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ROLE OF PROTEIN KINASES IN STRETCH-INDUCED REGULATION OF ELASTIN SYNTHESIS THROUGH INCREASED TRANSLATIONAL EFFICIENCY

By

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A thesis submitted in conformity with the requirements
for the degree of Masters of Science,
Graduate Department of Clinical Biochemistry
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DEDICATION

To my family

ABSTRACT

Under conditions of increased vascular wall stress such as is present in hypertension, the aorta responds by rapid production of additional elastin and collagen. This response can be mimicked *in vitro* using a model in which chick aortas are distended circumferentially with tubing. Stretch-induced increases in elastin production are not correlated with increases in elastin mRNA. In addition, stretch-induced increases in elastin production persist after pre-treatment with doses of the transcriptional inhibitor, DRB, that are capable of blocking stretch-induced increases in the transcriptional marker c-fos. These observations suggest that the response is not related to changes in transcriptional activity nor mRNA stability, but instead may be due to increased translational efficiency of existing elastin mRNA.

Although the signal transduction pathway for this response remains incompletely described, we have shown that vessel distention activates S6 kinase. Rapamycin inhibits the effects of stretch on both S6 kinase activity and elastin production. Similarly, wortmannin, an inhibitor of PI3 kinase, also blocks the stretch-induced response. In addition, the tyrosine kinase inhibitor tyrphostin 25 demonstrated an ability to block stretch-induced elastin synthesis. Tyrphostin 25 also blocks the stretch-induced increase in S6 kinase activity.

These results argue that stretch-induced production of aortic elastin is under translational control, and that the signaling pathway which transduces physical forces into increased elastin synthesis requires the early phosphorylation of an unknown protein by a tyrosine kinase, as well as the participation of PI3 kinase and S6 kinase.

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LIST OF ABBREVIATIONS

Ang II	angiotensin II
APS	ammonium persulfate
ATP	adenosine 5'-triphosphate
Ca	calcium
cAMP	cyclic adenosine monophosphate
cDNA	complimentary deoxynucleic acid
CNBr	cyanogen bromide
cpm	counts per minute
CsCl	cesium chloride
DEPC	diethyl pyrocarbonate
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxcytidine 5'-triphosphate
dGTP	deoxyguanisine 5'-triphosphate
dNTP	deoxynucleotide 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
DRB	5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
eIF	eukaryotic initiation factor
eEF	eukaryotic elongation factor
FRAP	FKBP-rapamycin associated protein
GSK-3	glycogen synthase kinase 3
GTC	guanidine thiocyanate
GTP	guanosine 5'-triphosphate
GF	growth factor
GH	growth hormone
HCl	hydrochloric acid
IGF-1	insulin-like growth factor 1
ILK-1	integrin linked kinase 1
IRE	iron response element
IRS	insulin receptor substrate
kDa	kilodaltons
KCl	potassium chloride
MAP	mitogen-activated protein
MEM	minimal essential medium
Mg	magnesium
MHC	myosin heavy chain
mRNPs	messenger ribonucleoprotein particles
mRNA	messenger ribonucleic acid
MOPS	morpholinopropanesulfonic acid
NaCl	sodium chloride
NaF	sodium fluoride
NaOH	sodium hydroxide
ODC	ornithine decarboxylase
PAGE	polyacrylamide gel electrophoresis
PDK1	3-phosphoinositide-dependent protein kinase-1
PH	pleckstrin homology
PMSF	phenylmethylsulfonyl fluoride
PI3 kinase	phosphatidylinositol-3-kinase

$PtdIns(3,4,5)P_3$	phosphatidylinositoltriphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
rRNA	ribosomal ribonucleic acid
RIPA	radioimmunoprecipitation
RPM	revolutions per minute
S6 kinase	$p70^{S6}$kinase
SDS	sodium dodecylsulfate
ssDNA	salmon sperm deoxynucleic acid
Ser	serine
SH	Src homology domain
tRNA	transfer ribonucleic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Thr	threonine
TOP	terminal oligopyrimidine tract
Tris	tris[hydroxymethyl]aminomethane
UTR	untranslated region
μg	microgram
μL	microlitre
γ	gamma

LIST OF COMPANIES

Ambion: The RNA Company	Austin, TX
Amersham Canada Ltd.	Oakville, ON
Becton Dickson Canada Ltd.	Mississauga, ON
Bio-Rad Laboratories	Hercules, CA
Calbiochem-Novabiochem Corportation	San Diego, CA
Gibco BRL Life Technologies	Gaithersburg, MD
ICN Biomedicals Inc.	Aurora, OH
Interscience	Markham, ON
Eastman Kodak	Rochester, NY
Molecular Dynamics	CA
Pharmacia Biotech Inc.	Baie d'Urfé, QUE
Promega Corporation	Madison, WI
Sigma-Aldrich Canada Ltd.	Oakville, ON
Stratagene	La Jolla, CA
Upstate Biotechnology	Lake Placid, NY

CHAPTER 1 - INTRODUCTION

I. Vascular Elastin Synthesis in Hypertension

1. General Characteristics of Vascular Elastin

Elastin and collagen are the extracellular matrix proteins which comprise the majority of the dry weight of large vessels such as the aorta, and provide the structural integrity required by these vascular tissues. Specifically, elastin makes up almost 50% by weight of the wall of these large vessels (Gerrity and Cliff, 1975), and imparts properties of extensibility and elastic recoil necessary for their normal function. Most of the elastin in the aorta is laid down in a period of rapid synthesis and accumulation during perinatal and early postnatal development (Gerrity and Cliff, 1975; Keeley 1979), after which time elastin synthesis and accumulation halts. Cessation of synthesis after completion of postnatal development is important since, once laid down in the extracellular matrix, elastin is extremely stable and does not turn over at any significant rate (Lefevre and Rucker, 1980).

2. Reappearance of Elastin Synthesis During Pathological Conditions

Although under normal conditions vascular elastin synthesis cannot be detected in the adult animal, there are several pathological situations where aortic elastin synthesis and accumulation reappears. One of the best examples of this is in hypertension, where elevated blood pressure results in structural changes to the arterial vessels. These structural changes include increased synthesis and deposition of both elastin and collagen in the arterial wall, which in turn increases the wall thickness (Wolinski, 1970; Cleary and Moont, 1976; Keeley and Johnson, 1986). This hypertrophy of the vessel wall results in a proportional accumulation of matrix components of the vessel such that, while the absolute contents of elastin and collagen in the vessels may increase substantially, their proportion by weight in the vessel may be essentially unchanged (Wolinski, 1970; Cleary and Moont, 1976; Keeley and Alatawi, 1991).

This response of arterial tissues to increased wall stress is present at all levels of the arterial tree (Keeley et al., 1991). Increased arterial wall thickness resulting from hypertension likely

serves to protect the vessel against the increased load since, according to LaPlace's Law, an increase in wall stress due to increased pressure can be restored to normal levels by increasing the thickness of the vessel wall (Peterson et al., 1960).

Although the initial effect of this thickening of the blood vessel wall is protective, it has been suggested that, in the longer term, these structural changes may contribute to the maintenance of hypertension (Folklow, 1978), since, because of their slow turnover, elastin and collagen accumulated during a period of hypertension may persist in the vessel wall even after blood pressure has returned to normal levels. Increased vascular stiffness in larger vessels, and decreased lumen size in smaller vessels as a result of these structural changes, have been suggested to create a positive feedback mechanism which will further elevate blood pressure (Folklow, 1978; Cox, 1982).

3. Effects of Physical Stimuli on Responses of Tissues and Cells

Mechanical load is believed to be an important factor in determining muscle mass and phenotype in skeletal and cardiac muscles *in vivo* (Vandenburgh and Kaufman, 1979; Sadoshima et al., 1993b).

Vandenburgh and Kaufman (1979) provided the first direct evidence that muscle cells were able to sense external load in the absence of neuronal or hormonal factors, demonstrating that cultured chick skeletal muscle cells grown on an elastic substrate undergo many of the biochemical changes seen in hypertrophy in response to static stretch. These include increased amino acid accumulation, increased incorporation of amino acids into cellular protein and increased accumulation of total proteins. This group later discovered that hypertrophy, in response to repetitive stretch/relaxation, was associated with increased production of prostaglandins (PG) E_2, and $F_{2\alpha}$, which are known to be modulators of protein turnover and cell growth (Vandenburgh et al., 1990; Vandenburgh et al., 1995a). Furthermore, they demonstrated that stretch-induced increases in these PGs were sensitive to extracellular Ca and lipase inhibitors, which inhibit liberation of arachidonic acid. Plus repeated mechanical stimulation increased the breakdown of

phospholipids, precursors of PG synthesis (Vandenburgh et al., 1993). This suggested that stretch might increase PG synthesis by increasing the amount of free arachidonic acid, which is required for PG synthesis. Additionally, stretch also appears to increase levels of diacylglycerol, second messenger which activates PKC (Vandenburgh et al., 1993). Starting in 1995 the group of Vandenburgh began investigating mechanical stimulation of cardiomyocytes. This group designed and improved a computerized mechanical cell stimulator device to study the growth response of neonatal cardiomyocytes in tissue culture subjected to unidirectional loads under conditions better reflective of an *in vivo* environment (Vandenburgh et al., 1995b; Vandenburgh et al., 1996).

Stretching of neonatal cardiac myocytes in culture results in increased protein synthesis and transcriptional activation of c-fos and other immediate-early genes, followed by cellular hypertrophy (Sadoshima et al., 1992; Sadoshima and Izumo 1993, a&b; Yazaki et al., 1993). Using c-fos gene expression as a nuclear marker for load-induced signal transduction, they showed that stretching of cardiac myocytes induced the tyrosine phosphorylation of intracellular proteins through a mechanism involving genistein-sensitive tyrosine kinases (Sadoshima and Izumo, 1993a). Stretch has also been shown to cause the rapid activation of several other second messengers PLC and PLD, $p21^{ras}$, PKC, MAP kinases ERK1&2 and MEK1, S6 kinase $pp90^{RSK}$, and various lipid products (diacylglycerol and arachidonic acids). (Sadoshima and Izumo, 1993a; Yamazaki et al., 1993). More recently Sadoshima and Izumo (1996) revealed that the effects of mechanical stretch of cardiac myocytes may be mediated through angiotensin II (AngII). This was demonstrated by several lines of evidence: (1) expression of immediate-early genes and increases in protein synthesis are completely blocked by AngII-specific receptor antagonists, (2) stretching *in vitro* causes the secretion of AngII and increased expression of the angiotensinogen gene, (3) AngII is sufficient to cause hypertrophy of cardiac myocytes in a manner that is indistinguishable from stretch-induced hypertrophy, and (4) AngII and stretch cause the activation of similar second messengers in cardiac myocytes (Sadoshima et al., 1992; Sadoshima and Izumo, 1993c; Sadoshima and Izumo, 1995; Sadoshima and Izumo, 1996).

In vitro cell and organ culture models have been used to investigate the effect of stretch on vascular smooth muscle cells, and in particular on the production of elastin in response to stretch (Sutcliffe and Davidson, 1990; Keeley and Bartoszewicz, 1995; Keeley and Alatawi, 1991). Our laboratory has developed an *in vitro* organ culture model in which explanted aortic tissue from neonatal chickens is distended with polyethylene tubing (Figure 1a). The tissue responds by increasing synthesis of both elastin and collagen. This response is rapid, with increased accumulation of insoluble elastin detected after two hours of stretch (Figure 1b), and elevated levels of tropoelastin, the soluble monomeric precursor of insoluble elastin, appearing within 30 minutes of stretch (Keeley and Bartoszewicz, 1995). The fact that a stretch-induced response can be seen in this *in vitro* organ culture model implies that this response must be intrinsic to the vessel itself and does not require circulating factors or neurohumoral influences.

Earlier investigations from our laboratory suggested a role for phospholipase C and PKC in stretch-induced elastin synthesis. In addition, both colchicine and cytochalasin D blocked the response, suggesting the importance of an intact cytoskeleton (Keeley and Bartoszewicz, 1995; Bartoszewicz, 1995). Furthermore, preliminary data indicated that inhibitors of tyrosine kinase activity (tyrphostin 25), abolished stretch-induced elastin synthesis (Keeley and Bartoszewicz 1995; Bartoszewicz, 1995). Other data from our laboratory indicated that the stretch-induced increase in synthesis of elastin in this organ culture model was not correlated with increased steady-state levels of mRNA for elastin. Lack of increase in aortic elastin mRNA in response to *in vivo* models of systemic hypertension was also evident in a study by Takasaki et al. (1990), who reported increased mRNA levels and protein synthesis for fibronectin under these conditions.

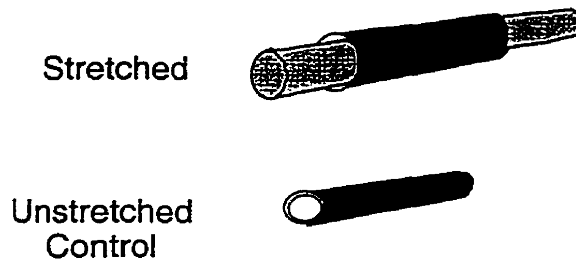

Figure 1A. The *in vitro* organ culture model. A symmetrical circumferential stress is applied by distending the aortas on polyethylene tubing of a known diameter. No tubing was inserted in the control vessels.

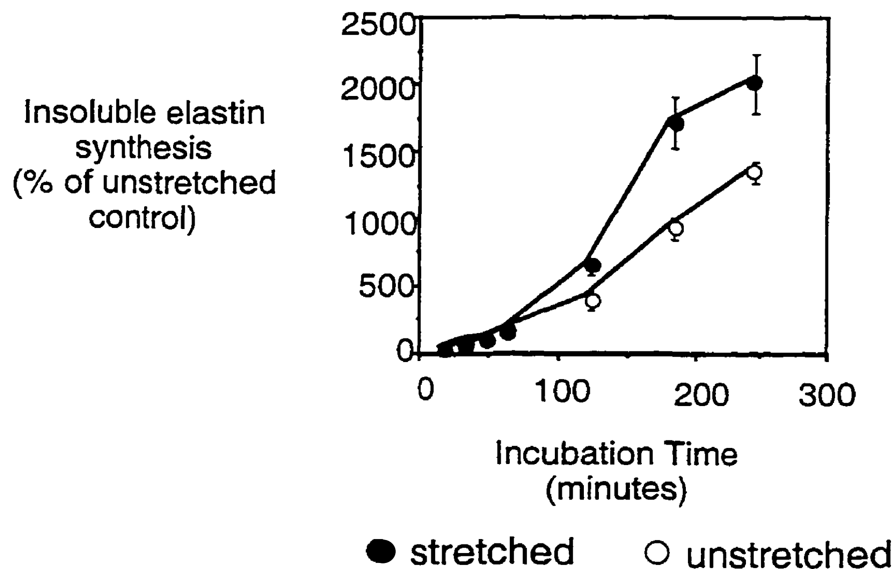

Figure 1B. The effect of distension of the aorta on insoluble elastin accumulation measured by the incorporation of [^{14}C]-Proline during a 4 hour incubation period. The control had no tubing inserted into the vessel. An increase in synthesis of insoluble elastin in response to stretch was first detected after 2 hours of incubation. However, an increase in synthesis of tropoelastin, the soluble monomer of elastin, can be detected as early as 30 minutes following stretch (data not shown).

II. Mechanisms for Regulation of Protein Synthesis

1. Translational Control of Protein Synthesis

For most proteins, synthesis rates are directly proportional to steady-state levels of mRNA for that protein, and regulation of synthesis is therefore at the transcriptional level, or by regulating the stability of its mRNA. However, in recent years several examples have emerged in which synthesis of proteins can be changed in the absence of changes in mRNA levels. Examples of increased protein synthesis in the absence of increases in mRNA include, stress from heat shock, cell state changes such as mitosis, and cellular responses to hormones and growth factors (Mathews, Sonenberg and Hershey, 1996). In these cases it appears that increased protein synthesis is due to increased efficiency of translation. A significant advantage of translation as a regulatory mechanism is that it allows for a rapid response to external stimuli without triggering nuclear pathways for mRNA synthesis and transport (Mathews, Sonenberg and Hershey, 1996).

Translational control is defined as a change in the number of completed protein products per mRNA per unit time (Mathews, Sonenberg and Hershey, 1996). At the translational level, protein synthesis rates can be accelerated by changes in capacity or efficiency. Translational efficiency refers to the efficiency with which a cell utilizes translational machinery such as mRNA, ribosomes, initiation factors and elongation factors (Figure 2) (Ivester et al., 1995). Translational capacity refers to the relative abundance of these translational components in the cell, especially ribosomes and translation factor pools (Ivester et al., 1995; Makhlouf and McDermott, 1998).

An important site for the control of translation is the association between ribosomes and mRNA. Several *cis*-acting regions on the mRNA and *trans*-acting factors (both protein and RNA) mediate this process. *Cis*-acting elements can be divided into those that act alone and those whose function depends on specific *trans*-acting factors.

