REGULATION OF FIBRONECTIN SYNTHESIS BY NITRIC OXIDE
DEPENDENT PHOSPHORYLATION OF LC3 AND BY A MAP KINASE
DEPENDENT MECHANISM

by

Caroline Fallery-Kinnear

A thesis submitted in conformity with the requirements for the degree of
Master of Science in the Graduate Department of Laboratory Medicine and
Pathobiology, in the University of Toronto

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Regulation of Fibronectin Synthesis by Nitric Oxide Dependent Phosphorylation of LC3 and by a MAP Kinase Dependent Mechanism

Master of Science, 2001
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ABSTRACT

Intimal cushion formation in the fetal ductus arteriosus (DA) requires fibronectin (FN)-dependent smooth muscle cell (SMC) migration. Nitric Oxide (NO) mediates increased FN mRNA translation in DA SMC through phosphorylation and binding of LC3, a microtubule-associated protein, to an AU rich element in the 3' UTR of FN mRNA. To investigate the mechanism whereby NO regulates LC3 phosphorylation, we assessed the influence of kinase inhibitors on FN synthesis. While extracellular signal-regulated kinase inhibition suppressed FN synthesis, this was not related to reduced LC3-mediated FN mRNA translation. We then sought to determine the specific NO-dependent phosphorylation site using an LC3 tagged green fluorescent protein (GFP) construct, transfected into DA SMC. In contrast to the perinuclear and microtubule associated distribution of native LC3, immunofluorescence was observed in vacuoles in LC3-GFP transfected cells and was increased by NO. Western immunoblotting revealed a truncated phosphorylated LC3-GFP, explaining the vacuolar distribution.
ACKNOWLEDGEMENTS

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I also thank my friends and family abroad for all their support. I have missed them tremendously. Lastly, I deeply thank my parents, Bernard and Odile Fallyery for their love, for letting me "leur petite derniere" settle far away, and for being proud of me and my achievements. I thank my mother for her daily e-mails which represented love and support, and helped me with being away from my family. I dedicate this thesis to my beloved parents.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENT</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>General Overview</td>
<td>1</td>
</tr>
<tr>
<td>I. The Blood Vessel Wall</td>
<td>2</td>
</tr>
<tr>
<td>Normal Structure</td>
<td>2</td>
</tr>
<tr>
<td>The Vascular Neointima in Disease</td>
<td>3</td>
</tr>
<tr>
<td>A Developmental Model of Neointimal Formation</td>
<td>3</td>
</tr>
<tr>
<td>II. Ductus Arteriosus</td>
<td>4</td>
</tr>
<tr>
<td>Physiological Role of the Ductus Arteriosus</td>
<td>4</td>
</tr>
<tr>
<td>Intimal Cushion Formation in the Ductus Arteriosus</td>
<td>5</td>
</tr>
<tr>
<td>Extracellular Matrix-Cell Interactions in Ductus Arteriosus Intimal Cushions</td>
<td>5</td>
</tr>
<tr>
<td>III. Fibronectin</td>
<td>6</td>
</tr>
<tr>
<td>Fibronectin and Cell Migration</td>
<td>6</td>
</tr>
<tr>
<td>Fibronectin in Neointimal Formation</td>
<td>7</td>
</tr>
<tr>
<td>Regulation of Fibronectin Synthesis</td>
<td>10</td>
</tr>
<tr>
<td>IV. Light Chain 3</td>
<td>11</td>
</tr>
<tr>
<td>Light Chain 3 Upregulates Fibronectin mRNA Translation</td>
<td>11</td>
</tr>
<tr>
<td>The N-Terminal of Light Chain 3 Upregulates Fibronectin mRNA Translation</td>
<td>14</td>
</tr>
<tr>
<td>Distribution and Phosphorylation of Light Chain 3</td>
<td>15</td>
</tr>
</tbody>
</table>
V. Nitric Oxide

| Nitric Oxide in Neointimal Formation of Vascular Disease | 16 |
| Nitric Oxide in Post-transcriptional Gene Regulation | 17 |
| Nitric Oxide in Light Chain 3-mediated Fibronectin Translational Upregulation | 17 |
| Nitric Oxide and phosphorylation | 21 |

VI. The Green Fluorescent Protein | 22 |

RATIONALE | 23 |

HYPOTHESIS | 24 |

OBJECTIVE I and II | 24 |

MATERIALS AND METHODS | 25 |

| Culture of Ductus Arteriosus Smooth Muscle Cells | 25 |
| Measurement of Fibronectin Synthesis | 25 |
| Preparation of Subcellular Fractions and Western Immunoblotting for LC3 | 27 |
| Northern Blotting | 28 |
| Molecular Cloning: Construction of LC3-GFP Expression Vector | 29 |
| Transfection of Cultured DA SMC | 29 |
| Transfection Efficiency | 30 |
| Preparation of Cell Lysates and Western Immunoblotting for GFP | 30 |
| Phosphatase Treatment of Cell Lysates Containing LC3-GFP | 31 |
| Immunofluorescence and Localization of LC3-GFP | 31 |
| Statistical Analyses | 31 |
RESULTS I

ERK Regulates Enhanced FN synthesis in DA SMC

ERK does not Mediate Expression of the Membrane-associated Form of LC-3 in DA SMC

ERK Regulation of FN Synthesis in DA SMC Involves Changes in Steady State mRNA Levels

ERK Might Influence FN mRNA Stability in DA SMC

PKG, PKA, PKC, Casein Kinases, PI3-K, S6K and Tyrosine Kinases do not Regulate FN Synthesis in DA SMC

Summary

RESULTS II

Production of LC3-GFP Fusion Protein

Transfection of Cultured DA SMC with the Negative Control Vector GFP and with LC3-GFP Expression Vector

Expression of LC3-GFP and GFP Vectors in Cultured DA SMC

Localization of LC3-GFP and Effect of NO in LC3-GFP Transfected DA SMC

Summary

DISCUSSION

FUTURE STUDIES

REFERENCES
## LIST OF FIGURES

1. Fibronectin-dependent mechanisms of intimal cushion formation in the DA. 9
2. FN is regulated at the post-transcriptional level. 13
3. Schematic summary of translational control of FN expression by mRNA sorting. 20
4. FN synthesis in DA SMC treated with NO modulators and a MEK inhibitor. 34
5. Expression of the membrane and the cytosolic-associated forms of LC3 in DA SMC treated with a MEK inhibitor. 37
6. Steady state levels of FN mRNA in DA SMC treated with the MEK inhibitor. 39
7. FN mRNA stability in DA SMC treated with the MEK inhibitor. 42
8. Proposed ERK-mediated mechanism of FN mRNA stability. 45
9. FN synthesis in DA SMC treated with aPKG inhibitor, a PKA inhibitor, and PKC inhibitors. 48
10. FN synthesis in DA SMC treated with casein kinases inhibitors, a PI3-kinase inhibitor, an S6 kinase inhibitor and tyrosine kinase inhibitors. 50
11. Summary of the effects of kinase inhibitors on FN synthesis in DA SMC. 53
12. Plasmid map of GFP and LC3-GFP expression vectors. 56
13. PCR amplification of LC3 coding region. 58
14. Restriction enzyme digestion (RED) of GFP and LC3 DNA. 60
15. Transfection efficiency of DA SMC transfected with GFP or LC3-GFP vectors (10X). 62
16. LC3-GFP and GFP expression in transfected DA SMC.

17. Immunofluorescence and localization of GFP and LC3-GFP transfected DA SMC treated with nitric oxide modulators (100X).

18. Quantification of immunofluorescence in LC3-GFP transfected DA SMC treated with SNAP or LNMMA.

19. Expression of LC3-GFP in transfected DA SMC treated with potato acid phosphatase and sequence of LC3 showing possible cleavage of LC3 and potential phosphorylation sites in the C-terminal region.
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>adenosine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ARE</td>
<td>adenosine uridine-rich element</td>
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<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine 3', 5'-monophosphate</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>DA</td>
<td>ductus arteriosus</td>
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<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRB</td>
<td>5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole</td>
</tr>
<tr>
<td>ecNOS</td>
<td>endothelial constitutive NOS</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis (β amino ethyl ether) tetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>FN</td>
<td>fibronectin</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony-stimulating factor</td>
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<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<td>------------------------------------</td>
</tr>
<tr>
<td>HA</td>
<td>haemagglutinin</td>
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<tr>
<td>HCl</td>
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<td>IL-1β</td>
<td>interleukin-1 β</td>
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<td>INF-γ</td>
<td>interferon-γ</td>
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<tr>
<td>kB</td>
<td>kilobase</td>
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<tr>
<td>kD</td>
<td>kilodalton</td>
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<tr>
<td>LC3</td>
<td>light chain 3</td>
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<tr>
<td>L-NMMA</td>
<td>N(^{\text{O1}})-monomethyl-L-arginine</td>
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<tr>
<td>MAP</td>
<td>microtubule associated protein</td>
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<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
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<tr>
<td>min</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>n</td>
<td>number of experiments</td>
</tr>
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<td>NaCl</td>
<td>sodium chloride</td>
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<tr>
<td>NaF</td>
<td>sodium fluoride</td>
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<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>P</td>
<td>phosphate</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAP</td>
<td>potato acid phosphatase</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
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<tr>
<td>PMSF</td>
<td>phenyl methyl sulfonyl fluoride</td>
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<td>PKA</td>
<td>protein kinase A</td>
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</tbody>
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PKC protein kinase C
PKG protein kinase G
PI3-K phosphatidylinositol 3-kinase
PVDF polyvinylidifluoride
RED restriction enzyme digestion
RER rough endoplasmic reticulum
RGD arginine, glycine, aspartate
RNA ribonucleic acid
rpm revolutions per minute
rRNA ribosomal ribonucleic acid
S sulphur
sec seconds
S6K S6 kinase
SE standard error
SDS sodium dodecyl sulphate
SEM standard error of the mean
SMC smooth muscle cell(s)
SNAP S-nitroso-N-acetylpencillamine
STAT signal transducer and activator of transcription
TBS tris buffered saline
TCA trichloroacetic acid
TGF-β transforming growth factor-β
TNF-α tumour necrosis factor-α
3'-UTR 3'-untranslated region
U uridine
UV ultraviolet
VEGF vascular epidermal growth factor
REGULATION OF FIBRONECTIN SYNTHESIS BY NITRIC OXIDE DEPENDENT PHOSPHORYLATION OF LC3 AND BY A MAP KINASE DEPENDENT MECHANISM

Caroline Fallery-Kinnear

2001
INTRODUCTION

General Overview
The intimal cushions which form in the late gestation fetal ductus arteriosus (DA), are similar to the neointima observed in a variety of vascular diseases including atherosclerosis, pulmonary hypertension, and restenosis. In the ductus arteriosus as well as in these vascular diseases, our laboratory has shown that formation of the neointima is characterized by increased synthesis of fibronectin (FN) by smooth muscle cell and this feature directs their migration from the media into the subendothelium. Using the ductus arteriosus as a model to study the molecular mechanism involved, we have observed that FN-dependent migration of DA smooth muscle cells is regulated by a post-transcriptional mechanism in which interaction of a microtubule-associated protein Light Chain 3 (LC3) with an adenosine-uridine rich element (ARE) in the 3' untranslated region of the FN mRNA upregulates FN mRNA translation through enhanced ribosome recruitment. Recent studies have suggested that this mechanism is dependent on nitric oxide (NO) which increases expression, and binding to the fibronectin mRNA ARE, of a membrane-associated phosphorylated form of LC3. The purpose of this study is to elucidate the mechanism whereby NO mediates LC3 phosphorylation by identifying the specific kinase involved and by investigating the specific phosphorylation sites on LC3. This will be important in addressing how phosphorylation of LC3 regulates its binding affinity to the ARE.
I. The Blood Vessel Wall

Normal Structure

In all blood vessels excluding precapillaries, capillaries and venules, three separate layers can be distinguished, the intima, media, and adventitia. The intima is composed of a single layer of endothelial cells lining the vascular lumen. A subendothelium consisting of one or at the most two layers of smooth muscle cells is present only in large elastic arteries such as the human aorta. The media is separated from the intima by the internal elastic lamina and consists of smooth muscle cells, and a rich extracellular matrix consisting of elastic laminae and bundles of collagen fibers interdigitating with glycoproteins and glycosaminoglycans. The adventitia is the most variable layer, containing fibroblasts, dense collagen, elastin fibrils, other extracellular matrix glycoproteins, blood vessels, and nerves. The two best characterized cell types in the vascular system are the endothelial cell and the smooth muscle cell. Because of their position, endothelial cells influence blood flow and hence are crucial to homeostasis in the vascular system. They secrete vasoactive substances that play a major role in the control of vascular tone (Furchgott and Zawadzki, 1980; Taylor and Weston, 1988; Yanagisawa et al., 1988). They also present an antithrombotic surface that resists platelet adhesion and does not activate coagulation (Gryglewski et al., 1988; Rosenberg and Rosenberg, 1984). Endothelial cells are held together by junctional complexes and this serves as a barrier, preventing or impeding highly mitogenic, thrombotic, or vasoactive substances from coming into direct contact with the underlying vascular smooth muscle (Svensjo and Grega, 1986). Smooth muscle cells (SMC) play a critical role in the arterial wall because of their capacity to maintain vascular tone. They have a well-developed contractile apparatus that includes α-actin, smooth muscle myosin and tropomyosin (Chamley-Campbell et al., 1981; Owens et al., 1986). In response to chemotactic agents and mitogens, SMC can be actively stimulated to migrate, replicate, and lay down new matrix (Campbell et al., 1988). The components of the matrix contribute to the biologic activities of vascular cells and these range from providing mechanical support,
to allowing cell adhesion, as well as affecting migration and proliferation (Liu et al., 1989; Wight, 1989; Davies and Hagen, 1994; Farhadian et al., 1996).

