effect of ATP stimulation. Sodium polyoxotungstate (POM)-1, an ENTPD inhibitor, not only enhanced the pro-fibrotic effects of exogenously added ATP but also increased basal expression of $\alpha$-smooth muscle actin ($\alpha$-SMA), plasminogen activator inhibitor (PAI)-1 and transforming growth
factor (TGF)-β, collagen synthesis and gel contraction. Furthermore, we found that adenosine, a product of ATP hydrolysis by ENTPD, acts via A2B receptors to counterbalance the pro-fibrotic response to ATP. Removal of extracellular adenosine or inhibition of A2B receptors enhanced pro-fibrotic ATP signaling. Together, these results demonstrate the contribution of basally released ATP in establishing the set point for fibrotic activity in adult rat CFs and identify a key role for the modulation of this activity by hydrolysis of released ATP by ENTPDs. These findings also imply that cellular homeostasis and fibrotic response involves the integration of signaling that is pro-fibrotic by ATP and anti-fibrotic by adenosine and which is regulated by ENTPDs.

5.2 Introduction

The regulation of the remodeling of extracellular matrix (ECM) is essential for tissue homeostasis during normal development and also, in tissue healing after injury (1, 2). In the heart, cardiac fibroblasts (CFs) maintain myocardial ECM turnover and are critical contributors to the remodeling that occurs after injury, such as myocardial infarction (3-5). Organization of myocardial structure by the ECM is also essential for normal cardiac contraction and electrical conduction (6, 7). Activation of fibroblasts by certain cytokines and hormones causes their transformation into pro-fibrogenic myofibroblasts and can result in tissue fibrosis through excessive deposition of collagens (primarily types I and III) and other ECM proteins (5, 8, 9).

Myofibroblasts express α-smooth muscle actin (α-SMA), a contractile protein that aids in wound contraction (10), and numerous pro-fibrotic cytokines, including
plasminogen activator inhibitor (PAI)-1 and transforming growth factor (TGF)-β. PAI-1 inhibits the activation of plasmin and matrix metalloproteinases (MMPs) and is linked to the development of tissue fibrosis (11, 12). TGF-β receptor/Smad signaling in fibroblasts is strongly pro-fibrotic and induces the synthesis of ECM and expression of α-SMA (13, 14).

Understanding tissue fibrosis requires identification of the stimuli that regulate the homeostatic phenotype of fibroblasts and that trigger fibrosis. Extracellular ATP has been shown to promote monocyte and inflammatory cell recruitment to sites of injury in the lung (15) and liver (16). Consistent with such effects, we have found that extracellular ATP and UTP and their signaling through P2Y2 receptors are pro-fibrotic in adult rat and mouse ventricular fibroblasts grown in primary culture (17). The activation of P2Y2 receptors by these nucleotides increases collagen synthesis and the expression of α-SMA, PAI-1 and TGF-β. Furthermore, ATP, released from CFs via connexin (Cx)-43 and Cx-45 hemi-channels, activates P2Y2 receptors in an autocrine/paracrine manner (18). Data in the latter study also revealed that the release of and signaling by ATP contribute to the basal fibrotic state of CFs: hydrolysis of extracellular ATP, by addition of the nucleotidase apyrase, decreased basal collagen synthesis and the expression of α-SMA stress fibers. Such data indicate that the release of cellular ATP and subsequent activation of P2Y2 receptors contribute to fibroblast phenotype and suggest that nucleotide signaling is an important pro-fibrotic mechanism in CFs and potentially in fibroblasts in other tissues.

Extracellular ATP catabolism is important in regulating signal transduction by nucleotides. Ectonucleoside triphosphate diphosphohydrolases (ENTPDs) are a family of
nucleotidases that hydrolyze tri- and di-phosphate nucleotides. ENTPD activity in vascular endothelial cells is essential for the regulation of inflammation and thrombosis (19, 20) and for vascular tone (21). Furthermore, ENTPD activity in hepatic portal fibroblasts may limit fibroblast and endothelial cell proliferation, thus implicating nucleotide catabolism in the regulation of liver fibrosis (22).

The strong anti-fibrotic effects of apyrase on activities of CFs led us to ask if CFs endogenously express nucleotidases that produce similar effects, and thereby provide a “brake” on the pro-fibrotic actions of released ATP. Of the four extracellular ENTPD isoforms (ENTPD-1, -2, -3, -8) (23), ENTPD-1 has been the most studied in the heart, but almost exclusively in the context of cardioprotection following injury (24). No previous information is available regarding expression of ENTPDs by CFs nor have the potential effects of ATP hydrolysis by ENTPDs been studied with respect to the transformation of CFs to pro-fibrogenic myofibroblasts.

We hypothesized that ENTPD activity is a mechanism that inhibits the pro-fibrotic autocrine/paracrine pathway stimulated by ATP-P2Y receptor signaling, such that both ATP release and its hydrolysis determine fibrotic response. In this study we investigated ENTPD expression in adult rat CFs, the effect of ENTPD inhibition on CF phenotype and the role of these enzymes in modulating pro-fibrotic nucleotide signaling. We also assessed whether adenosine, a product generated by the hydrolysis of ATP, activates adenosine receptors and also regulates CF phenotype. The results reveal a key integrative role for ENTPD in ATP-mediated pro-fibrotic and adenosine-mediated anti-fibrotic responses.
5.3 Materials and Methods

Isolation and culture of adult rat cardiac fibroblasts

Approval for the ethical care and use of animals for this study was granted by the UCSD Institutional Animal Care and Use Committee and was in compliance with the guiding principles of the American Physiological Society. CFs were isolated from adult (8-10 wk), male Sprague-Dawley (SD) rats, as previously described (8). Briefly, SD rats were anesthetized via intraperitoneal injection of 100 mg/kg ketamine, 10 mg/kg xylazine. The heart was removed, cannulated on a modified Langendorff apparatus and perfused with collagenase II (Worthington Biochemical Corp., Lakewood, NJ, USA). CFs were separated from cardiac myocytes by gravity separation and grown to confluency in 10-cm culture dishes at 37°C, 10% CO₂ in DMEM containing 10% FBS, 1% penicillin, 1% streptomycin. CFs were then split to appropriate-sized culture dishes, allowed to adhere overnight and serum-starved in DMEM/0% FBS for 24 h prior to treatment.

Extracellular ATP quantification

Growth media (100 µL) were carefully removed by placing a pipette as close to the fluid surface as possible to avoid perturbation of the cells. ATP concentration was measured using a luciferase-based ATP assay kit (ENLITEN ATP Assay; Promega, Madison, WI, USA) and TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA) according to the manufacturers’ instructions. Assays were conducted at room temperature. Standard curves using DMEM supplemented with known ATP
concentrations were generated and used to quantify ATP concentration in the experimental samples.

**Malachite green assay**

ATP hydrolysis was measured by quantifying inorganic phosphate accumulation using the SensoLyte malachite green assay kit (AnaSpec, Fremont, CA) following the manufacturer’s instructions. CFs were cultured in phosphate- and serum-free DMEM. One hour after addition of 30 µM exogenous ATP, media were gently removed and filtered (to remove free cells). Media samples (80 µL) or standards consisting of DMEM containing phosphate standards were mixed with 20 µL assay reagent in a 96-well plate. Following incubation at room temperature on a rotary shaker for 20 min, the absorbance was measured at 620 nm using a DTX 800 Multimode Detector (Beckman Coulter, USA). The concentration of inorganic phosphate in CF-conditioned media was calculated by comparison with standard curves.