Some examples of the first category include:

(1) the presence and availability of the 5' cap structure, m^7GPPPN (where N is any nucleotide), which facilitates ribosome binding;

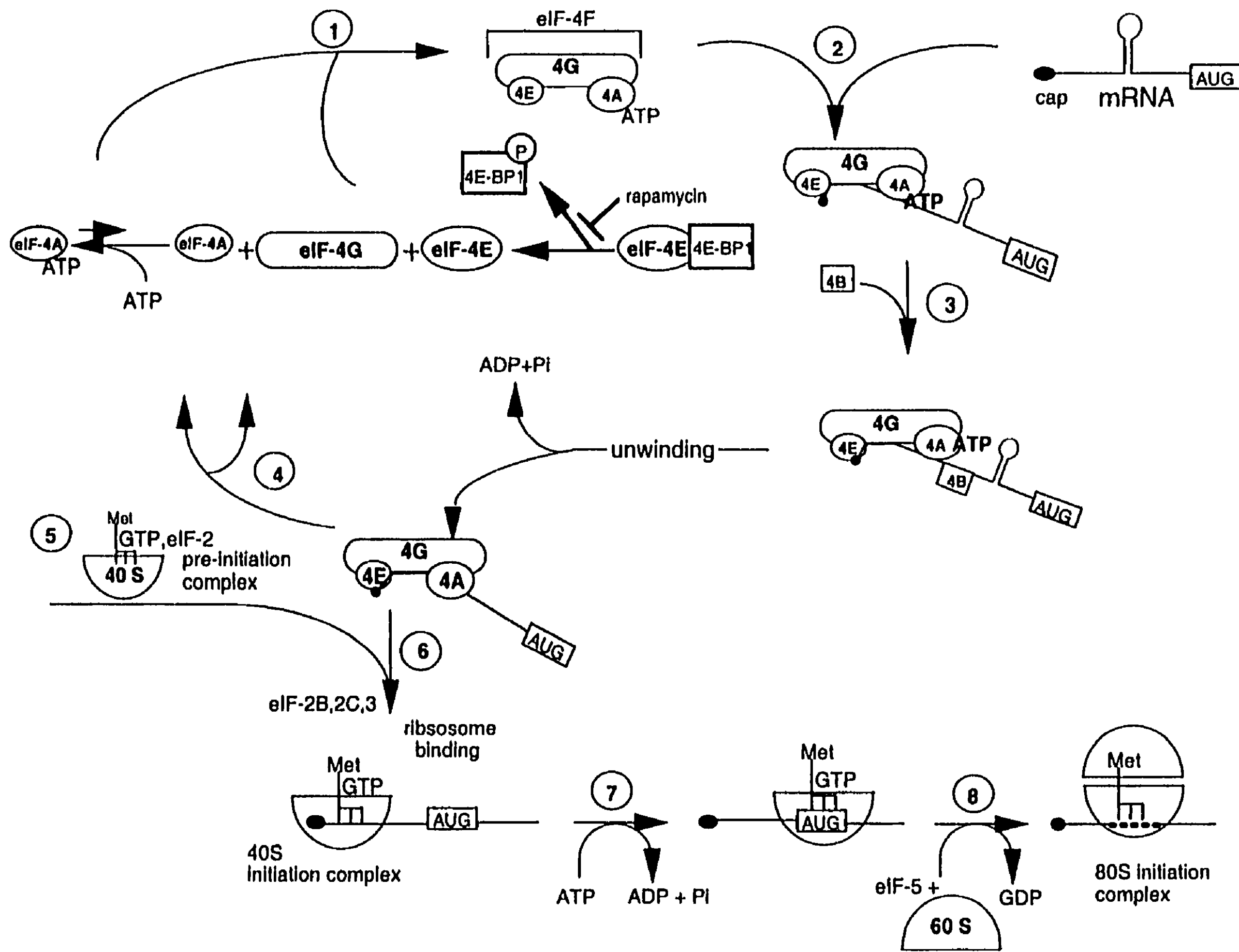

Figure 2. Overview of translation initiation (Sonenberg 1996 and Hershey 1991). Translation initiation can be subdivided into 8 stages. Once eIF-4A, -4G and -4E have associated to form eIF-4F, the complex binds to the 5' cap structure of mRNA. Then, eIF-4B binds to the mRNA, assisting -4A in unwinding of the 5' secondary structure. eIF-4A then recycles through eIF-4F, along with the formation of the pre-initiation complex. The pre-initiation complex binds to mRNA, giving rise to the 40S initiation complex, which allows the ribosome to scan the mRNA until it locates the initiation codon (AUG). At this point, there is an association between the 40S initiation complex and the 60S ribosomal subunit, completing the final stage of initiation with the formation of the 80S initiation complex.

(2) secondary structure, particularly in the 5' untranslated region (UTR), which inhibits ribosome binding;

(3) a 5' terminal oligopyrimidine tract (5' TOP), which has been implicated in translational regulation of at least some transcripts;

(4) consensus sequence preceding the initiator codon (AUG);

(5) presence and length of poly(A) tails at the 3' terminus has an important role as an enhancer of translation, but the requirement is not absolute (Mathews, Sonenberg and Hershey, 1996; Brown and Schreiber, 1996; Hershey, 1991).

Cis-acting elements of the second group include specific sequences affecting translation by interaction with *trans*-acting regulators. For example, the association between the iron response element (IRE) and the iron repressor protein inhibits synthesis of ferritin (Mathews, Sonenberg and Hershey, 1996).

The activity of many *cis*-acting elements in the 5' UTR of mRNA are also regulated by initiation and elongation factors. These factors promote formation of the ribosomal complex, mRNA binding to ribosomes (Figure 2), and elongation (eEF-2) of the nascent protein chain. Several of these factors are phosphorylated, and their phosphorylation state directly correlates with translation and growth rates of the cell (Hershey, 1991).

2. Rate Limiting Factors in Protein Synthesis

In order for the rate of protein synthesis to be changed, a cell must modify the kinetic parameters of the step that is rate limiting. The regulation of translation initiation is considered to be a rate limiting event in protein synthesis with initiation factor eIF-4F (Figure 2) specifically playing a key role in this regulation (Hershey, 1991; Mathews, Sonenberg and Hershey, 1996; Sonenberg, 1996; Whalen et al., 1996).

eIF-4F is composed of 3 subunits:

(1) eIF-4E, which binds directly to the N^7- methlyguanosine cap structure of mRNA, is thought to play a role in promoting protein synthesis and possibly in the selection of mRNAs to be translated;

(2) eIF-4A, has ATP-dependent RNA helicase activity and is thought to play a role in unwinding secondary structure at the 5' end of mRNA to allow the 40S ribosomal subunit to bind and/or scan the 5'-untranslated region (UTR) of the mRNA;

(3) eIF-4G (formerly known as p220), serves as a bridge between mRNA and the 40S ribosomal subunit since it contains binding sites for eIF-4E, and eIF-3 associated with the ribosome (Hershey, 1991; Sonenberg, 1996; Kimball et al., 1997).

eIF-4E is considered an excellent candidate as a key mediator in the regulation of translation and cell growth due to presence in limiting amounts in the cell, relative to other initiation factors, along with a direct correlation between its phosphorylation state and translation rates *in vivo* (Hershey, 1991; Sonenberg, 1996; Whalen et al., 1996). During translation initiation, eIF-4E interacts with eIF-4G through a conserved hydrophobic region in eIF-4G (Mader et al., 1995). A similar conserved sequence was also found in the protein 4E-BP1 which allows this protein to compete with eIF-4G for eIF-4E binding and therefore influence initiation of translation (Haghighat et al., 1995; Mader et al., 1995). It is also thought that the phosphorylation of other proteins, such as eIF-3, -4B, and ribosomal protein S6 may be important for translational regulation. (Hershey, 1991; Sonenberg, 1996).

III. Signal Transduction Cascade

The phosphorylation of proteins by various tyrosine kinases is a common early event which often serves as a route for the intracellular transmission of signals from extracellular stimuli in many signal transduction pathways. For instance, autophosphorylation of its receptor, as well as tyrosine phosphorylation of specific intracellular substrates, are the earliest detectable biochemical events elicited by insulin (Rosen, 1987).

Downstream of the action of the insulin receptor, both PI3 kinase and S6 kinase are also important to the insulin pathway modulating translational activity (Kimball et al., 1998), based on the inhibition of these steps with the kinase inhibitors wortmannin and rapamycin, respectively. Earlier results from our laboratory showed that wortmannin and rapamycin were also potent inhibitors of stretch-induced elastin synthesis in our *in vitro* organ culture model (see Figure 10). This raised the possibility of other similarities between the stretch-activated signaling pathway and that of the insulin cascade. Some of the other components of the insulin pathway include, Protein kinase B(PKB)/Akt kinase, FRAP kinase, and proteins eIF-4E and 4E-BP1.

1. Role of Phosphatidylinositol-3-kinase in the Activation of Various Kinase Systems

The cellular responses mediated by phosphatidylinositol-3-kinase (PI3 kinase) are diverse, including the inhibition of apoptosis, cell growth, actin cytoskeletal reorganization, and insulin-stimulated translocation of GLUT4 glucose transporter (Carpenter and Cantley, 1996; Toker and Cantley, 1997; Downward, 1998). PI3 kinase is a heterodimeric enzyme composed of a 85 kDa regulatory subunit and a 110 kDa catalytic subunit. The p85 subunit consists of multiple domains including a src homology 3 domain (SH3) and two src homology 2 domains (SH2), domains that recognize phosphorylated tyrosines enabling PI3 kinase to bind activated receptor tyrosine kinases as well as intracellular proteins that have been phosphorylated on tyrosine residues. PI3 kinase mediates cellular responses by phosphorylating the D-3 position of the inositol ring of phosphoinositides, generating PtdIns(3,4,5)P3 and PtdIns(3,4)P2 (Chen et al., 1996). These phosphoinositides then function directly as second messengers to activate downstream signaling molecules by binding to src homology 2 (SH2) and pleckstrin homology (PH) domains of downstream targets (Frech et al., 1997). The activation of PI3 kinase requires its translocation from the cytoplasm to the plasma membrane, which is accomplished by the association between the PI3 kinase p85 SH2 domains and phosphotyrosine-containing proteins, including tyrosine phosphorylated receptors or intermediate proteins, such as the insulin receptor substrates (IRS) and Gab1 (Carpenter and Cantley, 1996). It is also believed that binding of active Ras to the p110

catalytic subunit of PI3 kinase, in the presence of GTP, is also needed for complete activation of the enzyme (Klinghoffer et al., 1996). One tyrosine kinase that has been shown to directly interact with the p85 subunit is focal adhesion kinase (FAK) (Klippel et al., 1996; Chen et al., 1996). The primary action of the lipid products of PI3 kinases is the activation of PKB and S6 kinase and the inhibition of glycogen synthase kinase 3 (GSK-3) (Downward, 1998; Toker and Cantley, 1997). GSK-3 activity is inhibited by the presence of lipid products of PI3 kinases which is thought to contribute to insulin-induced dephosphorylation (activation) of glycogen synthase and initiation factor eIF-2B, therefore leading to stimulation of both glycogen and protein synthesis (Cohen et al., 1997).

2. Protein Kinase B as a Downstream Target of PI3 Kinase

Protein kinase B (PKB) or Akt is a serine/threonine kinase with sequence homology to both PKA and PKC (Coffer and Woodgett, 1991). PKB has been demonstrated to have roles in growth factor-mediated cell survival and insulin-mediated glucose uptake and glycogen synthesis (Marte and Downward, 1997; Delcommenne et al., 1998). PKB contains two primary domains, a catalytic kinase domain and a PH domain amino-terminal to the catalytic domain (Alessi and Cohen, 1998). Insulin and growth factors stimulate the rapid activation of PKB and this activation is inhibited by wortmannin (Burgering and Coffer, 1995).

The activation of PKB consists of three sequential steps. The first step is PH domain-dependent and growth factor- and PI3 kinase-independent, consisting of events that prepare the kinase for growth factor stimulation (Bellacosa et al., 1998). The second step, which is growth factor- and PI3 kinase-dependent, is characterized by the rapid translocation of PKB from the cytosol to the cell membrane. This occurs through the interaction of PKB's PH domain and PI3 kinase-generated D3 phosphoinositides (Bellacosa et al., 1998; Downward, 1998). Furthermore, these inositol lipid 'second messengers' appear to alter the conformation of PKB such that its major phosphorylation sites are accessible for phosphorylation (Alessi and Cohen, 1998). The final step is defined by the phosphorylation of PKB by at least two membrane-associated kinases

(Bellacosa et al., 1998). A recently identified kinase, PDK1, has been shown to phosphorylate PKB on Thr 308 (Alessi et al., 1997a; Alessi et al., 1998). This monomeric enzyme is composed of a catalytic domain that resembles the subfamily of protein kinase A, B, and C, and a carboxy-terminal PH domain (Alessi et al., 1997a). PDK1 is recruited to the membrane, as is PKB, through its interaction with PI3 kinase-generated phosphoinositides (Peterson and Schreiber, 1998). The phosphorylation of Thr 308 alone does not appear to be sufficient to fully activate PKB, as Ser 473 also needs to be phosphorylated (Alessi et al., 1997a; Alessi et al., 1998). More recently, Delcommenne et al. (1998) identified a serine/threonine kinase called integrin-linked kinase (ILK-1), which can interact with the cytoplasmic domain of the β_1, β_2, and β_3 integrin subunits. ILK activity can be rapidly stimulated by fibronectin association with integrins as well as by insulin, in a PI3 kinase- and PH-dependent manner. They further demonstrated that ILK directly phosphorylates PKB on Ser 473 *in vitro*, an event that is inhibited by treatment with wortmannin. These data implicate ILK as a kinase that can regulate PKB phosphorylation at its second critical phosphorylation site.

Therefore PKB resides downstream on the same signaling pathway as PI3 kinase. The pathway downstream of PKB is not clear, but it appears that PKB might play a role in FRAP activation (Scott et al., 1998). In addition, PKB activation appears to be insensitive to treatment with rapamycin, suggesting that PKB is likely either situated upstream of, or parallel to, the site of rapamycin action (Kauffmann-Zeh et al., 1997).

3. Rapamycin's Immunosuppressant Effects are Exerted through FRAP kinase

Rapamycin was initially characterized as an inhibitor of G1 cell cycle progression, but recently has been used to uncover growth factor regulated signaling pathways leading to enhanced translation of a specific subset of mRNAs (Brown and Schreiber, 1996). Jeffries et al. (1997) report that rapamycin selectively suppresses mitogen-induced translation of a class of mRNAs which contain an oligopyrimidine tract (TOP) in the 5' untranslated region just downstream of the methylguanosine cap.

The growth inhibitory effects of rapamycin require its initial association with its cellular receptor FKBP12 (Figure 3). This interaction creates a complex that strongly interferes with the intracellular signaling machinery (Sabers et al., 1995). In yeast, the rapamycin-FKBP12 complex targets two proteins called TOR 1&2 (Target Of Rapamycin) (Helliwell et al., 1994), but more recently Sabers et al. (1995) described a mammalian counterpart which was initially called mTOR but was later given the name FRAP (FKBP-Rapamycin-Associated Protein). FRAP belongs to the phosphatidylinositol kinase-related kinases family which has been implicated in events ranging from cell cycle regulation in response to stress, to DNA recombination (Brown and Schreiber, 1996). The literature has been inconclusive as to whether FRAP is a downstream element in the PI3 kinase-PKB pathway or in a separate signaling pathway. Recently, FRAP has been shown to phosphorylate 4E-BP1 *in vitro*, indicating that it has the ability to function as a protein kinase (Brunn et al., 1997). This discovery also provided a means to investigate the mechanisms involved in its activation. By using 4E-BP1 as a substrate, Scott et al. (1998) demonstrated that FRAP kinase activity is increased in response to insulin and growth factors, through promoting its phosphorylation via a pathway involving PI3 kinase and PKB. However, it cannot be concluded that PKB directly phoshorylates FRAP. As expected from its sensitivity to rapamycin, S6 kinase activity has been shown to be regulated by FRAP, suggesting that FRAP functions proximal to S6 kinase. (Chou and Blenis, 1995; Brown et al., 1995). vonManteuffel et al. (1997) showed that overexpression of S6 kinase inhibits the phosphorylation of 4E-BP1 by insulin, probably through the sequestering of an upstream activator. Since S6 kinase and 4E-BP1 are both inhibited by rapamycin, the common upstream activator could be FRAP.

4. The Involvement of $p70^{S6}$ Kinase in Translation

S6 is one of several proteins which comprise the mature 40S ribosomal subunit. It is the only ribosomal protein in this subunit shown to undergo phosphorylation in response to growth

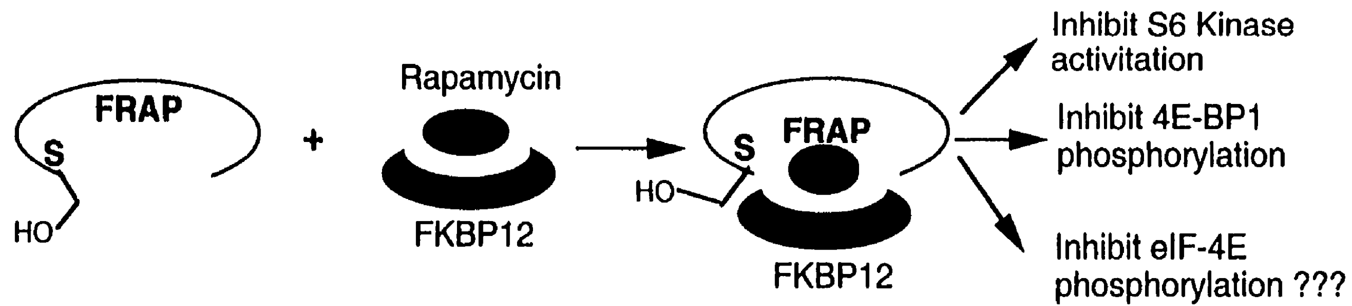

Figure 3. Mechanism of action of the immunosuppressant Rapamycin on FRAP kinase and untimately translation via its effects on S6 kinase and 4E-BP1 / eIF-4E. Rapamycin first binds FKBP12 forming an inihibitory complex that then can associate with FRAP, preventing its activation and the phosphorylation of downstream targets (Brown et al, 1995).

factor stimulation, and this phosphorylation has been directly linked to the initiation of translation as well as to increased rates of protein synthesis (Palen and Traugh, 1987; Stewart and Thomas, 1994). The phosphorylation of S6 has been suggested to increase the rate of protein synthesis by increasing the affinity of 40S subunits for stored messenger ribonucleoprotein (mRNP) particles early in translation (Jeffries and Thomas, 1996).