**The Vascular Neointima in Disease**

The production of a neointima is the hallmark of occlusive vascular diseases including atherosclerosis (Ross, 1986), pulmonary hypertension (Rabinovitch, 1998), restenosis (Gravanis and Roubin, 1989) and post-cardiac transplant coronary arteriopathy (Billingham, 1992). A thickened intima can be stimulated by various injuries such as electrical burning, radiation, angioplasty, venous and prosthetic bypass grafts, and endarterectomy (Chervu and Moore, 1990; Ip et al., 1990; Schwartz et al., 1995). Common features which characterize the formation of the neointima include fragmentation of elastic laminae and abnormal migration of SMC into a glycosaminoglycan-enriched subendothelium. The process of neointimal formation can be initiated by injury mediated alterations in endothelial cells which induce a cascade of intercellular and intracellular events (Libby et al., 1992). There is associated deposition of platelets, thrombus formation, migration of inflammatory cells and the release of various growth factors and cytokines such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), interleukin -1β (IL-1β), and transforming growth factor (TGF-β) from platelets, circulating monocytes, endothelial cells and SMC themselves. As a consequence of these events, the proto-oncogenes c-fos and c-myc, which have roles in promoting proliferation and differentiation of vascular SMC (Simons et al., 1992) are upregulated (Miano et al., 1990; Miano et al., 1993).

**A Developmental Model of Neointimal Formation**

Since neointimal formation is a major clinical problem associated with a wide spectrum of vascular disorders, several models have been developed to elucidate the cellular and molecular mechanisms involved. While the rat carotid balloon injury model was successful
in examining the proliferative characteristics of SMC, it has been less helpful in studying the features of SMC migration, or extracellular matrix production. Neointimal formation occurs as a normal developmental process in the fetal ductus arteriosus (DA). The biochemical and cellular mechanisms of intimal cushion formation in the DA are similar to pathologic neointimal formation: these include increased expression of growth factors, alterations in extracellular matrix production and a switch of the smooth muscle cell phenotype from a ‘contractile’ to a ‘synthetic’ and migratory state. In this respect, elucidating the mechanisms involved in intimal cushion formation in the DA should lead to new way to manipulate patency of this vessel in children with duct-dependent congenital heart defects and provide insight into the pathologic processes involved in vascular diseases.

II. Ductus Arteriosus

Physiological Role of the Ductus Arteriosus

Postnatally, the total cardiac output passes first through the lungs and then the systemic circulation. In the fetus, as the function of gas exchange is assumed by the placenta, only a small proportion of the combined ventricular output is directed to the pulmonary circulation, so that the metabolic role of the lung can be preserved. The ductus arteriosus, a blood vessel normally found in all mammalian fetuses connects the main pulmonary trunk with the descending aorta, and is necessary for the ventricular output to be diverted away from the lungs to the systemic circulation. Shortly after birth, the DA closes ensuring a proper transition from prenatal to postnatal circulation. Failure of the DA to close results in excess pulmonary blood flow and is associated with pulmonary hypertension, congestive heart failure and cardiac hypertrophy (Cassels and Moore, 1973). The ductus starts to close at birth with a strong vascular constriction that is triggered by the onset of breathing. The functional closure of the DA is, however, highly dependent on the formation of intimal cushions (Gittemberger-de Groot et al., 1985), a process which we observed is initiated
around 100 days of a 145-day gestation period in the fetal lamb DA, and is more or less completed by day 138 (Boudreau and Rabinovitch, 1991).

**Intimal Cushion Formation in the Ductus Arteriosus**

The formation of intimal cushions in the DA has been studied in humans (Gittenberger-de Groot et al., 1980; Silver et al., 1981) and in animal models including the lamb (Boudreau et al., 1992; Boudreau and Rabinovitch, 1991; Boudreau et al., 1991; Hinek et al., 1991; Hinek and Rabinovitch, 1993; Rabinovitch et al., 1988; Zhu et al., 1993), dog (De Reeder et al., 1988; De Reeder et al., 1989; Gittenberger-de Groot et al., 1985), and rabbit (Yoder et al., 1978). The process is initiated in the intima where it appears that accumulation of extracellular matrix components separates the endothelial cells from the internal elastic lamina. Smooth muscle cells (SMC) from the muscular media of the vessel wall then migrate into the matrix-enriched subendothelial region and contribute to the protrusion into the lumen of the thickened intima (intimal cushions), resulting in functional closure of the DA. The fetal lamb is the animal model used for the smooth muscle cell culture studies presented in this thesis. This animal model was chosen because it most closely resembles the human DA and it has been the model used for the majority of mechanistic studies to date, examining both intimal cushion formation as well as the physiology of the fetal and perinatal ductal circulation.

**Extracellular Matrix-Cell Interactions in Ductus Intimal Cushions**

Developmental studies of intimal cushion formation in the human and dog revealed that the first morphologic change observed in the DA is the deposition of extracellular matrix components, including fibronectin, collagen type III and mainly glycosaminoglycans (De Reeder et al., 1988; De Reeder et al., 1989; Gittenberger-de Groot et al., 1985; Rabinovitch et al., 1988; Slomp et al., 1992). The increased production of glycosaminoglycans, specifically hyaluronan acid by the endothelial cells and chondroitin
sulfate by smooth muscle cells, leads to their accumulation in the subendothelium (Boudreau and Rabinovitch, 1991). Hyaluronan acid provides the water-bound and matrix-enriched environment which favors SMC migration (Toole et al., 1984). The increased production of chondroitin sulfate from DA SMC has been shown to remove the elastin binding protein from the cell surface, leading to impaired assembly of elastin fibers (Hinek et al., 1991) and to the increased production of elastin peptides, favoring the movement of SMC into the subendothelium. Elastin peptides can directly influence the migration of SMC by inducing their production of the matrix glycoprotein fibronectin (Hinek et al., 1992).

III. Fibronectin

*Fibronectin and Cell Migration*

Fibronectin (FN) is a homodimeric secreted glycoprotein composed of subunits of 220-250 kD linked by disulfide bonds and it is found associated with the cell surface and incorporated into the extracellular matrix. Fibronectin plays a central role in cell adhesion, differentiation and migration (Hynes and Lander, 1992). It influences cell movement through its interaction with transmembrane extracellular matrix binding proteins, the integrins. Integrins consist of a family of $\alpha\beta$ heterodimeric transmembrane receptors which mediate cell-cell adhesion as well as cell-extracellular matrix interactions (Albelda and Buck, 1990). A specific RGD (arginine-glycine-aspartate) cell binding site in FN is recognized by primarily by $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins (Pierschbacher and Ruoslahti, 1984; Ruoslahti and Pierschbacher, 1987). Cell culture studies using blocking antibodies, specific to different integrin complexes, demonstrated that $\beta_1$ integrins were specifically required for DA SMC adhesion to FN while SMC migration required $\alpha_v\beta_3$ integrins (Clyman et al., 1992). Furthermore, immunohistochemical staining revealed expression of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins during intimal thickening in the DA (Clyman et al., 1996).
Fibronectin in Neointimal Formation

Fibronectin has been implicated in the regulation of SMC migration in neointimal formation in that the SMC migrate along a gradient of FN concentrations into the subendothelium via their interactions with integrins (Thyberg et al., 1990). Fibronectin gradient-dependent cell migration has also been demonstrated in the formation of the heart during embryogenesis and can be abrogated by antibodies to FN or RGD peptides, which block integrin binding to FN, resulting in inhibition of cardiac development (Linask and Lash, 1988a; Linask and Lash, 1988b). In primary SMC culture, FN promotes the transformation of SMC from a 'contractile' to a 'synthetic' phenotype. This process involves the loss of myofilaments, and cessation of the ability to contract. Moreover, in response to FN, an extensive rough endoplasmic reticulum and a large golgi complex are formed and SMC start to proliferate and produce extracellular-matrix components (Hedin et al., 1988; Hedin and Thyberg, 1987). This change to a 'synthetic' SMC phenotype is dependent on cell surface integrin-attachment to the RGD sequence of fibronectin (Hedin et al., 1989).

The FN-mediated-phenotypic change likely contributes to the enhanced SMC migration observed in the intimal cushion formation in the DA. Previous studies in our laboratory demonstrated that FN secretion by DA SMC from 100-day fetal lambs is twice that of aorta and pulmonary artery cells (Boudreau and Rabinovitch, 1991). In three-dimensional collagen gels, RGD peptides or FN antibodies reduce DA SMC motility (Boudreau et al., 1991). Increased FN therefore contributes to the enhanced DA SMC migration during the formation of intimal cushions in the DA (Figure 1). This is supported by the fact that FN synthesis is increased shortly after vascular injury and is deposited largely in the subendothelium where SMC are directed to migrate (Jones et al., 1997).
Figure 1. Fibronectin-dependent mechanisms of intimal cushion formation in the DA

At a 100 day gestation of the fetal lamb, the DA starts to undergo morphologic changes which leads to the formation of 'intimal cushions'. The cushions function to occlude this blood vessel following its constriction in the postnatal period. This process starts with the fragmentation of elastin and involves a FN-dependent vascular SMC migration from the media into the subendothelial space.
Ductus Arteriosus Intimal Cushion Formation

100 day fetal lamb DA

Term fetal lamb DA

Endothelium
Smooth Muscle
Fibronectin
Fragmented elastin
Increased FN expression has also been related to neointimal formation in human vascular diseases such as pulmonary hypertension (Botney et al., 1992; Jones et al., 1997; Molossi et al., 1993), restenosis (Clausell et al., 1995), atherosclerosis (Forsyth et al., 1997; Sanders, 1994; Shekhonin et al., 1987; Smith and Ashall, 1986; Stenman et al., 1980), and post cardiac transplant coronary arteriopathy (Molossi et al., 1993), the latter three involving FN induction by cytokines. In an experimental model of post cardiac transplant coronary arteriopathy, inhibition of FN-T cell interactions, resulted in reduced neointimal formation (Molossi et al., 1995b).