**siRNA transfection**

Targeted siRNA sequences for rat ENTPD-1 (s134179), ENTPD-2 (s134120) and negative control siRNA were purchased from Ambion (Grand Island, NY, USA). Cells were transfected with 5nM siRNA using RNAiMAX (Invitrogen, Grand Island, NY, USA) for 8 h according to the manufacturer’s instructions. The media containing transfection reagent were then replaced with fresh serum-free DMEM, and CFs were incubated for 24 h.
**Quantitative real-time PCR (qPCR)**

Total RNA was isolated by TRIzol extraction (Invitrogen), and cDNA was generated using the Superscript III cDNA synthesis system (Invitrogen) according to the manufacturer’s instructions. The qScript One-Step qRT-PCR kit (Quanta Biosciences, Gaithersburg, MD, USA) and a DNA Engine Opticon 2 (Bio-Rad, Hercules, CA, USA) qPCR machine was used for gene expression analyses. Primers for PCR amplification (Table 1) were designed based on the nucleotide sequences of the respective gene target using Primer3Plus software. When possible, each forward and reverse primer set was designed between multiple exons. Amplification efficiency of each primer pair was tested prior to analysis, and relative gene expression levels were determined using the ΔΔCT method with 18S as the reference gene (25).

**Immunoblot analysis**

Whole cell lysates were prepared in 150 mM Na$_2$CO$_3$ buffer (pH 11) and homogenized by sonication. Protein concentration was measured using a Bradford protein assay (Bio-Rad), and equal amounts of protein were separated by SDS/PAGE using 10% polyacrylamide precast gels (Invitrogen) and transferred to a poly(vinylidene difluoride) membrane with the iBlot system (Invitrogen). Membranes were blocked in PBS Tween (1%) containing 5% nonfat dry milk and incubated with primary antibody overnight at 4°C. Bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ECL reagent (Amersham Pharmacia, Pittsburg, PA, USA). Bands were compared to molecular weight standards to confirm migration of proteins at the appropriate size.
Quantification of the densitometry of protein expression was performed using ImageJ software (NIH). Antibodies for α-SMA were purchased from Invitrogen, PAI-1 from BD Biosciences (Franklin Lanes, NJ), GAPDH from Abcam, and p-ERK and total ERK from Cell Signaling (Danvers, MA, USA).

**Collagenase-sensitive [³H]proline incorporation**

Collagenase-sensitive [³H]proline incorporation assays were used to quantify collagen accumulation and were performed as previously described (8, 18). CFs cultured on 12-well plates were serum-starved for 24 h followed by the addition of 1 μCi/ml [³H]proline (PerkinElmer, Waltham, MA, USA; 1 Ci = 37 GBq) along with compounds of interest and incubated at 37°C for 24 h. Cells were lysed with 0.5 N NaOH, and following neutralization with HCl, protein was precipitated overnight with 10% trichloroacetic acid (TCA). Samples were pelleted and washed in 5% TCA, dissolved in 0.2 N NaOH and neutralized with HCl. Collagenase II (2 mg/ml; Worthington) in Tris-CaCl₂-N-ethylmaleimide buffer was added to each sample, which were incubated at 37°C for 1 h. Protein was then precipitated and centrifuged. The supernatant was collected and the radioactivity quantified using a liquid scintillation counter.

**Collagen gel contraction assay**

Collagen gels were prepared using rat-tail collagen 1 (BD Biosciences) neutralized with NaOH and supplemented with 1X DMEM (Sigma-Aldrich). Gels contained 2-3 mg/mL rat-tail collagen, depending on experimental protocols, in 0.5 mL volumes. CFs were seeded at a density of 1.8 x 10⁵ cells per gel, and the gels were
allowed to polymerize at 37°C for 1 h. Following polymerization, 0.5 mL serum-free DMEM was added, and the gels were detached and suspended in liquid culture. Treatments were done for 24 h, and images were taken for surface area quantification.

**Reagents**

ATP, adenosine, suramin, POM-1, H89, Rp-cAMPS, PSB 603 and SCH 442416 were purchased from Tocris Bioscience (Minneapolis, MN, USA). Apyrase and adenosine deaminase were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Statistical analysis**

Calculations and statistics were performed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Numerical values are presented as mean ± S.E.M. Analysis of numerical data from experiments with multiple comparisons were done using ANOVA with Tukey’s test. P<0.05 was considered significant.

**5.4 Results**

**5.4.1 ATP signaling regulates basal α-SMA expression and contractile tone in cardiac fibroblasts**

The expression of α-SMA is a hallmark of myofibroblast transformation (10) and confers a contractile phenotype to CFs (26). In previous studies we showed that ATP released from CFs regulates collagen synthesis and that addition of exogenous ATP increases formation of α-SMA-expressing fibers in CFs via P2Y$_2$ receptor signaling (17, 18). Those results suggested that extracellular ATP helps determine CF α-SMA
expression and contraction. To assess the extent of basally released ATP in regulating CF contractile tone, we seeded CFs into gels containing 2.5 mg/mL collagen, which CFs spontaneously contract. Hydrolysis of extracellular ATP, produced by addition of 1 U/mL apyrase, decreased this contraction and increased gel surface area (by 86%, p<0.001) while also decreasing basal α-SMA expression (by 42%, p<0.01) (Fig. 5.1A, B). Suramin (50 μM), a P2 nucleotide receptor inhibitor, also increased collagen gel surface area (by 67%, p<0.001). Thus, removal of ambient extracellular ATP by apyrase or blocking P2 receptors reduced basal α-SMA expression and CF contraction, indicating, in addition to our previous findings related to P2Y$_2$ signaling in CFs (18) that tonic ATP-P2Y$_2$ signaling promotes myofibroblast transformation of CFs. These results led us to ask if nucleotidase activity expressed by CFs may regulate CF homeostasis by hydrolyzing basally released ATP and attenuating this pro-fibrotic phenotype.

5.4.2 ENTPD expression in rat CFs: siRNA-mediated knockdown of endogenous NTPases increases extracellular ATP concentration and enhances the pro-fibrotic effect of ATP stimulation

CFs, along with numerous cell types in the myocardium, release ATP in response to physical or chemical stimuli (18, 27-29), but the fate of this released ATP is not well-defined. We hypothesized that CFs may endogenously express enzymes with nucleotide hydrolytic activity that might result in effects akin to those we observed by adding apyrase and thereby, provide a “brake” on pro-fibrotic ATP signaling. Kauffenstein, et al. described that the mouse vasculature expresses two NTPase isoforms, ENTPD-1 and -2, (21). Using real-time qPCR analysis of isolated rat ventricular CFs, we detected ENTPD-
1 and -2 at approximately equal expression levels (Fig. 5.2A). ENTPD-3 was not detected (not shown).

We used siRNA to selectively decrease expression of each ENTPD isoform. Though single knockdown of ENTPD-1 or -2 did not substantially change extracellular ATP concentration, knockdown of both ENTPD-1 and -2 decreased their expression by 57% and 54%, respectively, and significantly increased basal extracellular ATP concentration (by 2.5-fold, p<0.01) (Fig. 5.2B). The requirement to knock down both ENTPD-1 and -2 implies that both enzymes contribute to ATP hydrolytic activity in CFs. Assessment of changes in pro-fibrotic marker expression (by qPCR) was used to investigate the functional impact of knockdown of ENTPD-1 and -2, and thus of decreased ATP hydrolytic activity in CFs. Although we found increased expression of α-SMA, PAI-1 and TGF-β following ENTPD-1/-2 knockdown, these increases were not statistically significant (p>0.05). However, addition of 10 µM ATP to ENTPD-deficient CFs had a significantly greater stimulatory effect on α-SMA, PAI-1 and TGF-β expression (2.7-, 4.0- and 1.7-fold, respectively) than did ATP treatment of control siRNA-transfected CFs (Fig. 5.2C). Thus, a decrease in ENTPD expression in CFs enhanced the pro-fibrotic response to ATP.

