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UMI
THE ROLE OF HUMAN PROFILIN IN VASCULAR SMOOTH MUSCLE
CONTRACTILITY AND HYPERTENSION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
The Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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ABSTRACT

The ubiquitous G-actin binding protein profilin is believed to be a key regulator of actin polymerization in cells. In order to assess the role actin polymerization on vasculature, we have overexpressed transgenically either the wild-type cDNA of a the human profilin I gene (WT), or the dominant negative mutant isoform (88R/L) in the blood vessels of transgenic mice using mouse vascular smooth muscle α-actin promoter. Transgenic mice were identified by PCR analysis using specific primers that selectively recognize the transgene. RT-PCR analysis confirmed the selective transcription of profilin in smooth muscle, including blood vessels, spleen and intestine, while the transgene transcript was not detected in control mice. Histological analysis on aorta from profilin I WT, 88R/L mutant and non-transgenic for actin polymerization using Rhodamine staining showed that actin polymerization was high in profilin I WT transgenic, low in non-transgenic and lowest in 88R/L. We have assessed the migration profile of vascular smooth muscle cells (VSMCs) isolated from aorta of transgenic or non-transgenic mice. We found that VSMCs cells from profilin I WT migrate faster than those from 88R/L or non-transgenic controls. We also have monitored the blood pressure of profilin I WT and of non-transgenic. Our results showed that overexpression of profilin I WT in smooth muscle cells induced a chronic hypertensive response in these mice as compared to the non-transgenic. Increase actin polymerization in profilin I WT transgenic mice may increase the vasoconstriction of the blood vessels, which leads to
elevate the mean arterial pressure. This information should suffice as a foundation to further explore the potential effects of actin cytoskeleton in hypertension.
To my Parents, my Wife and little Daughter
ACKNOWLEDGMENTS

It was because of mercy and blessing of God Almighty this work was completed. I would like to express my sincere and heartfelt appreciation to my advisor, Dr. Robert L. Hamlin for his help and his thought provoking guidance and morale boosting encouragement during my study. My appreciation also goes to my research advisor Dr. Goldschmidt P.J for providing the opportunity to work on a research area that was direly needed for my country. I also appreciate my member of my advisory committee Drs. H. H. Hassanain, M. Yamagushi, M. Strauch for their invaluable help during the conduct of this study.

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Studies in Molecular of Cardiovascular System
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CHAPTER 1

GENERAL INTRODUCTION

1. INTRODUCTION

Blood pressures outside the physiological range represent a remarkably common trait in humans and account for a sizable portion of the cardiovascular disease burden in our society, in particular stroke, myocardial infarction and end-organ damage. Although for less than 10% of the patients, hypertension results from well established etiologies, such as primary aldosteronism, Cushing's syndrome and pheochromocytoma, for most individuals, the molecular basis of the heightened blood pressure remains unknown. It is established that both genetic and environmental factors contribute to the disease process (Garbers, 1999).

The vascular wall is an active organ composed of three layers: intima, media and adventitia. The wall of the blood vessels is subject to change its structure through a process known as remodeling. Remodeling is usually an adoptive process in response to long-term changes in hemodynamic conditions as occurs during development, but can also contribute to the pathophysiology of vascular and circulatory disease (Wang.
et al., 1996; Gibbons et al., 1994). Remolding is now considered to be a more complex process that include increased wall thickness, increased cross sectional area, and decreased lumen diameter, all charlatanic of hypertrophic or eutrophic inward remodeling (Baumbach et al.; 1989; Deng and Schiffrin 1991). This change of the wall of the blood vessels diameter will then increase the blood pressure and leading to heart disease as well (Hope & Schiffrin, 2001).

Angiotensin II (Ang II), the main peptide hormone of the renin-angiotensin system, plays an important role in the pathogenesis of cardiovascular diseases, including atherosclerosis, myocardial infarction, and hypertension (Vekshtein, et al., 1989). Ang II exerts hypertrophic and hyperplastic effects by activating a number of intracellular signal transduction pathways through a 7-transmembrane heterotrimeric G protein-coupled receptor called the Ang II type 1 (AT₁) receptor (Alexander, et al, 1991) Angiotensin II has been identified as one of the most important endogenous regulators. Angiotensin II binds to two types of receptors; AT₁ and AT₂ and shows multifunctional effects by influencing both contraction and growth of smooth muscle cells in blood vessels (Touyz et al., 1999; Jackson et al, 1992, Sauro et al., 1996). The AT₁ is better understood in terms of function and signal pathway, whereas, AT₂ receptors remain unclear. These activities of AT₁ couple to a variety of signal transduction pathways including mitogen-activated protein kinase (MAPK) or extracellular signal-regulated protein kinase (ERK) (Rhian et al, 1999; Manseau et al, 1996).
Smooth muscle cells are the major type of cells in the medial layer of the blood vessels either in the large or small arteries. Alteration of the function of the vascular smooth muscle cells has been implicated for a long time in the pathogenesis of hypertension (Hall et al, 1991; Strauch et al, 1999). Smooth muscle cells have been subject to study and to provide further understanding of biochemical mechanisms involved in the hypertension specially it cytoskeleton proteins such as actin protein (Geisterfer et al, 1988). Actin is the major cytoskeletal protein in eukaryotic cells. Actin is a highly dynamic network. It consists of actin filaments and a variety of associated proteins (Schmidt and Hall, 1998). Actin exists in two forms; a monomeric form (G-actin) or a polymeric form (F-actin). Each monomer of actin can bind to ATP, which hydrolyzes to ADP after incorporation of the actin molecule into an actin polymer (Engel et al, 1977). Actin mediated cells change shape during cells division. Thus is essential for several important activities, such as muscle contraction, controlling cell-cell and cell substrate interactions and participating in transmembrane signaling including endocytosis and secretion (Salmon 1989, Luna & Hitt 1992).

Many proteins associated with the actin cytoskeleton control actin assembly and disassembly. These proteins regulate actin assembly at multiple levels, including the organization of actin monomers into actin polymers (Schmidt & Hall 1998). One of key actin-regulatory protein is profilin which associates with polymerization of actin. Profilin is a ubiquitous small (12-15 kDa), actin-binding protein that plays an important role in the regulation of actin polymerization in a number of motility functions.
(Haarer& Brown 1990). The ability of profilin to bind to many ligands suggests that profilin is involved in signal transduction and may link transmembrane signaling to the control of the microfilament system (Elena et al, 1998). Early biochemical studies indicate that profilin regulates actin polymerization supported at the barbed end (Haarer& Brown 1990). Profilin interacts with actin in a 1:1 ratio and participates in the addition of monomers at the free barbed end of the filament then disassociates at the barbed end (Pantalonii& Carlier, 1993, Perelroizen et al 1996). Recent work has suggested several more functions of profilin aside from its monomer-sequestering ability. Profilin promotes the exchange of adenine nucleotide bound to actin monomer and also effectively lowers the critical concentration of monomer actin for polymerization of actin (Julie 1993, Borisy & Svitkina, 2000). It also promotes nucleotide exchange on an actin monomer by lowering the affinity of the actin monomer for its bound nucleotide by 1000-fold (Goldschmidt-Clermont et al, 1991b).

Studies have shown profilin interacts with three ligands; actin monomers, phosphatidylinositol 4,5-bisphosphate (PIP2) and Poly-L-Prolin through in vitro studies. By binding to PIP2, Profilin inhibits the hydrolysis of PIP2 by phospholipase CΥ1 (PLCY1) (Sohn et al., 1995). When PLCΥ1 is phosphorylated on specific tyrosine residues by extracellular growth factors binds to and activates tyrosine kinase receptors, PLCΥ1 is able to overcome the protective effects of profilin and hydrolyze PIP2 (Carlsson et al., 1976).
Profilin after being released from PIP2, profilin diffuses freely to the actin cytoskeleton where it acts as a regulator of actin polymerization (Sohn et al., 1995).

Despite the wealth of in vitro data referring to profilin function, the in vivo role is still under debate. The greatest complication of profilin is that it interacts with both actin and also a large number of proteins such as Rac, Cdc42 and Rho which may be an important aspect of profilin function (Witke et al., 2001). Rho, Cdc42 and Rac. In non-VSMCs as well as in VSMCs, Rho controls actin-myosin contractile function through the phosphorylation of myosin light chains (Hassanain and Goldschmidt-Clermont, 2000a) and suggested to mediate hypertrophic signals in cardiac myocytes (Moldovan, 1999).

Profilin I has been suggested to be a tumor suppressor, however no human diseases have been described as being linked to profilin and no mouse models are yet available (Witke et al., 2001). In order to assess the role of the human profilin in actin polymerization on vasculature, we have transgenically overexpressed both either the wild-type cDNA of the human profilin I gene (WT), and the dominant negative mutant isoform of the gene (88R/L) in the blood vessels of transgenic mice. This was done using the mouse vascular smooth muscle α-actin promoter.
1.2. Hypothesis:

The overexpression of the human profilin I gene in vascular smooth muscle cells of transgenic mice increases actin polymerization. This increase in actin polymerization may lead to vascular hypertrophy. And an increase in the resistance of blood flow through the blood vessels of these mice. Vascular hypertrophy and the increase of constriction of blood vessels may result in elevating the mean arterial pressure in these transgenic mice.
CHAPTER 2

LITREATURE REVIEW

2.1. REMODELIGN AND HYPERTENSION

High blood pressure resulting in heart attack or stroke is probably one of the most common causes of death due to heart disease. In humans normal blood pressure is 120-mmHg systolic and 80 mmHg diastolic. Blood pressure above 140 mmHg systolic or 90 mmHg diastolic is considered elevated. High blood pressure has been increasing in most countries including the western countries. For example in the United States the prevalence of patients with high blood pressure has increased especially in the adult population. The etiology of hypertension has remained unknown, however, it is known that no one single cause is responsible. High blood pressure is the end result of a large number of factors. These factors, including both genetic and environmental factors, may be quantitatively and qualitatively different between individuals. (Hypertension Medicine, by Michal A. Weber. Chapter #6)

The heart plays an important role in hypertension as it may be partially responsible for elevated arterial pressure and may suffer the consequences of elevated
arterial pressure. In fact the seriousness of hypertension may be predicted by the degree of increase in mass of the left ventricle. A large number of studies using echocardiography have reported this relationship between the left ventricle mass and blood pressure (Sybill et al., 2001).