**Regulation of Fibronectin Synthesis**

The molecular mechanisms regulating FN synthesis have been studied in oncogenically-transformed cells in which FN expression is decreased. An example is the transformation of chicken embryo fibroblasts with Rous sarcoma virus which leads to a 5-fold reduction in cell surface FN (Olden and Yamada, 1977). In contrast, increased expression of FN is observed in embryogenesis, wound repair, and tissue remodeling and is regulated at both transcriptional and post-transcriptional levels. Growth factors and cytokines such as epidermal growth factor (EGF), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF) and interferon-γ (IFN-γ) activate FN gene transcription (Blatti et al., 1988; Chen et al., 1977; Diaz and Jimenez, 1997). Interleukin-1 β (IL-1β) upregulates gene transcription in vascular SMC (Clausell and Rabinovitch, 1993; Molossi et al., 1995a) whereas tumor necrosis factor-α (TNF-α), which cooperatively interacts with IL-1β induces FN synthesis in coronary artery SMC at a post-transcriptional level (Mason, 1999). A number of growth factors and cytokines have been shown to regulate FN expression at a post-transcriptional level through modulation of mRNA stability or translational efficiency. TGF-β increases FN gene transcription in fibroblasts (Ignatov et al., 1987), alters the splicing pattern of FN mRNA in cultured normal human fibroblasts (Borsi et al., 1990) and increases mRNA stability in cultures human dermal fibroblasts
(Raghow et al., 1987). In contrast, IFN-γ mediated decreased FN synthesis is associated with decreased FN mRNA levels by a mechanism which involves increased transcription but destabilization of FN mRNA and repression of its translation (Diaz and Jimenez, 1997; Levine et al., 1990).

We demonstrated in DA compared to aortic SMC that increased FN synthesis was not associated with increased FN mRNA levels or with differences in mRNA stability or splicing (Boudreau et al., 1992), suggesting that it may be the result of increased translational efficiency. Zhou et al. (1997) and Zhou and Rabinovitch (1998) then elucidated a role for an adenosine-uridine rich element (ARE) (5'-UUAUUUAU-3') in the 3'-untranslated region (3'-UTR) of FN mRNA in enhanced recruitment of FN mRNA into polyribosomes in DA SMC. A protein binding to the FN ARE was purified from sheep aorta (Zhou et al., 1997), and identified as light chain 3 (LC3) of the microtubule-associated protein 1A and 1B.

IV. Light Chain 3

**Light Chain 3 Upregulates Fibronectin mRNA Translation**

Light chain 3 (LC3), a 16.4 kD protein, was identified originally as a component that copurified with microtubule-associated proteins (MAP) 1A and 1B from rat brain (Mann and Hammarback, 1994). It was thought to play a role in regulating the microtubule binding activity of MAP1A and MAP1B. Zhou and colleagues purified LC3 from the DA and Aortic SMC as a cytoplasmic factor which binds the AU-rich element (ARE) of the 3' UTR of the FN mRNA (Zhou et al., 1997). Ductus SMC express increased levels of LC3 in the cytosol and enhanced LC3/fibronectin mRNA complex formation relative to Aortic SMC. A role for LC3 in post-transcriptional FN upregulation was then established by demonstrating that transfection of Aortic SMC with LC3 results in enhanced FN mRNA translation to levels observed in DA SMC without altering FN mRNA levels (Zhou et al., 1997) (Figure 2).
Figure 2. FN is regulated at the post-transcriptional level

The interaction of light chain 3 (LC3), a cytoplasmic RNA binding protein, with an AU rich element in the 3'UTR of FN mRNA enhances FN mRNA translation in DA SMC.
Fibronectin (FN) mRNA

Translation
The importance of LC3 in the remodeling of the DA was later confirmed by a gene transfer approach whereby the fetal lamb DA was transfected \textit{in utero} with a plasmid encoding 'decoy' RNA to sequester LC3. This resulted in a reduction in FN synthesis, and in the inhibition of SMC migration and intimal cushion formation (Mason et al., 1999a). LC3 was also shown to facilitate recruitment of FN mRNA into polyribosomes, a process which requires intact microtubules (Zhou and Rabinovitch, 1998). Since LC3 is co-purified with microtubules \textit{in vivo} and associated with microtubules assembled from purified tubulin \textit{in vitro} (Mann and Hammarback, 1994), microtubules appeared to also be involved in translational regulation of FN (Zhou et al., 1997). This was confirmed in studies in which microtubule disruption with colchicine prevented FN mRNA translation in DA cells, and decreased the association of FN mRNA and LC3 protein with rough endoplasmic reticulum (RER) membrane-bound polysomes (Zhou and Rabinovitch, 1998).

\textbf{N-Terminal of Light Chain 3 Upregulates Fibronectin mRNA Translation}

To further investigate the site on LC3 which binds to the AU-rich element (ARE) on the 3' UTR of the FN mRNA, cyanogen bromide was used to generate LC3 peptides from the 16 kD recombinant LC3. Northwestern blot analysis was then carried out to assess the ARE-binding activity of these peptides using a $[^{32}\text{P}]$-radiolabeled ARE oligonucleotide probe (Lau, 1999). A 10 kD N-terminal peptide showed strong ARE-binding activity while a 6.8 kD N-terminal peptide showed no binding. This suggests that the 10 kD N-terminal region of LC3 possesses the ARE-binding activity, specifically from residues S61 to M88. Site-directed mutagenesis followed by northwestern blot analysis then demonstrated that 3 consecutive arginine residues contained in this region are responsible for ARE binding of LC3, probably via a charge-charge interaction. Stable transfection of wild-type versus mutant LC3 into LC3-null HT1080 fibrosarcoma cells, demonstrated the significance of the arginine-rich motif contained in the N-terminal of LC3 in regulating FN mRNA translation (Lau, 1999).
**Distribution and Phosphorylation of Light Chain 3**

LC3 distributes between two distinct pools within the cell: the membrane-associated pool, which was shown by polysomal analysis to associate with FN mRNA in the polyribosomes where translation occurs (Zhou and Rabinovitch, 1998), and a tubulin-associated cytosolic pool, consistent with LC3 as a microtubule-associated protein (Zhou and Rabinovitch, 1998; Mason et al., 1999b, Kabeya et al., 2000). Our group examined whether the two forms of LC3 might represent different phosphorylation states, as this might influence their localization and function (Mason et al., 1999b) and whether phosphorylation of LC3 might be associated with fibronectin mRNA recruitment to membrane bound polyribosomes.

Phosphorylation involves the formation of a phosphate derivative of a biomolecule, by a kinase which catalyzes the transfer of a phosphate group from adenosine triphosphate (ATP) to the hydroxyl groups of certain serine, threonine, and tyrosine residues. The phosphate groups thus add negative charges to these polypeptides. The involvement of tyrosine and serine/threonine kinases in cytokine-induced signal transduction and anti-apoptotic pathways has been intensively examined. For example, tyrosine, serine and threonine phosphorylation of STAT transcription factors, results in their ‘activation’ and in their subsequent translocation to the nucleus to modulate gene expression. Phosphorylation of these ‘signaling’ molecules has been implicated in the etiology of certain human cancers such as chronic lymphocytic leukemia (McCubrey et al., 2000). On the other hand, phosphorylation of a specific serine residue of the human p53 tumor suppressor protein, actually abrogates inappropriate growth that leads to the development of tumors (Ullrich et al., 1993). Reactive oxygen species can also directly stimulate a phosphotyrosine kinase signaling cascade. For example, Zent et al. (1999) have shown that this signaling mechanism alters mesangial cell-extracellular matrix interactions under both physiological and pathological conditions.
To investigate whether LC3 might be phosphorylated, Mason et al. treated DA SMC cell cytosolic and membrane extracts with potato acid phosphatase, and assessed changes in molecular weights by Western immunoblotting (Mason et al., 1999b). Many phosphorylated proteins exhibit either upward or downward shifts in apparent molecular weight on SDS PAGE relative to their unphosphorylated form (Payne and Dahmus, 1993; Taleghany and Oblinger, 1992; Weng et al., 1998). When membrane extracts containing both forms of LC3 were treated with potato acid phosphatase (PAP), the lower molecular weight band disappeared and the higher molecular weight band was increased in intensity, whereas no effect on molecular weight of the cytosolic form of LC3 was observed. This suggests that the membrane-associated form of LC3 is phosphorylated (Mason et al., 1999b). Further studies were carried out to address the mechanism of this phosphorylation and established a link with nitric oxide.

V. Nitric Oxide

Nitric Oxide in Neointimal Formation of Vascular Disease

Nitric oxide, N=0−, (NO) is a lipid soluble inorganic free radical molecule, synthesized by nitric oxide synthase enzymes from L-arginine by the transfer of an electron from molecular oxygen to the guanidinium nitrogen of the amino acid. NO has been shown to act as an intracellular and transcellular signaling molecule and as a cytotoxic molecule involved in host defense (reviewed in (Moncada and Higgs, 1991)). In the vasculature, NO can play a protective role against occlusive disease through cyclic guanosine 3', 5'-monophosphate (cGMP)-mediated inhibitory effects on platelet aggregation (Gries et al., 1998; Radomski et al., 1987) and platelet activation (Diodati et al., 1998) as well as vascular smooth muscle cell proliferation (Garg and Hassid, 1989). On the other hand, NO also plays a pathogenic role in vascular diseases in which the developing neointima is associated with elevated NO synthase enzymes (Akyurek et al., 1996; Arthur et al., 1997; Hogg et al., 1993; Ravalli et al., 1997). Furthermore, NO has been implicated as both a positive (Brown et al., 1998;
Poppa et al., 1998) and negative (Sarkar et al., 1996; Kibbe et al., 1999) regulator of migration for both smooth muscle cells and endothelial cells.

**Nitric Oxide in Post-transcriptional Gene Regulation**

NO regulates gene expression at transcriptional (Janssen et al., 1997; Morris, 1995) and post-transcriptional levels (Hartsfield et al., 1997; Hentze and Kuhn, 1996; Pantopoulos and Hentze, 1995). For example, NO has been implicated in the post-transcriptional control of iron metabolism by modulating interactions between RNA binding proteins and regulatory elements in the untranslated regions of transferrin mRNA thereby altering mRNA stability and translational efficiency (Hentze and Kuhn, 1996; Pantopoulos and Hentze, 1995). There is increasing evidence that intracellular reactive oxygen intermediates regulate the binding of proteins to ARE elements in the 3'-UTRs of messenger RNAs (Kuroki et al., 1996; Levy et al., 1996a; Levy et al., 1996b). Kuroki et al. (1996) demonstrated that vascular endothelial derived growth factor (VEGF) is increased in hypoxia and in response to reactive oxygen intermediates, largely due to increased VEGF mRNA stability. Experimental studies revealed that increased VEGF mRNA stability under hypoxic conditions is mediated by enhanced binding of proteins to an ARE in the 3'-UTR of VEGF mRNA (Levy et al., 1996a; Levy et al., 1996b).

**Nitric Oxide and Light Chain 3-mediated Fibronectin Translational Upregulation**

NO is produced in the developing DA, where it can act to decrease vascular tone (Coceani et al., 1994). However, its physiological role as a vasodilator in the DA has been shown to be less important than prostaglandin E2 (Fox et al., 1996; Smith and McGrath, 1993). Thus our group examined whether NO may play a role in remodeling of the developing DA during the formation of intimal cushions. Significantly increased NO production was observed in primary cultured DA compared to Aortic SMC, associated with increased neuronal NO synthase (nNOS) expression. In addition, NO was shown to enhance ductus
FN synthesis without modulating FN mRNA levels (Mason et al., 1999b). This indicated that NO might regulate the previously described post-transcriptional mechanism related to enhanced production and binding of LC3 to the ARE in the FN mRNA 3'UTR.

The effect of NO on LC3 expression was examined by using western immunoblot analysis of cytosolic and membrane DA SMC fractions treated with a NO donor, S-nitroso-N-acetylpenicillamine (SNAP) and a NOS inhibitor, Nω-monomethyl-L-arginine (L-NMMA). SNAP significantly increased total expression of LC3 while inhibition of endogenous NO production by L-NMMA did not affect overall LC3 expression but instead appears to cause a shift in LC3 localization away from the membrane-associated pool into the cytosolic pool (Mason et al., 1999b). RNA gel mobility shift assays were then carried out and showed a much stronger ARE-binding complex formation in the membrane fractions reflecting the phosphorylated form of LC3 when compared with the cytosolic fractions in which tubulin-associated LC3 was found. An increase with SNAP and a decrease with L-NMMA was detected in the membrane fractions (Mason et al., 1999b). Taken together, increased NO would appear to result in enhanced availability, phosphorylation and binding of LC3 to the ARE of the FN mRNA.