5.4.3 POM-1, an ENTPD inhibitor, enhances basal pro-fibrotic and ERK activity in CFs

Because of the partial knockdown that we achieved with siRNA and the need to target both ENTPD-1 and -2 to decrease ATP hydrolysis, we tested a second approach to
inhibit ENTPDs: treatment of CFs with a polyoxometalate-derived compound, which is an extracellular ENTPD inhibitor (30, 31). Sodium polyoxotungstate (POM)-1 prominently inhibits ENTPD activity in endothelial and smooth muscle cells and in mouse models of cardiac and renal ischemia (24, 32). Because POM-1 interferes with luciferase-based methods for ATP quantification, we used a malachite green (MG) assay to measure accumulation of inorganic phosphate resulting from ATP hydrolysis (31, 33-35). Addition of 30 µM POM-1 inhibited ATP hydrolysis by ~30% (p<0.001) (Fig. 5.3A); 100 µM POM-1 did not produce a significantly greater inhibitory effect. As a result of ENTPD inhibition, we found that treatment of CFs with POM-1 produced a concentration-dependent increase in α-SMA and PAI-1 protein expression and collagen synthesis (Fig. 5.3B, C), thus indicating that POM-1 enhances the basal transformation of CFs to myofibroblasts.

Previous data indicate that the P2Y2 receptor is the most highly expressed P2Y subtype in rat ventricular fibroblasts and that P2Y2 mediates the majority of pro-fibrotic responses of rat CFs in response to ATP stimulation (17, 18). P2Y2 receptor stimulation can lead to rapid and transient activation of MAPK/ERK (36, 37); rat CFs deficient in P2Y2 receptors lack this response to exogenously added ATP (18). Consistent with the ability of ENTPD inhibition to enhance ATP-mediated signaling, 1 h pre-treatment of CFs with 30 µM POM-1 increased basal phospho-ERK levels and prominently prolonged ATP-stimulated activation of ERK compared to the response of CFs treated with ATP alone (Fig. 5.3D). The P2 receptor antagonist suramin blocked the stimulatory effect of 30 µM POM-1 on collagen synthesis and on the protein expression of α-SMA and PAI-1
(Fig. 5.3E), indicating that the pro-fibrotic effects of POM-1 require P2, most likely P2Y2 receptor activation (17, 18).

5.4.4 POM-1 enhances the pro-fibrotic effects of ATP

In addition to stimulating basal pro-fibrotic signaling, POM-1 enhanced the pro-fibrotic effects of exogenously added ATP. Treatment with 30 µM POM-1 significantly increased ATP-promoted mRNA expression of α-SMA, PAI-1 and TGF-β by 1.9- (p<0.01), 3.0- (p<0.05) and 1.7-fold (p<0.01), respectively (Fig. 5.4A). Similarly, protein expression of α-SMA and PAI-1 were increased 30% (p<0.05) and 89% (p<0.01), respectively, and collagen synthesis was increased 73% (p<0.001) in CFs treated with POM-1 plus ATP compared with responses of cells incubated only with ATP (Fig. 5.4B & 5.5A). CF-mediated collagen gel contraction was also enhanced: POM-1 increased ATP-stimulated gel contraction by 23% (p<0.001) (Fig. 5.5B).

Together, these data indicate that the pro-fibrotic effects mediated by ATP are enhanced by inhibiting extracellular ENTPD activity. POM-1 increased the basal pro-fibrotic state of CFs and enhanced the stimulatory effect of ATP on collagen synthesis and the expression of pro-fibrotic markers, α-SMA, PAI-1 and TGF-β. Thus, ENTPD activity is a “brake” that regulates a basal pro-fibrotic pathway driven by constitutive nucleotide release and signaling.
5.4.5 Adenosine reverses the pro-fibrotic effects of POM-1

Adenosine signaling in rat CFs is anti-fibrotic, mediated by increased intracellular cAMP via activation of Gs-coupled A2 adenosine receptors (38-40). ENTPD activity, in combination with the activity of 5’-nucleotidases (e.g., CD73) and alkaline phosphatase, produces extracellular adenosine (24, 41, 42). Thus, we speculated that the generation of adenosine by ATP hydrolysis might represent a counterbalancing anti-fibrotic mechanism in CFs that functions via A2 receptor activation. Indeed, we found that addition of 100 µM adenosine significantly reduces the stimulatory effect of POM-1 on α-SMA mRNA and protein expression (p<0.05) (Fig. 5.6A, B).

The anti-fibrotic effects of adenosine in CFs occur via an increase in intracellular cAMP in response to Gs-coupled A2 receptor activation (38, 40). We therefore assessed PKA-dependent CREB phosphorylation resulting from ATP and adenosine treatment. Stimulation of CFs for 4 h with either 10 µM ATP or 30 µM adenosine significantly increased phospho-CREB levels in a PKA (and thus, cAMP)-dependent manner (Fig. 5.6C). Consistent with this idea, inhibition of PKA with 10 µM H89 or 50 µM Rp-cAMPS blocked CREB phosphorylation stimulated by ATP and adenosine. Furthermore, antagonism of A2B receptors with 300 nM PSB 603 blocked the stimulatory effect of ATP on CREB phosphorylation (p<0.05) (Fig. 5.6D), whereas the A2A receptor inhibitor SCH 442416 had no significant effect (p>0.05). These results show that ATP-stimulated CREB phosphorylation is dependent on A2B receptors and activation of the cAMP-PKA pathway. Thus, ATP can activate Gs-coupled A2B receptors, an effect resulting from the hydrolysis of extracellular ATP into adenosine.
5.4.6 Extracellular adenosine deamination and adenosine A2B receptor inhibition enhances pro-fibrotic ATP signaling

If the hydrolysis of pro-fibrotic ATP via the generation of adenosine produces a counterbalancing anti-fibrotic response in CFs, interventions that decrease extracellular adenosine should enhance the pro-fibrotic phenotype. To test this idea, we assessed α-SMA and PAI-1 protein expression in response to ATP in the presence of adenosine deaminase (ADA). ADA (1 U/mL) increased collagen synthesis by 38% (p<0.001) and the expression of α-SMA and PAI-1 protein by 2- and 3-fold, respectively (p<0.05) (Fig. 5.7A, B). Addition of ADA also enhanced response to ATP: incubation of CFs with ADA and ATP increased α-SMA and PAI-1 protein expression 42% (p<0.05) and 182% (p<0.001) more than to ATP alone (Fig. 5.7B).

As an alternative approach to block adenosine action, we incubated CFs with ATP or UTP in the presence of PSB 603. PSB 603 (300 nM) enhanced responses to ATP (10 µM): ATP-promoted increases in α-SMA and PAI-1 mRNA expression were 62% (p<0.01) and 69% (p<0.05) greater, respectively, in the presence of PSB 603, (Fig. 5.7C) and α-SMA protein levels were 33% (p<0.05) higher than if incubated only with ATP (Fig. 5.7D). By contrast, response to 10 µM UTP was not enhanced by the addition of PSB 603. This result is as expected because although ATP and UTP activate P2Y2 receptors with equal affinity (43, 44), UTP hydrolysis does not yield adenosine, thus A2B receptor inhibition should have no effect. Furthermore, PSB 603 did not potentiate the effects of POM-1 on α-SMA or PAI-1 expression (p>0.05) (Fig. 5.7E), indicating a robust response to ENTPD inhibition and the absence of adenosine signaling. Thus, pro-
fibrotic ATP signaling is amplified in CFs by interventions that block the counterbalancing anti-fibrotic pathway mediated by adenosine.

5.5. Discussion

Based on previous data that identified a role for constitutively released ATP in regulating fibrotic activity of CFs (18), we hypothesized that ATP hydrolysis by endogenous nucleotidases might be an important mechanism that controls the activation of pro-fibrogenic pathways by basal nucleotide signaling in CFs. We demonstrate results consistent with this hypothesis: endogenous ENTPD activity attenuates pro-fibrotic nucleotide signaling in CFs, thus establishing a stimulatory mechanism (i.e., ATP release) and an inhibitory mechanism (ATP hydrolysis by ENTPD) that work in concert to establish the set point for extracellular ATP levels and P2Y$_2$-mediated pro-fibrotic tone. Inhibition of ENTPD activity (such as with POM-1) increases pro-fibrotic autocrine/paracrine effects of basal nucleotide signaling, thus identifying the importance of these nucleotidases in attenuating this pathway.