Heart failure is a major problem in most countries including the western countries. Approximately 1-3 % of the population is suffering from heart failure. In early studies, before the introduction of antihypertensive treatment, hypertension was the most common cause of heart failure. (Long- guang Cao et al., 1992, Sohn 1994)

Elevation of systemic arterial pressure (SAP) to a systolic greater than 140 mmHg and/or a diastolic greater than 90 mmHg constitutes clinical “hypertension”, which is one of the most common forms of cardiovascular disease in humans, and leads to serious morbidity and mortality. Hypertension may be of familial origin (i.e. genetic), or may be a consequence of environmental factors. Part of the morbidity and mortality from hypertension stems from stroke, acceleration of atherosclerosis and heart attack, congestive heart failure, and retinal hemorrhage. Of course stroke and retinal hemorrhage result from elevation of cerebral vascular or retinal vascular pressures which may result in rupture of weakened cerebral or retinal vessels; while heart failure results from increased demand on the left ventricle, increased myocardial oxygen consumption, oxygen debt, reduction in ATP production, and decreased energy required for both contraction and relaxation.
Acceleration of atherosclerosis may occur because of intimal injury, which accelerates incorporation of fatty material into the tunica media and tunica intima.

Systemic arterial pressure consistent with Ohm’s law is calculated as the product of systemic vascular resistance (SVR) and cardiac output (CO). If either elevates, then SAP elevates. In children, hypertension is due principally to elevation of CO, whereas in adults, it is do principally to increase in SVR.

If the role of SVR is excluding from a determinant of CO, then CO is the product of stroke volume (SV) and heart rate (HR). When elevation in CO is responsible for hypertension, it is usually caused by elevation of HR. Heart rate depends upon the rate of discharge of the sino-atrial node (SAN), which is determined, principally, by a balance between parasympathetic (which slows) and sympathetic (which speeds) efferent traffic to the SAN.

If the role of CO is excluded from a determinant of SAP, then hypertension results from increase in SVR. Increased SVR depends upon excessive activation of arterial and arteriolar smooth muscle. This activation both decreases the sum total of the apertures of arterioles (increasing SVR), and stiffens the large arteries (increasing impedance).
The control of vascular smooth muscle is a very complex, multifactorial process. Smooth muscle is under the control of neurogenic (alpha-1, alpha-2, beta-2 adrenergic), endocrine (angiotensin II, vasopressin), and local factors (endothelin, nitric oxide, acetyl choline). The output of the vasomotor centers in the medulla in concert with "information" exchanged with the hypothalamus depends upon afferent influences coming over the vagus (from the aortic, high pressure baroreceptors) and the glossopharyngeal (from the carotid sinus, high pressure baroreceptors). Causes for hypertension may be defects in the high pressure baroreceptors or in the medulla/hypothalamus (altered "set-point"). However almost all therapy depends upon decreasing SVR by relaxation of vascular smooth muscle by one means (i.e. blocking neuro-endocrine signals) or another (i.e. blocking locally-produced substances) (Robert Berne, 1997).

Smooth muscle contraction is the result of a complicated cascade of physicochemical reactions. Calcium enters the cell and binds to calmodulin. The calcium-calmodulin complex phosphorylates light chains on the myosin cross-bridges, and this leads to contraction of smooth muscle. When an agonist (e.g. NO, beta-2-norepinephrine) binds to cyclic nucleotides (either cAMP or cGMP), smooth muscle inactivation (relaxation) occurs. Activation of the phosphatidylinositol system, as by alpha-1 adrenergic activity or endothelin, results in contraction of vascular smooth muscle (Hypertension Medicine, by Michal A. Weber. MD Chapter #6).
Smooth muscle cells are generally divided into two major types, multi-units of smooth muscle and single units of smooth muscle. Single units of smooth muscle are found in blood vessels. This type of smooth muscle is characterized by thousands of fibers that contract together as a single unit. These fibers are arranged in bundles and their cell membranes are adherent giving them the ability to transfer force from one unit to the next (Haarer&Brown, 1990).

Smooth muscle cells contain two major contractility proteins, myosin and actin. Smooth muscle is not striated, as is skeletal and cardiac muscle. Instead, actin is arranged in smooth muscle cells by binding to specialized structures called dense bodies. This type of actin is believed to be involved in the smooth muscle cells' contractility. Actin is interspersed with myosin in smooth muscle cells, and it is the myosin which plays a major role in contraction. Actin molecules bind to the cell membrane or are found moving freely in the cytoplasm of the smooth muscle cells. Actin in smooth muscle cells as in other cells is present in two forms, F-actin filament and G-monomer form interacting with all the necessary binding proteins (Guyton & Hall 2000).

Alteration in structure and function of vascular smooth muscle have been implicated for a long time in the pathogenesis of hypertension in spontaneously hypertensive rats. In this model, alterations were created which included both elevating rate of proliferation and increasing vascular responsiveness to different stimuli. (Keith
Smooth muscle cells were isolated from the aorta of this animal model and have provided further valuable understanding of biochemical mechanisms involved hypersensitivity of vascular smooth muscle cells (Geisterfer et al., 1988).

The function of arteries is to transport blood, under high pressure, to the tissues of the body. The high pressure in arteries requires strong vascular walls. Smooth muscle cells make up a majority of the wall of the arteries and is present mainly in the media (between intima and adventitia) (Guyton & Hall 2000; Berne 1997).

The arterial system acts like a pump as it converts the intermittent flow from the heart into more continuous flow to the organs (a windkessel effect). This functional behavior is due to the peculiar assembly of the cellular and extracellular components into the three distinct layer of the blood vessels: tunica intima, tunica media and tunica adventitia (Glasser, 2000). When the histological nature of these vessels changes in response to disease, the change is termed vascular remodeling (Mulvany, 2002, ).

Remodeling is usually an adaptive process in response to long-term changes in hemodynamic conditions (Wong L. C., et al., 1996). But it can also contribute to the pathophysiology of vascular disease (Gibbons, et al., 1994). The remodeling process of blood vessels can be triggers by many pathophysiological forcings, and this remodeling may increase stiffness and result in elevation of blood pressure (Glasser, 2000).
Remodeling results in increased wall thickness increases cross section area and decrease lumen diameter (Baumbach et al., 1989). These morphological adaptations often result in modifications of the functional properties of the affected vessels (Deng et al., 1991).

In hypertension, blood vessels undergo hypertrophic remodeling. In hypertension rats exhibit predominantly inward eutrophic remodeling characterized by the increase in the medial-lumenal ratio (Intengan, 2001; Mulvany, 1996). Increases in stiffness of the blood vessels results in loss of arterial compliance, the ability of the vessels to store blood volume temporarily as it is ejected with each systole. Remodeling of blood vessels can lead to vascular stiffness, elevation of blood pressure, and atherosclerosis (Glasser, 2000; Rizzoni et al., 2000)

Increases in local vascular resistances may lead to maldistribution of blood flow to or within various organs. Alteration in renal plasma flow may causes further elevation of systemic arterial pressure, thus a viscous cycle of positive feedback amplifies the level of hypertension. (Guyton & Hall, 2000)

Recently, experiments in transgenic mice have evaluated genetic factors that influence blood pressure. In humans, essential hypertension is a complex disease and several syndromes have been found to influence blood pressure. Researchers commonly use animals (mostly rats or mice) as a model of hypertension.
Many researchers have studied rats, which develop naturally either high or low blood pressure to provide animals for the study of the disease (Change, et al., 1996). Most of the available animal models for hypertension (called genetic hypertension GH) are based on genetic information. These animals are then crossed with genetically hypertensive rats. This type of model is selectively bred based on blood pressure, and dietary and/or environmental factors are used as provocative or amplifying stimuli. (Julie et al., 1993).

Another model for hypertension is the Dahl salt-sensitive (S) and the Dahl salt-resistant (R) rats. These rats models were bred on the basis of their blood pressure after being fed a high-salt diet. Many other models are available commercially or in Private labs. However, these models might not help to determine the role of specific genes and proteins in the pathophysiology of hypertension (Change, et al., 1996).

2. 2. **Cellular and molecular events in cardiovascular system.**

2. 2. 1. **Actin cytoskeleton**

The cellular cytoskeleton in general is composed of three types of filaments: microfilaments, microtubules and actin filaments (Julei et al., 1993). Actin cytoskeleton is a key player in cell motility, cell differentiation as well as the progressing of cell cycle. Furthermore, it participates in many cell signals, endocytosis and secretion (Luna & Hitt 1992; Bretscher 1993 and Nobes & Hall 1995). During mitosis, the actin...
mediates the separation of daughter cells by forming the contractile ring, process called cytokinesis (Lama&Hitt1992; Gotteib et al 1993). Moreover, actin is a dynamic protein is essentially involved in cell movement and is highly controlled by myosin protein. Myosin is the motor protein, which interacts with polymers of actin to produce mechanical force. Myosin transduces the energy produced by hydrolyzing ATP to ADP into mechanical force and movement. At the gene level, actin gene has been highly conserved throughout evolution (Titus 1993; Goodson et al., 1997).

Actin exists in two forms: the monomeric (G-actin) and the polymeric form (F-actin). The monomeric form (G-actin) is able to bind to ATP which triggers the incorporation of G-actin to the polymeric form. The assembly of the actin cytoskeleton is highly organized. There are many actin regulatory proteins that are involved in actin organization such as profilin as well GTP-binding proteins (Goodson et al., 1997).

2.2.2 Actin regulatory proteins

GTP-binding proteins are members of the Ras superfamily regulate a wide variety of cellular activities. These proteins function as molecular switches that cycle between the active GTP-bound state, which interacts with downstream targets, and the inactive GDP-bound state. The Ras-superfamily can be subdivided further based on sequence similarities into Ras, Rho/Rac, Rab, Ran, Rad and Arf subfamilies. These subfamilies can be associated with specific different biological functions of their members (Schmidt and hall 1998).
2.2.3. Profilin as a major monomer actin regulatory protein

Profilin is a ubiquitous, actin-monomer binding protein found in many organisms from yeast to humans. Profilin is a small 12-14 kDa protein and is necessary for normal cell growth and function. (Julei, 1993). Profilin gene is a well-conserved gene across species ranging from fungi to trees. Profilin was originally identified as a component of cell extracts that has both negative and positive effects on the growth of actin filaments in vitro (Haarer & Brown 1990). Studies have shown that profilin interacts with three ligands: actin monomer, Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and Poly-L-Prolin (PLP) (Gar Boris et al., 2000; Sohn et al., 1995).

Profilin binds to actin monomer in a ratio of 1:1 thereby decreases the critical concentration of actin monomer. Profilin actin complexes have been shown to add to the barbed end of the growing filament. Profilin however has a lower affinity to the barbed end, causing it to then dissociate and leave the actin filament. In the model suggested by Julie of profilin mixed with actin, there are two pathways for barbed end elongation: either the monomer will add directly to the barbed end of the growing filament, or the profilin-actin complex will add to the barbed end of the filament and the profilin will dissociate (Figure 1 c).