These data support that the post-transcriptional mechanism of elevated fibronectin synthesis in DA smooth muscle cells is NO-dependent and involves increased expression, and binding to the fibronectin mRNA ARE of a membrane-associated phosphorylated form of LC3 (Figure 3). The mechanism by which NO enhances phosphorylation of LC3 was however, unknown.
Figure 3. Schematic summary of translational control of FN expression by mRNA sorting

FN mRNA is sorted onto RER by nascent signal peptide, and translation is then initiated. High constitutive levels of NO involve increased expression, and binding to the ARE of FN mRNA of a membrane-associated phosphorylated form of LC3. Binding of phosphorylated LC3 to FN mRNA and to microtubules facilitates sorting of FN mRNA onto RER and FN translation.
NO Regulates LC3 Production & Phosphorylation & Fibronectin mRNA Recruitment to Ribosomes
Nitric Oxide and Phosphorylation

NO has been implicated in a variety of different mechanisms leading to protein phosphorylation. Nakaya et al., (2000) reported that NO increases the functional activity of p53 by phosphorylation of particular residues, distinct from those that are phosphorylated following exposure to gamma-irradiation, UV light, and adriamycin. The mechanism whereby NO induces a specific kinase to phosphorylate p53 has not, however, been elucidated. In another study, peroxynitrite, the reaction product of NO and superoxide, was shown to activate phosphorylation of p38 mitogen activated protein kinase (MAPK). This induces growth arrest and DNA damage-inducible mRNA and protein in human neuroblastoma SH-SY5Y cells (Oh-Hashi et al., 2001). A role for NO in a MAPK signaling pathway has also been shown in endothelial cell proliferation. NO was shown to activate extracellular signal-regulated kinase (ERK) 1/2 by stimulating guanylate cyclase and the formation of cGMP and this was necessary for VEGF-induced endothelial cell growth (Parenti et al., 1998).

NO has also been shown to prevent phosphorylation. Llovera et al., (2001), demonstrated that endogenous production of NO inhibits signaling pathways activated by IFN-γ in the macrophage cell line J774. This was due, at least in part, to direct nitration of tyrosine residues in STAT1 which prevented its phosphorylation. Protein tyrosine nitration results from the generation of reactive nitrogen species, peroxynitrite and nitryl (nitronium) chloride (Halliwell, 1997). NO has been shown to repress ERK phosphorylation in vascular SMC, inhibiting the transcription of elastase, a proteolytic enzyme implicated in various cardiovascular diseases (Mitani et al., 2000). The mechanism is related to the NO mediated induction of protein kinase G (PKG) which interferes with the Ras/Raf pathway.

Thus, in elucidating the role of NO in the phosphorylation of LC3 as this relates to enhanced FN mRNA translation, it will be necessary to link an NO dependent kinase with a
specific pattern of phosphorylation. A variety of kinase inhibitors are available which can be used to pinpoint candidate phosphorylation sequences in LC3 and peptide mapping of the phosphorylation site can be carried out following immunoprecipitation of LC3. In the absence of antibodies which will effectively precipitate the LC3, a molecular strategy can be used to engineer an LC3–tagged fusion protein. An antibody to the tagged region can then be used to immunoprecipitate the protein. In considering the creation of this fusion protein, it is necessary to assure that the function is not altered, and that additional dynamic information about the protein might be gained.

VI. The Green Fluorescent Protein
The green fluorescent protein (GFP) is a spontaneously fluorescent polypeptide of 27 kD from the jellyfish Aequorea victoria that absorbs UV-blue light and emits in the green region of the spectrum (Sacchetti et al., 2000). As a fusion tag GFP can be used to immunoprecipitate proteins but since it can also be used to study their localization and dynamics in living cells, it has quickly become a primary tool in cell biology (Margolin, 2000).

For example, GFP has enabled visualization of the movement of an essential protein constituent of the mRNA transport machinery in living neurons. The fusion of Staufen, an RNA-binding protein characterized in rat hippocampal neurons, with GFP has revealed a microtubule-dependent transport pathway involving RNA-containing granules with Staufen as a core component (Kohrmann et al., 1999). GFP tagging also allowed investigators to document the phosphorylation of CLN3, the gene product associated with Batten disease. In vivo labeling of a GFP-CLN3 transfected ovary cell line with $[^{32}\text{P}]$ was followed by immunoprecipitation with anti-GFP antibody and phosphoamino acid analysis demonstrated that CLN3 is phosphorylated on serine and threonine residues (Michalewski et al., 1999).
Previous studies in our laboratory demonstrated that nitric oxide (NO) mediates increased fibronectin (FN) translation in ductus arteriosus (DA) SMC through enhanced phosphorylation and binding of light chain 3 (LC3) to an AU rich element in the 3' UTR of FN mRNA (Zhou et al., 1997; Mason et al., 1999b). In view of the importance of protein phosphorylation in regulating gene expression, this report investigates the mechanism of LC3 phosphorylation involved in increased FN mRNA translation.

Computer-based analysis of LC3 protein structure indicates that it contains many serine, threonine and tyrosine residues that can be phosphorylated by different kinases. The kinase involved can be identified by evaluating the influence of kinase inhibitors on constitutive and exogenous NO-dependent FN synthesis in DA SMC, as analyzed by gelatin-sepharose extraction following [35S]-metabolic labeling. Further studies can then determine whether this correlates with decreased expression of phosphorylated LC3.

Another approach could be to map the NO dependent phosphorylation site following immunoprecipitation. Since two previously produced antibodies to LC3 were unsuccessful in immunoprecipitating this protein, the strategy of expressing LC3 fused with the green fluorescent protein (GFP) would allow us to immunoprecipitate the complex with an anti-GFP antibody. This would give us the added advantage of localizing the GFP labeled LC3 in living cells. Therefore, transient transfection of ductus arteriosus SMC with LC3 fused to GFP in the presence and absence of NO could be used as a strategy to identify the phosphorylation sites important for enhanced NO dependent FN mRNA translation.
HYPOTHESIS

A specific kinase, regulated by NO, phosphorylates LC3 in the regulation of FN mRNA translation. The amino acids phosphorylated can be identified.

OBJECTIVES

1. Determine which kinase phosphorylates LC3 in the regulation of FN mRNA translation, taking into consideration, kinases known to be modulated by NO as well as phosphorylation consensus sequences in LC3.

2. Transfect DA SMC with an LC3-GFP expression vector so that the product (LC3-GFP) can be immunoprecipitated and the amino acids phosphorylated, identified.
MATERIALS AND METHODS

**Culture of Ductus Arteriosus Smooth Muscle Cells**

DA SMC were isolated for primary cultures from fetal lambs at 100 days of gestation. The vessel was opened, rinsed in Dulbecco's phosphate-buffered saline (PBS) containing 3% antibiotics/antimycotics (GIBCO BRL, Burlington, ON, Canada) and the adventitia was removed. Endothelial cells were removed by scraping the vessels with a No. 11 scalpel blade. The media was cut into 1 mm square pieces which were allowed to adhere to 100 mm culture dishes before the addition of Medium 199 (Cell Grow, Hearndon, VA) containing 1% antibiotics/antimycotics and 10% fetal calf serum (Cell Grow). SMC were allowed to grow out from the explants for three to four weeks in culture before passaging. DA SMC propagated from these explants were used for experiments after two passages.

**Measurement of Fibronectin Synthesis**

Semi-confluent DA SMC plated in 35 mm dishes at passage 2 were incubated in 2 ml serum-free and cysteine/methionine-free DMEM (ICN Biomedicals Inc., Aurora, OH) containing 1% bovine serum albumin (BSA) for 1 h. This media was then replaced and 10 μCi/ml of [35S]-methionine (Amersham, Baie d'Urfé, QC, Canada) were added with the inhibitors and donors described below. The conditioned media were collected for analysis of FN protein after 4 h. Triplicate assessments of total protein synthesis were obtained from 50 μl aliquots of culture medium precipitated in 2% BSA/25% trichloroacetic acid (TCA) and analyzed by liquid scintillation spectrometry. To measure FN synthesis, 1 ml aliquots of culture medium were incubated with 100 μl gelatin 4B-sepharose beads (Pharmacia Biotech, Uppsala, Sweden) overnight at 4°C. The beads were washed three times in PBS and bound FN eluted from the beads into 60 μl SDS sample buffer (5% β-mercaptoethanol, 2% SDS, 10% glycerol, 62.5 mM TRIS-HCl pH 6.8) by boiling for 5 min. The samples were then separated on a 6% SDS polyacrylamide gel electrophoresis.
(PAGE), corrected for total protein synthesis. Gels were fixed in 5% acetic acid/10% methanol for 30 min and soaked in Amplify (Amersham) for 15 min before drying under vacuum at 80°C on a Model 443 slab dryer (Bio-Rad, Hercules, CA). Dried gels were exposed to Kodak X-OMAT AR film for 2 to 6 days and the 220 kD band corresponding to FN was excised from the gel and counted by liquid scintillation spectrometry. Identification of this 220 kD band as FN was confirmed in our laboratory by western blotting (Boudreau and Rabinovitch, 1991).

FN synthesis was measured in the presence of the following compounds:

a. Nitric oxide (NO) donor, SNAP (100 μM) (BIOMOL Research Lab, Plymouth meeting, PA)
b. NO synthase inhibitor, L-NMMA (250 μM) (SIGMA, St Louis, MO)
c. MAPK/ERK kinase (MEK) 1/2 inhibitor, PD 98059 (100 μM) (Calbiochem, La Jolla, CA)
d. Protein kinase G (PKG) inhibitor Rp-8-pCPT-cGMP (20 μM) (Alexis Corp., San Diego, CA)
e. Protein kinase A (PKA) inhibitor, H89 (500 nM) (Calbiochem)
f. Protein kinase C (PKC) inhibitors:
   - chelerytrine (1 μM) (SIGMA)
   - staurosporine (10 nM) (SIGMA)
   - N-myristylated peptide 19-27 (20 and 40 μM) (Calbiochem)
   - Bisindolylmaleimide I (1 μM and 100nM) (Calbiochem)
g. Casein kinase I inhibitor, CKI-7 (10 μM and 100 μM) (Seikagaku Corp. Tokyo, Japan)
h. Casein kinase II inhibitor, DRB (6 μM and 500 nM) (Calbiochem)
i. PI3-kinase inhibitor, wortmannin (10 nM and 50 nM) (Calbiochem)
j. S6 kinase inhibitor, rapamycin (10 nM and 50 nM) (Calbiochem)
k. Tyrosine kinase inhibitors:
   - PD 153035 (1μM and 100 nM) (Calbiochem)
   - Genistein (50 μM and 10 μM) (Calbiochem)
The concentrations for each compound were chosen according to what has been used in the literature, mostly in vascular smooth muscle cells. SNAP (Ceneviva et al., 1998; Wang et al., 1999). LNMMA (Mason et al, 1999b). PD (Parenti et al., 1998; Gorenne et al., 1998). Rp-8-pCPT-cGMP (Hichami et al., 1996; Yamakage et al., 1996). H89 (Liu et al., 2000; Fawzi et al., 2000). Chelerythrine (Yoshida et al., 1999; Kutz et al., 1998). Staurosporine (Kageyama et al., 1991; Du et al., 1996). N-myristylated peptide 19-27 (Bergman et al, 1997; Eichholtz et al., 1993). Bisindolylmaleimide I (Weissmann et al., 1999; Grataroli et al., 2000). CKI-7 (Ankrapp et al., 1996; Tang et al., 1995). DRB (Kee et al., 1998; Critchfield et al., 1997). Wortmanin (Misawa and Yamaguchi, 2000; Imai and Clemmons, 1999). Rapamycin (Seidel and Ragan, 1997; Roques and Vidal, 1999). PD 153035 (Lange et al., 1997; Bos et al., 1997). Genistein (Sun et al., 1999; Utz et al., 1999).