Previous reports have described the use of POM-1 as an ENTPD inhibitor (24, 31, 32) and one study showed that POM-1 inhibits cardioprotection via adenosine generation (24). However, previous studies have not addressed whether ENTPD inhibition perturbs fibrogenic responses. We found that treatment of CFs with POM-1 inhibits hydrolysis of extracellular ATP by CFs, increases basal expression of the pro-fibrotic markers α-SMA, PAI-1 and TGF-β and stimulates collagen synthesis. We did not observe statistically significant effects on basal CF gene expression with siRNA-mediated ENTPD
knockdown, likely because we only achieved partial knockdown of ENTPD expression. Unfortunately, we found that ENTPD siRNA concentrations higher than 5 nM were not well tolerated by CFs. Even so, CFs transfected with siRNA targeting ENTPD-1 and -2 were significantly more pro-fibrotic in response to exogenously added ATP than were control siRNA-transfected CFs.

The effects observed with POM-1 treatment were blunted by P2 receptor inhibition, consistent with the involvement of extracellular nucleotide-P2Y receptor signaling in the pro-fibrotic response to POM-1 (17, 18). Incubation with POM-1 also enhanced and prolonged the transient effects of ATP on ERK activation, indicating that POM-1 enhances ATP signaling. Consistent with this idea, treatment with POM-1 significantly increased the ATP-promoted increase in expression of α-SMA, PAI-1 and TGF-β, cellular collagen synthesis and CF-mediated gel contraction.

In addition to attenuating pro-fibrotic ATP signaling, nucleotidase activity, via the generation of adenosine, initiates a counterbalancing, anti-fibrotic response mediated by A2B receptor activation. Numerous studies have found that ENTPD activity facilitates the production of adenosine and that ENTPD inhibition decreases extracellular adenosine concentration (24, 31, 32, 45). We found that ATP stimulates PKA-dependent CREB phosphorylation, a response that is sensitive to inhibition of A2B receptors, indicating that these effects are a result of adenosine generation and signaling. Furthermore, treating CFs with agents that blunt adenosine levels (ADA) or response (an A2B receptor antagonist) enhanced the pro-fibrotic activity of CFs, increasing the pro-fibrotic effect of ATP on α-SMA and PAI-1 expression. Thus the anti-fibrotic component of ATP signaling is initiated by ecto-nucleotidase-mediated hydrolysis. Consistent with this mechanism, we
found that A2B inhibition did not further potentiate the pro-fibrotic effects of POM-1, indicating a strong induction of ATP signaling by ENTPD inhibition and the absence of adenosine signaling.

Together, our findings indicate that a decrease in ENTPD expression or activity in CFs will enhance pro-fibrogenic activity and potentially promote the development of cardiac fibrosis, which can result from two effects: prolonged ATP signaling and decreased adenosine generation. Of note, such effects are not limited to the heart. Similar mechanisms may regulate hepatic stellate cell and portal fibroblast proliferation and activity in the liver (22, 46). Our data demonstrate that autocrine/paracrine signaling that results from nucleotide release and hydrolysis appear to be essential for determining the set point of the CF phenotype and perhaps in other tissues as well.

In the cardiovascular system, further studies are required to investigate whether disease settings result in altered extracellular ATP catabolism and the impact of this effect on CF activity. Mice that overexpress ENTPD1/CD39 are protected from ischemia-reperfusion injury as a consequence of adenosine production and signaling in cardiac myocytes (24, 33). Furthermore, differential ENTPD1/CD39 activities have been observed in patients with coronary artery disease and ischemic heart disease (47, 48). Our data suggest that ENTPD up-regulation may protect against cardiac fibrosis in vivo and that conversely, down-regulation or decreased activity of ENTPD may exacerbate the fibrotic phenotype.

Together with previous findings (18, 22, 46, 49), the data presented here demonstrate the important role of basally released ATP and nucleotide hydrolysis in mediating cellular homeostasis and physiology (e.g., proliferation, contraction, migration,
collagen synthesis) (**Fig. 5.8**). ENTPDs play an essential role in the attenuation of ATP signaling but also initiate ADP (and UDP) and adenosine-driven responses (20, 49). We find that the generation of adenosine, an anti-fibrotic mediator in CFs (38, 40), also helps determine the set point of CFs. The release of cellular nucleotides and the activity of ENTPDs and possibly other ecto-nucleotidases thus mediate counteracting ATP-P2Y and adenosine-P1 pathways that regulate cellular homeostasis.
5.6 Acknowledgements

This work was supported by research grants from the U.S. National Institutes of Health (NIH) and support from the University of California–San Diego Academic Senate (to P.A.I.). D.L. was supported by National Research Service Award 1F31AG039992-01. The authors have no conflicts of interest to declare.

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5.7 Figures

Table 5.1: Primer sequences for real-time qPCR.

<table>
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<th>Gene</th>
<th>Forward, 5’-3’</th>
<th>Reverse, 5’-3’</th>
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<tr>
<td>α-SMA</td>
<td>CATCAGGAACCTCGAGAAGC</td>
<td>TCGGATCTTCAGGGTCAAGG</td>
</tr>
<tr>
<td>PAI-1</td>
<td>GGAGAAGCGAAACAGGAGTG</td>
<td>TCCAGAAGGGATATGTTGC</td>
</tr>
<tr>
<td>TGF-β</td>
<td>CCTGGAAAGGGCTCAACA</td>
<td>GTTGTTGTAGAGGGCAAGG</td>
</tr>
<tr>
<td>ENTPD-1</td>
<td>AGGAGCTGAAGGCTACCC</td>
<td>GTCGTATTAGGCGACGAA</td>
</tr>
<tr>
<td>ENTPD-2</td>
<td>CTCGGGATGACCCAGAGA</td>
<td>CAGCAGGTAGTTGCGATCTCA</td>
</tr>
<tr>
<td>18S</td>
<td>GTAACCCTGGACCTCAT</td>
<td>CCATCCAATCGGTAGTAGCG</td>
</tr>
</tbody>
</table>