The enzyme responsible for the phosphorylation of the S6 protein is S6 kinase (Jeffries and Thomas, 1996). Rapamycin treatment results in the rapid inactivation of S6 kinase and the dephosphorylation of S6. This inhibition must be indirect since the effects of the FKBP12-rapamycin complex are exerted on FRAP (Brown et al., 1995). However, no direct interaction between S6 kinase and FRAP has been established, suggesting that there are components of this signaling pathway yet to be identified (Brown and Schreiber, 1996). As mentioned previously, rapamycin is suspected to inhibit the translation of only a subset of mRNAs, those containing 5' TOP elements, suggesting that the role of S6 phosphorylation is not general for translation (Brown and Schreiber, 1996; Jeffries and Thomas, 1996).

Earlier signaling events involved in S6 kinase regulation have been elucidated through the use of the potent inhibitor of PI3 kinase, wortmannin. Treatment with wortmannin prevents the growth factor-regulated increase in S6 kinase activity and appears to function on the same signaling pathway as rapamycin, since wortmannin treatment inhibits the same set of phosphorylation sites on S6 kinase as does rapamycin (Han et al., 1995). Furthermore, evidence has indicated that PI3 kinase is an upstream regulator of S6 kinase, as illustrated by research on mutants of the PDGF receptor (Chung et al., 1994). The mechanism for propagation of the signal from PI3 kinase to S6 kinase involves PDK1 in a similar fashion as with PKB (Alessi and Cohen, 1998). If S6 kinase, which does not possess a PH domain, is regulated by its proximity to PDK1, there must an alternative method for its translocation to the plasma membrane. Alessi et al. (1998) report that this may occur through Rac1 and/or Cdc42. These proteins are localized at the membrane and have been shown to bind S6 kinase in addition to being required for its activation (Chou and Blenis, 1996). PKB and S6 kinase are highly complementary in regions surrounding their phosphorylation

sites (Peterson and Schreiber, 1998). Two important phosphorylation regions are common to both kinases, the activation loop phosphorylation sites, Thr 308 in PKB and Thr 252 in S6 kinase, (Alessi et al., 1998) and phosphorylation sites carboxy-terminus to the kinase domain, Ser 473 in PKB and Thr 412 in S6 kinase (Alessi et al., 1998; Peterson and Schreiber, 1998). These data suggest that PDK1 may serve as a point of bifurcation in the PI3 kinase pathway, but this issue still requires further investigation.

5. Involvement of 4E-BP1 in Translation

As discussed previously, the protein 4E-BP1 competes with eIF-4G for eIF- 4E binding, inhibiting the formation of the eIF-4F complex, which binds to the mRNA and initiates translation (Pause et al., 1994; Mader et al., 1995; Sonenberg, 1996). 4E-BP1 is a heat and acid stable eIF-4E binding protein (otherwise known as PHAS-I) whose activity is regulated by its phosphorylation state in response to insulin and other growth factors (Lin et al., 1994; Pause et al., 1994).

It had been previously postulated that 4E-BP1 was a direct target of MAP kinase (Lin et al., 1994). However, various studies, including those using wortmannin and rapamycin, instead implied the involvement of the PI3 kinase-FRAP kinase-S6 kinase pathway (Graves et al., 1995; Lin et al., 1995; Beretta et al., 1996; vonManteuffel et al., 1996). Some other role for MAP kinase in the regulation of 4E-BP1 regulation cannot be ruled out at the present time (Lin et al., 1995). Brunn et al. (1997) demonstrated that FRAP was capable of phosphorylating 4E-BP1 *in vitro*, with PKB implicated as an upstream regulator of FRAP activation (Scott et al., 1998). In addition, data demonstrating that motifs flanking potential 4E-BP1 phosphorylation sites differ from the rapamycin-sensitive S6 kinase sites, indicate that S6 kinase and 4E-BP1 are likely not phosphorylated by the same kinase (Moser et al., 1997). By this pathway, 4E-BP1 appears to mediate translational activity, and therefore protein synthesis through the PI3 kinase-PKB-FRAP pathway.

6. Importance of eIF-4E Phosphorylation to Translational Control

An additional mechanism implicated in regulation of translation is phosphorylation of eIF-4E itself, with several research groups reporting increased eIF-4E phosphorylation under conditions where translation is enhanced, including treatment with phorbol esters, hormones, and growth factors (Sonenberg, 1996). The role of eIF-4E phosphorylation remains unknown, but speculations include a higher affinity for the mRNA cap structure, and increased association with other components of the eIF-4F complex (Bu et al., 1993; Minich et al., 1994). Several pathways for this phosphorylation have been suggested, including PKC (Whalen, 1996), MAP kinase (Flynn and Proud, 1996), and PI3 kinase-S6 kinase pathway (Kimball et al., 1998). Whatever the mechanism, increases in eIF-4E phosphorylation appears to be correlated with increases in translational efficiency.

IV. Examples of Regulation of Translational Efficiency

1. Effect of Insulin

The response of cells to insulin is one the best examples of a growth factor signaling pathway leading to increased translational efficiency. A schematic representation of this pathway is found in Figure 4.

Insulin is an anabolic hormone which promotes a wide variety of biological responses *in vivo,* including glucose transport, glucose metabolism and regulation of protein synthesis (Chipkin et al., 1994). Stimulation of protein synthesis by insulin results from effects on both transcriptional and translational activities (O'Brien and Granner, 1991). In tissues such as heart, skeletal muscle, adipose tissue and liver, insulin induces increases in total protein synthesis over and above levels expected from the increase in mRNA levels, indicative of increased mRNA translational efficiency (O'Brien and Granner, 1991).

As indicated above, recent studies demonstrate that insulin exerts its effects through the PI3/S6 kinase pathway (Okada et al., 1994; Kimball et al., 1998). Okada et al. (1994) showed that treatment of rat adipocytes with insulin resulted in a significant increase in PI3 kinase activity

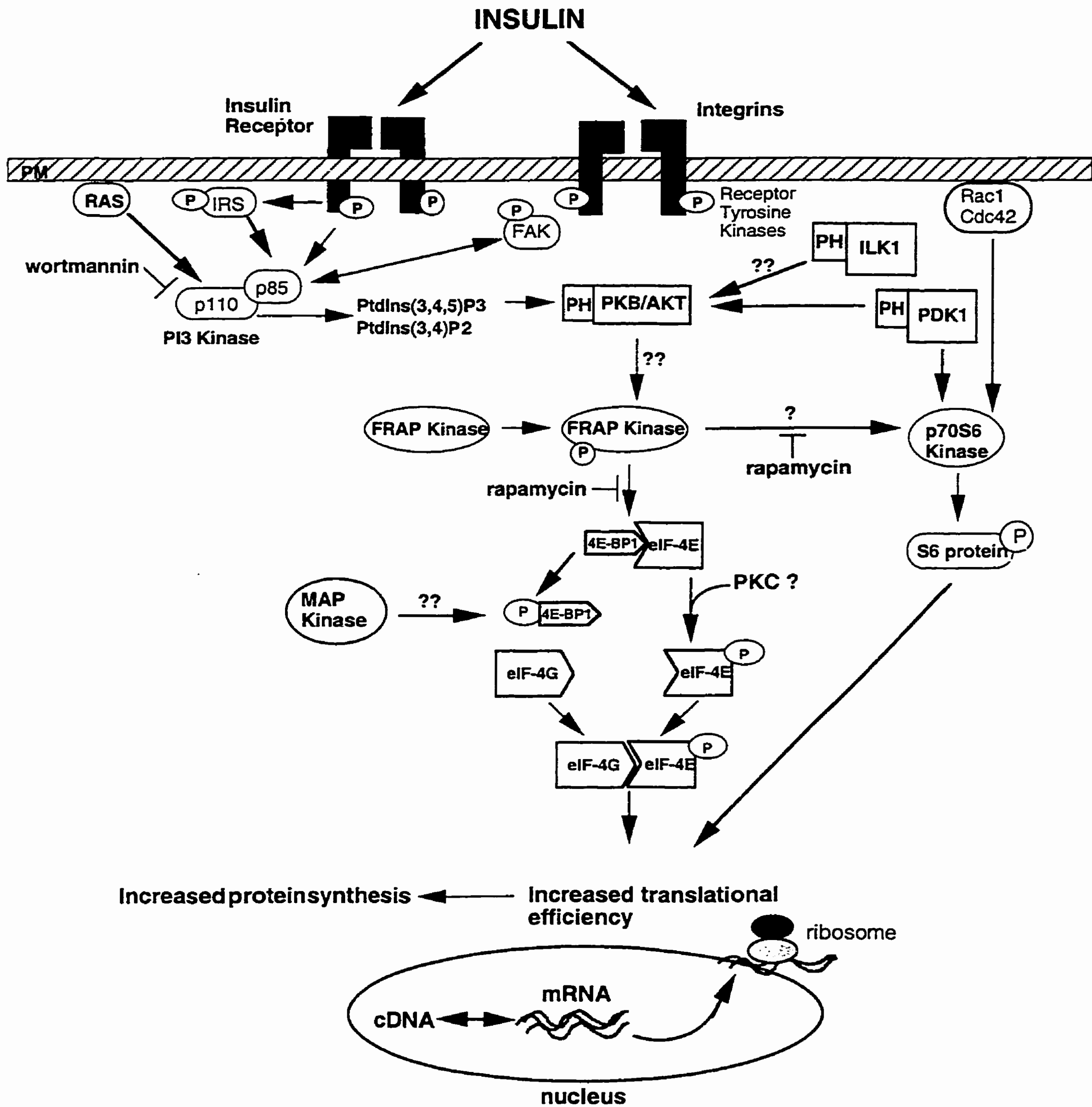

Figure 4. The proposed signal transduction pathway from the activation of the insulin receptor resulting in increased translational efficiency and increased protein synthesis (Gingras et al., 1998; Peterson and Schreiber, 1998; Scott et al., 1998).

which was completely inhibited by pre-treatment with wortmannin. In addition, they showed that wortmannin was unable to inhibit insulin receptor tyrosine kinase activity. Furthermore, the SH2 domains of PI3 kinase have been shown to bind to tyrosine phosphorylated IRS's (Carpenter and Cantley, 1996), suggesting that tyrosine phosphorylation is upstream of PI3 kinase in the insulin signaling pathway. In addition, studies have indicated that PI3 kinase plays a role in glucose uptake, glycogen synthesis and protein synthesis, the three most important biological functions of insulin (Okada et al., 1994; Beretta et al., 1996; vonManteuffel et al., 1996; Kimball et al., 1998).

Ueki et al. (1998) provided the first key evidence that PKB activity was sufficient to activate glucose uptake, glycogen synthesis and protein synthesis *in vivo*. In addition, these responses to insulin, and the insulin-induced activation of PKB were inhibited by wortmannin (Ueki et al., 1998), suggesting that PKB might be the molecular link between PI3 kinase and these biological effects by insulin. This was further supported by studies demonstrating that insulin stimulates ILK activity in a PI3 kinase-dependent manner and that ILK has been implicated as a kinase that regulates PKB activity by phosphorylation (Delcommenne et al., 1998). S6 kinase has also been shown to be an important mediator of insulin stimulation due to the ability of rapamycin to abolish insulin-induced stimulation of protein synthesis (Kimball et al., 1998) and due to the ability of insulin to directly increase S6 kinase activity and S6 phosphorylation (Alessi and Cohen, 1998).

Insulin has been shown to increase the phosphorylation of 4E-BP1, a response which increases the efficiency of translational initiation, and which is inhibited by both wortmannin and rapamycin (Kimball et al., 1998). Furthermore, FRAP has been implicated as the 4E-BP1 kinase and appears to be phosphorylated itself in response to insulin, an event that increases its activity (Scott et al., 1998). These observations suggest that the regulation of insulin-induced 4E-BP1 phosphorylation likely occurs through the PI3 kinase-FRAP kinase pathway. A second 4E-BP1 phosphorylation event has been described which is rapamycin-insensitive and does not promote the disassociation of the 4E-BP1/eIF-4E complex (Diggle et al., 1996). This phosphorylation event is

sensitive to wortmannin, indicating that rapamycin-insensitive phosphorylation is also under the influence of PI3 kinase (Diggle et al., 1996).

Once eIF-4E has been released from the 4E-BP1 complex, translation initiation proceeds with the binding of eIF-4E to eIF-4G (Mader et al., 1995; Kimball et al., 1997). Kimball et al. (1997) demonstrated that insulin caused a significant increase in the amount of eIF-4G coprecipitating with eIF-4E, an effect that neither wortmannin, rapamycin nor the MAP kinase inhibitor PD98059 was able to inhibit. This implied that insulin induces the release of eIF-4E through the PI3 kinase-FRAP kinase pathway but likely promotes the association of eIF-4E and -4G through some alternative pathway not involving PI3 kinase-S6 kinase nor MAP kinase.

2. Cardiac Hypertrophy and Synthesis of Myosin Heavy Chain

Ivester et al. (1995) used an *in vitro* model of electrically stimulated contraction of adult cardiocytes to study the anabolic changes that occur during hypertrophy of the adult myocardium. They demonstrated that electrically stimulated contraction resulted in an acute acceleration of both total protein and myosin heavy chain synthesis (MHC) rates (Wada et al., 1996). This increase was believed to be a result of increased translational efficiency, since protein synthesis rates increased even when transcription was blocked. In addition, these effects were observed quickly (within one hour), and rates of MHC synthesis accelerated in the absence of an increase in steady-state levels of mRNA for MHC (Ivester et al., 1995; Wada et al., 1996; Makhlouf and McDermott, 1998). In this system, message levels for eIF-4E, and phosphorylation of this factor were increased in response to electrical stimulation (Wada et al., 1996; Makhlouf and McDermott, 1998).

3. Overexpression of eIF-4E and Increased Synthesis of Ornithine Decarboxylase

Overexpression of eIF-4E has been shown to result in enhanced translation of growth-promoting proteins, such as growth factors and growth factor receptors (Sonenberg, 1996). Translational enhancement of these proteins appears to be related to their long and highly structured

5'-UTRs which can inhibit ribosome binding and translation initiation (Pelletier and Sonenberg, 1985). Since formation of the eIF-4F complex, which possesses RNA helicase activity, depends on the participation of eIF-4E, it is postulated that increasing levels of eIF-4E may relieve inhibition of translation of these mRNAs (Sonenberg, 1996). Ornithine decarboxylase (ODC) is an example of a protein with a structured 5'-UTR whose expression has been shown to increase in response to eIF-4E overexpression (Rousseau et al., 1996)

4. Protein Synthesis Stimulated by IGF-1 in Sepsis

Sepsis induces significant changes in protein metabolism primarily through decreased protein synthesis of skeletal muscle proteins and increased protein degradation (Jurasinski et al., 1995). Growth hormone (GH) has been shown to reverse sepsis-induced changes in skeletal muscle degeneration through its ability to increase protein synthesis (Jurasinski et al., 1995). However, the anabolic actions of GH are mediated by a second hormone, IGF-1 (Gluckman et al., 1991; Fan et al., 1994). Using a rat model of skeletal muscle sepsis, Jurasinski and Vary (1995) demonstrated that exogenous IGF-1 treatment stimulated protein synthesis in the absence of increased levels of total RNA, suggesting an increase in the efficiency of translation. Furthermore, the enhanced translational efficiency was accompanied by a decrease in the abundance of both 40S and 60S ribosomal subunits, indicating that the mechanism whereby IGF-1 modifies protein synthesis is through accelerated peptide-chain initiation.

V. Rationale for the Study

Earlier results from our laboratory showed that in an *in vitro* organ culture model (Figure 1a), arterial vessels respond to increases in wall stress mimicking *in vivo* responses to increased blood pressure in hypertension. In addition, we have shown that changes in insoluble elastin synthesis and accumulation, in this *in vitro* model, are rapid (Figure 1b) (Keeley and Bartoszewicz, 1995). Furthermore, preliminary data from our laboratory has suggested that the stretch-induced increase in synthesis of insoluble elastin is not correlated with increases in steady-

state levels of elastin mRNA (Keeley and Bartoszewicz, 1995). Thus, stretch-induced elastin synthesis was hypothesized to be post-transcriptionally regulated.

The signal transduction mechanisms whereby arterial cells sense a deformation and respond by increasing the production of elastin have not been well characterized. Initial studies from our laboratory indicated that some tyrosine kinase inhibitors can block the increase in elastin synthesis seen in response to stretch (Keeley and Bartoszewicz, 1995). In addition, the kinase inhibitors wortmannin and rapamycin have also been shown to abolish stretch-induced increases in insoluble elastin synthesis (see Figure 10) (unpublished data).

This thesis therefore addresses the mechanism by which aortic tissues perceive and transduce increases in wall stress into increased production of vascular insoluble elastin.

VI. Hypotheses

The increase in aortic elastin production with increased pressure is transduced through a post-transcriptional pathway involving an elevation in the efficiency of translation of elastin mRNA. This stretch-induced increase in aortic elastin is mediated through a pathway involving tyrosine kinases, phosphatidylinositol-3- kinase and $p70^{S6}$kinase.