Preparation of Subcellular Fractions and Western Immunoblotting for LC3

Semi-confluent DA SMC were harvested by scraping into PBS and spun at 2000 rpm (1000xg) in a TJ-6 centrifuge (Beckman, Mississauga, ON, Canada) for 10 min. Cells were resuspended in two volumes of hypotonic buffer (0.1 mM EDTA, 25 mM Tris-HCl pH 7.9) with a phosphatase inhibitor, sodium vanadate (1 mM) (SIGMA), and lysed by three cycles of freeze-thaw. Cytosolic extracts were isolated by centrifugation for 30 min at 16000Xg at 4°C. Pellets were solubilized in 1% SDS. Protein concentration was determined using the BCA protein assay (PIERCE, Rockford, IL) followed by spectrophotometry at 595 nm. Protein extracts (15 µg) from DA SMC in Laemmli sample buffer (5% β-mercaptoethanol, 2% SDS, 10% glycerol, 62.5 mM TRIS-HCl pH 6.8) were separated by SDS PAGE (8-16% Tris-Glycine gel, NOVEX, San Diego, CA) and transferred to a polyvinylidifluoride (PVDF) membrane. Gels were stained with Coomassie Blue to visualize bands and confirm equal protein loading.
Membranes were blocked for 1 h in Tris-buffered saline (TBS) containing 0.5% tween-20 and 5% non-fat dry milk and probed overnight at 4°C with rabbit antiserum to LC3 (1:2000) (supplied by Dr. J. Hammerback, Department of Neurobiology and Anatomy, Bowman Gray School of Medicine, Winston-Salem, NC). Blots were washed 4 times for 1 h at room temperature using TBS containing 0.5% tween-20 and then incubated with peroxidase-conjugated goat anti-rabbit IgG (1:3000) (Amersham). The blots were washed 4 times as described above before developing using enhanced chemiluminescence western blotting detection reagents (Amersham). The intensity of immunoreactive bands was analysed using Multi Analyst (Biorad) software.

**Northern Blotting**

Total RNA was isolated from cultured DA SMC using TRIZOL reagent (GIBCO BRL). Cells were lysed in TRIZOL (1 ml/100 mm dish) by repeated pipetting. The cell lysate was extracted with chloroform and RNA was precipitated with isopropyl alcohol and dissolved in 0.5% SDS in 0.1% diethylpyrocarbonate (DEPC)-treated water. Ten μg of total RNA from each sample were resolved on a 1% agarose gel containing 6% formaldehyde and transferred to nitrocellulose membrane. Membranes were probed with [32P]-dCTP (10⁶ cpm/ml) (Amersham) labeled human FN cDNA probe (1.4 kB) (GIBCO BRL) in hybridization buffer (6XSSC, 5X Denhardt's reagent, 0.5% SDS, 100 μg/ml denatured fragmented salmon sperm DNA, 50% deionized formamide) overnight at 42°C. Membranes were washed twice in 2 X SSC/0.1% SDS at 55°C for 30 min and once with 0.2 X SSC/0.1% SDS at 65°C for 1 h, before exposure to Kodak X OMAT film. Ethidium bromide staining of 28S and 18S ribosome RNAs served to control for loading conditions.

To investigate the stability of FN mRNA, actinomycin D (SIGMA) was added to cultured DA SMC at a concentration of 5 μg/ml, and cells were incubated from various times, from
6 to 24 h before extraction of total RNA. Northern blot analyses were then carried out as described on each sample as described above.

**Molecular Cloning: Construction of LC3-GFP Expression Vector**

A cDNA encoding LC3 (142 amino acids) was obtained by polymerase chain reaction (PCR) from rat total cDNA with the forward primer 5'-GGGAAGCTTCCCATATGACGTCCGAG-3' and the reverse primer 5'-CCCGGGCCGCAGCATGG-3'. The GFP expression vector, pEGFP-N1 (Clontech Laboratories, Palo Alto, CA), and LC3 cDNA were digested with Hind III and APA I restriction enzymes. LC3 cDNA was then ligated to the vector proximal to the N-terminal of GFP by incubating the purified gel products with ligation buffer and T4 DNA ligase (1 unit) overnight at 12°C. Transformation was then performed by adding 5 µl of ligation reaction to 50 µl of Top10F competent cells (Invitrogen, Carlsbad, CA). Bacteria cells were incubated on ice for 30 min, heat shocked for 30 sec at 42°C and placed on ice again. 250 µl of SOC medium were added and bacteria were shaken in a rotary shaker for 1 h at 225 rpm at 37°C. Transformed cells (100 and 200 µl) were plated separately onto agar plates containing 30 µg/ml kanamycin and incubated at 37°C overnight. Plasmid DNA was isolated from individual clones and the presence of the correct insert was confirmed by restriction enzyme digestion with Hind III and APA I and gel electrophoresis. Purified DNA was sent for sequence analysis (DNA sequencing facility, Biotechnology Centre, The Hospital for Sick Children, Toronto, ON, Canada) to confirm the correct sequence of LC3-GFP.

**Transfection of Cultured DA SMC**

Twenty-four h prior to transfection, DA smooth muscle cells were plated at a density of 2 x 10^5 cells/60-mm dish in Medium 199 containing 1% antibiotics/antimycotics and 10% fetal calf serum. DA cells were transiently transfected with either the LC3-GFP expression
vector or the GFP expression vector as a negative control using Effectene Transfection Reagent (Quiagen, Mississauga, ON, Canada). Four μg of either the LC3-GFP or the GFP expression vector were incubated with 80 μl Effectene Reagent to transfect each dish for 16 h. Two μg of pSV-β-gal vector (Promega, Madison, WI) were used to co-transfect the cells. Mock transfection was performed under the same conditions as the above constructs in the absence of DNA. Cells were washed with warm PBS and then incubated in culture media containing serum and antibiotics. Each fusion construct was tested at least three times to optimize the conditions of transfection.

Transfection Efficiency
Twenty-four to 48 h post-transfection, living cells were examined with a fluorescence microscope using a 10X lens (Nikon, Eclipse E1000). The GFP fluorescence signal of the transfected cells allowed us to acquire images to observe the transfection efficiency. Cells co-transfected with LC3-GFP or GFP vectors and the pSV-β-gal vector were washed twice in PBS and fixed in 1% formaldehyde and 0.2% glutaraldehyde for 5 min at room temperature. Cells were washed four times and stained for β-galactosidase at 37°C overnight with an X-gal solution.

Preparation of Cell Lysates and Western Immunoblot for GFP
Forty-eight hours post-transfection, DA SMC were washed twice in cold PBS and once in 1 mM sodium orthovanadate prior to lysis. Cells were then incubated for 10 min at 4°C with 0.5 ml lysis buffer (50 mM TRIS-HCl (pH 7.4), 150 mM NaCl, 1 % Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EGTA), containing the phosphatase inhibitors (1 mM sodium orthovanadate and 1 mM NaF), and protease inhibitors (1 mM PMSF, 2 μg/ml aprotinin and 2 μg/ml leupeptin). Cell debris was collected by centrifugation for 20 min at 16 000g at 4°C, and protein content was determined with the BCA protein assay. Western blotting was carried out as described above except that blots were blocked overnight at 4°C.
in PBS containing 0.5% tween-20 and 5% non-fat dry milk, probed with the monoclonal anti-GFP antibody (1:500) (Clontech Laboratories) for 2 h at room temperature and incubated with peroxidase-conjugated goat anti-mouse IgG (1:10 000) (Amersham).

**Phosphatase Treatment of Cell Lysates Containing LC3-GFP**

Cells lysates (10µg) from LC3-GFP, GFP and Mock transfected cells were incubated with 0.3 units of potato acid phosphatase (PAP) (SIGMA) at 37°C for 1 h in TRIS buffer, pH 6.8. Lysates were then analyzed by SDS-PAGE, followed by western immunoblotting for GFP expression.

**Immunofluorescence and Localization of LC3-GFP**

Forty-eight hours after transfection with LC3-GFP and GFP vectors, DA SMC were incubated in serum-free and cysteine/methionine-free medium 199 containing 1% BSA for 1 h. This media was then replaced and the nitric oxide (NO) donor, SNAP and the NO synthase inhibitor, L-NMMA were added for 4 h. Cells were then washed twice with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, washed again and mounted on glass coverslips with antifade reagent (Molecular Probes Inc., Eugene, OR). Confocal microscopy was used to localize LC3-GFP in transfected cells. Quantification of immunofluorescence was performed on fixed LC3-GFP transfected DA SMC treated with SNAP and L-NMMA using Image-Pro Plus software.

**Statistical Analyses**

A one way analysis of variance (ANOVA) was used to detect differences between the treatments of DA SMC. Post hoc comparison of individual groups was carried out using a Fisher's test. A level of P<0.05 was accepted as statistically significant. Data are represented as mean ±SEM. The number of experiments is given in the figure legends.
RESULTS I

ERK Regulates Enhanced FN Synthesis in DA SMC

We previously showed that enhanced SMC FN synthesis involves increased mRNA translation through recruitment of FN mRNA to the polyribosomes as a result of nitric oxide (NO)-mediated binding of a phosphorylated form of LC3 to the ARE of FN mRNA (Zhou et al, 1997; Zhou and Rabinovitch, 1998; Mason et al, 1999b). To elucidate which kinase phosphorylates LC3 in the regulation of FN mRNA translation, we evaluated the influence of kinase inhibitors on constitutive and exogenous NO-dependent FN synthesis in DA SMC. LC3 contains several potential sites for serine/threonine phosphorylation by MAP kinases, so we investigated the role of ERK 1/2, a subfamily of MAP kinases, in NO-induced fibronectin expression. To this end, DA SMC were treated with the NO donor (SNAP) ± the MAPK/ERK kinase (MEK) 1/2 inhibitor PD 98059 (PD), with PD alone and with the NO synthase inhibitor, L-NMMA. Cells were metabolically labeled with [35S]-methionine for 4 h and newly-synthesized FN secreted into the culture media was measured. Equal amounts of proteins in culture media, as determined by TCA precipitation, were incubated with gelatin sepharose beads to affinity-purify FN, which was eluted and resolved by SDS-PAGE (Figure 4).

Treatment of DA SMC with SNAP for 4 h caused a onefold increase in FN synthesis above basal levels (P<0.05), and incubation with L-NMMA caused a ~50% decrease in FN synthesis relative to levels in control cells, as previously shown by Mason et al (1999b). When cells were treated with SNAP along with PD, the MEK inhibitor abrogated the NO-induced FN synthesis. Furthermore, PD alone inhibited DA SMC FN synthesis (P<0.05) below basal levels. This indicates, in DA SMC, that phosphorylation of ERK is necessary in mediating NO dependent FN expression.
Figure 4: FN synthesis in DA SMC treated with NO modulators and a MEK inhibitor.

Representative autoradiographs of secreted FN resolved by SDS PAGE following [35S]-methionine labeling and gelatine sepharose extraction. FN expression in untreated DA SMC (Con) and after 4h treatment with a nitric oxide donor: SNAP +/- PD 98059 (PD), a MEK 1/2 inhibitor, PD alone and a nitric oxide synthase inhibitor, L-NMMA. The increase in FN synthesis with SNAP (P<0.05) was abolished by PD. Both L-NMMA and PD inhibited DA SMC FN synthesis (P<0.05) but PD decreased FN synthesis below basal levels. Each bar represents the mean of 4 experiments ±SEM. * denotes P<0.05 versus control cells.
ERK Does Not Mediate Expression of the Membrane-Associated Form of LC3 in DA SMC

To determine whether PD 98059 reduction of NO-induced FN synthesis correlates with decreased expression of phosphorylated LC3, we carried out western immunoblot analyses followed by densitometry (Figure 5). Two different molecular weight forms of LC3 have been identified, a tubulin-containing cytosolic form and a membrane-associated form which was shown to be phosphorylated and responsible for FN mRNA recruitment onto polyribosomes (Mason et al., 1999b). To evaluate the effect of ERK on LC3 expression, we treated DA SMC with PD 98059 (PD) for 4 h and used freeze and thaw and centrifugation of cell lysates to separate cytosolic extracts in the supernatant from cell membranes in the remaining cell pellet. The higher molecular weight form of LC3 that co-localizes with tubulin in the supernatant was increased by PD, but this form is not related to the mechanism of LC3 dependent post-transcriptional regulation of FN. The lower molecular weight membrane-associated and phosphorylated form in the lysed cell pellet was unaffected. *In vitro* phosphorylation assays also failed to demonstrate LC3 phosphorylation by ERK-2 (data not shown). Thus, the PD dependent decrease in FN synthesis appears independent of the previously described post-transcriptional mechanism which is associated with LC3 phosphorylation.