Figure 5.1: Basal ATP signaling stimulates α-SMA expression and CF contraction. (A) CFs seeded in 2.5 mg/mL rat tail collagen spontaneously contracted the collagen gels by 24 h. Subsequent 24 h treatment with apyrase (1 U/mL) and P2 inhibition with suramin (50 µM) reversed spontaneous collagen gel contraction and increased collagen gel surface area compared to 48 h untreated controls by 65% and 67%, respectively. (B) Apyrase (1 U/mL for 24 h) decreased α-SMA protein expression by 42%. **p<0.01, ***p<0.001 vs. untreated controls; quantitative data are presented as mean +/-SEM of 3 independent experiments.
Figure 5.2: Simultaneous siRNA knockdown of ENTPD1 (E1) and ENTPD2 (E2) increases extracellular ATP concentration and enhances the pro-fibrotic effect of ATP. (A) ENTPD-1 and -2 were detected in similar abundance in rat ventricular CFs. Co-transfection with siRNA for ENTPD-1 and -2 (E1/E2) decreased expression by 57% and 54%, respectively. (B) ENTPD-1/-2 knockdown increased basal extracellular ATP concentration by 2.5-fold. (C) Knockdown of ENTPD-1/-2 significantly enhanced the pro-fibrotic effect of 10 μM ATP on CFs: CFs transfected with siRNA targeting both ENTPD-1/-2 up-regulated α-SMA, PAI-1 and TGF-β expression by 2.7-, 4.0- and 1.7-fold, respectively, in response to 4 h incubation with ATP as compared to control siRNA-transfected CFs incubated with ATP. *p<0.05, **p<0.01, ***p<0.001 vs. identically transfected, untreated samples; +p<0.05, ++p<0.01, +++p<0.001 between groups indicated. Gene expression data are presented as mean +/- SEM of 3 independent experiments; ATP assay data are presented as mean +/- SEM of 6 independent experiments.
Figure 5.3: Inhibition of endogenous NTPase activity by POM-1 is pro-fibrotic. (A) ATP hydrolysis was measured using a malachite green assay. Addition of 30 µM POM-1 inhibited ENTPD-mediated hydrolysis of exogenously added ATP (30 µM) after 1 h. POM-1 treatment for 24 h increased CF α-SMA and PAI-1 protein expression (B) and collagen synthesis (C) in a concentration-dependent manner. (D) POM-1 pretreatment (1 h) increased basal ERK phosphorylation and prolonged 10 µM ATP-stimulated ERK phosphorylation. (E) The stimulatory effects of 30 µM POM-1 on collagen synthesis and expression of α-SMA and PAI-1 protein were blocked by 50 µM suramin (sur). *p<0.05, **p<0.01, ***p<0.001 vs. untreated controls; +++p<0.001 between groups indicated. Quantitative data are presented as mean +/- SEM of at least 3 independent experiments.
Figure 5.4: POM-1 enhances the effects of ATP on pro-fibrotic marker expression of CFs. (A) Incubation of CFs with POM-1 together with ATP for 4 h increased expression of α-SMA, PAI-1 and TGF-β mRNA by 1.9-, 3.0- and 1.7-fold, respectively, compared to CFs incubated only with ATP. (B) Incubation of CFs with POM-1 for 24 h increased α-SMA and PAI-1 protein expression by 30% and 89%, respectively, as compared to CFs incubated only with ATP. *p<0.05, **p<0.01, ***p<0.001 vs. untreated controls; +p<0.05, ++p<0.01 between groups indicated. Data are presented as mean +/- SEM of at least 3 independent experiments.
Figure 5.5: POM-1 enhances the stimulatory effect of ATP on collagen synthesis and gel contraction. (A) Incubation of CFs with 30 μM POM-1 increased the effect of 10 μM ATP on collagen synthesis 73% after 24 h as compared to CFs incubated only with ATP. (B) POM-1 increased the 12% reduction in collagen gel surface area in response to a 24 h incubation with 10 μM ATP to 32%. *p<0.05, **p<0.01, ***p<0.001 vs. untreated controls; +++p<0.001 between groups indicated. Quantitative data are presented as mean +/- SEM of 3 independent experiments.
Figure 5.6: Adenosine signaling counteracts the pro-fibrotic effects of ATP. Adenosine (ado) decreased POM-1-stimulated α-SMA mRNA (A) and protein (B) expression in a concentration-dependent manner. (C) Incubation with ATP (10 µM) and adenosine (ado, 30 µM) for 4 h increased CREB phosphorylation, an effect that was blunted with the PKA inhibitors, H89 (10 µM) and Rp-cAMPS (50 µM). (D) Inhibition of A2B adenosine receptors with PSB 603 (300 nM) blocked ATP-stimulated CREB phosphorylation; A2A receptor inhibition (300 nM SCH 442416) had no significant effect. *p<0.05, **p<0.01 vs. untreated controls; +p<0.05, ++p<0.01, +++p<0.001 between groups indicated, or as compared to control CFs not treated with inhibitors (B). Data are presented as mean +/- SEM of at least 3 independent experiments.
Figure 5.7: Extracellular adenosine deamination and A2B receptor inhibition enhances pro-fibrotic ATP signaling. Incubation with adenosine deaminase (ADA) increased collagen synthesis by 38% (after 24 h) (A) and the expression of α-SMA and PAI-1 protein 2- and 3-fold, respectively (after 4 h) (B). Incubation of CFs with ADA enhanced the stimulation produced by 10 µM ATP in the expression of α-SMA (42%) and PAI-1 (182%). (C) Incubation with 300 nM PSB 603 (PSB) enhanced the stimulation by ATP (10 µM, 4 h treatment) of α-SMA and PAI-1 gene expression by 62% and 69%, respectively. (D) Incubation for 24 h with PSB produced a 33% increase in ATP-promoted enhancement in α-SMA protein levels. PSB did not enhance the UTP-promoted increases in α-SMA or PAI-1 expression. (E) The effects of 30 µM POM-1 were not enhanced by pretreatment with PSB. *p<0.05, **p<0.01, ***p<0.001 vs. untreated controls; +p<0.05, ++p<0.01, +++p<0.001 between groups indicated. Data are presented as mean +/- SEM of 3 independent experiments.
Figure 5.8: Model of the counterbalancing pro-fibrotic ATP-P2Y \((\text{P2Y}_2)\) and anti-fibrotic adenosine-P1 signaling pathways initiated by cellular ATP release and regulated by ENTPD and nucleotidase activity.
5.8 References


contributes to cardiac fibrosis after myocardial infarction. *Am J Pathol* **164**, 449-456


CHAPTER 6:

SUMMARY OF FINDINGS AND CONCLUDING DISCUSSION

While the physiological roles of cellular nucleotide release and signaling have garnered much attention, still much remains unknown regarding the complex pathways of release and mechanisms of signaling. The results presented here demonstrate the ability of ATP to be released from ventricular CFs in vitro via Cx hemi-channels and that released ATP activates P2Y$_2$ receptors in an autocrine/paracrine manner to drive CF transformation. Once released ATP, can be hydrolyzed by endogenous nucleotidases (ENTPDs) present on the cell surface that attenuate pro-fibrotic nucleotide signaling and generate adenosine, a potential anti-fibrotic mediator in CFs (Fig. 6.1). Though the data shown here are derived from studies in CFs and focus largely on the implications in cardiac fibrosis, the findings are likely applicable to fibroblasts in other tissues, as well as other cell types. The following sections detail the significance of these findings and propose future directions to further explore underlying mechanisms.

6.1 ATP is released via Cx43 and Cx45 hemi-channels in CFs

The data presented in Chapter 4 demonstrate that ATP is released from primary rat ventricular CFs via Cx43 and Cx45 hemi-channels. In those experiments, hypotonic
stimulation, designed to provoke cell swelling, was used as the physical stimulus to invoke ATP release. I attempted to use an alternative model involving ~10% static biaxial stretch of CFs plated onto a deformable silicone membrane (as implemented by Lee et al. (1)) to assess the effects of mechanical stretch on ATP release. Though 10 min static stretch increased extracellular ATP concentration by ~8 fold (data not shown), those results were inconsistent, and might have been secondary to cell lysis resulting from excessive stretch. Nevertheless, 10 min static stretch did increase ERK activation (Fig. 6.2), which was blunted by the addition of apyrase, akin to the findings from CFs challenged with hypotonic media (Fig. 4.6). Thus, static stretch of CFs may also release physiologically relevant concentrations of ATP that can participate in autocrine/paracrine signaling.

Carbenoxolone and probenecid, both pharmacological gap junction blockers, reduced ATP release in CFs, thus implicating the involvement of hemi-channels. These results were confirmed with selective siRNA knockdown of Cx43 and Cx45, which substantially inhibited the release of cellular ATP. Of note, probenecid is reportedly specific for Panx hemi-channels (2), suggesting a role of Panxs in ATP release from CFs. However, we detected very low Panx1 expression in CFs, and siRNA knockdown had no effect on ATP release. Nonetheless, I cannot unequivocally rule out a role for Panx1 in ATP release from CFs. Interestingly, Dolmatova et al. recently demonstrated that hypoxic conditions can up-regulate Panx1 expression in cardiomyocytes, which functions as an ATP release pathway that stimulates the paracrine activation of fibroblasts (3). It may be possible that Panx1 can be up-regulated in CFs under certain conditions or pathologies and play a larger role as a nucleotide release conduit than my current results indicate.
Differential expression of Cx and Panx hemi-channels have been observed in various disease states. Notably, CFs isolated from infarcted rodent hearts exhibited up-regulation of Cx43 expression and intracellular coupling (4), while myocytes appear to lose Cx43 expression following MI (5). In our studies, rat ventricular CFs exposed to hypoxic conditions (95% N₂, 5% O₂) for 24 h did not alter expression of Panx1 or Cx45, but Cx43 was significantly up-regulated (Fig. 6.3). Furthermore, increased Cx43 expression correlated with an increase in ATP release in response to hypotonic challenge (Fig. 6.4). Together, these data suggest that pathologic conditions such as ischemia or hypoxia may alter the expression of Cx and Panx hemi-channels, potentially leading to alterations in nucleotide release and subsequent signaling. Hypoxia or ischemia may stimulate the release of ATP by opening hemi-channels as well as by up-regulation of Cx or Panx expression, thus representing potential nucleotide-dependent mechanisms in the initiation of fibrotic response and remodeling. Additional work examining the effects of pro-fibrotic agents (e.g., TGF-β, Ang II) and pathologies (e.g., MI, pressure overload, age-associated fibrosis) on hemi-channel expression and ATP release are warranted, so as to address the possibility of differential ATP release as a mechanism in regulating CF response to disease conditions.