The human genome contains two types of profilin genes: a gene coding for profilin I and a gene coding for profilin II (Moldovan et al, 1996, Honore et al., 1993,
Kwiatkowski et al., 1988). There is a great amount of available genetic data to support the idea that profilin is necessary to promote the formation or the stability of the actin filaments. For example, the elimination of an ovary-specific profilin in *Drosophila* results in female sterility. This demonstrates the importance of the cytoskeleton actin network for proper oocyte development (Cooley et al., 1992). In mice, it has been shown that the absence of profilin was lethal (Lu & Pollard, 2001; Lanier et al., 1999).

Profilin binds to many potential proteins ligands with Prolin-rich sequences including VASP (Vasodilator-stimulated phosphoprotein), and other proteins which supports the biological significance of the role of profilin and its interactions (Janke et al., 2000; Lanier et al., 1999). VASP is a member of a family of proteins involved in regulation of actin reorganization (Bachmann et al., 1999).

Profilin also binds to PIP2, which indicates its role in cell signaling. Until recent years no one know the binding site of profilin on PIP2 until early the nineties when Pollard and Rimm started studying this in *Acanthamoboa*. And the later, Vinson, et. All, 1994, proposed the PIP2-binding sites. The study that has been done by Sohn, et. al, has identified the important amino acids sequences in PIP2 that are involved in PIP2 binding profilin. They found that a crucial binding site for PIP2 on human profilin is contained within five amino acids, one of them is arginine at position 88. The mutation in this arginine amino acid will prevent profiling from binding to PIP2 as well monomer actin which will inhibit actin polymerization.
Rapid actin reorganization as a response to external stimuli is thought to be a process of accumulation of Rho-family small G proteins. These proteins such as Cdc42, Rac and Rho are essential for the reorganization of actin, leading to the formation of filopodia, lamellipodia and stress fibers. (Hall, A., 1998).

Furthermore, Shiro Sutsugu have shown that profilin is essential for ruffling formation and microspik formation and that the mutation in profilin was able to suppress Cdc42-induced microspik formation and Rac-induced membrane ruffling (Shiro Suetsugu et al., 1999). The ability of the mutant profilin to inhibit the cytokinases of these proteins might suggest that profilin works downstream of Rho, Cdc42, Rac and other regulatory molecules (Ostrander et al., 1999).

2.2.4. Profilin and cells motility

Cell activity, such as motility, is highly dependent on the ability of the actin cytoskeleton to undergo rapid reorganization. This function of actin is highly modulated in vivo by profilin and other actin-binding proteins. In yeasts lacking profilin, cells grow slow and lack detectable actin cables (Amy K. Wolven et al., 2000, Purqne & Bretshe, 2000). This dual function of profilin in cell motility is thought to be essential for rapid filament turnover such as in the growing end of fast moving lamellipodia of fibroblasts (Lambrechts et al., 1997; Small, 1995). In addition, it has been shown in vitro that expression of exogenous human profilin I affect growth, cytoskeletal organization, cell spreading and motility and tumorigenicity. Injecting a
tumorigenic breast cancer cell line, CAL51, in nude mice after being transfected with human profilin I cDNA shows suppressed tumorigenicity. In other breast cancer cells, immunohistochemical analysis revealed low levels of profilin I. These results suggest the role of profilin in cell signaling and tumor suppression of the breast cancer cells. (Janke et al., 2000).

2.2.5. Profilin and hypertrophy pathway

The renin/angiotensin/aldosterone (RAS) pathway is a key modifier of vasoconstriction, sodium retention, and vascular cell proliferation, whose activity counteracts the agonists of vasodilation/natriuresis mediated by cyclic GMP (Weir et al., 1999; Masatsugu et al., 1999; Garbers et al., 1999; McIntyre et al., 1999). Angiotensin II has been identified as one of the most important endogenous regulators. Angiotensin II binds to two types of receptors; AT₁ and AT₂ and shows multifunctional effects by influencing both contraction and growth of smooth muscle cells in blood vessels (Rhian et al., 1998, Sauro et al., 1996, Griendling et al., 1997). The AT₁ is better understood in terms of function and signal pathway, whereas, AT2 receptors remain unclear.

These activities of AT₁ couple to a variety of signal transduction pathways including mitogen-activated protein kinase (MAPK) or extracellular signal-regulated protein kinase (ERK). (Griendling et al., 1997).
Angiotensin II (Ang II), the main peptide hormone of the renin-angiotensin system, plays an important role in the pathogenesis of cardiovascular diseases, including atherosclerosis, myocardial infarction, and hypertension (Berk, B.C., Vekshtein, et al., 1989). Ang II exerts hypertrophic and hyperplastic effects by activating a number of intracellular signal transduction pathways through a 7-transmembrane heterotrimeric G protein–coupled receptor called the Ang II type 1 (AT₁) receptor (Murphy, T.J., Alexander et al., 1991). New data indicate that Ang II plays an important role in the generation of reactive oxygen species (ROS) by activation of NADPH oxidase, a plasma membrane–bound protein (Zafari, 1996; Lander, Hajja et al., 1997). In the pathway activated by Ang-II, as well as for any putative pathway that requires the production of \( \cdot O_2^- \) by NADPH oxidase to induce hypertension (Fujita, N., and Nagao, 1991), the final common step is the switching on of the small guanyl binding protein Rac that has been known to be activated by profilin (Figure 1) (Suetsugu et al., 1999).

The extracellular signal-regulated protein kinase (ERK) is one of the mammalian mitogen-activated protein kinase (MAPKs) in the hypertrophy pathway. It is regulated by many factors such as Ras. It has been demonstrated that in vascular smooth muscle cells of human peripheral arteries resistance partially depends on the ERK activities (Rhian et al., 1998). The MAP kinase isoform ERK1/2 is expressed in most cells if not all (Yanhua et al., 1999). These proteins are identified initially as two protein kinases with a molecular weight of 42-44 Kda. ERK1/2 is phosphorylated on
tyrosin in a response to growth factors and stimuli (Ray LB et al., 1988). The signal pathway that is mediated by ERK1/2 is a multisteped cascade of phosphorylation. This pathway includes the activation and phosphorylation of other regulatory proteins. Also, ERK-mediates the signal pathway to the level of gene expression and has been reported to be involved in proliferating cells and cell differentiation in vitro (Strugil et al., 1988, Tamemoto et al., 1992).

Another important kinases in the hypertrophy pathway C-jun-N-terminal kinase (JNK), which is also known as the stress activated protein kinase (SAPK). The JNK pathway has been found to be active mainly in apoptosis. However, the cellular function of JNK is not clear and it has been reported to be active mainly as a stress response pathway (Banes et al., 2001). So the activation of JNK by protein stress is important for many pathological processes. These processes include ischemia and reperfusion injury associated with cardiovascular disease. In vascular smooth muscles, it has been noted that the JNK pathway can be activated by either vascular injury or by G-protein coupled receptor agonist, notably angiotensin II (Ohashi et al., 2000, Schmitz et al., 1998).

Recent findings suggest that the small Rho plays a crucial role regulation of blood pressure in vivo. Rho may e regulated by cytoskeletal structure that play an important role mechanotransduction of pressure in blood vessels (Henrion D., et al., 1997). With that concept, several in vitro studies have shown that activated Rho plays a
role in smooth muscle contraction, myosin light-chain phosphorylation and stress fiber formation (Aman M, et al., 1997). In addition, Rho involves in activating hypertrophy pathway by activating ERK1/2 and the contraction induced by Ang II by activating the intact actin filaments network (Matrougui K, et al., 2001).

Studies have shown that profilin as a key regulator of actin reorganization function downstream of Rac and Rho signaling cascade, therefore, could be an important mediator in the hypertrophy pathway. Profilin could play a role in the activation of members of MAPK kinase in the hypertrophy pathway.

In order to study the role of profilin in actin polymerization and in vascular hypertrophy, we have expressed transgenically the cDNA of active mutant of human profilin I (wild type), using smooth muscle α-actin promoter to direct expression of the transgenic to smooth muscle cells. Our data indicating that the ability of human profilin I to enhance actin polymerization in the vascular smooth muscle cells in culture and altering the normal function of the cardiovascular system in vivo.
Figure 1. Three levels of regulation by profilin

A) Profilin promotes depolymerization by binding to monomer

B) Profilin promotes polymerization by increasing the rate of nucleation exchange

C) Profilin promotes polymerization by lowering the critical concentration

(Juie A et al., 1993)
Three levels of Regulation by Profilin

Figure 1: Three level of regulation by profilin
Figure 2. Dendritic model of profilin in cell motility and actin polymerization

Profilin increases actin polymerization by facilitating nucleotide exchange. Profilin Interacts with other proteins (WASP, Arp2/3, Cdc42 and Rac) and then activate actin polymerization at the barbed end.
Figure 2. Dendritic model of profilin in cell motility and actin polymerization
CHAPTER 3

MATERIALS AND METHODS

3.1. Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and were of the best grade available. Rhodamine-phalloidin is from Molecular Probes (Eugene, OR).

3.2. Animals

Dr. Hamdy Hassanain (Department of Surgery at the Ohio State University) has engineered a transgenic mouse models that over-expresses the human profilin gene (wild type) or the dominant negative mutant (88R/L) in FVB/N mice, using the mouse smooth muscle a-actin promoter containing all elements known to be required for optimal transcription of the smooth muscle α-actin gene. The smooth muscle α-actin promoter was used to induce selective over-expression of human wild type or 88R/L profilin mutant in smooth muscle cells. The genome of the mice incorporated the cDNA of WT-profilin or the mutant including their polyadenylation sites. Smp-8 plasmid that contains a 3.6 kb segment of the 5'-region of the mouse smooth muscle α-actin

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promoter containing all the elements known to be required for optimal transcription, was cut with XhoI and EcoRI, then the cDNA of human profilin I WT or 88R/L including its polyadenylation tail (with XhoI and EcoRI ends) was cloned within these sites. A SphI/EcoRI fragment of this plasmid that has the promoter and the profilin cDNA, was isolated and microinjected into mouse fertilized eggs of FVB/N females. Single cell embryos derived from superovulated FVB/N females were used for the microinjection procedure. Surviving microinjected embryos were implanted into pseudopregnant CBA/B6 foster mothers (Hogan et al., 1986). PCR of tail genomic DNA with specific primers for the transgene was used to identify the transgenic founders. Three transgenic founders were selected and confirmed on the basis of positive southern blot analysis, and the one having the highest number of human gene copies was used to establish a stable transgenic line by breeding it with non-transgenic FVB/N mates.

3.3. Extracting of genomic DNA from the tails of mice

Polymerase Chain Reaction (PCR) was used to identify the transgenic mice. Genomic DNA was isolated from tail clips and incubated with 500 μl of lysis buffer overnight at 60 °C. 