CHAPTER 2 - METHODS

I. *In Vitro* Organ Culture Model

Stretch-induced elastin synthesis was measured using intact aortic tissue obtained from day old chicks. Isolated aortic segments approximately 5 mm in length were carefully guided onto a short piece of polyethylene tubing (OD=2.08 mm, Becton Dickson Canada Inc.) producing a sustained, symmetrical, circumferential distension. Control aortas were incubated without tubing inserted. Our laboratory had previously shown that controls consisting of no tubing and those using tubing which did not distend the vessel, resulted in comparable levels of elastin synthesis. Aortas were incubated in minimal essential tissue culture medium (Table 1), without added serum, at 41° C.

To monitor drug effects on stretch-induced elastin synthesis, aortas were pre-treated, prior to stretch, for one hour in medium supplemented with appropriate doses of drugs. The tissues were then transferred to fresh medium, with or without drugs, and subdivided into stretched and unstretched groups. Wortmannin, rapamycin, tyrphostin 25, and genistein were purchased from Calbiochem-Novabiochem Corporation. DRB was purchased from Sigma-Aldrich Canada Ltd..

II. Determination of Insoluble Elastin Synthesis

Insoluble elastin synthesis was determined by incubation of the tissues, usually for 4 hours, in the presence of either [^{14}C]-Proline (50 µCi/mL) or [^{3}H]-Valine (5 mCi/mL), both purchased from Amersham Canada Ltd.. After incubation the tissues were blotted and weighed, then placed in a sealed test tube containing 1.0 mL of cyanogen bromide (CNBr) solution (50 mg/mL in 70% formic acid, bubbled with nitrogen). Digestion was carried out overnight at room temperature. Since elastin does not contain methionine residues, it resists CNBr digestion and remains as an insoluble residue during this procedure. All other aortic

TABLE I. Volumes of Stock Solutions Used to Make Minimal Essential Tissue Culture Medium

Stock Solution	Volume (mL/100 mL medium)
Hanks' Balanced Salt Solution 10X [modified] without Sodium Bicarbonate, ICN Biomedicals Inc.	7.6
100X MEM Nonessential Amino Acids [modified], ICN Biomedicals Inc.	3.0
50X MEM Amino Acids [modified] without L-Glu, ICN Biomedicals Inc.	2.0
Glutamine (14.7 mg/mL)	2.0
Ascorbic Acid (2.5 mg/mL)	2.0
100X MEM Vitamins, ICN Biomedicals Inc.	1.0
Penicillin-Streptomycin 5000 I.U./mL and 5000 μg/mL, ICN Biomedicals Inc.	2.0
Sodium HEPES (40.1 mg/mL)	24.0
** pH to 7.6 with HCl	
Distilled Water	To 100 mL

proteins are solubilized during this procedure, leaving an insoluble residue of pure elastin (Keeley et al., 1992). After digestion was complete, the residual elastin was rinsed twice with approximately 500 μL of hot distilled water and solubilized in 500 μL of 5.7 N HCl at 110° C for approximately 6 hours. Replicate aliquots from each sample were placed in scintillation vials containing 5.0 mL of scintillation fluid, counted, replicate results averaged, and insoluble elastin was calculated as cpm/mg wet weight of tissue.

III. RNA Extraction

To assess the effect of stretch on elastin mRNA levels, total RNA was extracted from chick aortas by guanidine thiocyantate (GTC), followed by ultracentrifugation through a CsCl cushion.

Aortic tissues were homogenized in 2.5 mL of GTC buffer (25 mM sodium citrate buffer, pH 7.0, containing 4 M guanidine thiocyanate, 0.2% sarcosyl, and 200 mM β-mercaptoethanol) using a polytron homogenizer. An additional 500 μL of GTC buffer was used to rinse the polytron probe. RNA was purified by CsCl centrifugation (Glisin et al., 1974). One gram of CsCl was added to each 3.0 mL homogenate and the mixture was then layered over a 1.2 mL 5.7 M CsCl/0.1 M EDTA cushion. The samples were centrifuged overnight at 33,000 RPM, in a SW 50.1 rotor at 4° C to pellet the RNA. The supernatant was removed and the RNA pellet was resuspended using TES and TE buffers. RNA was extracted sequentially with 100% phenol, followed by 50:50 phenol/chloroform and lastly with 24:1 chloroform/isoamyl alcohol. To precipitate the RNA, 0.1 vol of 3 M sodium acetate, pH 5.2, and 2.2 vol of 100% ethanol was added, and the samples were stored at -20° C overnight. The pellets were washed once with 70% ethanol, dissolved in DEPC-treated water and stored at -70° C. Total RNA was estimated by diluting 4 μL of sample into 1 mL of DEPC-treated water, and reading the absorbance at 260 nm.

IV. Northern Analysis

For northern analysis, equal quantities of total RNA were reprecipitated by ethanol precipitation, as described above. In preparation for gel loading, deionized formamide was added

to the RNA followed by incubation at 60° C in 50/50 1X MOPS/37% formaldehyde to denature the RNA. Tracking dye (6X Tracking Dye: 0.25% Bromophenol Blue, 0.25% Xylene Cyanol, and 30% Glycerol) was added and the samples were electrophoresed at 60 - 100V in a 1X MOPS buffer through a 1% agarose gel, containing ethidium bromide. After electrophoresis was complete, the gels were viewed and images recorded under UV light. RNA was transferred onto Hybond-N membrane (Amersham Canada Ltd.), using a capillary transfer technique, and the membrane was UV-cross linked in a UV Stratalinker (Stratagene).

Hybridization used a cDNA of chicken elastin (pCEL - obtained from Dr. Judith Foster (Dept. Biochemistry, Boston University School of Medicine, Boston). The cDNA was random prime labeled in a reaction consisting of 40-50 ng cDNA, 3 μg primer, 2.5 μL 10X Klenow Polymerase Buffer (0.5 M Tris-pH 7.5, 0.1 M magnesium chloride, 10 mM DTT, and 0.5 mg/mL BSA), 2.5 μL of a dNTP mix (0.5 mM each of dATP, dTTP, dGTP), 6.4 units Klenow Polymerase (Pharmacia Biotech Inc.), and 50 μCi of [^{32}P]dCTP (10 μCi/μL, Amersham Canada Ltd.). The reaction was incubated at room temperature for 2-4 hours and terminated by the addition of 2 μL 0.25 M EDTA, and 3 μL 10 mg/mL tRNA. The probe was diluted from 30 to 100 μL with TE buffer, pH 8, purified through a Sephadex G-50 spin column, and activity determined in a liquid scintillation counter.

The RNA-bound membranes were hybridized with 1×10^7 CPM of radiolabelled probe in 10 mL of hybridization buffer (2.5 mL of 20X SSC, 5 mL deionized formamide, 100 μL 10% SDS, 1 mL 50X Denhardts, and 100 μL ssDNA) at 42° C for a period of 16-18 hours in a Hybaid hybridization oven (Interscience Inc.). After hybridization was complete, the blots were washed using a multi-step technique: 5 minute wash with 2X SSC at room temperature followed by a 20 minute wash with 2X SSC, 0.1% SDS at 50° C, and ending with another 20 minute wash with 0.2X SSC, 0.1% SDS at 50° C. This final wash was repeated until the general level of radioactivity on the membrane, determined with a geiger counter, reached an acceptable background level. The membranes were then subjected to autoradiography using K-OMAT x-ray

film (Eastman Kodak). mRNA levels for elastin were corrected for loading using ethidium bromide staining of 28S ribosomal RNA on the gels.

V. Assay of S6 Kinase Activity in Aortic Tissues

Approximately 8-10 aortas were minced and suspended in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM each of EDTA, PMSF, NaF, and sodium orthovanadate, and, 1 μg/mL each of aprotinin, leupeptin, and pepstatin). The tissue was then homogenized using a pre-cooled polytron homogenizer in 30 second bursts. The homogenate was cooled for one minute between bursts to maintain the activity of the enzyme. This process was repeated until the homogenate was smooth. The homogenate was then transferred to 1.5 mL centrifuge tubes and centrifuged at 14,000 x g for 3-5 minutes. The above steps were performed at 4° C. The pellet was discarded and supernatant collected. The protein concentration in the supernatant was determined in triplicate using the Bradford Protein Assay (Bio-Rad Laboratories).

The effect of aortic stretch on $p70^{S6}$kinase activity was determined using an assay kit obtained from Upstate Biotechnology. Aliquots of aortic homogenates containing equal amounts of total protein were combined with S6 kinase substrate peptide, serine/threonine kinase inhibitors, and assay dilution buffer. A phosphorylating mixture was prepared by diluting 10 μL of [γ^{32}P]-ATP (10 mCi/mL, Amersham Canada Ltd.) to 100 μL with the unlabelled Mg/ATP solution provided with the kit. The reaction was initiated by the addition of this ATP mixture to the aliquots of homogenate. For internal controls, substrate peptide was replaced with assay dilution buffer. These served to indicate phosphorylation levels of endogenous substrates in the aortic tissue homogenate. The samples were then incubated at 30° C for 10 minutes and incubation terminated by transferring a portion of each reaction mixture to P81 phosphocellulose paper. Phosphorylated S6 substrate peptide was separated from residual [γ^{32}P]-ATP by its binding to the phosphocellulose paper. Unbound radioactivity was removed by 10 2-3 minute washes with ortho-phosphoric acid (0.75%), followed by a single 5 minute wash with acetone. The phosphocellulose papers were placed into scintillation vials containing 5 mL of scintillation fluid. The samples were

counted, and S6 kinase activity was expressed as experimental - background counts, divided by the amount of total homogenate protein used the assay.

VI. Isolation of Polysomes Using Sucrose Density Gradient Centrifugation

The technique used was essentially identical to that described by Jain et al. (1997). Equal numbers of aortas were homogenized in a polysome buffer (10 mM Tris, pH 8.6, containing 5 mM magnesium chloride, 0.5% Nonident P- 40, 100 mM KCl, 100 μg/mL cycloheximide, 200 units/mL RNAGuard (Pharmacia Biotech Inc.), and 1 mg t-RNA) using a pre-chilled polytron apparatus at 4° C. The homogenate was transferred to 1.5 mL centrifuge tubes, and centrifuged at 12,000 x g for 5 minutes to pellet the nuclei. 15-40% sucrose gradients were prepared using stock solutions of 0% sucrose containing, 10 mM Tris, pH 8.6, 5 mM magnesium chloride, and 100 mM KCl, and 40% sucrose containing 10 mM Tris, pH 8.6, 5 mM magnesium chloride, and 100 mM KCl, mixed in the appropriate proportions to produce 40, 35, 30, 25, 20, and 15% sucrose solutions, which were poured into six 750 μL layers and left to equilibrate at 4° C overnight. The supernatant was then carefully layered onto the gradient, followed by ultracentrifugation at 41,000 RPM for 2 hours at 4° C in a SW 50.1 rotor. The gradient was separated into 18 250 μL fractions using a semi-automated fraction collector. In some cases a portion of each fraction (50 μL) was collected for spectrophotometric readings at 254 nm, after dilution to 1 mL with distilled water. This allowed analysis of the ribosomal RNA distribution in each gradient. The remainder of each fraction was transferred to Trizol for RNA extraction (see below).

VII. Total RNA Isolation Using Trizol Reagent

The Trizol technique (Gibco BRL Life Technologies) was utilized to extract total RNA both from polysomal fractions and from samples used for c-fos mRNA quantitation. This method is based on the single-step RNA extraction procedure modified from Chomczynski and Sacchi (1987).

a) Preparation of RNA from sucrose density gradient fractions:

Sucrose density gradient fractions were added directly to 1 mL of the Trizol reagent. The samples were then incubated at room temperature for 10 minutes to permit complete disassociation of nucleoprotein complexes. Then 200 µL of chloroform was added to each tube, and the samples were vortexed using three 7 second bursts, and incubated again at room temperature for 10 minutes. The samples were centrifuged at 14,000 x g for 15 minutes at 4° C to separate the mixture into the lower organic, phenol-chloroform (red) phase and the upper, aqueous (colourless) phase, which contains the RNA. The aqueous phase was then transferred to 1.5 mL centrifuge tubes containing 500 µL of isopropanol to precipitate the RNA. The samples were incubated at -20° C for 2 - 18 hours, and centrifuged at 14,000 x g for 15 minutes at 4° C to pellet the RNA. The supernatant was removed and the pellets were washed once with 70% ethanol. Pellets were redissolved in 10 µL of DEPC-treated water.

b) Preparation of RNA from aortic tissue:

For experiments investigating the effects of DRB on c-fos mRNA, aortic tissue were homogenized in 1 mL of Trizol Reagent using a pre-chilled (4° C) polytron apparatus, and subsequently treated as described above. The final RNA pellets were redissolved in 50 µL of DEPC-treated water and total RNA estimated by dilution in DEPC-treated water and reading absorbance at 260 nm.

VIII. Nuclease Protection Assay

The Multi-Nuclease Protection Assay from Ambion was utilized to detect and quantitate mRNA species from chick aortic total RNA extracts. For the purpose of this technique, two oligonucleotide probes were used, a 55 oligomer specific for chicken c-fos (5'-GCTGGAGAA GGAGTCCGCCGGGGACGGGTAGTAGGTGAGGCTGTCCCCGGCTCCG-3') and a 64 oligomer specific for chicken elastin (5'-AGCTTGGGATTTACATTCTGAACCATGCA TAGCACCAAAGTTCATGAGGTCATCGGTGATGGG-3'). The two probes were 5' end-labelled in a forward labeling reaction using the T4 Polynucleotide Kinase Labeling Reaction kit

(Gibco BRL Life Technologies). The reaction mixture consisted of 10 pmol of dephosphorylated DNA oligonucleotide, 5 μL of 5X Forward Reaction Buffer, 10 units of T4 Polynucleotide Kinase, and 2.5 μL [γ^{32}P]-ATP (10 μCi/μL). The reaction mixture was incubated for 10 minutes at 37° C, and the reaction was terminated by heat inactivation at 65° C for 5 minutes. The radiolabelled oligonucleotide probes were gel purified through a 10% acrylamide/ 8 M urea gel, and exposed to film to enable cutting out the band of interest, and eluted into 350 μL of probe elution buffer.

Appropriate amounts of total RNA (20 μg for the c-fos mRNA determination or the entire precipitated samples of polysome RNA obtained from sucrose gradient fractions) were mixed with 10^5 CPM of radiolabelled probe, and concentrated by ethanol precipitation. For each probe, two control reactions were included containing the same amount of labeled probe used in the experimental reactions, with added yeast RNA equivalent to the highest amount of each sample RNA. The pellets consisting of probe and RNA were then dissolved in hybridization buffer and incubated at 90° C for 5 minutes to denature and solubilize the RNA. Samples were incubated overnight at room temperature to assure complete hybridization of mRNA with the probe.

A combination of DNA- and RNA-specific nucleases (provided in the kit) were added to each experimental reaction, and to one of the yeast control RNA reactions. Samples were then incubated at room temperature for 30 minutes to digest any unhybridized probe and RNA. The nuclease reaction was terminated by the addition of 40 μL of inactivation buffer. Precipitation of the probe-mRNA complex was accomplished by addition of 600 μL of 100% ethanol and incubation at -20° C for 20 minutes.

The pellets were dissolved by heating in gel loading buffer at 90° C for 5 minutes, and separated by gel electrophoresis through a 10% acrylamide/ 8 M urea gel. The gel was dried on a Bio-Rad Gel Dryer for three hours and exposed to K-OMAT x-ray film (Eastman Kodak) or a phosphorimager cassette (Molecular Dynamics).

IV. Statistical Analysis

Results were analyzed using a unpaired student t-test and implemented on InStat statistical analysis software (InStat for Macintosh Version 1.12 (1992) GraphPad Software). Error bars represent the Standard Error of the Mean.

CHAPTER 3

STRETCH-INDUCED SYNTHESIS OF ELASTIN IS TRANSDUCED THROUGH A POST-TRANSCRIPTIONAL MECHANISM

I. Steady-State Elastin mRNA Levels are Unaffected by Stretch

Previous experiments from our laboratory had demonstrated that distension of aortic vessels in an organ culture model (Figure 1a) resulted in an increase in insoluble elastin synthesis (Figure 1b). Measuring increased production of insoluble elastin as an end point, this increase in synthesis was first detected after 2 hours of incubation. However, increased synthesis of the soluble monomer, tropoelastin, could be seen as early as 30 minutes after initiation of the stretch. The rapidity of this response suggested that post-transcriptional mechanisms might be involved. Preliminary evidence from our laboratory also suggested that this increase in synthesis of elastin did not correlate with an increase in steady-state levels of elastin mRNA.

To establish that increases in steady-state mRNA levels for elastin were not necessary for this response, we designed an experiment in which insoluble elastin synthesis levels and steady-state elastin mRNA levels could be measured in the same aortic tissue (Figure 5a and 5b). Day old chick aortas were distended in organ culture in media supplemented with [^{14}C]-Proline for a period of 4 hours. After incubation the aortas were divided into two portions. One portion of each aorta was utilized to extract total RNA for northern analysis. The second portion was reserved for measurement of stretch-induced elastin synthesis. As expected from previous experiments, there was approximately an 80% increase in insoluble elastin synthesis in the stretched vessels as compared to unstretched controls (Figure 5a). At the same time, in the same vessels, there was no significant increase in steady-state levels of elastin mRNA (Figure 5b). These results were in agreement with the earlier preliminary observations, and established that the rapid increase in synthesis of elastin in response to stretch was not correlated with increased mRNA levels for elastin.