Another question from results in this thesis is whether ATP is released from homomeric or heteromeric Cx43 and Cx45 channels in CFs. I observed a decrease in ATP release when either Cx was knocked down, but a decrease in expression of either Cx isoform conceivably may alter the formation and function of heteromeric channels. Studies expressing Cx43 and Cx45 in HeLa cells noted heteromeric channel formation (6), but similar studies have not been done in primary fibroblasts. Further investigation
could utilize immunofluorescent staining for Cx43 and Cx45 and assess for colocalization. Additionally, CFs could be lysed in non-denaturing conditions and the lysates used in co-immunoprecipitation experiments to detect physical interactions between the two Cx isoforms as components of a heteromeric connexon.

6.2 P2Y₂ receptor activation by released nucleotides is pro-fibrotic

The findings presented in Chapters 4 and 5 show that released ATP functions as a paracrine signaling molecule by activation of P2Y₂ receptors on CFs. P2Y₂ receptor activation by ATP (and UTP) stimulates the expression of α-SMA and pro-fibrotic markers, increases collagen production and stimulates CF proliferation, migration and gel contraction. P2Y₂ receptor stimulation rapidly results in ERK phosphorylation, and the activation of ERK in response to mechanical perturbations is blunted by Cx hemi-channel inhibition, apyrase or P2Y₂ receptor inhibition, indicating that physiologically relevant amounts of nucleotides are released from CFs. Furthermore, ERK inhibition with U0126 abolished the stimulatory effects of ATP/UTP on collagen synthesis and up-regulation of PAI-1 and MCP-1, indicating that these responses depend on MAPK/ERK signaling. Stimulation of P2Y₂ receptors on CFs also induces RhoA activation, and the inhibition of the RhoA/ROCK pathway with fasudil blocks nucleotide-stimulated α-SMA up-regulation and collagen gel contraction. Thus, P2Y₂ activation seems to mediate its downstream effects via MAPK/ERK-dependent collagen production and pro-fibrotic marker expression and RhoA/ROCK-dependent α-SMA expression and cell contraction.
In rat ventricular CFs, P2Y<sub>2</sub> receptors are the most highly expressed P2Y subtype and among the most highly expressed known GPCRs, as assessed by gene array analysis (7). Importantly ATP and UTP failed to elicit pro-fibrotic effects from rat CFs transfected with siRNA targeting P2Y<sub>2</sub> receptors and from CFs isolated from P2Y<sub>2</sub>−/− mice (Figs. 3.7, 4.7, 4.9), indicating that P2Y<sub>2</sub> is the predominant receptor involved in ATP/UTP signaling. Furthermore, P2X receptor inhibition by pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) did not inhibit pro-fibrotic ATP/UTP signaling, but may enhance the pro-fibrotic effects of ATP/UTP (Fig. 6.5). This preliminary result suggests a potential anti-fibrotic role of P2X receptor activation. Additional studies using P2X receptor inhibition or knockdown will be necessary to define a role for those receptors in the response of CFs to released ATP.

Nishida et al. described fibrotic actions mediated by P2Y<sub>6</sub> receptors in mouse cardiomyocytes (8). Though the P2Y<sub>2</sub> receptor is the predominant P2Y receptor in rat CFs, I found the P2Y<sub>6</sub> subtype is also highly expressed in mouse CFs. Thus, although P2Y<sub>2</sub>-mediated responses to ATP/UTP predominate for nucleotide-mediated pro-fibrotic action in rat CFs, UDP-mediated responses may play a relatively larger role in mouse CFs. The presence of numerous P2Y subtypes on the cell surface suggests the integration of complex signaling pathways dependent on the release of various nucleotides and their subsequent hydrolysis. Unfortunately, due to the difficulty in acquiring viable human cardiac samples for the isolation of primary human CFs, my ability to apply similar studies in human tissues was limited.

Although both ATP and UTP are strongly pro-fibrotic, UTP stimulation of rat CFs often elicits more potent effects than does ATP. The reason for this differential effect
was not rigorously studied here, but several potential explanations exist. Most likely, as discussed in Chapter 5, the generation of adenosine from ATP hydrolysis may activate a counterbalancing signaling pathway via $G_s$-coupled adenosine receptors, which are anti-fibrotic in CFs (9, 10). In contrast, UTP hydrolysis does not generate an analogous anti-fibrotic signaling molecule. Furthermore, kinetic studies of extracellular ENTPDs, the primary nucleotidases that hydrolyze ATP and UTP, described a lower affinity of ENTPD-1 and -2 for UTP as compared to ATP. For mouse ENTPD1, $K_m$ values for ATP and UTP are 12 µM and 49 µM, respectively. For mouse ENTPD2, $K_m$ values for ATP and UTP are 37 µM and 49 µM, respectively (11). The same study noted the substantial accumulation of UDP as a result of ENTPD-mediated UTP hydrolysis. In contrast to adenosine generation from ATP, the accumulation of UDP may enhance the perceived effects of UTP-dependent signaling via the simultaneous activation of pro-fibrotic P2Y$_6$ receptors. A rigorous examination of the relative contribution of these P2Y subtypes is difficult due to the lack of subtype-specific pharmacologic inhibitors. However, the data in Chapter 5 demonstrating that adenosine A2B receptor inhibition enhanced the pro-fibrotic effect of ATP supports this hypothesis.

Another potential cause for the differential potency of ATP and UTP on CF P2Y$_2$ receptors is the possibility that though the receptor binds each nucleotide with equal affinity (12), the downstream effects of receptor activation may differ. Differences in receptor conformational changes dependent on ATP or UTP binding may stimulate differential intracellular signaling cascades. However, our findings did not observe differences in RhoA or MAPK activation by either ATP or UTP. Nonetheless, differential responses or agonist-induced desensitization could conceivably account for the
differential effects I observed. Further studies could employ fluorescent intracellular Ca\(^{2+}\) assays (e.g., using Fura-2AM) to measure possible differences in the extent of P2Y\(_2\)-G\(_q\) activation in response to ATP and UTP.