Lysis buffer components:

50 mM ..............................................Tris pH 8.0

50 mM ..............................................NaCl
50 mM ..................................EDTA
37.5 µl ..................................Proteinase K (100µg/ml)

The lysate was cleaned with phenol: chloroform: isooamy alcohol (25:24:1) two times followed by one chloroform extraction. The supernatant was removed and placed into a new tube after each of the three extraction steps. The supernatant was cleaned and was free from any contamination of the lower-organic phase or the protein inter-phase. Equal volume of ice cold Ethanol (100%) was used to precipitate DNA followed by centrifugation at 14,000 rpm for 20 minutes at 4 °C. The DNA was then washed twice with alcohol (70%) for at least 5 minutes each time and centrifuged for ten minutes at 4 °C to recover DNA. The DNA pellets were dried from any trace of Ethanol using spin vacuum. Thirty-five µl of H2O was used to dissolve DNA in each centrifuge tube.

The PCR reaction was run using specific primers for the human profilin gene. The forward EX2-primer is TCT CTG CAG AAC CCT GAG AC derived from the smooth muscle α-actin promoter located at the 5’ end of profilin cDNA. The reverse primer sequence is (GAG GTC GAC GGT ATC GAT AAG CTT G) derived from the 3’ end of human profilin cDNA. From each sample, 1 µl of genomic DNA was used for the PCR and incubated with nineteen µl H2O at 95°C for 15 – 20 minutes to in activate proteinase K. The PCR reaction was set as following:

5 µl ................................. PCR buffer
1.5 µl ...................................... 50 mM MgCl₂
1 µl ........................................ dNTPs (10 mM)
1 µl (12 pmole) ......................... EX2-primer
1 µl (12 pmole) ......................... Reserve-primer
0.5 µl (2.5 units) ....................... Tag polymerase
20 µl ..................................... ddH₂O

(All PCR reagents were purchased from Gibco-BRL)

The final volume for the reaction was 50 µl. The PCR cycle was as following;

94 °C for 45 second .................. Denaturation
54 °C for 1 minute .................... Annealing
70 °C for 2 minutes .................. Extension

The reaction was run for 35 cycles and followed by one extension step at 70 °C for 10 minute to assure all the PCR products are double-stranded DNA.

The PCR products were separated on 1.5% agarose gel using TAE buffer (0.04 M Tris-Bis, 0.002 M EDTA). The agarose gel was run under electric field of 95 voltages for 2 hours. The PCR products on the agarose gel were visualized using ethidium bromide (0.5 µg/M).
3.4. Reverse Transcriptase Polymerase Chain Reaction RT-PCR

Since there is no antibody for profilin available, we have used the reverse transcriptase polymerase chain reaction (RT-PCR) to confirm the expression of profilin in vascular smooth muscle cells.

Tissues isolated from mice were immediately frozen in liquid nitrogen. The frozen tissue was pulverized in liquid N2 to a fine powder using a mortar and pestle. The powder was transferred to 50 ml plastic blue-capped tube. The RTL lysis buffer (QIAGEN) was added to each sample (350 µl to 600 µl) and the sample was homogenized. Total cellular RNA was extracted using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The cDNA template was obtained utilizing Superscript II (Gibco-BRL) then amplified using Taq-DNA polymerase (Gibco BRL) with 35 cycles of denaturation (94°C, 45 s), primer annealing (54°C, 30 s) and extension (72°C, 2 min). The profilin-specific primers (as shown above) were used for PCR.

3.5. In Situ RT-PCR

In order to identify the selective expression of the profilin I gene in the layers of the blood vessels, we used the In situ RT-PCR. The aorta from transgenic (profilin I WT or 88R/L) and control mice were fixed in 10% buffered formalin, then embedded in paraffin. Three 4 micron sections were placed on individual silane coated glass slides to facilitate the performance of the in situ PCR reaction. RT in situ PCR was performed on aortic specimens from transgenic and nontransgenic animals concurrently using specific
primers specific for profilin (as mentioned above). The in-situ RT-PCR technique was performed in collaboration with Dr. Nuovo (Associate Professor of Medicine, Department of Pathology at Ohio State University). The protocol used for these experiments has been previously described (Nuovo, 1997, 1998). Briefly, optimal protease digestion time was first determined using as the guide nonspecific incorporation of the reporter nucleotide (10 μM digoxigenin dUTP). Optimal protease digestion was followed by overnight incubation in RNase-free DNase (10 U per sample, Boehringer Mannheim, Indianapolis, IN), and one step RT/PCR using digoxigenin dUTP. After 20 cycles, the slides were washed at high stringency (60°C for 10 minutes in 15 mM salt with 2% bovine serum albumin). The digoxigenin labeled target specific cDNA was detected using the antidigoxigenin-alkaline phosphatase conjugate (Boehringer Mannheim, 1:200 in PBS for 30 minutes at 37°C) followed by exposure to the chromogens nitroblue tetrazolium and bromochloroindoly1 phosphate (NBT/BCIP) (EnzoBiochemicals, Farmingdale, NY). Nuclear fast red, which stains negative cells pink, served as the counterstain. As negative controls, in situ RT-PCR was performed similarly either with irrelevant human papillomavirus (HPV) specific primers or with RNase pretreatment of the slides prior to the in situ RT-PCR with specific profilin primers. For the positive control, the same HPV primers were used without prior DNase treatment on the slides. Specimens from transgenic mice were run concurrently with non-transgenic mice.
3. 6. *Quantitative analysis of profilin with Polyproline-Sepharose Beads*

3. 6. 1. Preparation of affinity columns from CNBr-activated sepharose 4B

Washing the beads:

One gram of beads (dry powder, about 3.5 ml final gel volume) was suspended in 1 mM HCl for about one minute. The gel was washed first over a sintered filter with 200 ml of 1 mM HCl. The gel was washed a second time with same volume of dd H2O then with about 200 ml of 0.1 M NaHCO3 buffer at pH 8.3

3. 6. 2. Coupling of the protein:

Approximately 5 – 10 mg protein/1 ml gel was used. The protein was dissolved in the binding buffer (NaHCO3), using 5 ml per gram dry powder. The dissolved protein was added over the gel and was rotated gently for 1 hour at 22° C. The excess unbound protein was washed out with minimum 5 gel volumes of binding buffer. The remaining active groups in the gel were blocked with 1 M Ethanolamine at pH 8 or with 0.1 M Tris-HCl at pH 8 and then the gel was rotated gently at 22° C for 2 hours. The gel was then washed with 3 times (minimum 5 volumes of the gel) of each of the following binding buffers:

1. 100 mM NaAc buffer, pH 4.0, with 0.5 M NaCl.
2. 100 mM Tris-HCl buffer, pH 8.0, with 0.5 M NaCl.

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3. 6. 3. Poly(L)Proline-Sepherose 4B column preparation

One gram of poly-L-prolin (MW 12000-15000) was dissolved in 100 ml dH₂O by stirring at 4° C for 2 days, then 500 ml of CNBr-activated beads (Pharmacia) were added and stirred at 4° C for 20 hours (as described in the manufacture’s protocol). Large volumes of double distilled water were used for washing in a large beaker. We then washed again with a cold buffer, the composition of the buffer is as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.1 M</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.1 M</td>
</tr>
<tr>
<td>DTT</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>Tris HCl</td>
<td>0.01 M at pH 7 (adjusted at 4° C)</td>
</tr>
</tbody>
</table>

The gel was poured into the columns and the columns were then equilibrated as described in details by (Kaiser, Cell Motil Cytoskel 14,251; Tuderman, Eur j Biochem 52, 9). In order to isolate profilin, smooth muscle cells isolated from profilin WT, 88R/L or non-transgenic control mice were grown in 6 well-plate then lysed with lysis buffer contains protease inhibitor cocktail (CLAP from Sigma). The lysed cells were then sonicated for 20 seconds and centrifuged for 10 minute at 4° C. The pellet was discarded and the supernatant was kept and small volume was used for protein assay using BCA reagent (Pierce). The protein assay was performed as described in the Pierce protocol.
In order to isolate profilin from cell lysates, equal amounts of cell lysates was added to 50 μl of Poly-L-Proline/sephrose beads and kept on a shaker for 1 hour at 4 °C to insure mixing. The tubes were centrifuged at 1000 rpm at 4° C and the supernatant was discarded. The beads were washed twice with PBS buffer. Thirty micro liters of sample buffer was added to each sample tube and then boiled for 30 seconds in water. The samples were centrifuged and the supernatant was collected and profilin was separated on SDS-PAGE. Twenty microliters of samples was used to run the SDS gel. Platelets were used as a positive control due to its high content of profilin protein. Platelets by centrifuging about 100 μl of blood at 750 rpm/ 18 minute of blood to remove blood cells. The supernatant (plasma) was collected and 2 µl prostaglandin was added plasma then centrifuged again at 1500 rpm for 10 minutes and the pellet (platelets) were washed with Tyrodes twice by adding drops to the wall of the tube to avoid activating the platelet.

3. 6. 4. Staining with Coomasie Blue

Protein bands were visualized on the SDS-polyacrylamide gel using coomasie blue staining as described in current protocols in Molecular biology (Current Protocol in Molecular Biology, V.2).
3. 7. Preparation of primary culture from aortic vascular smooth muscle cells

3. 7. 1. Collagen coating:

Sixty-eight µl of collagen (Upstate biotechnology) was added to 5 ml of phosphate buffered saline with calcium (PBS plusCa²⁺ pH 7) to 6-well tissue culture plates (Falcon, Becton Dickinson, Oxnard, CA). The collagen was allowed to set for 1 to 2 hours at room temperature or overnight at 4 C. Collagen was removed and the tissue culture plates were washed twice with PBS. One ml of SmGm media (Insulin 0.5 ml, HFGF-B 1.0 ml, GA-1000 0.5 ml, FBS 25 ml and hEGF 0.5 ml) was added to the plates and they allowed to set for 30 minutes at 37 °C before the introduction of the cell explants.

3. 7. 2. Tissue Explants:

Adult male mice (Profilin I, 88R/L transgenic and non-transgenic control) were sacrificed by CO₂ asphyxiation. The thoracic aorta was removed and placed in a cold Hanks' balanced salt solution (HBBS) (GIBCO). Clotted blood and connective tissues and were removed from the lumen and the outer layer of the blood vessels. Gently the tunica adventitia was removed, and the lumen was opened and gently layer was scraped. The removal of the tunica adventitia as well as the endothelial layer to insure isolation pure smooth muscle cells from the media. The media layer was then cut to small portions and placed in the media with the collagen gel. The dish was then placed at 37°C for two hours and then another 1 ml media was added to the explants and left undisturbed in the incubator for 2 to 3 days. The media was changed every 2 to 3 days.