ERK Regulation of FN Synthesis in DA SMC Involves Changes in Steady State mRNA Levels

Having reported that ERK induces DA SMC FN synthesis without activating LC3 phosphorylation, we next elucidated the role of ERK in FN synthesis by performing northern blot analyses followed by densitometry (Figure 6). We measured FN mRNA levels with respect to 18S and 28S rRNA in untreated DA SMC and after a 4 h treatment with the MEK inhibitor PD 98059 (PD). PD decreased (P<0.05) FN mRNA levels with respect to 18S rRNA as compared to control. Our group previously showed that despite
Figure 5: Expression of the membrane and the cytosolic-associated forms of LC3 in DA SMC treated with a MEK inhibitor.

Representative western blot of LC3 in the cell membrane (pellet) and cytosolic protein extracts (supernatant) from DA SMC (Con) and cells treated with PD 98059 (PD) for 4 h in culture. Densitometric analyses illustrate that the cytosolic form of LC3 was increased by PD and that the membrane associated LC3 was not affected by PD. Each bar represents the mean of 4 experiments ±SEM. * denotes P<0.05 versus control cells.
Figure 6: Steady state levels of FN mRNA in DA SMC treated with the MEK inhibitor.

Top: Representative autoradiograph of northern blot probed with $^{32}$P dCTP labeled human FN cDNA using total RNA extracts from untreated DA SMC (Con) and cells treated with PD 98059 (PD). Ethidium bromide stained 18S and 28S ribosomal RNA were used to measure FN mRNA levels according to equivalent loading. Bottom: Densitometric analyses of FN mRNA levels depict a significance difference in FN mRNA levels relative to 18S ribosomal RNA following PD treatment. Each bar represents the mean of 4 experiments ±SEM. * denotes p<0.05 versus control cells.
increased FN synthesis in DA compared to aortic SMC, steady state levels of FN mRNA were not elevated (Boudreau et al., 1992). Here we demonstrate that ERK influences FN mRNA levels in DA SMC. This confirms that ERK does not regulate the previously described post-transcriptional control mechanism related to enhanced production and binding of LC3 to the ARE in the FN mRNA 3'UTR. Instead, ERK regulates FN at a transcriptional level or by influencing stability of the mRNA.

**ERK Might Influence FN mRNA Stability in DA SMC**

We then addressed whether the differences in FN mRNA state levels in the presence of the MEK inhibitor could be attributed to alterations in mRNA stability. Transcription was inhibited by actinomycin D and the FN mRNA remaining at various time-points ranging from 6 to 24 h was analyzed by northern blot. Any differences in total RNA loading were corrected by standardizing to 18S rRNA. mRNA decay curves were then generated by plotting the remaining RNA of three experiments to related time-point (Figure 7). The same pattern of mRNA destabilization is observed with a calculated mRNA half-life of approximately 24 h for untreated DA SMC and 18 h for PD treated cells. Fisher's test confirmed that the MEK inhibitor (PD) did not affect (P<0.05) FN mRNA levels over the different time points compared with FN mRNA levels in untreated DA SMC.

We showed that PD decreased FN mRNA levels after a 4 h incubation (Figure 6), however actinomycin D which abrogates transcription did not significantly reduce mRNA levels in the presence or absence of PD after 6 h (Figure 7). This suggests that PD reduction in FN mRNA levels is not related to a decrease in FN mRNA transcription. PD might therefore influence FN mRNA stability. No significant differences were however detected in PD treated cells as compared to untreated cells, indicating that the effect of PD might be blocked by actinomycin D.
Figure 7: FN mRNA stability in DA SMC treated with the MEK inhibitor.  
Top: Northern blot analysis of total RNA extracts from untreated DA SMC (Con) and cells treated with PD 98059 (PD) following actinomycin D inhibited transcription at various time points. Membranes were probed with $[^{32}P]$ dCTP labeled human FN cDNA. Ethidium bromide stained 18S and 28S ribosomal RNA from each sample demonstrates equal loading of the lanes. Bottom: Line graph depicting the natural logarithmic (LN) values of FN mRNA versus time after standardizing to 18S ribosomal RNA. The error bars represent the SE for triplicate samples. No significant difference (P<0.05) in FN mRNA levels was detected following PD treatment versus control cells over the different time points. mRNA half-life was determined by simple linear regression analysis.
FN mRNA: The figure shows the expression levels of FN mRNA over time for control and PD groups. The data indicate that PD leads to a decrease in FN mRNA expression compared to control, with a halflife of 18 hours for PD and 24 hours for control.

- **Control**: Time = 0, 6, 12, 24 hours
- **PD**: Time = 6, 12, 24 hours

The graph plots FN mRNA levels (normalized to 18S) against time (h), showing a logarithmic decrease over time.
There is growing evidence that actinomycin D may have significant effects on mRNA turnover. The addition of actinomycin D to NIH 3T3 cells stabilized transgenic c-fos and GM-CSF ARE-containing mRNAs (Chen et al., 1995). Similarly, the levels of glial glutamate transporter mRNAs did not decrease even after 24 h of treatment with actinomycin D (Zelenaia and Robinson, 2000). Also, cis elements, which normally destabilize c-fos (Shyu et al., 1989), c-myc (Wisdom and Lee, 1991) or erythropoietin mRNAs (Goldberg, et al., 1991) are nonfunctional in the presence of actinomycin D. While the underlying mechanisms for these effects are unclear, they cast significant doubt on the validity of mRNA decay measurements in the presence of this transcriptional blocker.

For example, it is possible that PD activates a factor which would normally destabilize FN mRNA, but actinomycin might inhibit the transcription of this destabilizing factor and therefore result in stable mRNA. Taken together, we suggest that ERK stabilizes FN mRNA in DA SMC by repressing a destabilizing factor (Figure 8). Alternatively, it is possible that ERK inhibits a factor that represses transcription that is also inhibited by actinomycin.

PKG, PKA, PKC, Casein Kinases, PI3-K, S6K and Tyrosine Kinases do not Regulate FN Synthesis in DA SMC

To pursue the identification of the kinase that phosphorylates LC3 in the regulation of FN mRNA translation, we evaluated the influence of other kinase inhibitors on FN synthesis in DA SMC. NO activates guanylate cyclase resulting in the formation of cyclic GMP, a signaling molecule which mediates the effects of nitric oxide as a vasodilator and inhibitor of SMC proliferation (reviewed in (Cohen 1995)). To determine whether protein kinase G (PKG) is implicated in enhanced NO-dependent FN expression, we compared FN synthesis in untreated DA SMC (C) to those treated with the cGMP-PKG inhibitor
Figure 8: Proposed ERK-mediated mechanism of FN mRNA stability.

Top: ERK might stabilize FN mRNA by repressing a destabilizing factor. The MEK inhibitor, PD 98059, stimulates this factor which destabilizes mRNA. Bottom: Actinomycin D inhibits transcription of the destabilizing factor, blocking the effect of PD 98059 and therefore stabilizes FN mRNA.
Rp cGMP (Figure 9, A). Each bar represents the mean of 3 experiments ±SEM. Rp cGMP did not alter FN synthesis in DA SMC.

The LC3 amino acid sequence contains phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC). *In vitro* phosphorylation of the LC3-GST fusion protein was assessed and showed that LC3-GST is phosphorylated by PKA and PKC *in vitro* (data not shown, but carried out by Dr Alan Mak, Queens University, Kingston, ON). To further determine whether PKA or PKC regulates FN synthesis in DA SMC, we measured FN expression in untreated cells, compared to cells treated with PKA and PKC inhibitors (Figure 9). Each bar represents the mean of 4 experiments ±SEM. The PKA inhibitor H89 did not have a significant effect on FN synthesis (Figure 9, B). The PKC inhibitor staurosporine only slightly decreased FN synthesis (P<0.05) (Figure 9, D); however, the three other PKC inhibitors used did not inhibit FN synthesis (Figure 9, C, E, F), indicating that the staurosporine inhibition might represent a toxic effect which was confirmed using a slightly higher dose.

LC-3 also contains potential phosphorylation sites for casein kinase I and II, and tyrosine kinases, so we investigated the role of these kinases in FN expression. The casein kinase II inhibitor DRB (Figure 10, A), the casein kinase I inhibitor CKI-7 (Figure 10, B), and the tyrosine kinase inhibitors PD 153035 (Figure 10, E) and genistein (Figure 10, F) did not influence FN synthesis in DA cells.

PI3-kinase and S6-kinase are central enzymes in the signal-dependent control of mRNA translation. Rapamycin, an S6-kinase inhibitor causes reduction in the initiation rate of "Polypyrimidine Tract" synthesis, as measured both by [35S] methionine incorporation into protein and by recruitment of 80S ribosomes into polysomes (Jefferies, et al., 1994). Wortmanin inhibits translation of tumor necrosis factor-alpha (TNF-α) in superantigen-
Figure 9: FN synthesis in DA SMC treated with a PKG inhibitor, a PKA inhibitor, and PKC inhibitors.

Representative autoradiographs depict FN synthesis in untreated DA SMC(C) and after 4h treatment with a PKG inhibitor: RpcGMP (A), a PKA inhibitor: H89 (B), and PKC inhibitors: chelerythrine (Ch) (C), staurosporine (St) (D), peptide 19-27 (E), and bisindolylmaleimide I (Bis) (F). Bars represent the mean ±SEM of 3 to 4 different experiments. The PKG and PKA inhibitors did not alter FN synthesis (A, B). Staurosporine slightly decreased FN synthesis (*P<0.05) (D), however, the other PKC inhibitors did not affect FN synthesis(C, E, F).
Figure 10: FN synthesis in DA SMC treated with casein kinases inhibitors, a PI3-kinase inhibitor, an S6 kinase inhibitor and tyrosine kinase inhibitors.

Representative autoradiographs shown above depict FN synthesis in untreated DA SMC(C) and after 4h treatment with a casein kinase II inhibitor: DRB (A), a casein kinase I inhibitor: CKI-7(B), a PI3-kinase inhibitor: wortmamin (C), an S6 kinase inhibitor: rapamycin (D), and tyrosine kinase inhibitors: PD 153035 (E) and genistein (F). Densitometric analyses demonstrate that there is no significant difference in FN synthesis following inhibitor treatments compared to control cells. Each bar represents the mean ±SEM of n=4 different experiments.
activated T cells (Ramirez, et al., 1999). We therefore studied the effect of these two kinase inhibitors on FN synthesis in DA SMC. Neither wortmanin nor rapamycin altered FN synthesis in the cells (Figure 10, C and D).

SUMMARY I

In these studies we showed that the MEK inhibitor PD 98059 decreases fibronectin (FN) synthesis in DA SMC, but has no effect on the phosphorylation of LC3. PD 98059 decreases FN mRNA levels and might influence mRNA stability. We propose that ERK regulates the stability of FN mRNA in DA SMC by repressing a destabilizing factor. This is in keeping with previous studies which show that ERK regulates mRNA stability. For example, the profound increase in p21 protein levels has been shown to require ERK-mediated stabilization of p21 mRNA and p21 protein in hepatocytes (Park et al., 2000).

PKA, PKC, casein kinases, and tyrosine kinases can phosphorylate LC3, PKG can be regulated by nitric oxide and PI3-K and S6K promote mRNA translation. However, none of these kinases were found to phosphorylate LC3 or affect FN mRNA translation (Figure 11). We therefore conclude that another and perhaps a novel kinase, phosphorylates LC3 in the NO-dependent regulation of FN mRNA translation and to determine the nature of this enzyme would require careful mapping of the NO dependent LC3 phosphorylation site.
Figure 11: Summary of the effects of kinase inhibitors on FN synthesis in DA SMC.
<table>
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<th>Phosphorylation sites in LC-3 sequence</th>
<th>Decreased FN synthesis</th>
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RESULTS II

Production of LC3-GFP Fusion Protein

In order to map the NO-dependent phosphorylation site in LC3, it is necessary to immunoprecipitate LC3. The strategy of expressing LC3 fused with the green fluorescent protein (GFP) would allow us to immunoprecipitate the complex with an anti-GFP antibody and would give us the added advantage of localizing the GFP labeled LC3 in living cells. We therefore generated a plasmid in which the coding region of LC3 was fused to GFP under the transcriptional regulation of the CMV promoter (Figure 12). LC3 cDNA was amplified by PCR using sequence specific primers containing Hind III and APA I restriction enzyme sites (Figure 13). GFP vector and LC3 cDNA were digested with these same restriction enzymes (Figure 14, A) and LC3 was then ligated into the GFP vector. Competent bacteria were transformed and the presence of the correct insert was confirmed by restriction enzyme digestion and gel electrophoresis of plasmid DNA isolated from individual clones (Figure 14, B lane 3 and 9). Purified DNA was sent for sequence analysis which confirmed the correct sequence of LC3-GFP.