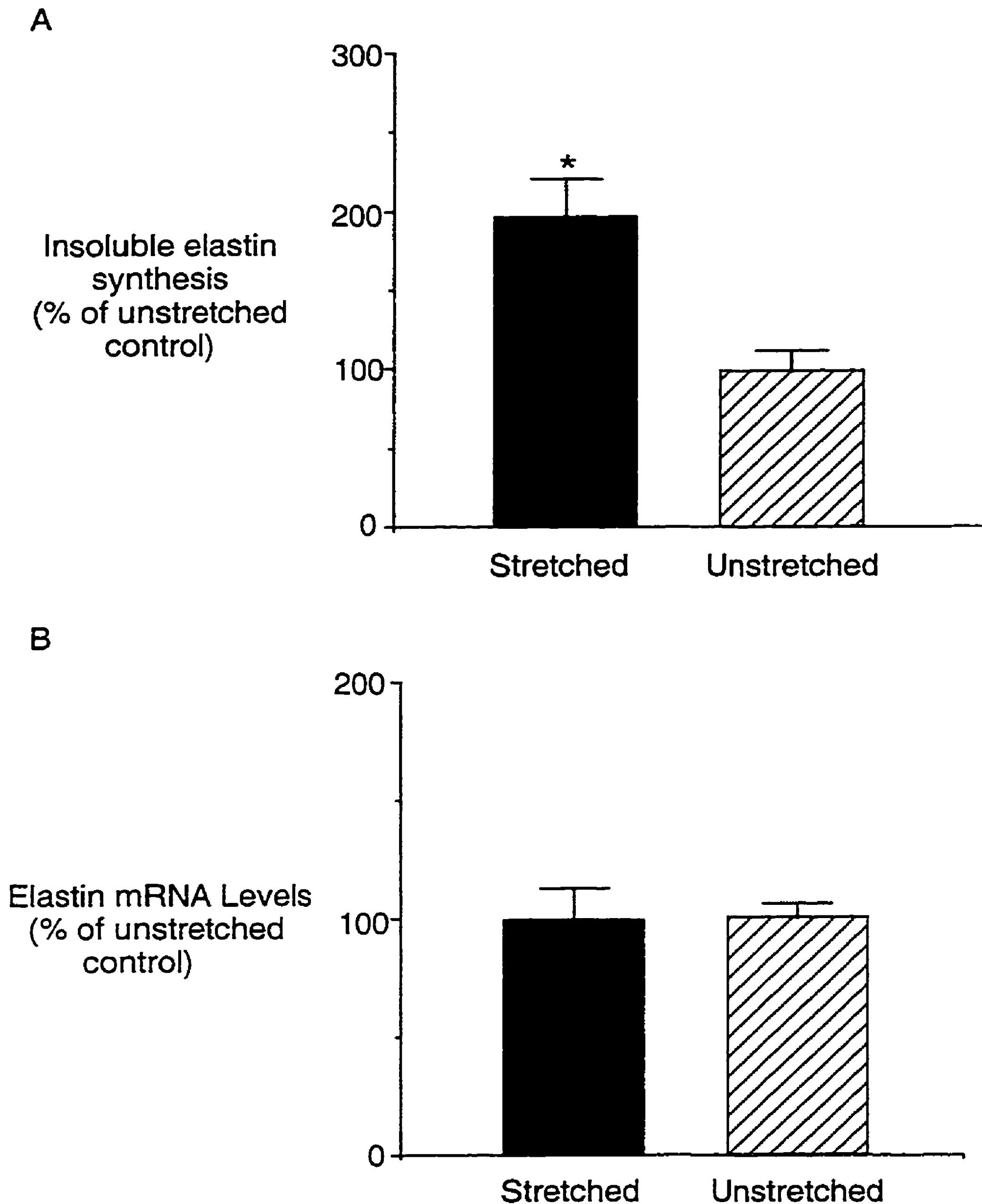

Figure 5. Insoluble elastin synthesis and steady-state mRNA levels for elastin were measured in the same aortic tissue. Day old chick aortas were distended for a period of 4 hours in medium supplemented with [^{14}C]-Proline, using an in vitro organ culture model developed in our laboratory. After incubation, the aortas were divided into two sections. One portion was utilized for measurement of stretch-induced elastin synthesis (A). The other portion was used to extract total RNA for northern analysis (B). Elastin synthesis was measured by [^{14}C]-Proline incorporation into cyanogen bromide-insoluble elastin, and expressed as a percentage of synthesis in unstretched controls. A significant increase in elastin synthesis was observed after 4 hours of stretch. Steady-state mRNA levels were expressed as a percentage of levels in unstretched controls. Loading of RNA was corrected for by ethidium bromide staining. No significant increase in elastin mRNA was observed in response to stretch. Results are combined from 2 individual experiments with values of n being 2-3 per experimental group * indicates significant difference at $P<0.05$

II. Effect of an Inhibitor of Transcription on Stretch-Induced Elastin Synthesis

In order to confirm that elevated transcriptional activity was not required for the stretch-induced response of elastin synthesis, we investigated the effect of the transcriptional inhibitor, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) on this response.

Aortas were pre-incubated for one hour in the presence or absence of DRB, followed by an additional four hour incubation, under the same conditions, in the presence of [^{3}H]-Valine and either stretched or unstretched. Synthesis of insoluble elastin was measured as outlined in the Methods and expressed as a percent of synthesis in unstretched control vessels (Figure 6). The results showed that the presence of 60 μM DRB, a concentration shown to inhibit transcriptional activity in other systems (Zandomeni et al., 1986), does not diminish the stretch-induced response. This was consistent with the hypothesis that increased transcriptional activity was not required for increased elastin synthesis in response to stretch.

In order to confirm that this concentration of DRB was sufficient to inhibit transcription in this organ culture system, it was necessary to determine the effect of DRB on transcription of another gene which was known to be upregulated by stretch. Rapid increases in steady-state mRNA levels for c-fos have been reported after stretching of cardiac myocytes (Sadoshima and Izumo, 1993a). We therefore investigated the effect of this concentration of DRB on stretch-induced steady-state levels of c-fos mRNA. Aortas were pre-incubated in the presence or absence of 60 μM DRB for 1 hour, followed by a 30 minute incubation under the same conditions, either stretched or unstretched. Approximately 10 aortas were pooled for each treatment group. Steady-state levels of c-fos were determined using a nuclease protection assay (see Methods for details). Results were expressed both as absolute counts recorded by the phosphorimager and as a percent of unstretched controls.

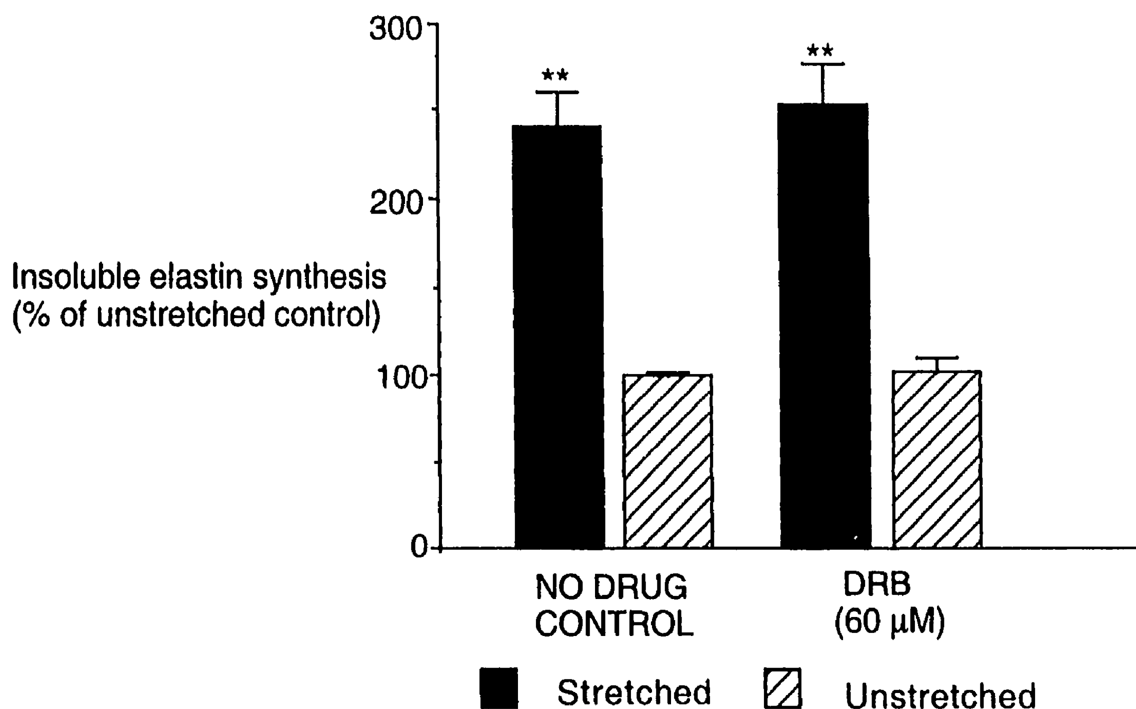

Figure 6. Effect of the transcriptional inhibitor, DRB, on stretch-induced elastin synthesis in our in vitro organ culture model. Aortas were pre-incubated for one hour with or without DRB, followed by a 4 hour incubation under the same conditions, but in the presence of the [^{3}H]-Valine. During the 4 hour incubation the aortas were divided into stretched or unstretched groups. Elastin synthesis was measured by incorporation of [^{3}H]-Valine into cyanogen bromide-insoluble elastin. Insoluble elastin synthesis was expressed as a percentage of the unstretched controls. A significant increase in insoluble elastin synthesis was observed in both the DRB-treated and DRB-untreated aortas. Results were combined from four individual experiments with n values between 5-7 per treatment group. ** indicates significant difference at $P<0.001$.

The initial experiment indicated an increase in c-fos steady-state mRNA after 30 minutes of stretching which was inhibited by pre-incubation for 60 minutes in 60 μM DRB (Figure 7). However, in one DRB treatment group, when the message was expressed as a percent of unstretched controls, there appeared to be a residual increase in c-fos mRNA in response to stretch. In contrast, comparing stretched and unstretched samples as absolute phosphorimager counts, it was clear that this effect was not due to an increase in the stretch group, but rather a decrease in the control group, suggesting that this apparent difference might be due to underloading of the control lane. In order to confirm this, the experiment was repeated (Figure 8). In this case, each stretched DRB treatment group showing no increase in c-fos mRNA relative to control. These results confirmed that a concentration of 60 μM DRB was capable of inhibiting a transcriptional response in our organ culture model. The fact that the same concentration of DRB had no effect on stretch-induced elastin synthesis provides additional evidence that this response does not require increases in transcriptional activity.

III. Direct Measurement of Translational Activity Using Polysome Analysis

The fact that increased elastin synthesis, induced by stretch, does not appear to be regulated by increased transcriptional activity, or increased mRNA half life, suggests the involvement of translational mechanisms in regulating this response. One method for directly measuring translational activity is by analysis of the distribution of mRNA in a polysome size profile (Hershey, 1991).

Aortas were incubated for 4 hours under either stretched or unstretched conditions. After incubation, aortic tissues were pooled, homogenized in a polysome lysis buffer (see Methods) and the extract centrifuged through a 15-40% sucrose density gradient. A total of 18 fractions were collected. In some cases, a small portion of each fraction was removed for absorbance readings at 254 nm (Figure 9). The large peak situated in the middle of the gradient, indicated by the arrow, corresponds to the monosomal RNA unit or 80S peak, usually the most abundant form of ribosomal RNA. Dimer, trimers and higher polysome

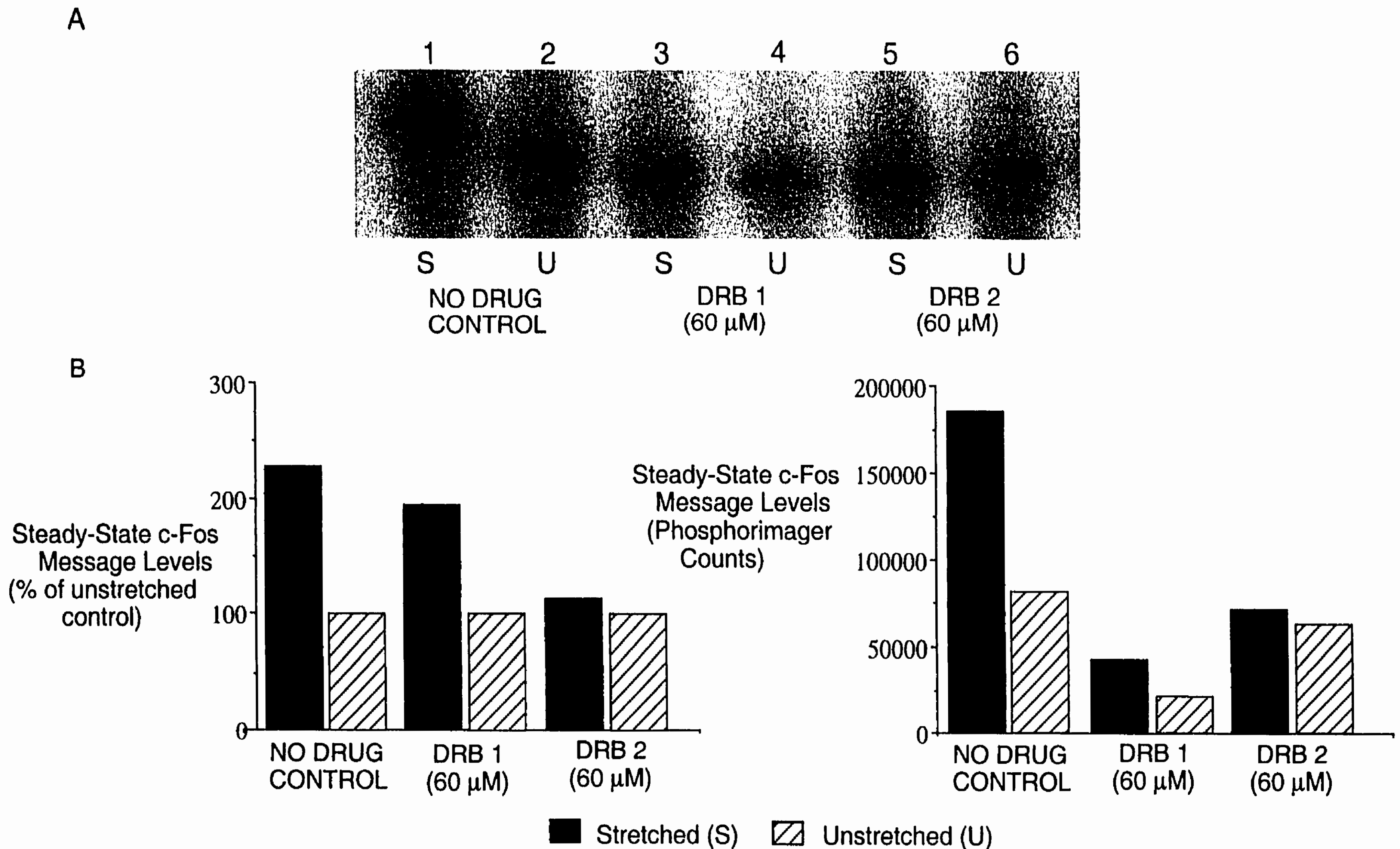

Figure 7. Effect of stretch on steady-state levels of c-fos mRNA in the presence and absence of the transcriptional inhibitor DRB. Aortas were pre-incubated in the presence of DRB for 1 hour, followed by a 30 minute stretch. c-fos levels were assessed using a nuclease protection assay. Results are represented from one experiment with two DRB treatment group (stretched and unstretched) repeats.

A. Image from phosphorimager. Lanes 1 and 2 are non DRB-treated. Lanes 3 to 6 are treated with DRB.

B. Steady-state levels of c-fos mRNA are expressed as a percentage of levels in unstretched controls and as total phosphorimager counts. In non DRB-treated samples there was a significant increase in c-fos message in response to stretch. However, when DRB was added 60 minutes prior to stretch, this increase was abolished.