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until the cells were 100% confluent. The cells were then passed to T 25 dish for exponentially growth. Cultures were removed from 6 well dishes by tryptic digestion (0.05% trypsin, 0.025% EDTA in Ca^{2+}, Mg^{2+} free phosphate buffered saline (PBS), pH 7.4 at 36° C). One cells were confluent in T25 flasks they were passed tp T75 flasks to be used for experiments or preparing freezing stocks.

3. 7. 3. Confirming the purity of the vascular smooth muscle cells

The isolated cells were stained for the vascular smooth muscle α-actin to confirm their identity as smooth muscle cells. Cells were then fixed in paraformaldehyde 3% (3g/1000ml, pH 7.1-7.4) using 7 ml for T-75. The samples were incubated for 15 minutes on ice. The samples were centrifuged at 1000 rpm for 5 minutes at 4° C and the supernatant was discarded. The samples were then resuspended again with 4 ml cold PBS buffer and centrifuged at 1000 rpm for 5 minutes. The suspended samples were then kept at 4° C. The samples were left at 4° C (samples + PBS + paraformaldehyed) and then centrifuged at 1000 rpm for 10 minutes at 4° C. The pellet was kept and the supernatant was discarded. The cells were treated with saponin to increase their permeability to the antibody. Saponin was made by dissolving 0.15 g of dry ml of 1X PBS without Ca^{2+}, and then 0.5g of 1% goat serum albumin was added (Vector labs S-1000) and then filtered. Saponin solution was added to each sample in the amount of 1000 μl, then rinsed and centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded with care not to disturbed the pellet. For each sample, a control tube was labeled. Saponin was added again in the amount of 200 μl to each
tube and mixed by pipette until the cells were spread throughout the solution. One hundred microliters were taken from each tube of sample and added to the labeled control tube for each sample. The tubes remained on ice the entire time. The primary antibody was the mouse anti α-actin antibody (Sigma A-2547). Primary antibody was prepared by 1:100 dilution (99 μl saponin solution + 1 μl antibodies). For each sample, 100 μl total solution was used except for the negative control which does not contain the primary antibodies. The tubes were left on ice for 40 minutes. After that, the samples were washed with saponin solution twice and centrifuged for 5 minutes at 1000 rpm at 4°C and the supernatant was discarded. A secondary labeled antibody (goat α-mouse anti bodies-FITC) was added in the amount of 100 μl, including the control tubes. The tubes were left on ice and in the dark for 30 minutes.

The samples were then washed twice with saponin solution and spun after each wash. Cells responded with saponin solution (250 μl) and then put in FACS tubes and 10 μl LDS, for labeling the dead cells. The tubes with samples were then read by Flow Cytometry. (Keith L. Hall et al., 1991, Geoffrey Pickering J. et al., 1992, and Donald C. Macleod et al., 1994).
3. 8. Staining with Rhodamine-Phalloidin

Smooth muscle cells from profilin I, 88R/L and non-transgenic control were grown on coverslips placed in 35 mm plates. The cells were fixed in 3.7% formaldehyde in PBS for 10 minutes at room temperature after reaching 60-70% confluency. Tritron X-100 (TX) in PBS was then added to the coverslips for 10 minutes at room temperature. Rhodamine-Phalloidin (100 μl in 1 ml PBS) was added to each samples and incubated for half hour in the dark. The coverslips were washed with PBS for 30 minutes. A Nikon Eclipse 800 fluorescence microscope was used to visualize and assess the actin filaments stained with Rhodamine-Phalloidin.

3. 9. Cell migration assay using fluorescence microscope

Smooth muscle cells from WT profilin, 88R/L and control were grown in 6-well dishes on the top of cover slips. The Cells were left until they become confluent and then wounded with a pipette tip. The confluent monolayer on the cover slip was placed in the chamber containing the growth media. The chamber was placed on a warm stage of the Nikon Eclipse 800 fluorescence microscope that used to monitor the migration of the cells. Time-lapse microscopy was performed in a closed bath-imaging chamber (Warner Instrument Corp) on wounded monolayer. Images were acquired at 60 second for at least 4 hours. Individual cells were tracked with the "track object" menu of the MetaMorph image analysis system. At the end of the assay, individual cells were tracked with the "Track object" menu of MetaMorph, with the selector placed on the nucleus of each tracked cell and the mean speed of the individual cells (μm/min)
was computed by the program. The module of the resultant vector of the cell path was
determined by measuring the distance between the start and end points. Many cells in
each experiment were traced by the same program and the experiment was repeated
many times for each line as described (Leni Moldovan et al., 2000)

3. 10. Immunohistochemistry

Mice from the tow lines (profilin I WT, 88R/L transgenic, and non-transgenic
control) were sacrificed and the aorta was collected. Tissue samples were placed in
small cassettes and fixed in 10% neutral buffer formalin embedding. Ethanol 70% was
added to the samples in the container and shook for 30 minutes. The old ethanol was
discarded and the cassettes were left in fresh ethanol 70% and left on the shaker for 30
minutes. The cassettes were transferred to the container with fresh ethanol 95% for 30
minutes on the shaker. The ethanol was changed once and placed on the shaker for
anther 30-minutes. Finally, 100% ethanol was used for 30 minutes on the shaker and
then transferred to a container with fresh histoclear solution for 30 minutes on the
shaker. Samples in the cassettes were placed in containers with melted paraffin. The
cassettes were left in hot paraffin for 30 minutes. The cassettes were then placed in a
second batch of paraffin and allowed to stand for 30 minutes. The samples embedded
in paraffin were then cut to 3 micron sections and placed in slides for
Immunohistochemistry and H&E staining.
The samples were circled with pap pen on the slides. The sections were treated with 2% paraformaldehyde for 15 minutes and then were rinsed with a PBS squirt bottle. The sections were then submerged in PBS for 2 minutes. The sections were incubated with 10% normal mouse serum at 37 °C for 30 minutes. The serum was then tapped off of the slides and blotted dry. PBS was placed in the control section. Primary antibodies (anti actin smooth muscle) were placed on the rest of the sections, except the control in dilution of 1:1500 in PBS and incubated at 37 °C for 30 minutes. The sections were rinsed with a PBS squirt bottle and rinsed with PBS two times for 2 minutes each. Secondary antibodies in dilution of 1:1500 were applied to all sections. With the secondary antibodies, the sections were incubated for 30 minutes at 37 °C. Streptavidin of concentration of 1:2000 in PBS was applied to all sections and incubated for 30 minutes at 37 °C. The samples were then washed with PBS and then checked under the light microscope to insure staining. A vector DAB kit was used for 5 minutes to develop the slides (2 drops buffer, 4 drops DAB solution, 2 drops H₂O₂ in 5 ml ddH₂O). The reaction was stopped by rinsing the slides in warm running water. The slides were dipped 5 times in hematoxylin and rinsed immediately in water. The slides were then dehydrated and covered with permout.

3. II. Hematoxylin and Eosin (H&E) staining

A paraffin section of 3 microns were used to stain samples from the aorta of the profilin I WT, non-transgenic control and 88R/L. The sections were placed in the superfrost plus slides and left until the sections were adhered completely onto the slides.
The slides with sections were deparaffinized and dehydrated by alcohols and then rinsed in running tap water. The slides were then placed in hematoxylin for 3 minutes and then rinsed in running tap water. The slides were placed in clarifier for 1 minute and rinsed again in running tap water. Ten to twenty drops of ammonia water was used on the slides which were then rinsed in running tap water. Ten to twenty drops of alcohol 70% was used on the slides. Eosin was then added to the slides for 3 minutes and rinsed in running tap water. The slides with the sections were then dehydrated and cleared with histoclear solution. Slides were mounted in permount and coverslips.

3. 12. Western Blot

The cells were washed twice with cold 1x PBS after removing the medium. At the time of lysing the cells, protease inhibitor cocktail was added to the lysis buffer at 10 μl for each ml (Sigma). The cells were lysed for 15-20 minutes at room temperature.

The composition of the lysis buffer is as follows:

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Stock</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Sodium Phosphate (pH 7.2)</td>
<td>1 M</td>
<td>10 ml</td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>5 M</td>
<td>30 ml</td>
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<tr>
<td>10 mM NaF</td>
<td>1 M</td>
<td>10 ml</td>
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<tr>
<td>0.5% deoxycholate</td>
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<td>5 g</td>
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<tr>
<td>10 mM EDTA (pH 8.0)</td>
<td>0.5 M</td>
<td>20 ml</td>
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<tr>
<td>0.1% SDS</td>
<td>20%</td>
<td>5 ml</td>
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1% Nonidet P40 or Igepal CA-630 
Water 

10 ml 
915 ml 

Cell lysate was transferred to 1.5 ml eppendorf tube and sonicated for 15 minutes. The tubes were spun for 10 minutes 10,000 rpm and the supernatant was saved. A protein assay was assessed by Pierce BCA protein assay kit. The standard curve was done using BSA as described Pierce protocol.

3.12 Preparing SDS-Polyacrylamide gel:
3.12.1 Preparing the separating gel:

We used 7.5% gel that was determined according to the MW of our target proteins. For 7.5% gel we have prepared two gels as follows;

30% Acrylamide/Bis (30%, 0.8%) 
Separating buffer (1.5m Tris-HCl pH 8.8) 
(From National Diagnostic) 
SDS (10%) 
Water 
10%Ammonium persulphate (fresh) 
TEMED 

0.3 ml 
7.5 ml 
7.5 ml 
0.3 ml 
15 ml 
0.1 ml 
0.02 ml 

The mixture was gently mixed after adding all the components. The gel solution was loaded with a transfer pipet between the two glass plates up to the mark (1 cm
mark). Isopropanol was added to the top of the gel to make sure the surface of the gel was straight. The gel was left for one hour to polymerize.

3. 12. 2. Preparing the stacking gel:

In a 15 ml fraction tube, the stacking gel was prepared as followed:

- Acrylamide/Bis (30%, 0.8%) 2.66 ml
- Stacking buffer (0.5M Tris-HCl pH 6.8) 5 ml
- (From National Diagnostic)
- 10% SDS 0.2 ml
- Water 12.2 ml
- Freshly prepared 10% Ammonium persulphate 100 µl
- TEMED 5 µl

The gel stacking solution was left for 45 minutes for polymerization.

The running buffer is as follows:

- Trizma (0.025 M Tris-HCl pH 8.3) 12 g
- 0.192 M Glycine 57.6 g
- 0.1% SDS 40 ml
- Water Up to 4 liter
The frozen samples were thawed out and 5 μl of 2X loading buffer was added to each 25μl samples.

The loading buffer was prepared by:

Stacking buffer 2.5 ml
10% SDS 4 ml
Glycerol 2 ml
2-mercaptoethanol 1 ml
Water 10 ml

Equal amounts of protein were taken from each sample and the volume was adjusted with water to keep the volume equal for each sample. The samples were boiled for 5 minutes to denature the protein then cooled at room temperature. The samples were centrifuged and then loaded onto the gel.