Transfection of Cultured DA SMC with the Negative Control Vector GFP and with LC3-GFP Expression Vector

Expression of GFP and LC3-GFP vectors was observed 24 h post-transfection in living cells (Figure 15, C and D). On this basis, transfection efficiency was determined to be approximately 25% in vector transfected cells (Figure 15, C) and only 3% in LC3-GFP transfected cells (Figure 15, D). Cells were 70% confluent in both groups (Figure 15, A and B). To explore whether the difference in transfection efficiency between the two vectors was due to a problem in the transfection method, we co-transfected the cells with GFP or LC3-GFP and half the concentration of pSV-β-gal vector. Transfection efficiency is similar in GFP-pSV-β-gal (Figure 15, E) and LC3-GFP-pSV-β-gal (Figure 15, F).
Figure 12: Plasmid map of GFP and LC3-GFP expression vectors.

The LC3 cDNA was ligated in the pEGFP-N1 vector under the transcriptional regulation of the CMV promoter. LC3 was expressed as a fusion to the N-terminus of GFP (right panel). SV40 polyadenylation signals downstream of the GFP gene direct proper processing of the 3' end of the GFP mRNA. The pEGFP-N1 vector was used as a negative control (left panel).
Figure 13: PCR amplification of LC3 coding region.

Top: LC3 cDNA was obtained from total cDNA by polymerase chain reaction (PCR) using sequence specific primers containing unique restriction enzyme sites for Hind III and APA I, as underlined. Bottom: The PCR product was resolved on a 1.5% agarose gel containing ethidium bromide to visualize the right molecular size of LC3, 0.42 Kb.
Forward primer:

5' GGGAAGCTTCCCCATATGACGTCCGAG 3'
Hind III

Reverse primer:

5' CCCGGGCCCCGCAAGCATGG 3'
APA I
Figure 14: Restriction enzyme digestion (RED) of GFP and LC3 DNA.
The GFP vector and LC3 cDNA were digested with Hind III and APA I.  
A) RED prior to insertion of LC3 cDNA into the pEGFP-N1 vector, to allow for subsequent ligation.  
B) RED after ligation and transformation of LC3-GFP to identify the positive clones (lanes 3 and 9).  
1.5% agarose gels containing ethidium bromide confirm the right molecular size of LC3, 0.42 Kb and GFP, 4.7 Kb.
Figure 15: Transfection efficiency of DA SMC transfected with GFP (A, C, E) or LC3-GFP (B, D, F) vectors (10X).

The density of GFP and LC3-GFP transfected cells, visualized under the bright light, was similar (A and B). Green fluorescence shows positive transfection and expression was found in 25% in vector transfected cells (C) and 3% in LC3-GFP transfected cells (D). Cells were then co-transfected with GFP or LC3-GFP and 1/2 pSV-β-gal vector. Blue staining shows positive transfection and efficiency was the same in GFP- pSV-β-gal (E) and LC3-GFP-pSV-β-gal (F) transfected cells.
transfected cells. This indicates that the difference of transfection efficiency is not a function of the methodology used but that expression of LC3-GFP is lower than GFP alone as LC3-GFP does not translate very effectively or is rapidly degraded.

Expression of LC3-GFP and GFP Vectors in Cultured DA SMC
Western immunoblotting of cell lysates 48 h after transfection with the control GFP vector was assessed using a monoclonal antibody against GFP and demonstrated an immunoreactive band of the appropriate molecular weight: ~27kD (Figure 16, A, lane3). LC3-GFP transfected cells show a slightly higher molecular weight band around ~30kD (Figure 16, A, lane2). However, the expected size of the LC3-GFP fusion protein is ~43kD (~16kD LC3 and ~27kD GFP). Mock transfected cells were used as a negative control. We then probed the same membrane with our polyclonal antibody directed against the N-terminal region of LC3. The LC3 antibody detected the two forms of endogenous LC3 but did not recognize LC3-GFP (Figure 16, B). We therefore speculate that only the C-terminal of LC3 is expressed in the fusion protein since (1) LC3-GFP is in frame and LC3 is fused to the N-terminal of GFP, (2) the molecular size of LC3-GFP is only ~30kD and (3) the fusion protein is not recognized by our N-terminal LC3 antibodies. It is possible that LC3 is cleaved close to the C-terminal region by some cellular protease when it is GFP labeled at the C-terminal. A report showed that Apg8/Aut7p, an LC3 homologue, is cleaved immediately after Gly 116, which corresponds to Gly 120 in LC3, and this proteolytic processing is required for autophagosome formation (Kirisako et al., 2000). LC3 cleavage after Gly 120 would result in a protein of 22 amino acids long, or 2.4kD (Figure 19, B) which is consistent with the size of LC3 in our LC3-GFP fusion protein (Figure 16, A, lane2).
Figure 16: LC3-GFP and GFP expression in transfected DA SMC.
Representative western blots of LC3-GFP and GFP expression 48h post-transfection using A) an anti-GFP antibody and B) an anti-LC3 antibody. Mock transfected cells were used as a negative control. LC3-GFP transfected DA SMC showed expression of a 30 kD protein and GFP transfected cells demonstrated a 27 kD band (corresponding to the size of GFP) (A). Only the two molecular weight forms of endogenous LC3 were detected with the antibody against LC3 (B).
Localization of LC3-GFP and Effect of NO in LC3-GFP Transfected DA SMC

Even though only a small LC3 peptide-GFP fusion protein was produced by SMC, we were interested in localizing LC3 as it might have a similar function to the proteolytically processed LC3 homologue described above. We addressed this by confocal microscopy. Vector transfected cells (Figure 17, A, C, E) showed a diffuse green fluorescence in the cytoplasm compared to the punctate staining of LC3-GFP transfected cells (Figure 17, B, D, F). In addition, there was clumping of immunoreactivity in what could be autophagosomes, a pattern previously described by immunoelectron microscopy in studies carried out at the same time as ours by another group using GFP-LC3 transfected HeLa cells (Kabeya et al., 2000).

The NO donor SNAP increased the intensity of LC3-GFP staining (Figure 17, D), whereas L-NMMA had no effect (Figure 17, F). Quantification of the fluorescent staining demonstrated a significant increase of LC3-GFP staining with SNAP (Figure 18). NO has previously been shown to mediate phosphorylation of endogenous LC3, as revealed by a shift in apparent molecular weight in SDS-PAGE relative to its unphosphorylated form (Mason et al., 1999b). To investigate whether the NO-induced increase of LC3-GFP staining correlates with phosphorylation of LC3 at the C-terminal, we treated LC3-GFP transfected DA SMC lysates with potato acid phosphatase (PAP), and assessed changes in LC3 molecular weight by Western immunoblotting (Figure 19, A). The appearance of a lower molecular weight form of our samples following phosphatase treatment suggests that the LC3 peptide-GFP fusion protein is phosphorylated (Figure 19, A). Computer-based analyses of this C-terminal region indicated that it contains as many as 4 threonine (T), 1 serine (S) and 1 tyrosine (Y) residues that can undergo phosphorylation. This region also contains a potential protein kinase C phosphorylation site (threonine-X-arginine(R)), and a casein kinase II phosphorylation site (threonine-X-X-glutamate[E]) (Figure 19, B).
Figure 17: Immunofluorescence and localization of GFP and LC3-GFP transfected DA SMC treated with nitric oxide modulators (100X).

Expression of GFP (A, C, E) and LC3-GFP (B, D, F) vector transfected cells in untreated DA SMC (A and B) and after 4 h treatment with the nitric oxide (NO) donor SNAP (C and D), and the NO synthase inhibitor L-NMMA (E and F). Vector transfected cells showed a diffuse green fluorescence in the cytoplasm compared to the punctate and vacuolar staining of LC3-GFP transfected cells. SNAP increases the intensity of LC3-GFP staining (D) whereas L-NMMA does not affect LC3-GFP staining (F).
Figure 18: Quantification of immunofluorescence in LC3-GFP transfected DA SMC treated with SNAP or L-NMMA.

Images of fixed LC3-GFP transfected DA SMC treated with SNAP and L-NMMA were analyzed for fluorescent quantification using the Image-Pro Plus software. Bars represent mean ±SEM for n=10 cells for control (Con) n=13 for SNAP and n=12 for L-NMMA. LC3-GFP was significantly increased by SNAP (*P<0.05) but not affected by L-NMMA.
Con SNAP L-NMMA

Fluorescence

Con SNAP L-NMMA
Figure 19: Expression of LC3-GFP in transfected DA SMC treated with potato acid phosphatase and sequence of LC3 showing possible cleavage of LC3 and potential phosphorylation sites in the C-terminal region.

A) Western blot using the monoclonal antibody against GFP demonstrates that treatment (+) of expressed LC3-GFP fusion protein with potato acid phosphatase (PAP) results in a doublet of LC3-GFP (arrows) suggesting that the protein is phosphorylated. B) Amino acid sequence for the C-terminal region of LC3 showing the possible cleavage of LC3 after glycine 120 (G). The potential phosphorylation sites serine (S), threonine (T) and tyrosine (Y) residues are underlined. This region of LC3 also contains a potential protein kinase C (PKC) phosphorylation site (threonine-X-arginine (R)), and a casein kinase II phosphorylation site (threonine-X-X-glutamate (E)).
A

PAP + -

172 79 49 36 24 19 13

LC3-GFP

B

120 VYASQETFGTALAVTYMSALKATATGREPCL

PKC (P) SITE

CKII (P) SITE
SUMMARY II

In our studies we documented LC3-GFP fusion protein expression 24 h after DA SMC transfection. We suspect that only the C-terminal region of LC3 is present in our GFP fusion protein. LC3 might undergo a cleavage process when attached to GFP such as its homologue Apg8/Aut7p (Kirisako et al., 2000). LC3-GFP appears to localize to vacuoles. In studies carried out concurrent with ours, GFP-labeled LC-3 localized to autophagosomes (Kabeya et al., 2000).

Previous studies have shown that NO increases LC3 expression in DA SMC and appears to increase its phosphorylation (Mason et al., 1999b). We showed that NO increases LC3-GFP expression and that the LC3-GFP fusion protein is phosphorylated. NO-dependent phosphorylation of LC3 in the C-terminal region could influence FN mRNA translation indirectly since previous studies have shown that the N-terminal region of LC3 binds to the ARE of FN mRNA. That is, NO dependent phosphorylation could facilitate the function of LC3 as an RNA binding protein by altering its stability or its conformation. The NO-dependent phosphorylation site in the C-terminal of LC3 could be identified to investigate its importance in the regulation of FN mRNA translation in DA SMC, however, it will be more relevant to create another LC3 fusion protein to ensure expression of the N-terminal region of LC3 as well as the C-terminal.
DISCUSSION

Recent studies in our laboratory have implicated the microtubule-associated protein LC3 as an RNA binding protein involved in the post-transcriptional regulation of FN, an extracellular matrix glycoprotein which is critical in vascular smooth muscle migration (Zhou et al., 1997). LC3 binds FN mRNA at the AU consensus sequence (ARE) and this is associated with increased FN mRNA translation into protein. NO has been shown to mediate this process by increasing binding and phosphorylation of LC3 to the ARE of the FN mRNA (Mason et al., 1999b). Phosphorylation of some RNA binding proteins is associated with their binding to ribonucleoprotein particles such that mRNA recruitment into polyribosomes is facilitated (Standart et al., 1990; Walker et al., 1996). Therefore, we investigated the sites of NO-dependent LC3 phosphorylation to elucidate how they would determine the mechanism of LC3 dependent FN mRNA translation.