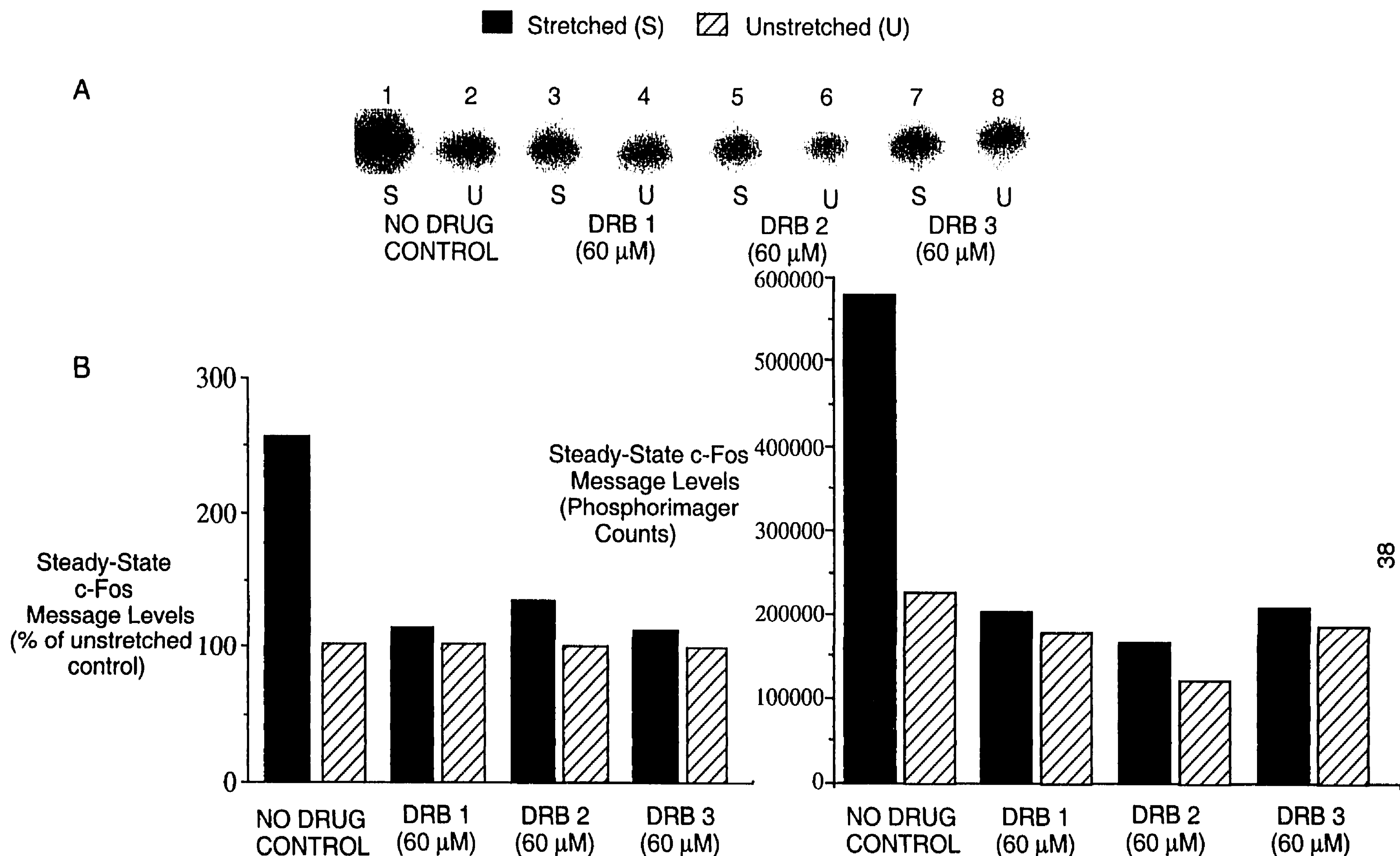

Figure 8. Effect of stretch on steady-state levels of c-fos mRNA in the presence and absence of the transcriptional inhibitor DRB. Aortas were pre-incubated in the presence of DRB for 1 hour, followed by a 30 minute stretch. c-fos levels were assessed using a nuclease protection assay. Results are represented from one experiment with three DRB treatment group (stretched and unstretched) repeats.
A. Image from phosphorimager. Lanes 1 and 2 are non DRB-treated. Lanes 3 to 8 are treated with DRB.
B. Steady-state levels of c-fos mRNA are expressed as a percentage of levels in unstretched controls and as total phosphorimager counts. In non DRB-treated samples there was a significant increase in c-fos message in response to stretch. However, when DRB was added 60 minutes prior to stretch, this increase was abolished.

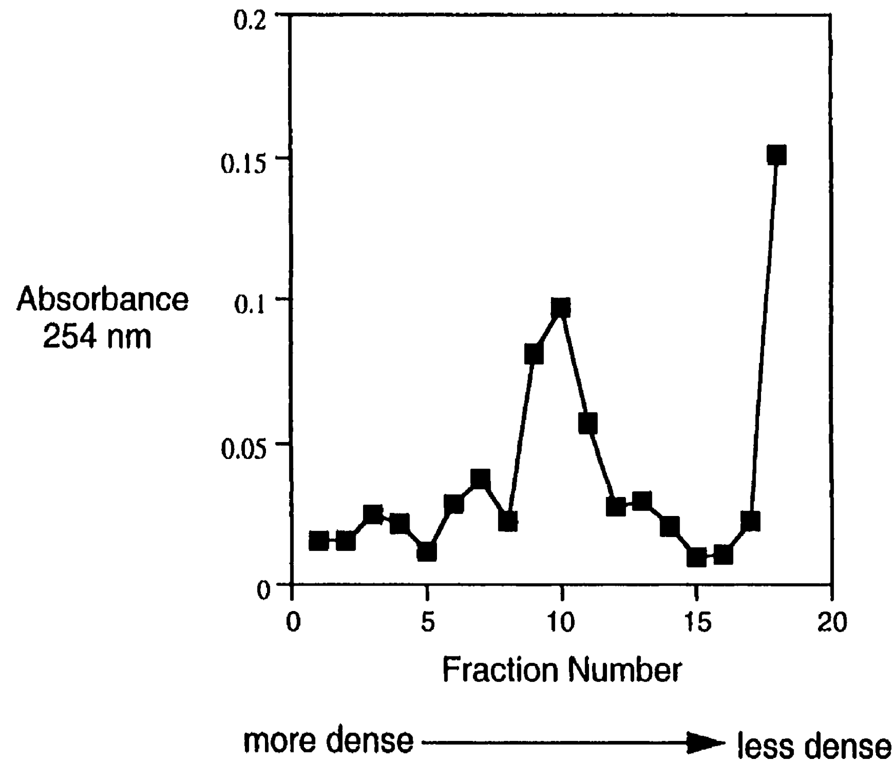

Figure 9. Typical polysome profile obtained from aortic tissue. The 15-40% sucrose gradient was subdivided into 18 fractions from top (fraction #1) to bottom (fraction #18). Absorbance at 254 nm was monitored to detect the ribosomal RNA/mRNA complex. The peak in the center of the gradient (fractions 8-12) represents monosomal RNA units or the 80S peak. Dimers, trimers and higher polysomal oligomers are distributed between this peak and the dense end of the gradient.

oligomers should be distributed between this peak and the dense end of the gradient. mRNA for elastin was determined in the gradient fractions by a nuclease protection assay. Results from these studies were inconclusive (data not shown).

CHAPTER 4

SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN STRETCH-INDUCED ELASTIN SYNTHESIS

I. Aortic p70^{S6}Kinase Activity is Increased in Response to Stretch

Previous data from our laboratory demonstrated that the stretch-induced response of increased elastin synthesis could be blocked by rapamycin (Figure 10), an immunosuppressant reported to inhibit S6 kinase activation (Brown et al., 1995). Our data suggesting the role of translational activity in this response, and the known involvement of this kinase in pathways affecting translational efficiency (Jeffries et al., 1997; Kimball et al., 1998) prompted further investigation.

Using an S6 kinase assay kit, the effect of stretch on S6 kinase activity was determined (Figure 11). The results indicated that stretch of the aortic tissue resulted in a rapid (within 5 minutes) increase in S6 kinase activity as compared to unstretched controls. This increased activity was transient, returning to control levels after one hour of stretch. These results were consistent with S6 kinase being a component of the stretch-induced signaling pathway.

As indicated previously, rapamycin is a potent inhibitor of the activation of S6 kinase and the subsequent phosphorylation of ribosomal protein S6. We therefore investigated the effect of rapamycin on the response of S6 kinase activity to stretch (Figure 12). Results of these experiments confirmed that the dose of rapamycin which blocked stretch-induced elastin synthesis was also capable of inhibiting the stretch-induced increase in S6 kinase activity in these aortic tissues. This increase in S6 kinase activity was also blocked by doses of wortmannin which had been previously shown by our laboratory to inhibit the stretch-induced synthesis of elastin (Figure 10). Wortmannin is widely used as an inhibitor of PI3 kinase. PI3 kinase has been implicated as an upstream component of translational pathways involving S6 kinase (Chung et al., 1994; Han et al., 1994). This suggests that activation of PI3 kinase may also play a role in the stretch response in aortic tissue.

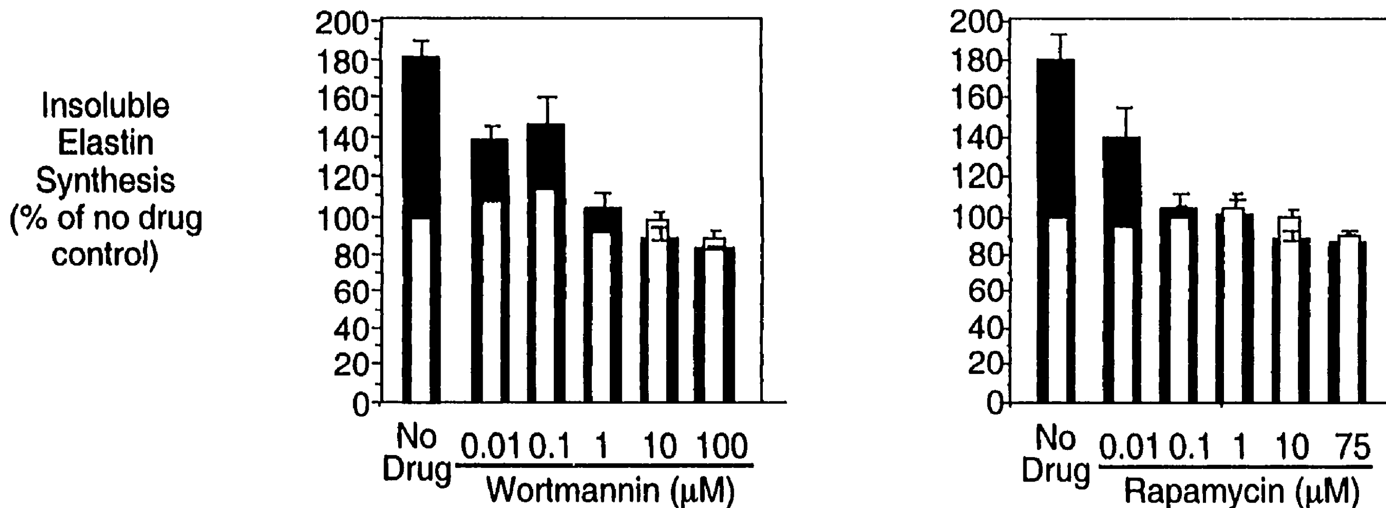

Figure 10. Effect of wortmannin and rapamycin, inhibitors of PI3 kinase and S6 kinase, on stretch-induced elastin synthesis in an *in vitro* organ culture model. Aortas were pre-incubated for one hour with or without inhibitor followed by a 4 hour incubation under the same conditions but in the presence of [^{14}C]-Proline. During the 4 hour incubation the aortas were either stretched or unstretched. Elastin synthesis was measured by incorporation of [^{14}C]-Proline into cyangoen bromide insoluble elastin. Insoluble elastin synthesis was expressed as a percentage of the unstretched drug free control. In the drug free control there was approximately an 80% increase in stretch-induced elastin synthesis. Each of these inhibitors was able to block stretch-induced elastin synthesis in a dose-dependent manner.

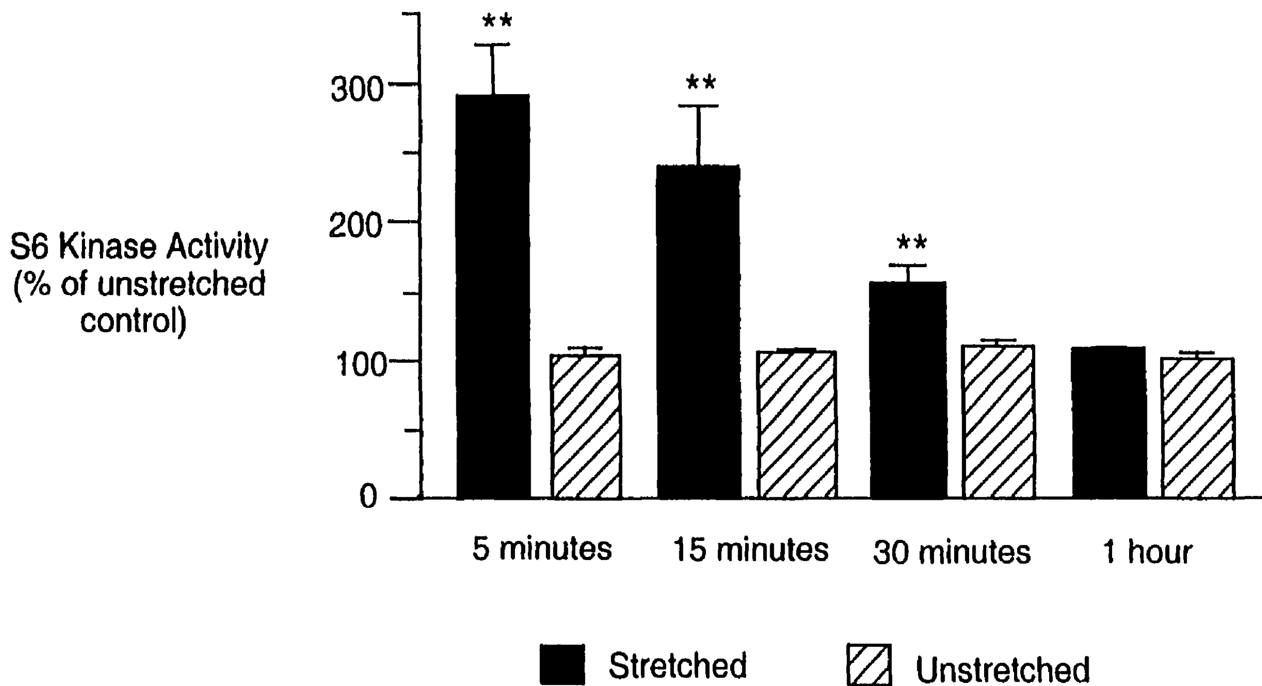

Figure 11. Effect of stretch on aortic S6 kinase activity. S6 kinase activity was measured using protein extracts obtained from stretched or unstretched aortas after 5, 15, 30 minutes and 1 hour of incubation. S6 kinase activity was assayed by incorporation of [γ32P] ATP into a substrate peptide for the enzyme. S6 Kinase activity is expressed as a percentage of activity in unstretched controls. Stretch of aortic tissue resulted in a rapid (5 minutes) increase in S6 kinase activity compared with the unstretched controls. This increase in activity returned to control levels after 1 hour of stretch. Results are combined from 5 experiments for the 5 minute group, 3 experiments for the 15 minute group, and 2 experiments for both the 30 minute and 1 hour groups with values of n being 4-6 per experimental group. ** indicates significant difference at $P<0.001$.

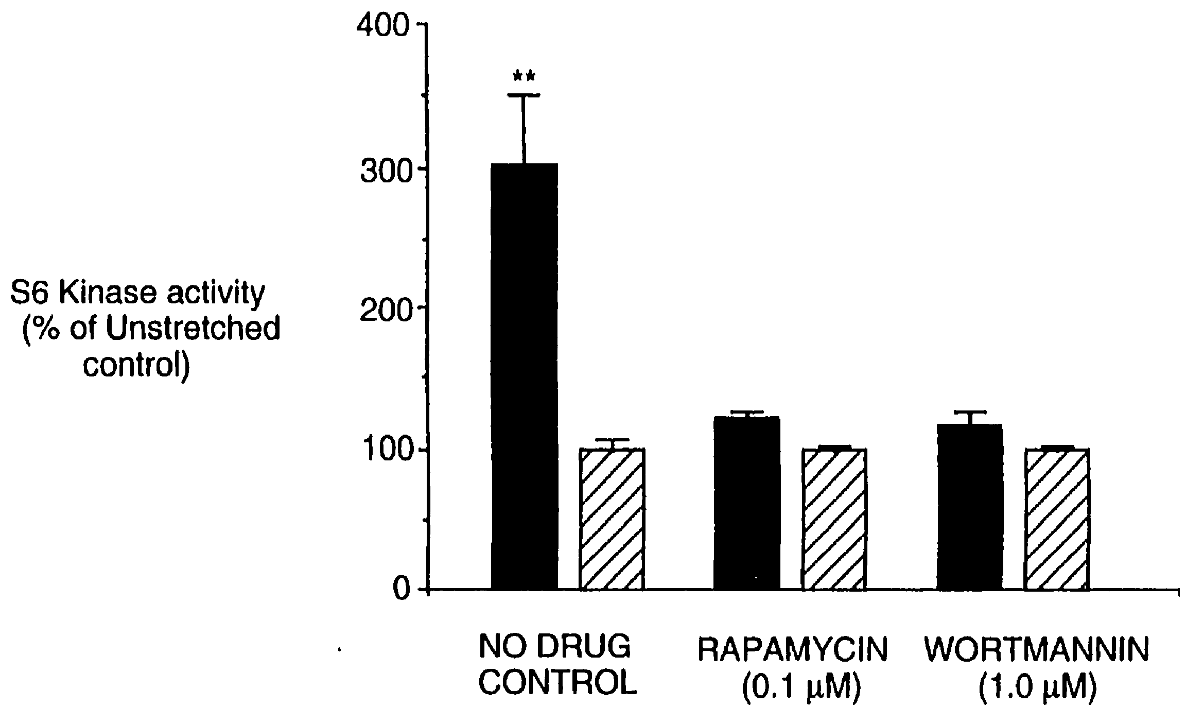

Figure 12. Effect of rapamycin and wortmannin on stretch-induced S6 kinase activity. Aortas were pre-incubated with or without inhibitor for one hour, followed by a 5 minute incubation under the same conditions with aortas either stretched or unstretched. S6 kinase activity was measured and expressed as in Figure 12. Both rapamycin and wortmannin abolished the stretch-induced increase in S6 kinase activity . Results are combined from 5 individual experiments for the no drug treatment group, 4 experiments for the rapamycin treatment group, and 3 experiments for the wortmannin treatment group with values of n being 4-6 per group. ** indicates significant difference at $P<0.001$

II. Effect of Tyrosine Kinase Inhibitors on Stretch-Induced Elastin Synthesis

Tyrosine phosphorylation by protein tyrosine kinases is an early event in many signal transduction pathways. Furthermore, tyrosine kinases have been identified as upstream components of pathways known to affect translational activity (eg. the insulin receptor) (Rosen, 1987). Preliminary data from our laboratory had suggested that inhibition of this activity might block the stretch-induced effect on elastin synthesis (Bartoszewicz, 1995). However, this observation had not been confirmed. In addition, no investigation was made of the relationship of this tyrosine phosphorylation event to other possible elements of a signal transduction pathway. We therefore investigated the effect of two tyrosine kinase inhibitors, tyrphostin 25 and genistein, on stretch-induced elastin synthesis and activation of S6 kinase by stretch in these aortic tissues in several independent experiments. Initially doses of 100 µM were used for these inhibitors, based on data in the literature and previous experience in our laboratory.