The gel was ran at 80 voltages. After the samples were interred into the separating gel, the voltage was increased to 90 voltages. The gel was allowed to run until the lower tracking dye close to end of the gel.

3.12.3. Preparing the membrane and transfer buffer:

During this time, the membrane and filter papers were cut into 9cm X 5.5 cm blocks. The membrane and filter papers were soaked in transfer buffer for about 15 minutes. The composition of the transfer buffer is as follows:

Methanol 200 ml
10X Wet transfer buffer (3.02g Trizma and 143g Glycine)  
Water

3.12.4. Transfer proteins from the gel to the membrane:

Once the gel is ready, the stacking gel was removed and the gel was placed submerge in a small plastic container with the transfer buffer for about for 10 minutes with a slow agitation. The gel was assembled in the cassette of the transfer apparatus with the membrane in contact with the gel and facing the positive electrode. The transfer was run at 70 voltages for 3 hours in the cold room.

3.12.5. Immunoblotting:

After the gel was transferred, the cassette was removed and the membrane was transferred to a container and soaked in blocking solution (1X PBS-Tween with 2% of non-fat milk) for 2 hours. The membrane was rinsed twice with 1X PBS-TWEEN-20 (15 minute each time) then the membrane was hybridized the primary antibody added in the blocking solution at a concentration of 1:1000 for two hours. Then the membrane was washed once with 1X TBS-Tween for 15 minutes and three times with 1XPBS-TWEEN-20 for 5 minutes each. Secondary antibodies were prepared with the same blocking solution at the dilution of 1:4000 and incubated with the membrane for one hour. After the hybridization with the second antibody, the membrane was washed three times as mentioned above. The protein bands were visualized by using Super Signal West Pico Chemiluminescent kit from Pierce Co. according to the kit protocol.
3. 13. *Animal Surgery:*

The detailed techniques for catheter insertion are described in Dr. Morris’ publications (Merrill, D.C et al., 1996). Briefly, mice are anesthetized with a ketamine:xylazine mixture (70:6 mg/kg, im) and surgery is conducted with the aid of a dissecting microscope. The carotid artery and jugular vein are isolated and cannulated. A polysulfone button is attached to the back muscle and the catheters are passed through into a stainless steel spring attached to a swivel at the top of the cage. Heparinized saline (20 U/ml) is continuously infused (25 μl/h) into the arterial catheter using a syringe pump to maintain catheter patency. The mice are housed individually in specialized cages (Instech Laboratories Inc). Arterial catheters are prepared from Microrenathane tubing (0.025 OD x 0.012 I.E., Braintree Laboratories) with the end heat-stretched and a polyethylene collar used to secure the catheter in place. Animals will be allowed to recover from surgery before the experiments begin (normally 3-4 days for vascular catheters). This is extremely important in order to obtain non-stressed, baseline cardiovascular data.


Monitoring blood pressure technique was performed and under the supervision of Dr. Morris Wright State University. A flow-through blood pressure transducer is calibrated and connected to a computerized data acquisition system (BIOPAC Systems Inc.). Systolic, diastolic arterial blood pressure, and heart rate are recorded using a...
Pentium based computer with a removable hard drive. Mean arterial blood pressure (MAP) is calculated from systolic and diastolic measurements. Data can be collected long term for circadian analysis or for shorter periods for evaluation of stimulus-induced changes. The arterial catheter is used for the collection of blood samples and the venous catheter for injections.

3.15. Statistics and Data Analysis:

Mean arterial pressure (MAP) and heart rate are converted to text files for plotting and analysis. The night/day patterns will be determined using "Circadia" (a program designed for analysis of rhythmic patterns). It is able to handle the large amount of data, which is generated with 24h monitoring of cardiovascular and intake parameters. Statistical analysis of the experimental paradigms will be performed using the appropriate analysis of variance coupled with post-hoc testing. Statistical analysis of electrophysiological data testing two-sample hypothesis will be performed with the Student's t test, to determine the differences between control, profilin I WT, and 88R/L mice.
CHAPTER 4

RESULTS

4. 1. Generation of transgenic mice;

We have engineered a transgenic mouse model that overexpresses the cDNA of the human profilin I gene (wild type isoform) or the dominant negative mutant (88R/L) in FVB/N mice, using the mouse smooth muscle α-actin promoter containing all elements known to be required for optimal transcription of the SM α-actin gene (Strauch et al., 1992, 1995). The genome of the mice incorporated the cDNA of profilin I WT transgenic or 88R/L mutant including its polyadenylation sites. Founder mice were identified using southern blot analysis of tail genomic DNA. Three transgenic founders were selected on the basis of positive southern blot analysis, and the one confirmed as having the highest number of human profilin I WT transgenic or 88R/L copies were used to establish a stable transgenic line by breeding it with nontransgenic FVB/N mates. Polymerase chain reaction analysis of genomic DNA from tails of F1 generation was performed to identify the transgenic primers that can specifically recognize the transgene (Fig.4).
The positive samples were confirmed using specific restriction enzyme that cut through
the transgene (Figure # 5).

4. 2. Expression of human profilin gene in transgenic mice

To confirm the expression of profiling I WT or 88R/L mutant in transgenic mice, various tissues were analyzed using Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR). The cDNA was amplified using primers that selectively recognize the transgene (profiling 1 or 88R/L mutant). RT-PCR analysis confirmed the selective transcription of human profilin in smooth muscle, including blood vessels, spleen, uterus and intestine (Figure # 6), while the transgene transcript was not detected in control mice (data not shown), nor in the in the heart and brain of transgenic mice (Figure # 6). The kidney was also positive for profilin I WT transgenic mRNA as the mesangial cells of glomeruli express SM α-actin (Johnson et al., 1992).

4. 3. In Situ RT-PCR

Since there is no commercially available antibody for profilin to conduct Western blotting or immunohistochemistry analyses, we sought to confirm the expression of profiling with In Situ RT-PCR. The advantage of this new technique is that it is a quantitative assay and can be performed on paraffin embedded sections of desired tissues allowing us to determine the sites of expression. Therefore, we developed an in situ-reverse transcriptase PCR assay (IS-RT-PCR) to analyze paraffin embedded sections of aortas from profiling I WT transgenic, 88R/L and non-transgenic
mice. Using *in situ*-RT-PCR, we have localized RNA molecules coding for the transgene using specific sets of primers in profilin I WT and 88R/L transgenic mice. Expression of RNA for human profilin was undetectable in the aorta of non-transgenic mice. Our results showed that 70% of human profilin I WT or 88R/L messages were expressed in the media layer (smooth muscle cells) and about 30% were found in the adventicia (Figures #7, 8 & 9).

4.4. Poly-L-Prolin Assay

The activity of a particular gene is mainly determined by its ability to produce a functional protein. Due to the lack of profilin antibody to confirm protein expression, we have adopted an alternative method that has been used to analyze the expression of profilin in cell extracts. This new method has been widely used to detect the expression of profilin by its affinity to bind to poly-L-prolin. Poly-L-prolin beads have been used widely to extract profilin from extracts based on its ability to bind to poly-L-Prolin. The experiment was performed by adding poly-L-prolin beads to the aortic protein extracts from aortas of transgenic mice (profilin I WT or 88R/L) or non-transgenic mice and mixed for two hours at 4°C, then the extracted proteins from the beads were analyzed on SDS gel electrophoresis. A positive control for profilin was also included in SDS gel. The polyacrylamide gel electrophoresis has revealed the presence of profilin protein of approximately 12-15 kd which is the size of profilin from the profilin I WT or 88R/L transgenic mice.
The level of the extracted profiling from the beads was high in the aortic extracts from the transgenic mice (profilin I WT or 88R/L), but low in non-transgenic control (Figure # 10).

4. 5. Smooth muscle cells analysis by flow Cytometry

We have developed a primary aortic VSMCs from thoracic aortas isolated from male profilin I WT or 88R/L transgenic mice, or non-transgenic mice. The aortas were treated with collagenase type II (2 mg/ml) for 10 minutes to loosen the adventitia then both endothelial and adventitial layer are carefully removed by gentle peeling with two pairs of fine forceps under a dissecting microscope. The muscle layers were transferred to fresh collagenase, cut into small pieces in a gently stirred orbital shaker for 1 hour at 37°C, and dispersed into single cells. The cells were incubated in DMEM supplemented with 10% calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and were passaged twice a week by harvesting with trypsin:EDTA and seeding into 75-cm² flasks. Smooth muscle cells analysis by flow Cytometry after vascular smooth muscle α-actin staining confirmed the purity of the isolated aortic VSM cells. Our results showed that more than 97.5 % of the cells were smooth muscle cells α-actin positive as in Figures # 11, 12 and 13.

4. 6. Profilin induces actin organization

The classical role of profilin in actin polymerization is to facilitate the ATP and ADP exchange and loading ATP-monomer actin to the barbed end of the growing
filament. In our transgenic models, as a result of overexpression of profilin I WT cDNA or the dominant negative mutant in vascular smooth muscle cells, it is anticipated that significant effects on actin organization. In order to assess actin organization and stress fiber formation we have stained confluent monolayer of smooth muscle cells from transgenic mice or non-transgenic controls with Rhodamine-phalloidin. Our results showed significant increase in stress fibers and membrane ruffling in VSM cells from profilin I WT transgenic mice as compared to non-transgenic controls (Figure # 14 and 15). The dominant negative 88R/L cells, however, showed significant decrease in stress fiber formation and ruffling than the non-transgenic controls (Figure # 16). Our results clearly showed the direct effects of profilin on actin reorganization and suggested a possible role of actin in vascular hypertrophy and in changing the vascular tone.

4. 7. Profilin induces cell migration

The role of profilin in actin polymerization and membrane ruffling supports its critical role in cell migration and possible contribution in developing vascular lesions i.e. arteriosclerosis (Shiro Suetsugu et al., 1999). In order to determine the role of profilin in smooth muscle cells motility we introduced a wound on monolayes of VSM cells from transgenic or control cells. The confluent monolayer of smooth muscle cells was scratched with a glass tip to create a wound and the movement of cells was monitored. The smooth muscle cells close to the wound margin began to move to the space provided by the glass tips to restore the confluence of the monolayer. Time-lapse
fluorescence microscopy of the monolayer closest to the margins of the wound shows that the smooth muscle cells from the profilin I WT were more actively motile with a mean speed of $0.23 \pm 0.03 \, \mu \text{m/min}$ (Figure #18) ($P < 0.05$). In contrast, smooth muscle cells from the 88R/L line were slower (Figure #19) than profilin1 cells with a mean speed of $0.17 \pm 0.03 \, \mu \text{m/min}$. There was no statistical difference in the speed found between the 88R/L and non-transgenic control (Figure #17). The figure also showed clearly huge increase in ruffling in profilin1 cells during their movement to restore the confluence of the monolayer as compared to 88R/L or non-transgenic VSM cells. These results again confirmed the role of profilin I in induce cell migration by increasing ruffling formation. In order to confirm increase of ruffling in VSM cells from profilin I WT transgenic mice we have stain cells with rhodamin-phallidine prior introducing wounds in the confluent monolayers (Figure # 15). This figure shows the significant increase in ruffling in profilin I WT cell compare to 88R/L and control cells.