We first attempted to identify the kinase that phosphorylates LC3 in the regulation of FN mRNA translation by evaluating the influence of kinase inhibitors on constitutive and exogenous NO-dependent FN synthesis in DA SMC, as analyzed by gelatin-sepharose extraction following $[^{35}\text{S}]$-metabolic labeling. We examined the involvement of kinases that could potentially phosphorylate LC3, kinases known to be modulated by NO and kinases that regulate mRNA translation. None of the kinases investigated were found to phosphorylate LC3 in association with enhanced translation of FN mRNA. It is possible that a very selective inhibitor or a different class of inhibitor is required to suppress this process or that the concentrations of inhibitors used which were determined according to other studies using different cells, might not have been adequate in our primary cells. A protein kinase classification has been provided by Steven Hanks (Hanks and Quinn, 1991), who organized the known members of the eukaryotic protein kinase superfamily into distinct families that share basic structural and functional properties. In our studies, we used inhibitors which should suppress the activity of four out of the five families: the AGC
group which include protein kinase A, C, G and S6; the CMGC group which contains cyclin dependent kinase, MAP kinase, glycogen synthase kinase 3, casein kinase II and CDC28/cdc2-like kinase (CLK); the PTK group which represents the protein-tyrosine kinases; and the OPK group which refers to other protein kinases not falling in major groups such as casein kinase I family. We did not test the effect of an inhibitor of the CaMK group which contains Ca2+/calmodulin-regulated protein kinase primarily because the consensus sequence which it phosphorylates is unknown. For all three NO synthase isoforms, however, NO synthesis depends upon the enzyme’s binding of the ubiquitous calcium regulatory protein calmodulin. For ecNOS and nNOS, increases in resting intracellular Ca2+ concentrations are required for their binding calmodulin and, consequently, for their becoming fully activated (Michel and Feron, 1997). Increased nNOS and ecNOS expression resulted in elevated NO production in the intact DA (Mason et al., 1999b). It is therefore conceivable that a kinase regulated by Ca2+/calmodulin becomes activated upon NO elevation and phosphorylates LC3. Another possibility is that LC3 is phosphorylated by a novel kinase.

ERK regulates FN synthesis but did not increase the expression of the membrane-associated LC3, which is the phosphorylated form of LC3 (Mason et al., 1999b). On the other hand, the MEK inhibitor caused an increase in the levels of the microtubule-associated nonphosphorylated form of LC3, suggesting that ERK could play a role in LC3 localization by preventing LC3 from going into the tubulin-associated-cytosolic pool. MAP kinase phosphorylation of ERK was then found to increase FN mRNA levels. We therefore examined whether ERK could be regulating FN mRNA stability using the transcription blocker actinomycin D. Our results, however, were confounded by the properties of actinomycin D. Actinomycin D is commonly used to block transcription prior to mRNA decay determination, but this agent has also been shown to stabilize ARE containing mRNAs (Chen et al., 1995). The mechanism might be related to the significant two- to threefold accumulation of a U-rich-sequence-binding protein (URBP), termed AU-
A, as well as heterogeneous nuclear ribonucleoprotein (hnRNP) A1 protein, observed in the cytoplasm after the treatment of cells with Actinomycin D or 5,6-dichloro-1-b-D-ribofuranosyl-benzimidazole (DRB) for 3 h (Katz et al., 1994; Piñol-Roma and Dreyfuss, 1992). After the arrest of nuclear transcription by ActD or DRB, certain hnRNP and probably some other URBPs might be transported from the nucleus to the cytoplasm which could in turn result in displacement of the binding of authentic cognate ARE-binding proteins in the cytoplasm or alternatively in a change of the cytoplasmic equilibrium of binding between these proteins and AREs (Chen et al., 1995). As a result, this might lead to disruption of the functional ARE-protein complex necessary for ARE-directed mRNA decay. Thus, the inhibition of transcription could lead to mRNA stabilization. A group has developed a novel system to measure mRNA decay rates in the absence of transcriptional blockade. They successfully used a particle-mediated gene transfer (PMGT) to deliver in vitro transcribed mRNAs directly into cells and assessed their decay kinetics (Rajagopalan and Malter, 1996). mRNAs introduced into cells by PMGT are appropriately localized, translated, and degraded.

The investigations of mRNA stability with transcriptional inhibitors must be interpreted with extreme care, especially in the case of ARE-mediated decay. The role of ERK on FN mRNA levels in DA SMC is not confirmed and implies that ERK regulates either transcription or stability of the FN mRNA in DA SMC. Different studies have demonstrated that ERK could regulate gene transcription or mRNA stability. Transcriptional activation of the TNF-alpha gene has been reported to require MAP kinase in selected macrophage populations (Means et al., 2000) and MAP kinase regulates transcription of the Apo CIII gene, a key regulator of serum triglyceride levels (Reddy et al., 1999). On the other hand, the profound increase in p21 protein levels has been shown to require ERK-mediated stabilization of p21 mRNA and p21 protein in hepatocytes (Park et al., 2000).
Our attempt to identify the kinase that phosphorylates LC3 in the regulation of FN mRNA translation using different kinase inhibitors was not successful. Instead of pursuing this search using other candidates, we created an LC3-green fluorescent protein (GFP) fusion protein so that we would be able to immunoprecipitate LC3-GFP and identify the amino acids phosphorylated. In contrast to the perinuclear and microtubule-associated distribution of native LC3, immunofluorescence in LC3-GFP transfected DA SMC showed punctate staining and LC3-GFP was prominently distributed in vacuoles. Western immunoblotting of lysates from LC3-GFP transfected cells using an anti-GFP antibody revealed that LC3-GFP was truncated. A possible explanation is that LC3, when fused to GFP is cleaved by some cellular process and targeted to vacuoles for degradation. We speculate that only the C-terminal of LC3 is expressed in the fusion protein since LC3-GFP is not recognized by an N-terminal LC3 antibody and this features is also compatible with the cleavage at the C-terminal of Apg8/Aut7p, an LC3 homologue (Kirisako et al., 2000).

A group recently GFP-labeled LC3 and reported that LC3 associates with autophagosome membranes (Kabeya et al., 2000). Kabeya et al. first observed endogenous LC3 enriched in the autophagic vacuole fraction prepared from rat liver. Hela cells were then transfected with a construct encoding LC3 fused to GFP and the gene product was detected by immunogold and silver enhancement method using an anti-GFP antibody. The silver-enhanced gold particles showing the presence of GFP-LC3 were associated with autophagosome membranes (Kabeya et al., 2000). Immunoblot analysis of GFP-LC3 in transfected cells was not shown, so it is possible that the size of LC3 was similar to the truncated form we observed in autophagosomes. Furthermore, full length LC3 is not observed in autophagosomes in normal SMC. We therefore suggest that this might be a degradation or default pathway of a truncated LC3. It is therefore important to produce a construct in which LC3 is fused to a different peptide tag to preserve its normal distribution and function.
Our finding that NO regulates LC3-GFP and that LC3-GFP is phosphorylated is however, consistent with our previous study which indicated that NO mediates increased FN synthesis through the binding to the FN ARE of a phosphorylated form of LC3 (Mason et al., 1999b). It is of interest that phosphatase treatment of native LC3 shifted its molecular weight upward (Mason et al., 1999b) whereas phosphatase treatment of LC3-GFP resulted in a downward shift. The difference in mobility could be the result of an alteration in charge engendered by a different number of dephosphorylation processes, or from an altered conformation caused by the presence of GFP.

The increase in LC3-GFP with the NO donor SNAP as revealed by immunocytochemistry and confocal microscopy could be the direct result of the phosphorylation of LC3-GFP. That is, NO-mediated phosphorylation might repress ubiquitination and protein degradation allowing LC3 to remain in the cytoplasm.

Since we showed that NO influences LC3-GFP expression and that the LC3-GFP fusion protein is phosphorylated, we could purify the small 22 amino acid peptide that likely corresponds to the C-terminal of LC3 and subsequently identify the NO-dependent phosphorylation site. Previous studies have, however, shown that the N-terminal region of LC3 regulates binding to the ARE of FN mRNA and results in the increased FN synthesis (Lau, 1999). NO-dependent phosphorylation of LC3 in the C-terminal region could nonetheless alter the stability or conformation of LC3 and thereby influence its binding to the ARE in association with FN mRNA translation. A clam oocyte protein p82, has been related to Xenopus CPEB, an RNA-binding protein that interacts with the U-rich cytoplasmic polyadenylation elements (CPEs) of maternal mRNAs and promotes their polyadenylation (Walker et al., 1999). Cloned clam p82/CPEB showed extensive homology to Xenopus CPEB, and related polypeptides from mouse, goldfish, Drosophila and Caenorhabditis elegans, particularly in their RNA-binding C-terminal halves. Phosphorylation of p82 in the N-terminal region by cdc2 kinase has been found to be required for translational activation of maternal mRNA (Walker et al., 1999).
FUTURE STUDIES

Our hypothesis that a kinase, regulated by NO, phosphorylates LC3 in the upregulation of FN mRNA translation is based on previous experiments implicating LC3 phosphorylation in the formation of intimal cushions in the DA. In this thesis, we investigated the kinase involved in this post-transcriptional mechanism and the NO-mediated phosphorylated site on LC3. We were not able however to determine the NO-mediated kinase implicated in phosphorylation of LC3 by examining the effect of kinase inhibitors on FN synthesis. We were also unsuccessful in creating an LC3-GFP fusion protein that would replicate the distribution and function of endogenous LC3. The generation of another fusion protein will be critical in determining the amino acids phosphorylated directly or indirectly by NO. A construct could be engineered in which the small haemagglutinin (HA) tag is fused to LC3 and this is widely used as a strategy for localization and immunoprecipitation of proteins. It will be necessary to assure that HA-LC3 function is not altered. Immunoprecipitation of the HA-LC3 using an anti-HA antibody would be performed followed by phospho-amino analyses and peptide mapping to depict the phosphorylated sites. This will also allow us to elucidate whether NO-dependent LC3 phosphorylation involves the C-terminal region suspected in our experiments with LC3-GFP or whether there are other sites in the N-terminal. Then, site-directed mutagenesis could be used to mutate the phosphorylation site(s) in LC3 to confirm that this site is critical to the regulation of FN mRNA translation. This can be achieved by transfecting the wild type and mutated constructs in DA SMC followed by examination of LC3 phosphorylation and localization by western blotting and immunofluorescent microscopy, as well as assays of FN synthesis and cell migration. Transfection of HT1080 fibrosarcoma cells may prove to be particularly informative since these cells normally lack FN synthesis and exhibit a rounded contact inhibited cell phenotype. Stable transfection of wild type LC3 has been shown
previously to both increase FN synthesis and cause reorganization of the cytoskeleton allowing for cell adherence and spreading of these cells (Lau, 1999).

Our findings that ERK regulation of FN synthesis is associated with enhanced steady-state levels of mRNA beg exploration of the possible mechanisms involved. The role of ERK could be addressed by further assessing rates of transcription of mRNA by nuclear run-on. If ERK was found to stimulate FN mRNA transcription, it would be interesting to examine the binding domains for transcription factors on the FN promoter in our model. In rat vascular SMC, the FN promoter contains a putative activating protein (AP-1) binding site (rFN/AP-1), and the results of a transient transfection and electrophoretic mobility shift assay showed that angiotensin II enhanced rFN/AP-1 activity. Using inhibitor studies, angiotensin II was reported to activate transcription of the FN gene via the activation of AP-1 by a signaling mechanism dependent on PKC and PTK (Tamura et al., 1998).

Another possibility is that ERK phosphorylation stabilizes FN mRNA by repressing a destabilizing factor. RNA gel mobility shift assays could be performed to examine the binding complexes in the presence and absence of the MEK inhibitor PD 98059 and actinomycin D. If in fact ERK stabilizes FN mRNA, we would expect that PD 98059 treated cells will result in an increase of the FN mRNA/destabilizing complex binding, but that treatment of the cells with an ERK activator would reduce the binding complex formation. We could also examine the role of actinomycin D in reducing the expression of this destabilizing factor. Exploration of the ERK dependent binding site of the destabilizing factor on the FN mRNA would then be of great interest. The 3'UTR would probably be the first region to investigate because of laboratory experience (Zhou et al., 1997; Zhou and Rabinovitch, 1998). It is also entirely possible and consistent with other studies that post-transcriptional regulatory elements are present within the 5'UTR (Linz et al., 1997) or even within the coding region of the mRNA (Shetty et al., 1997).
Our studies therefore reveal that there are two different mechanisms orchestrating FN synthesis which is critical to intimal cushion formation in the DA and potentially in vascular pathobiology. NO mediates post-transcriptional upregulation of FN by an unknown kinase which phosphorylates LC3, and phosphorylation of ERK by a MAP kinase also regulates FN mRNA levels.
REFERENCES