Aortas were pre-incubated for 1 hour in the presence or absence of the tyrosine kinase inhibitors, followed by an additional four hours under the same conditions but either stretched or unstretched and in the presence of a [^{3}H]-Valine. As before, synthesis of insoluble elastin was determined as CPM incorporated into insoluble elastin per mg of tissue, and expressed as a percentage of synthesis in the unstretched control group. At doses of 100 µM, tyrphostin 25 inhibited the stretch-induced increase in elastin synthesis but had little or no effect on control synthesis of elastin (Figure 13). A similar dose of genistein was ineffective at blocking the stretch-induced effect.

A similar experiment using three concentrations of tyrphostin 25 demonstrated that the effect of this tyrosine kinase inhibitor to block stretch-induced elastin synthesis was dose-dependent (Figure 14). No effect of tyrphostin 25 was detected at a concentration of 1.0 µM, a partial effect was seen at 10 µM, and a concentration of 100 µM resulted in full inhibition of the stretch-induced effect.

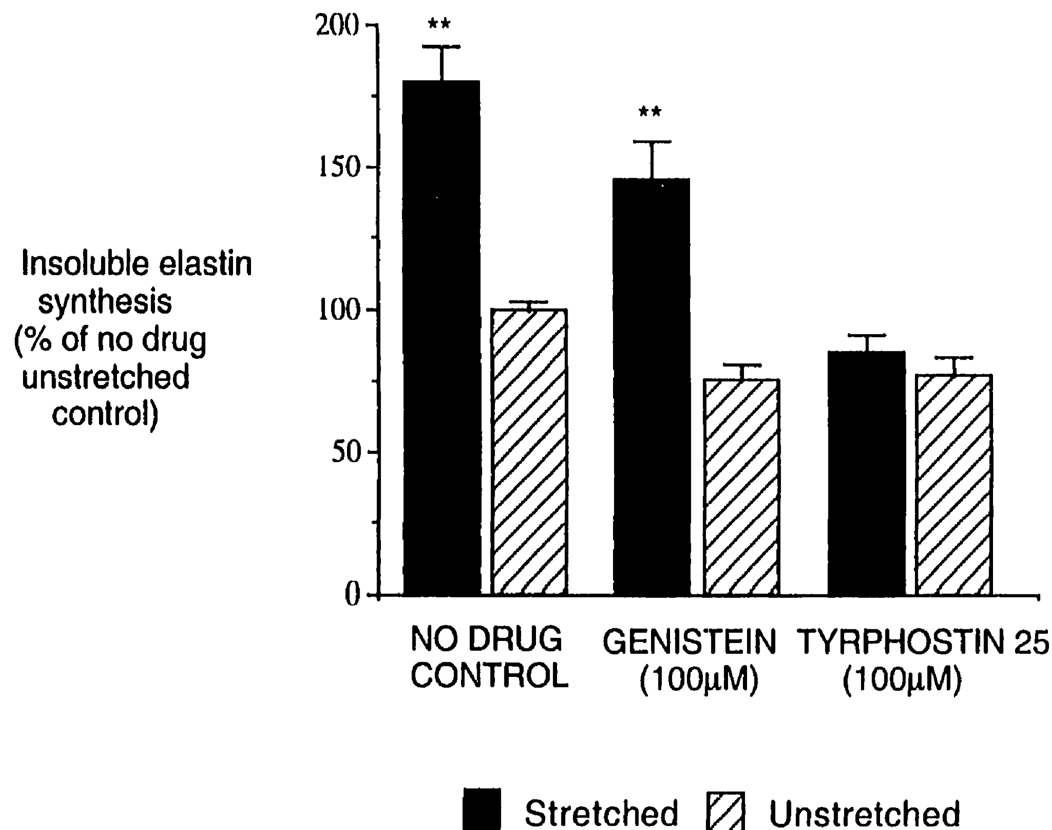

Figure 13. Effect of tyrosine kinase inhibitors, genistein and tyrphostin 25, on stretch-induced elastin synthesis. Aortas were pre-incubated for one hour with or without inhibitor followed by a 4 hour incubation under similar conditions, either stretched or unstretched. Elastin synthesis was measured by the incorporation of $[^3H]$-Valine into cyanogen bromide-insoluble elastin, and expressed as a percentage of the no drug unstretched control. A significant increase in insoluble elastin synthesis was observed in the aortas not exposed to inhibitor and in the genistein treated aortas. However, in aortas treated with tyrphostin 25, stretch-induced elastin synthesis was inhibited. Results are combined from 3 individual experiments with values of n being 5-7 per treatment group. ** indicates significant difference at P<0.001

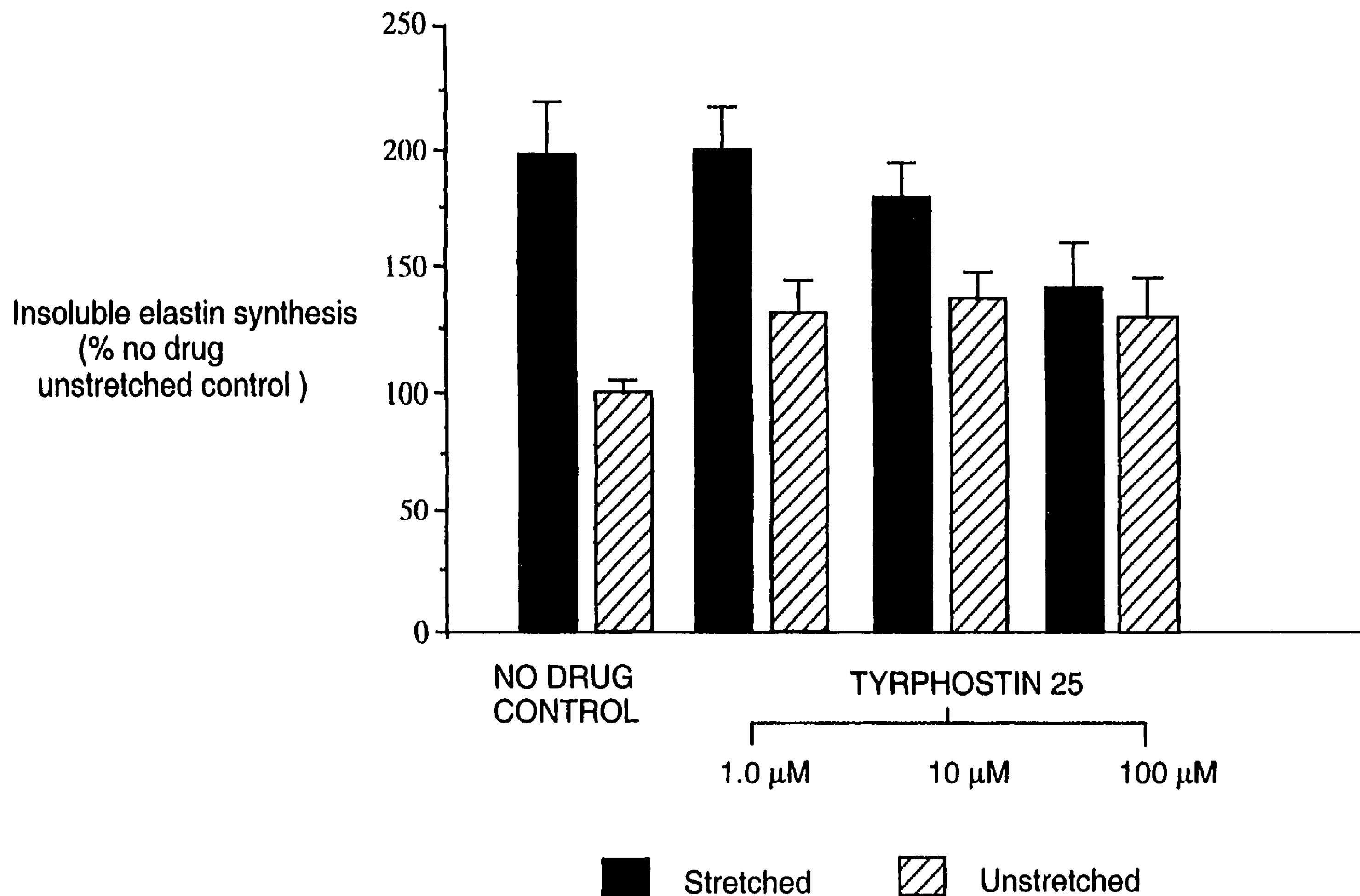

Figure 14. Effect of varying doses of tyrphostin 25 on stretch-induced insoluble elastin synthesis. The aortas were treated as in Figure 14. Elastin synthesis was measured by incorporation of [^{3}H]-Valine into cyanogen bromide-insoluble elastin. Insoluble elastin synthesis was expressed as a percentage of the no drug unstretched control. Tyrphostin 25 inhibited stretch-induced elastin synthesis in a dose-dependent manner. Results are from 1 individual experiment with values of n being 5-7 per treatment group.

III. Effect of Tyrphostin 25 on Stretch-Induced p70^{S6}Kinase Activity

We had previously shown that stretching of blood vessels caused increased activity of S6 kinase and that either direct or indirect inhibition of this kinase with rapamycin or wortmannin blocked both stretch-induced increases in S6 kinase activity and stretch-induced increases in elastin synthesis. Our results also demonstrated that the tyrosine kinase inhibitor, tyrphostin 25, was able to inhibit stretch-induced elastin synthesis. Therefore we investigated whether this effect of tyrphostin 25 might be working through blocking the effect of stretch on the activation of S6 kinase.

Aortas were pre-incubated for 1 hour in the presence or absence of 100 μM of tyrphostin 25 followed by a further 5 minute incubation for which the tissue was divided into stretched and unstretched groups. S6 kinase activity was determined as described above, and expressed as a percent of activity in untreated, unstretched aortas. The results (Figure 15) showed that tyrphostin 25 blocked the stretch-induced increase in S6 kinase activity, implying that this S6 kinase activation by stretch is downstream of the activation of tyrphostin-inhibitable tyrosine kinase.

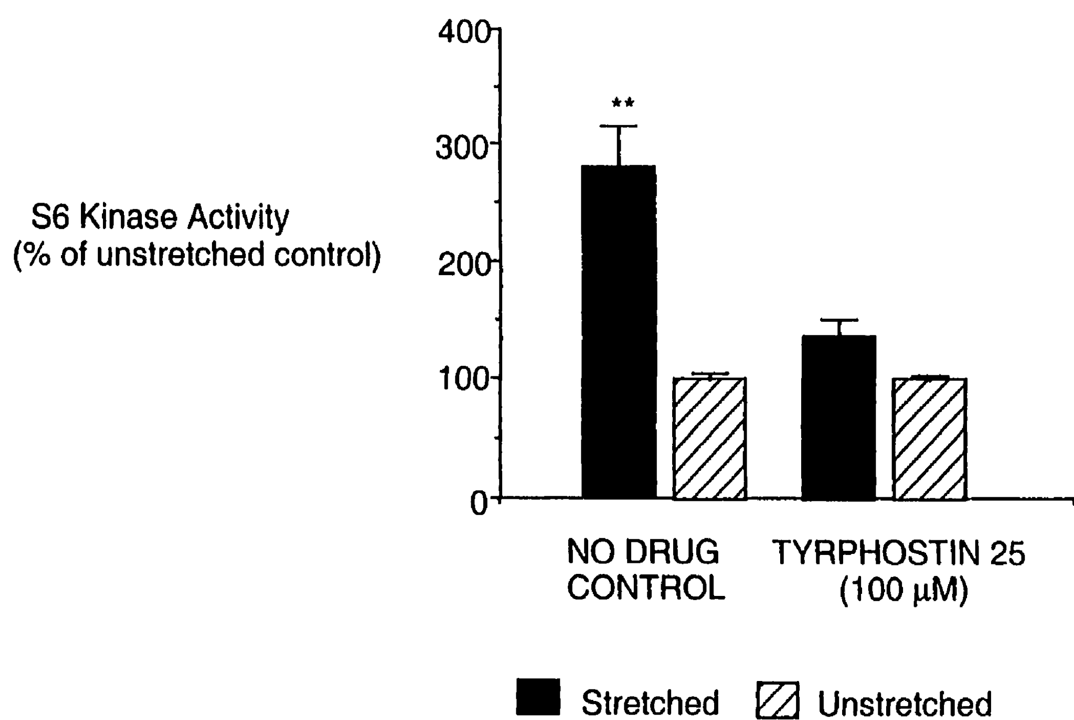

Figure 15. Effect of tyrosine kinase inhibitors, tyrphostin 25, on stretch-induced S6 kinase activity. Aortas were pre-incubated with or without inhibitor for one hour followed by a 5 minute incubation under the same conditions either stretched or unstretched. S6 kinase activity was determined and expressed as in Figure 13. Treatment aortas with tyrphostin 25 abolished the stretch-induced increase in S6 kinase activity. Results are combined from 5 individual experiments for the no drug treatment group and 2 experiments for the tyrphostin 25 treatment group with values of n being 4-6 per treatment group. ** indicates significant difference at $P<0.001$

CHAPTER 5 - DISCUSSION

I. General Discussion

1. Distended Aortic *In Vitro* Organ Culture Model

Previous studies have demonstrated that mechanical forces, particularly stretch, induced the reproducible stimulation of tropoelastin synthesis (Sutcliffe and Davidson, 1992; Keeley and Bartoszewicz, 1995). However, very little has been done to elucidate mechanisms whereby stretch induces such an intracellular change in elastin synthesis. In the present study our aim was to clarify the mechanism whereby the cell recognizes and responds to this external load.

Our laboratory designed an *in vitro* organ culture model to investigate elastin synthesis in aortas explanted from day old chickens. The *in vitro* technique involves circumferentially distending the aorta with tubing (Figure 1a). *In vitro* systems are often more amenable for studying the complex interactions of signal transduction because they allow the manipulation of each variable separately, without interference of other factors that might be encountered *in vivo*. This model has several advantages which make it an appropriate technique for this study. Keeley and Bartoszewicz (1995) indicated that in this *in vitro* model, the aorta responds to an increase in wall stress in a similar fashion as *in vivo* responses to increased blood pressure in hypertension. Furthermore, this model allows for insoluble elastin synthesis to be readily measured as an end point, and the effect of stretch on elastin synthesis is significant, rapid, and easily reproducible (Keeley and Bartoszewicz, 1995). Mature elastin is essentially insoluble in all reagents except for those that break peptide bonds. This insolubility is accounted for by the polymeric structure of elastin, which is maintained by desmosine and isodesmosine cross-links formed from its soluble monomeric precursor, tropoelastin. Cyanogen bromide was used as a chemical treatment for the isolation of mature, insoluble aortic elastin since it digests proteins by cleaving at methionine residues. Mature elastin is unusual in that, unlike most other proteins, it does not contain any methionines. Therefore essentially all other aortic proteins are readily digested and solubilized following cyanogen bromide treatment, leaving a residue of mature insoluble elastin.

2. Stretch-Induced Synthesis of Elastin is Transduced Through a Post-Transcriptional Mechanism

There are several mechanisms which could account for the increase in elastin synthesis in response to stretch. For example, an increase in steady-state levels of elastin mRNA can be due to either increased rates of transcription, without a change in mRNA stability, decreased turnover of mRNA without a change in rates of transcription, or some combination of the two. However, if there are no changes in steady-state elastin mRNA levels, the response is likely related to increases in translational efficiency.

Preliminary experiments in our laboratory had suggested that stretch of aortic vessels in our *in vitro* organ culture model resulted in increased insoluble elastin synthesis without a corresponding increase in steady-state elastin mRNA levels (Keeley and Bartoszewicz, 1995; Bartoszewicz, 1995). This observation was confirmed by analyzing the effect of stretch on steady-state elastin mRNA levels and insoluble elastin synthesis and accumulation in the same aortic tissue. The results obtained from this study supported earlier observations, suggesting a mechanism for increased protein synthesis resulting from increased translational efficiency.

The rapidity of the stretch-induced response also supports the possibility that translational efficiency is important in this mechanism. Increases in the production of insoluble elastin in response to stretch were detected after 2 hours of incubation. Furthermore, increased synthesis of the soluble elastin monomer, tropoelastin, could be detected as early as 30 minutes. For most proteins, it would be expected that increased protein synthesis, based on transcriptional activation, would require a longer response time. A major benefit of translational regulation is that it can induce rapid changes in protein content in response to external stimuli (Mathews, Sonenberg and Hershey, 1996).

If stretch-induced elastin synthesis is a consequence of increased translational efficiency, inhibition of transcription should not affect the response. The adenosine analogue 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) is commonly used to inhibit transcription by inhibiting RNA Polymerase II (Zandomeni et al., 1986). The reported DRB concentration required for 50% inhibition of transcriptional activity was 4-10 µM, with a dose of 60 µM providing almost

complete transcriptional inhibition (Zandomeni et al., 1986). Pre-treatment of aortas in the presence of 60 μM DRB did not abolish the increase in elastin synthesis in stretched aortas.