4. 8. Immunohistochemistry staining for the aorta

We used immunohistochemistry (IHC) to examine the integrity of the blood vessels in profilin I WT and 88R/L transgenic as compared to matched control littermates. Paraffin sections were also stained for SMα-actin and many slides were examined. Our results showed the actin density was greater in paraffin sections from profilin I WT transgenic as compared to 88R/L and non-transgenic controls. These results suggested the increase in actin polymerization in aortic sections of profilin I WT compared to 88R/L and non-transgenic (Figures #, 29, 30 and 31).
4. 9. **H & E Staining**

The H&E staining showed clear sign of remodeling and vascular hypertrophy in aorta of profilin I WT transgenic mice that was evident by enlargement of VSM cells and increase the size of nucleus in these cells (Figure # 27). There were no differences, however, between 88R/L and non-transgenic control aortic sections (Figures # 26 and 28). Our data clearly suggested that actin dynamic is an important process and could lead to hypertrophy and heat diseases in general when this process is misregulated.

4. 10. **Profilin induces the hypertrophy signaling cascades**

Angiotensin II (Ang II), the main peptide hormone of the renin-angiotensin system, plays an important role in the pathogenesis of cardiovascular diseases, including atherosclerosis, myocardial infarction, and hypertension (Berk, B.C., et al., 1989). Ang II exerts hypertrophic and hyperplastic effects by activating a number of intracellular signal transduction pathways through a 7-transmembrane heterotrimeric G protein-coupled receptor called the Ang II type 1 (AT₁) receptor (Murphy, T.J., et al., 1991). This pathway is called also the hypertrophy pathway and includes the activation a family of MAPK kinases. The MAPKs are a family of serine/threonine kinases that control cellular responses to growth, apoptosis, and stress signals. There are 4 main MAPKs, including extracellular signal–regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNKs, also termed SAPKs), p38 MAPKs, and big MAPK-1. Activation of this pathway will also lead to the activation of important transcription factor such as AP1 that regulate the expression of growth related genes (Amy KL., et al. 2001).
Since we have seen clear signs of vascular hypertrophy in the aorta of profilin I WT transgenic, we sought to assess the activation of the hypertrophy pathway in these mice as compared to 88R/L transgenic mice and the non-transgenic controls using Western blot analysis. We assessed two important kinases (i.e. ERK1,2 and JNK) in the hypertrophy pathway. Western blot analysis was performed on protein extracts isolated from VSM cells from aortas of profilin I WT, 88R/L and non-transgenic controls using anti phospho-tyrosin-ERK1/2 that recognizes the activated ERK proteins. Our results showed increase phosphorylation of ERK1,2 in profilin I WT extracts as compared to non-transgenic control. However, the phosphorylation of activation of ERK1, 2 in 88R/L protein extracts was completely inhibited (Figure # 23). Another blot was hybridized with anti-ERK1, 2 antibody and the results showed no change in the ERK1,2 protein levels among profilin I WT, 88R/L and control extracts (Figure # 22). These results suggest that increase actin polymerization by increase the expression of profilin1 gene results in the activation of the ERK1, 2 in the hypertrophy pathway.

We also assessed the activation of JNK in smooth muscle cells from of the aorta of profilin I WT, 88R/L and the non-transgenic control mice using western blot and anti phospho-JNK antibody. Our results showed that JNK was activated in VSM cells of profilin1 transgenic, however, JNK activation was inhibited in 88R/L and non-transgenic extracts (Figure # 25). In addition, Western blot on profilin I WT, 88R/L and control protein extracts using anti-JNK antibody showed no changes in the protein.
levels among these extracts (Figure # 24). The activation of RRK1, 2 and JNK is clear evidence that the hypertrophy pathway is activated in blood vessels of profilin I WT transgenic. These results are in agreement with the vascular remodeling seen in profilin I WT mice (Figure # 27 & 30).

4. II. Circadian monitoring of blood pressure and heart rate

Blood pressure is physiologically maintained and can be affected by many factors including the change in the blood vessels structure. In order to evaluate the physiological consequences of the expression of the human profilin in the transgenic mice, we assessed by monitoring the circadian pattern of blood pressure. Circadian blood pressure that allows continuous monitoring of pressure and heart rate in conscious, non-stressed mice was performed by cannulation of the external carotid artery, both on transgenic and control mice (Merrill et al., 1996; Mattson et al., 1998). This study was conducted in collaboration with Dr. Mariana Morris at Wright State University, Dayton Ohio. An external carotid arterial catheter was used and heparin was used during the time of the experiment to prevent thrombotic complications that might be induced by the catheter. Five males from the profilin I WT transgenic mice were used in our study at the age of 11 to 14 months and compared with matching age and gender of control non-transgenic mice. After mice recovered from surgery and showing a stabilized pattern of food and water intake, the blood pressure was monitored in both the profilin I WT and non-transgenic control mice. The mean arterial pressure (MAP) of the profilin I WT mice was significantly and consistently higher comparison to the
MAP of the non-transgenic control mice (Figures #32, 33 and 34). The mean arterial pressure in the profilin I WT mice fluctuated within 130-134 mmHg. Whereas, the mean arterial pressure in the non-transgenic control mice fluctuated within 104-112 mmHg. These results clearly indicate that vascular hypertrophy in media of profilin I WT transgenic mice led to increased resistance of blood flow and development of hypertension.
Figure 3: Creating transgenic line.

Two transgenic lines used to approach the goal of our study. One line was overexpressed with the active form of human profilin I "WT". The second line was overexpressed with the dominant negative human profilin 88R/L. Non-transgenic mice were used as a control. Mice were selectively bred on the basis of the expression of the human profilin I and the α-actin smooth muscle promoter without any dietary or environmental provocative stimuli.
Figure 3: Creating transgenic line.
Figure 4: Polymerase Chain Reaction.

Samples were collected by clipping the tail of the mice at the age of one month.

The genomic DNA then was extracted from the offspring of the mice and Polymerase Chain reaction was performed to select the positives.
Figure 4: Polymerase Chain Reaction (PCR).
Figure 5: Attacking the target band by Restriction enzymes of the founders.

One µl from the positive samples was incubated for three hours with restriction enzymes such as Sam by 0.5 µL. The target band was dissected to two small bands, which insure that the band shown in the PCR was the right band of profilin.
Figure 5: Attacking the target band by Restriction enzymes of the founders.
Figure 6: Reverse Transcriptase Polymerase Chain Reaction RT-PCR.

RT-PCR analysis of mRNA isolated from various tissues of profilin I "wild type" transgenic mice. Note that the aorta, gut and spleen were positive while the liver and brain were negative, consistent with the targeted overexpression of profilin under the control of the smooth muscle α-actin promoter.
Figure 6: Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).
Figure 7: In situ RT-PCR for Control non-transgenic mice.

In situ RT-PCR was performed on four-micron sections of the aorta from specimens from non-transgenic control mice using profilin I specific sets of primers. We found no expression of profilin in the aorta of the control mice.
Figure 7: In situ RT-PCR for Control non-transgenic mice.
Figure 8: In situ RT-PCR for Profilin I Wild type transgenic mice.

In situ RT-PCR was performed on four-micron sections of the aorta from specimens from the profilin I wild type transgenic mice using profilin specific sets of primers. We have found that close to 70% of profilin transcripts, black arrows, were localized in the smooth muscle cells of the aorta and 30% were localized in the adventitia.
Figure 8: In situ RT-PCR for Wild type transgenic mice.
Figure 9: In situ RT-PCR for 88R/L transgenic mice

In situ RT-PCR was performed on four-micron sections of the aorta from specimens from the 88R/L transgenic mice line using profilin-specific sets of primers. We have found that close to 70% of profilin transcripts, black arrows, were localized in the smooth muscle cells of the aorta and 30% were localized in the adventitia.
Figure 9: In situ RT-PCR for 88R/L transgenic mice
Figure 10: Quantitative analysis of profilin with Polyprolin-Sepherose Bead

Profilin was extracted from the smooth muscle cells of the aorta from the profilin I WT transgenic, 88R/L and non-transgenic control mice. Equal amounts of cells lysates were incubated with Poly-L-Proline-Sepharose beads. The bound proteins were then separated on SDS PAGE and then stained with Coomassie blue. The results show that the profilin I WT and the 88R/L transgenic mice have high amounts of human profilin I in their aorta compared to the non-transgenic control mice. An arrow is pointing toward the profilin bands.
Figure 10: Quantitative analysis of profilin with Polyprolin-Sepherose Beads.
Figure 11: Analysis of Smooth muscle cells by flow Cytometry of our non-transgenic mice control.

Smooth muscle cells were isolated from the aorta of the control non-transgenic mice for establishing primary cultures. They were analyzed at the passage 4 by Flow Cytometry to confirm origin of the smooth muscle cells. The results showed that 97.6% of these cultures are smooth muscle cells, α-actin positive.
Figure 11: Analysis of Smooth muscle cells by flow Cytometry of our control non-transgenic mice.
Figure 12: Analysis of Smooth muscle cells by flow Cytometry of our profilin 1 Wild type.

Smooth muscle cells were isolated from the aorta of the wild type mice for establishing primary cultures. They were analyzed at the passage 4 by Flow Cytometry to confirm the origin of the smooth muscle cells. The results shows that 99.5% of these cultures are smooth muscle cells, α-actin positive.
Figure 12: Analysis of Smooth muscle cells by flow Cytometry our profilin I WT type.
Figure 13: Analysis of Smooth muscle cells by flow Cytometry of our R88L/L.

Smooth muscle cells were isolated from the aorta of the 88R/L mice for establishing primary cultures. They were analyzed at the passage 4 by Flow Cytometry to confirm the smooth muscle cells origin. The results shows that 99.7% of these cultures are smooth muscle cells α-actin positive.
Figure 13: Analysis of Smooth muscle cells by flow Cytometry of our 88R/L
Figure 14: The expression of actin cytoskeleton for the non-transgenic control mice.

Actin polymerization was assessed in the smooth muscle cells that were cultured from the aorta of the control non-transgenic mice using Rhodamine-Phalloidin staining. Our results show expression of actin polymerization in the aorta of the non-transgenic control mice but not at much as the next figure shows.
Figure 14: The expression of actin cytoskeleton for the non-transgenic control mice.
Figure 15: The expression of actin cytoskeleton for the Profilin I Wild type transgenic mice.