6.3 Endogenous nucleotidases hydrolyze released nucleotides and contribute to counterbalancing adenosine receptor signaling

In the course of these studies, I observed prominent effects of extracellular nucleotide hydrolysis on CF phenotype. Addition of apyrase substantially decreased basal α-SMA expression and collagen production, and suppressed the myofibroblast phenotype. This discovery led me to hypothesize that constitutive nucleotide release contributes to the regulation of basal CF phenotype. Subsequent work, described in Chapter 5, revealed that rat ventricular CFs express the extracellular nucleotidases ENTPD-1 and -2, and that inhibition of these nucleotidases is pro-fibrotic via the enhancement of nucleotide signaling. Those experiments revealed that ENTPD activity plays an important role in attenuating pro-fibrotic responses resulting from P2Y receptor activation. Furthermore, the generation of adenosine, a product of ATP hydrolysis by ENTPDs, activates G\(_i\)-coupled adenosine A\(_2B\) receptors to promote an anti-fibrotic response. Deamination of extracellular adenosine with ADA and A\(_2B\) receptor inhibition both enhanced the pro-fibrotic effect of ATP. Thus, nucleotide signaling is pro-fibrotic in CFs but is blunted by ENTPDs that generate adenosine. Cellular release of ATP, which activates pro-fibrotic P2Y\(_2\) receptors, thus can also signal via anti-fibrotic receptors for adenosine, a molecule generated by endogenous ENTPD activity. Importantly, these findings demonstrate that
basal autocrine/paracrine nucleotide signaling can regulate cellular tone and basal phenotype, implying that the interplay between release and hydrolysis represents a homeostatic regulatory mechanism.

In addition to further elaborating on a novel mechanism for the regulation of basal CF phenotype and response to injury or stress, these data imply that nucleotide signaling, degradation and adenosine generation may be a general pathway for homeostatic maintenance and injury response in a variety of cell types and tissues. Adenosine generation via ENTPD activity is known to mediate renal and cardiac protection from ischemia (13, 14) and attenuate inflammation and thrombosis (15, 16). Increasing evidence indicates a role for ENTPDs in paracrine nucleotide signaling pathways. ENTPD2 activity in liver portal fibroblasts regulates bile duct epithelial cell proliferation, with implications for liver fibrosis and cholangiopathy (17, 18). Thus the regulatory axis we observed in CFs may be a general mechanism responsive to soluble and mechanical cues in regulating basal tissue homeostasis as well as response to injury.

Several questions remain regarding the role of ENTPDs in pathologic states. Treatment of rat CFs with TGF-β or Ang II decreased ENTPD2 expression, whereas treatment with forskolin (FSK) or 1-(6-Amino-9H-purin-9-yl)-1-deoxy-N-ethyl-β-D-ribofuranuronamide (NECA), an adenosine receptor agonist, increased its expression (Fig. 6.6). ENTPD1 expression was largely unchanged. These data are of interest because they imply that a pathologic state, such as myofibroblast transformation, may alter the ability of cells to hydrolyze extracellular nucleotides, potentially altering cell phenotype. A decrease in ENTPD2 expression with TGF-β or Ang II, and an increase in expression with FSK or NECA, argues for potential positive feedback loops. A canonically pro-
fibrotic agonist such as TGF-β, by decreasing nucleotide hydrolysis, may further drive myofibroblast transformation in a feed-forward manner by increasing P2Y receptor activation. Furthermore, because we find that ATP/UTP signaling up-regulates TGF-β expression in CFs, nucleotide release (for example, resulting from cellular stress or apoptosis) might induce TGF-β production, thereby integrating two distinct pro-fibrotic pathways and enhancing TGF-β-mediated responses. Conversely, cAMP-elevating agonists such as FSK or NECA may have the opposite effect by increasing the rate of nucleotide hydrolysis. Unfortunately, preliminary experiments that I undertook to quantify ATP degradation in extracellular media were largely negative: extracellular ATP concentration decreased with similar kinetics in the conditioned media of CFs treated with TGF-β or FSK as compared to control (Fig. 6.7). However, several caveats exist for this preliminary experiment. The data presented show the kinetics by which total extracellular ATP is removed from the media and not necessarily the kinetics of ENTPD-mediated ATP hydrolysis. ATP can be removed from the media, for example, potentially by uptake into CFs via as-yet unidentified pathways, ecto-nucleoside pyrophosphatases and alkaline phosphatase, which are extracellular nucleotidases that can hydrolyze ATP and have yet to be characterized in CFs (19).

Further studies are necessary to more closely examine the potential effects of differential ENTPD expression on the regulation of cellular function. An HPLC-based system to assess ATP hydrolysis would be more effective and sensitive for measurement of ATP itself and its hydrolytic products. Using CF conditioned media for future HPLC-based experiments may identify potential differences in nucleotide hydrolysis in response to model disease states. Furthermore, HPLC would be able to quantify UTP/UDP release
and assess the relative contributions of those nucleotides to autocrine/paracrine signaling pathways. Finally, with the existence of ENTPD1/CD39 knockout mice, in vivo studies examining the effects of ENTPD1 deficiency may be extremely enlightening. Consistent with the in vitro data presented here, ENTPD1 deficiency in vivo may exacerbate cardiac fibrosis in mice subjected to experimental MI or pressure overload hypertrophy. Similar mechanisms may also contribute to fibrosis and inflammation in other tissue types with ENTPD1 deficiency.

6.4 In vivo studies examining the effect of P2Y2 receptor deficiency in mice

P2Y2−/− mice were used to study the effects of P2Y2 receptor deficiency in fibrotic responses to pressure overload hypertrophy. In a pilot study conducted by Oscar Braun, WT and P2Y2−/− mice were subjected to 4 weeks of transverse aortic constriction (TAC) banding, whereby surgical ligation of the aorta with a suture causes chronic pressure overload of the heart, eventually resulting in ventricular hypertrophy, heart failure and cardiac fibrosis (20). Unfortunately, these initial studies were inconclusive. No differences in survivability or hemodynamic function were observed between WT and P2Y2−/− mice (data not shown). Histological staining with Sirius red to visualize collagen detected no significant differences in the amount of fibrosis between the experimental groups (p>0.05) (Fig. 6.8). Because of the variability and low statistical power of the data, I conducted a subsequent in vivo study utilizing an isoproterenol-induced model of cardiac hypertrophy (see Chapter 6 Appendix). However, this model failed to produce substantial cardiac fibrosis.
These in vivo results suggest that pro-fibrotic actions in response to extracellular nucleotides may be largely mediated by several parallel pathways, and deficiency of a single subtype (P2Y_2) may not be sufficient for protection against injury-induced fibrosis. Perhaps another P2Y receptor, such as P2Y_6 receptors, compensate for P2Y_2 deficiency. It is also conceivable that additional factors mediated by mechanical cues (e.g., activation of integrins and focal adhesions) or chemical signaling (e.g., TGF-β, Ang II) may obscure and “override” the lack of pro-fibrotic P2Y_2 receptor signaling. Finally, the nature of whole-body receptor knockouts could introduce additional confounding variables.

Previous studies have documented that P2Y_2^{−/−} mice are hypertensive (21), an observation supported by these studies (Fig. S6.1). Chronic hypertension may cause reactive perivascular fibrosis via mechanisms separate from those underlying interstitial fibrosis, the focus of this study. Thus, these data do not unequivocally disprove the hypothesis that nucleotide receptors to respond to other models of injury such as ischemia or hypoxia, and further studies are required to fully understand the in vivo physiological role of P2Y receptors in these cardiac pathologies.

6.5 Conclusions and Implications

In sum, the findings presented in this dissertation describe a novel autocrine/paracrine signaling pathway initiated by the release of cellular ATP via Cx hemi-channels on CFs in response to physical stimulation. Released ATP, and possibly UTP, activate P2Y_2 receptors on the same and neighboring CFs to increase ECM synthesis and promote myofibroblast transformation. This signaling axis contributes to
the regulation of basal CF phenotype and response to conditions that increase extracellular ATP. The results also demonstrate that the extracellular nucleotidases, ENTPD-1 and -2, play an essential role in attenuating pro-fibrotic nucleotide signaling. Hydrolysis of extracellular ATP blunts P2Y receptor activation while initiating a counterbalancing pathway via the generation of adenosine, which activate anti-fibrotic Gs-coupled adenosine receptors.

These findings suggest that pathologic conditions such as myocyte death, myocardial stress, hypoxia or vascular shear forces recruit CFs to sites of injury via nucleotide release and purinergic (P2Y2) receptor activation. Indeed, analogous mechanisms have been shown to mediate lung inflammation (22, 23). These studies also support the notion that my findings identify general mechanisms for cellular homeostasis and response to stress or injury. The data add substantially to the current knowledge regarding localized, nucleotide-mediated paracrine mechanisms of inflammation, fibrosis and remodeling.