In order to confirm that this concentration of DRB was sufficient to block transcription in the aortic organ culture model, it was important to investigate the effect of DRB on the transcription of another gene that has been reported to be induced by stretch. c-fos was chosen for this purpose since its expression had been shown to be increased in cardiac myocytes following stretch (Sadoshima and Izumo, 1993a). Steady-state levels of c-fos mRNA were analyzed in the presence and absence of DRB under both stretched and unstretched conditions. It was expected that stretch would result in increased transcription of the c-fos gene in the absence of DRB. However, with a dose of 60 μM DRB, there should be no increase in steady-state c-fos message levels. Two replicate experiments showed that stretch resulted in a significant increase in steady-state levels of aortic c-fos, and that this effect was blocked by pre-treatment of aortas with 60 μM DRB. The fact that this dose of DRB was unable to inhibit stretch-induced elastin synthesis suggested that increased transcriptional activity is likely not required for this response.

These data indicate that stretch-induced elastin synthesis is likely not a result of increased transcriptional activity. We also attempted to provide direct evidence for increased translational efficiency under these conditions.

Analysis of the distribution of mRNA species in polysome profiles has been employed as an approach to investigate increased rates of translation in various *in vivo* and *in vitro* systems (Hershey, 1991). A polysome is defined as a cluster of ribosomes that are simultaneously translating a single piece of mRNA. The basis of this technique is that cytoplasmic RNAs exist in two functionally distinct states. mRNA in the process of being translated exists bound to polysomes, while inactive mRNA is found associated with messenger ribonucleoprotein particles (mRNPs). Increased translation of a message is identified by the association of that mRNA with increased numbers of ribosomes, forming larger polysomal clusters (Hershey, 1991). Sucrose density gradient centrifugation is used to size separate active polysomes from nontranslating 80S particles and ribosomal subunits (Hershey, 1991). A change in the rate of translation of a specific

protein can be readily detected by analyzing the mRNA/polysome distribution pattern following hybridizing with a specific DNA or RNA probe. Therefore, a shift of the position of elastin mRNA in the polysomal profile to larger polysomes, might provide support for increased translational activity.

The distribution of elastin message in sucrose gradient fractions was assessed by a nuclease protection method using a chick elastin oligonucleotide probe. Attempts to detect elastin message in the gradient fractions by northern analysis were not successful because of some inevitable RNA degradation, resulting in smearing of the blots. The nuclease protection method was therefore utilized because it is able to detect mRNA for elastin which was not necessarily completely intact. For this reason, the nuclease protection technique is commonly used in studies of polysome profile mRNA distribution patterns (Jain et al., 1997).

Results of these experiments were inconclusive, preventing any conclusions to be drawn for the data. There are several possible explanations for the non-reproducibility of this technique. First, background levels of elastin synthesis in day old chicks are already high. In most circumstances where this technique has been successful, the translation of the mRNA in question is shifting from a state of low activity to high activity (Bennett and Adams, 1987; Rousseau et al., 1996; Jain et al., 1997). The technique may not be sufficiently sensitive to detect changes related to only a 2 fold increase in elastin synthesis over this already high background. It is possible that improvements to the technical protocol, in order to limit mRNA degradation, might allow stretch-induced differences in the polysome distribution pattern of elastin mRNA to be detected.

3. Signal Transduction Pathways Involved in Stretch-Induced Elastin Synthesis

The second phase of this study involved understanding how the onset of stretch is rapidly transduced through the cell into an increase in elastin synthesis. Reference was made in the introduction to the insulin signaling pathway, with emphasis on aspects relevant to increased translational efficiency, including protein tyrosine kinases, PI3 kinase, PKB/Akt kinase, FRAP kinase and S6 kinase, proteins of the eIF-4F complex, and binding protein 4E-BP1.

Previous studies from our laboratory identified some apparent similarities between the insulin pathway and the pathway involved in stretch-induced elastin synthesis. For example, two kinase inhibitors, wortmannin, a specific inhibitor of PI3 kinase (Saito et al., 1996) and rapamycin, a potent inhibitor of S6 kinase activation (Brown et al., 1995), were shown to inhibit stretch-induced elastin synthesis, suggesting the involvement of S6 kinase in the signaling pathway. The fact that S6 kinase is an important factor in the regulation of translation (Palen and Traugh, 1987) is consistent with our hypothesis that stretch alters elastin synthesis through effects on translational efficiency. The effect of wortmannin on elastin synthesis also suggested that PI3 kinase might play a role in the signaling pathway in response to stretch. These observations focused our research on the PI3 kinase-S6 kinase signaling pathway.

An S6 kinase assay was used to directly evaluate the effect of aortic stretch on S6 kinase activity, anticipating that stretch should cause an increase in the activity of this kinase. Data showed that stretch resulted in a rapid increase in S6 kinase activity (within 5 minutes). The response of increased S6 kinase activity to stretch was transient, returning to control levels after one hour of stretched incubation. This rapid increase in activity was consistent with the rapidity of the response of elastin synthesis, and suggested that S6 kinase was a likely component of the pathway leading to increased synthesis of elastin in response to stretch.

Both rapamycin and wortmannin had been shown to fully inhibit stretch-induced elastin synthesis at doses of 0.1 μM and 1.0 μM respectively. These doses were somewhat higher than those required to block S6 kinase and PI3 kinase activity in response to insulin . However, partial inhibitory effects were seen at concentrations of rapamycin and wortmannin of 0.02 μM and 0.1 μM respectively, doses very close to those used in studies involving insulin (Kimball et al., 1998; Scott et al., 1998). These differences in sensitivity may be related to the fact that our studies involved organ cultures, as compared to the cell cultures used for the insulin studies. We therefore investigated the effect of these agents on S6 kinase activity in response to stretch. As expected, pre-treatment of aortas with rapamycin abolished the stretch-induced increase in S6 kinase activity. Similar effects were also seen for wortmannin, an agent which inhibits PI3 kinase activity.

Wortmannin has been reported by others to be capable of indirectly blocking S6 kinase activity through its effects on PI3 kinase (Brown and Schreiber, 1996) suggesting that both PI3 kinase and S6 kinase are on the signal transduction pathway for stretch-induced elastin synthesis, with PI3 kinase being upstream of S6 kinase. The data also suggests that increased elastin synthesis requires participation of S6 kinase.

Increased tyrosine kinase activity is known to be involved as an early upstream event in signal transduction pathways leading to increased translational efficiency (Okada et al., 1994). We therefore investigated the effect of tyrosine kinase inhibitors on stretch-induced elastin synthesis and on the induction of S6 kinase activity by stretch. Both genistein and tyrphostin 25 were used in this study.

Tyrphostins are a series of low molecular weight synthetic compounds that have been synthesized for the purpose of inhibiting the activity of tyrosine kinases (Wolbring et al., 1994; Bilder et al., 1991). Tyrphostins are widely used in the range of 10-100 μM in studies *in vitro* (Gazit et al., 1989) and *in vivo* (Yamazaki et al., 1993) to assess the potential role of protein tyrosine kinase activity in signal transduction. Several tyrphostins are available, with tyrphostins 23, 25 and 47 being most commonly used (Wolbring et al., 1994). Tyrphostin 25 is a competitive inhibitor of substrate binding to EGF receptor-family of protein tyrosine kinases (Gazit et al., 1989; Bilder et al., 1991). Genistein also inhibits EGF receptor-family of protein tyrosine kinases but through the prevention of ATP binding to the kinase (Hidaka and Kobayashi, 1992). However, genistein is considered to be a much more general inhibitor than tyrphostin 25 (Akiyama et al., 1987; Kim et al., 1995). Studies *in vivo* demonstrated that high doses of genistein (IC_{50}=120 μM) prevents autophosphorylation of the EGF-receptor mediated by EGF (Akiyama et al., 1987). Doses of the two inhibitors used in this study were therefore based on concentrations used commonly in the literature, as well as concentrations used in previous preliminary experiments in our laboratory (Bartoszewicz, 1995). While tyrphostin 25 completely inhibited stretch-induced elastin synthesis, genistein had little or no effect. This was consistent with previous data from our laboratory (Bartoszewicz, 1995). Neither inhibitor, at these concentrations,

substantially affected control levels of elastin synthesis, arguing against a general toxic effect of these concentrations of inhibitors on the aortic tissue. In addition, inhibition of stretch-induced elastin synthesis by tyrphostin 25 showed a clear dose-response relationship. These data suggested that the phosphorylation of unknown protein by tyrosine kinase activity is necessary to transduce stretch into increased elastin synthesis.

To determine whether this tyrosine kinase activity was linked to the increases in S6 kinase activity previously demonstrated in stretched aortic tissues, we investigated the effect of tyrphostin 25 on stretch-induced S6 kinase activity. If activation of a tyrosine kinase is a required upstream event for stretch-induced increases in S6 kinase activity, then tyrphostin 25 should inhibit this increase in S6 kinase activity. Data showed that, in the presence of tyrphostin 25, stretch was not able to activate S6 kinase. These results are consistent with a pathway which involves activation of a tyrosine kinase as an early event, and a dependent downstream activation of S6 kinase.

These observations, together with current information on the insulin-dependent signaling pathway, are summarized in Figure 16. Increased vascular wall stress, induced by a circumferential aortic stretch, results in a rapid increase in synthesis and accumulation of arterial elastin. The absence of corresponding changes in steady-state elastin mRNA levels, together with the inability of DRB to attenuate elastin synthesis in response to stretch, suggests that stretch-induced elastin synthesis occurs through increased translational efficiency. Additional efforts are necessary to establish a direct connection between stretch-induced elastin synthesis and translational efficiency.

Although the signal transduction pathway linking these events remains incomplete, several similarities to the pathway linking the activated insulin receptor to increase translation of proteins have been identified. Both pathways require as a downstream event, activation of S6 kinase, an enzyme closely linked to mechanisms altering processes important for translation, and in both cases downstream events are blocked by rapamycin, an inhibitor of S6 kinase activation. Both pathways also appear to involve activation of PI3 kinase upstream of S6 kinase, as indicated by the inhibitory effect of wortmannin. Finally, both pathways appear to require activation of a tyrosine

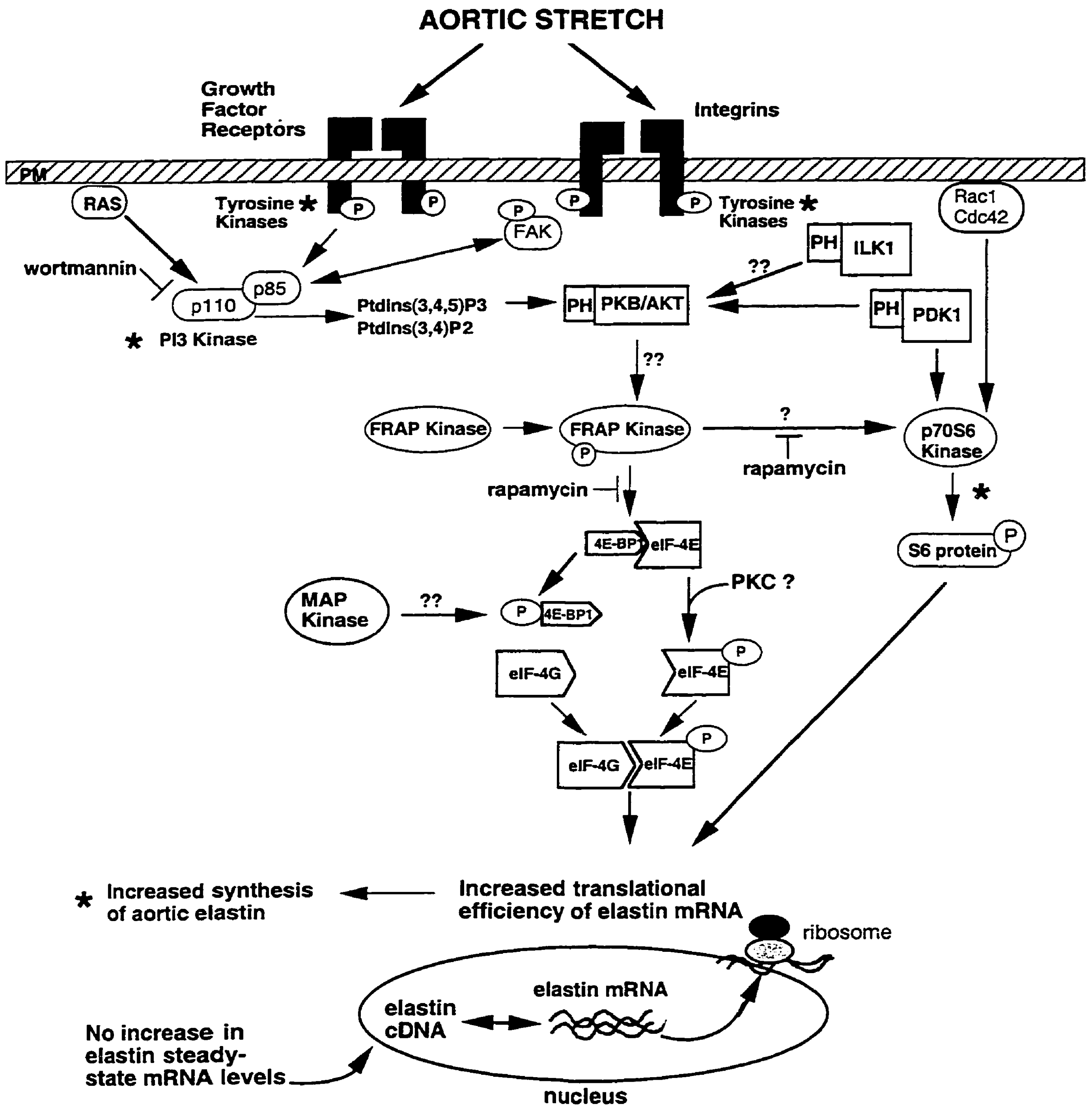

Figure 16. A proposed signal transduction pathway for stretch-induced elastin synthesis in aortic tissue. * indicate steps in the pathway either directly or indirectly investigated in this thesis(Gingras et al., 1998; Peterson and Schreiber, 1998; Scott et al., 1998).

kinase as an early event. In the case of the insulin pathway, this kinase may be the insulin receptor itself. However, in the case of stretch-induced elastin synthesis, the identity of this upstream tyrosine kinase is not known, nor are the events leading to its activation by increased wall stress understood.

II. FUTURE DIRECTIONS

The data presented in this thesis still leaves several unanswered questions, and suggest further experimental approaches. These include:

1. Modifications Increasing the Sensitivity of Detection of Shift in Polysome Profiles

Although, as discussed earlier, the two-fold increase in elastin synthesis caused by stretch on the background of an already high level of elastin synthesis in these aortas may not result in a detectable shift in elastin mRNA in the polysome profile, there are at least two improvements to the assay system which could allow more sensitive detection of these changes. These would include steps to minimize RNA degradation during the extraction and sucrose gradient centrifugation procedures, such as increasing contents of RNAGuard and tRNA in the extraction buffer, and changes to the technical procedure to minimize the time during which the RNA may be exposed to conditions under which digestion might take place. Secondly, changes in polysome profile may be hidden by differences in RNA loading in the sucrose density gradient fractions. Elastin mRNA in each fraction can be normalized by the use of 28S rRNA probes, in addition to the elastin probe (Ivester et al., 1995). Correcting for 28S RNA may make it possible to detect a relatively small shifts in the elastin mRNA population in response to stretch.

2. The "Black Box" in the Signal Transduction Pathway

We have assumed that PI3 kinase is involved in the stretch response on the basis of inhibition of the response by wortmannin, and by analogy to the insulin signaling pathway. However, it would be important to confirm directly that PI3 kinase activity is increased in response to stretch using a PI3 kinase assay.

If PI3 kinase is indeed a key player then it would be of interest to establish how the stretch signal is conveyed from PI3 kinase to S6 kinase. By analogy to known steps in the insulin signaling pathway, a connection may also exist between PI3 kinase and PKB kinase in the stretch signaling pathway. We could therefore determine the effect of stretch on PKB activity and the effect of inhibitors (wortmannin, rapamycin, and tyrphostin 25).

There remains some controversy on the role, if any, of MAP kinase activation in the insulin signaling pathway. A preliminary examination of the effect of stretch on MAP kinase activity indicated a stretch-induced effect which was less rapid than that seen for S6 kinase. These preliminary results should be investigated further.

3. Determine if Stretch Influences the Phosphorylation of eIF-4E and its Binding Protein 4E-BP1

Because of the known role of phosphorylation of 4E-BP1 and eIF-4E in regulation of translation, and the fact that this phosphorylation appears, at least in case of insulin to be controlled by a PI3 kinase - S6 kinase pathway, it would be important to investigate the effect of aortic stretch on phosphorylation of these proteins. Antibodies to 4E-BP1 and eIF-4E are available and could be used for these studies, providing that these antibodies were able to cross-react with and immunoprecipitate these factors in chicken. Identification a stretch-induced response would be followed by investigation of the effects of inhibitors (wortmannin, rapamycin, and tyrphostin 25) on the response.

4. *In Vitro* Translation Studies to Confirm Increased Translational Efficiency

Additional evidence to support increased translational efficiency might be obtained using *in vitro* translation studies. Vayda et al. (1995) demonstrated that it was possible to assess translational activity in tissue following environmental stimuli. This group analyzed protein synthesis in potato tubers under stressed and unstressed conditions by run-off translation of polysomes. When translation studies are undertaken with polysomes as a template, it is possible that all the factors, including binding and regulatory proteins, would be present in the polysome

complex. Therefore, it might be expected that a stretch-induced increase in translational activity of elastin mRNA might be evident in the *in vitro* translation assay. Although preliminary experiments using *in vitro* translation of isolated elastin mRNA were done, the absence of a good immunoprecipitating antibody for chicken elastin prevented these experiments from proceeding further at the time.

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