In future studies, assessment of possible age- or disease-related changes in the expression profiles of hemi-channel proteins, nucleotide/nucleoside receptors and ENTPD enzymes will be of great interest. Changes in expression of those components would suggest that the mechanisms described here may not only represent an acute response, but may be linked to chronic pathologies. My data demonstrating that basal P2Y signaling regulates CF tone suggests that chronic increases in ATP release and/or P2Y signaling or decreases in nucleotidase activity may have causative (as opposed to reactive) roles in tissue fibrosis, inflammation or proliferative diseases.
In conclusion, this work identifies a novel autocrine/paracrine nucleotide signaling component regulating CF phenotype and functional responses. These findings not only expand knowledge of the pathophysiological mechanisms underlying cardiac fibrosis, but they may have wide-ranging, analogous roles in the remodeling of other tissues. Preliminary data also imply that disease-associated alterations in expression or activity in the pathways of nucleotide release, signaling and hydrolysis may be causative in adverse remodeling responses. Future investigators will hopefully address these open questions and their compelling implications.
6.6 Figures

**Figure 6.1:** Schema of ATP release, pro-fibrotic P2Y₂ receptor activation and anti-fibrotic P1 receptor activation via ENTPD-mediated generation of adenosine.

**Figure 6.2:** Biaxial static stretch of CFs increases phosphorylated ERK levels in a nucleotide-dependent mechanism. p-ERK levels were significantly increased in CFs subjected to 10 min static stretch (str). However, treatment with apyrase (0.6 U/mL) blunted the stimulatory effect of stretch on ERK activation. *p<0.05; data presented as means ± SEM of 2 independent experiments.
Figure 6.3: CFs exposed to 24 h hypoxia up-regulate Cx43 expression. CFs were incubated in 5% O₂ for 4 h and 24 h (Hx). qRT-PCR analysis indicated that Cx43 expression was increased after 24 h Hx compared to normoxic controls (Nx). **p<0.01; data presented as means ± SEM of 2 independent experiments.

Figure 6.4: ATP release in response to hypotonic challenge is increased in CFs exposed to hypoxic conditions for 24 h. CFs cultured in 5% O₂ (Hx) for 24 h release ~60% more ATP with hypotonic challenge as compared to CFs cultured in normoxic conditions (Nx). **p<0.01, **p<0.001; data presented as means ± SEM of 3 independent experiments.
Figure 6.5: P2X receptor inhibition with PPADS does not block pro-fibrotic ATP/UTP signaling. PPADS (10 µM) did not block the stimulatory effect of (4 h, 10 µM) ATP or UTP treatment on pro-fibrotic gene expression. Data presented as means ± SEM of 2 independent experiments.

Figure 6.6: Myofibroblast transformation reduces ENTPD2 expression. CFs were treated for 24 h with either TGF-β (10 ng/mL), Ang II (100 nM), FSK (10 µM) or NECA (10 µM). TGF-β and Ang II decreased expression of ENTPD2 while FSK and NECA increased its expression. ENTPD1 expression levels were not substantially altered with any treatments. Data presented as means ± SEM of 2 independent experiments.
Figure 6.7: Rate of decrease of extracellular ATP in the conditioned media of CFs treated with TGF-β or FSK. CFs were treated with 10 ng/mL TGF-β or 10 µM FSK for 24 h. Afterwards, 4 nM ATP was added into the culture media. Extracellular ATP concentrations in bulk conditioned media was measured at times indicated. Graphs indicate the kinetics by which extracellular ATP concentration decreased in CF-conditioned media. Data presented as means ± SEM of 2 independent experiments, each time point was measured in triplicate.
Figure 6.8: Analysis of interstitial collagen content after TAC banding. Cross sections of WT and P2Y$_2^{-/-}$ hearts were taken after 4 wk TAC banding and stained with Sirius red. Though TAC banding resulted in substantial interstitial fibrosis, no significant differences were seen between WT and KO groups. (WT-sham groups were not included in this pilot study). [n=9 KO TAC, n=6 WT TAC, n=4 KO sham]
6.7 Appendix

6.7.1 Isoproterenol-induced cardiac hypertrophy and fibrosis

Chronic infusion with the β-adrenergic agonist isoproterenol (iso) is a commonly used technique to induce cardiac hypertrophy and fibrosis in rodents (24-26). Animals receive either via daily intraperitoneal injections or subcutaneous implantation of osmotic drug delivery pumps. Though the treatment strategy varies, typically iso is administered at a dose of 5-20 mg/kg/day for a period of 2-4 weeks. For this study, osmotic minipumps obtained from Alzet (Cupertino, CA) were subcutaneously implanted in WT or P2Y₂⁻/⁻ mice on a C57BL/6 background. Isoflurane anesthetic was delivered by inhalation via vaporizer, and buprenorphine was used as an analgesic post-surgery. Minipumps delivered iso at a dose of 15 mg/kg/day for 14 days. Control animals received minipumps delivering vehicle dissolved in saline.

Chronic iso infusion causes acute chronotropic and inotropic effects on the heart, which induces myocyte hypertrophy, fibrosis and heart failure over time (25). In this preliminary study, heart rate (HR) and blood pressure (BP) were measured to verify the action of iso infusion (Fig. S6.1). Iso significantly increased heart rate in P2Y₂⁻/⁻ mice compared to vehicle controls. However, no significant difference was seen between WT mice receiving either vehicle or iso. The elevation of HR across all groups was likely a result of stress on the animals and may indicate flawed data collection techniques. BP, however, was consistently elevated in P2Y₂⁻/⁻ mice regardless of treatment as compared to WT controls. Iso infusion significantly increased BP in both WT and P2Y₂⁻/⁻ mice as
compared to vehicle controls. Though preliminary and potentially flawed (i.e., animals were stressed), these measurements suggest that iso infusion did indeed have acute effects in the elevation of HR and BP. However, future studies should include hemodynamic measurements in addition to HR and BP. HR and BP were measured in all animals on three separate days spaced evenly throughout the treatment regime.

After 2 weeks, the mice were sacrificed, and the hearts were assessed for hypertrophy and fibrosis. Though the sample size was limited, iso infusion did not induce cardiac hypertrophy as assessed by heart weight normalized to tibia length (Fig. S6.2). Furthermore, histological staining of the myocardium with Sirius red did not detect significant development of fibrosis with iso infusion (data not shown). No differences in heart size or interstitial collagen content were seen between WT and P2Y2−/− groups.

This study is largely inconclusive due to the failure of iso infusion to elicit cardiac hypertrophy and fibrosis. It is probable that considerable optimization of iso concentration and treatment time would be necessary to determine the ideal treatment regime to induce hypertrophy and fibrosis within a dynamic range applicable to this study. The ability to collect hemodynamic and echocardiographic data throughout the course of treatment would greatly assist in the optimization of these parameters.
Figure S6.1: Heart rate and blood pressure in mice implanted with osmotic pumps delivering vehicle or isoproterenol. P2Y$_2^{++}$ mice receiving iso had significantly higher HR as compared to P2Y$_2^{-/-}$ mice receiving vehicle. However, no difference was observed between WT groups. Both WT and KO groups receiving iso had significantly higher systolic and diastolic BP compared to respective veh controls. P2Y$_2^{++}$ mice had consistently higher BP as compared to WT mice, regardless of drug infusion. ***p<0.001 compared to vehicle control, +++p<0.001 compared to similarly treated WT animals; n=3 per group. Data presented as means ± SEM and were collected from each animal, in quadruplicate, on 3 separate days throughout the treatment regime.

Figure S6.2: Isoproterenol infusion did not result in cardiac hypertrophy. No significant differences in total heart weight normalized to tibia length were seen after 2 weeks iso infusion. n=3 per group.
6.8 References