Actin polymerization was assessed in the smooth muscle cells that were cultured from the aorta of the control non-transgenic mice using Rhodamine-Phalloidin staining. Our results show the expression of the actin polymerization in the aorta of the profilin I wild type transgenic mice were more in comparison to the non-transgenic control and the 88R/L transgenic mice.
Figure 15: The expression of actin cytoskeleton for the Profilin I Wild type transgenic mice.
Figure 16: The expression of actin cytoskeleton for the 88R/L transgenic mice.

Actin polymerization was assessed in the smooth muscle cells that were cultured from the aorta of the control non-transgenic mice using Rhodamine-Phalloidin staining. Our results show that the expression of actin polymerization in the aorta of the 88R/L transgenic mice were much less than that in the profilin I WT transgenic or in the control non-transgenic mice.
Figure 16: The expression of actin cytoskeleton for the 88R/L transgenic mice.
Figure 17: Motility of Smooth muscle cells in the non-transgenic control mice.

Confluent monolayer smooth muscle cells isolated from the aorta of the control non-transgenic mice were wounded with a pipette tip and cell migration to the wound was followed up by lapse-time microscopy. Images were obtained using the 40x objective, and the MetaMorph software, on a SenSys digital camera, with no binning. The parameters were as follows: images were recorded every 60 sec, at 490 nm excitation wavelength, 1 sec exposure, with the ND8 and ND16 filters in the light pass. Images were recorded every 60 sec for 5 hr, using the 40x objective and the MetaMorph software. A black arrow is pointing toward one cell in each experiment.
Figure 17: Motility of Smooth muscle cells in the non-transgenic control mice.
Figure 18: Motility of smooth muscle cells motility and ruffling formation in the Profilin I Wild type transgenic mice.

Confluent monolayer smooth muscle cells isolated from the aorta of the profilin I wild type transgenic mice were wounded with a pipette tip and cell migration to the wound was followed up by lapse-time microscopy. Images were obtained using the 40x objective, and the MetaMorph software, on a SenSys digital camera, with no binning. The parameters were as follows: images were recorded every 60 sec, at 490 nm excitation wavelength, 1 sec exposure, with the ND8 and ND16 filters in the light pass. Images were recorded every 60 sec for 5 hr, using the 40x objective and the MetaMorph software. A black arrow is pointing toward one cell in each experiment. Note the intense ruffling of most of the migrating cells at the wound margin indicated by red arrows.
Figure 18: Motility of smooth muscle cells and ruffling formation in the Profilin I Wild type transgenic mice.
Figure 19: Motility of smooth muscle cells motility in the 88R/L transgenic mice.

Confluent monolayer smooth muscle cells isolated from the aorta of the 88R/L transgenic mice were wounded with a pipette tip and cell migration to the wound was followed up by lapse-time microscopy. Images were obtained using the 40x objective, and the MetaMorph software, on a SenSys digital camera, with no binning. The parameters were as follows: images were recorded every 60 sec, at 490 nm excitation wavelength, 1 sec exposure, with the ND8 and ND16 filters in the light pass. Images were recorded every 60 sec for 5 hr, using the 40x objective and the MetaMorph software. A black arrow is pointing toward one cell in each experiment. Note the reduced speed migration of the cells and the ruffling activity is also much reduced at the wound margin.
Figure 19: Motility of smooth muscle cells motility in the 88R/L transgenic mice.
Figure 20: Ruffling formation in the smooth muscle cells of the profilin I WT

Random movement of smooth muscle cells extracted from the aorta of the profilin I WT. The random movement of the smooth muscle cells shows the formation of the membrane ruffling at the direction of movement of the cells. The red arrows are pointing to the site of membrane ruffling at the direction of movement of the selected cells.
Figure 20: Ruffling formation in random movement of smooth muscle cells in Profilin I WT
Figure 21: The effect of the expression of profilin on smooth muscle cell motility.

The effect of the overexpression of profilin on the speed of the migrated cells at the edge of wound. Three experiments were performed and 3 to 7 cells were tracked for each experiment. Data is expressed as means of all cells tracked in individual conditions (± SEM).
Figure 21: Smooth muscle cells migration (μm/min).
Figure 22: Western blot analysis for ERK1/2 total protein expression in smooth muscle cells.

Western blot analyses were performed on the aortic lysates from the control non-transgenic, profilin I wild type transgenic and 88R/L transgenic mice. We used anti ERK1/2 antibody “total protein”. Our results show no differences in terms of total protein expression in the three lines.
Figure 22: Western blot analysis for ERK1/2 total protein expression in smooth muscle cells.
Figure 23: Western blot analysis for ERK1/2 Phospho-Tyrosin expression in smooth muscle cells.

Western blot analyses were performed on the aortic lysates from the control non-transgenic, profilin I wild type transgenic and 88R/L transgenic mice. We used anti-Phospho-Tyrosin for ERK 1/2. Our results showed the activation of this pathway in the aorta of the profilin I WT transgenic mice.
Figure 23: Western blot analysis for ERK1/2 Phospho-Tyrosin expression in smooth muscle cells.
Figure 24: Western blot analysis for JNK total protein expression in smooth muscle cells.

Western blot analyses were performed on the aortic lysates from the control non-transgenic, profilin I wild type and 88R/L transgenic mice. We used anti JNK antibody “total protein”. Our results showed no differences in the expression of the total JNK protein between the three lines.
Figure 24: Western blot analysis for JNK total protein expression in smooth muscle cells.
Figure 25: Western blot analysis for JNK Phospho-Tyrosin expression in smooth muscle cells.

Western blot analyses were performed on the aortic lysates from the control non-transgenic, profilin I wild type and 88R/L transgenic mice. We used anti Phospho-Tyrosin for antibody for JNK. Our results showed an increase in the activity of the JNK protein in the profilin I wild type in comparison to the control non-transgenic and 88R/L transgenic mice.
Figure 25: Western blot analysis for JNK Phospho-Tyrosin expression in smooth muscle cells
Figure 26: H&E staining for the Aorta of the non-transgenic control mice.

H&E staining for the aorta of the control non-transgenic mice. The picture shows the normal structure of the aorta for the 9 months old mice.
Figure 26: H&E staining for the Aorta of the Control non-transgenic mice.
Figure 27: H&E staining for the aorta of the profilin I Wild type transgenic mice.

H&E staining for the aorta of the 9-month-old mice. The series of sectioning for the aorta showed the signs of hypertrophy in the aorta of the profilin I wild type transgenic mice. The arrows are pointing toward some enlarged cells in the aorta.
Figure 27: H&E staining for the aorta of the profilin I Wild type transgenic mice.
Figure 28: H&E staining for the aorta of the 88R/L transgenic mice.

H&E staining for the aorta of the 88R/L transgenic mice. Many sections were examined for four mice at the age of 9 months. Our results showed no differences between the aorta of the control non-transgenic and 88R/L transgenic mice.
Figure 28: H&E staining for the aorta of the 88R/L transgenic mice.
Figure 29: Actin staining for the smooth muscle cells of the aorta of the non-transgenic control mice.

Smooth muscle cells actin staining of the aorta of the non-transgenic control mice. The expression of actin in the aorta shows the normal expression of actin in comparison to its expression in the profilin I wild type and 88R/L transgenic mice.
Figure 29: Actin staining for the smooth muscle cells of the aorta of the Control non-transgenic mice.
Figure 30: Actin staining for the smooth muscle cells of the aorta of the profilin I wild type transgenic mice.

Smooth muscle cells actin staining confirmed the integrity of the smooth muscle cells and actin expression in the aorta of the profilin I wild type transgenic mice in comparison to the control non-transgenic mice.
Figure 30: Actin staining for the smooth muscle cells of the sorta of the Profilin I wild type transgenic mice.
Figure 31: Actin staining for the smooth muscle cells of the aorta of the 88R/L transgenic mice.

Smooth muscle cells actin staining of the aorta of the 88R/L transgenic mice shows the expression of the actin is less than that expressed in the profilin I wild type transgenic and control non-transgenic mice.
Figure 31: Actin staining for the smooth muscle cells of the aorta of the 88R/L transgenic mice.
Figure 32: Twenty four hours monitoring of the blood pressure of one of the profilin I WT mice.

Blood pressure monitored in one male mouse at age of 11 to 14 months from the profilin I WT group. The pressure shown in the table shows the blood pressure with then 24 hour. It is clear that the blood pressure was fluctuating around 135 mmHg.
Figure 32; Twenty four hours monitoring of the blood pressure of one of the Profilin I WT mice.
Figure 33: Twenty four hours monitoring of the blood pressure of one of the non-transgenic control mice.

Blood pressure monitored from one male mouse from the non-transgenic control mice at age of 11 to 14 months. The 24 hour monitoring of the pressure shows that the mouse’s blood was fluctuating around 110 mmHg.
Figure 33; Twenty four hours monitoring of the blood pressure of one of the non-transgenic control mice.
Figure 34: Circadian pattern of blood pressure

The circadian pattern of blood pressure has been determined in the profilin I wild type transgenic vs. control non-transgenic mice. Five conscious mice age of 11 to 14 months from each group were used to determine the blood pressure for 8 days. Data correspond to mean±SEM $P<0.05$ for profilin I WT vs non-transgenic control at 5 hours. The mean arterial pressure (MAP) of the profilin I wild type transgenic mice was significantly and consistently higher across the day and night cycle, compared to the control non-transgenic mice.

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Figure 34: Circadian pattern of blood pressure of 5 Profilin I WT mice and 5 non-transgenic control mice.
CHAPTER 5

DISCUSSION

The arterial system acts like a pump as it converts the intermittent flow from the heart into continuous flow to the organs. This functional behavior is due to the peculiar assembling of the cellular and extra cellular components into the three distinct layer of the blood vessels: tunica intima, tunica media and tunica adventia (Glasser, 2000). This dynamic activity of the blood vessels, which is controlled by its wall’s components, is subject to be affected by process of stiffening which occurs via vascular remodeling (Wong, et al., 1996).

Vascular remodeling is usually an adaptive process in respond to long-term changes in homodynamic conditions, as occurs during development (Wong, and Langille, 1996). But it can also contribute to the pathophysiology of vascular disease (Gibbons, and Dzau, 1994). The vascular wall is an active organ composed of endothelial cells, SMCs, and adventitial cells, which are coupled to each other. The wall is able to change its structure through a process known as remodeling. Over the past
decade, there has been extensive research concerning the structure of vessels and the process of remodeling in both physiological and pathological conditions and with special interest at the level of the resistance vessels (Wong, and Langille, 1996). Remodeling is now considered to be a more complex process that may include increased wall thickness, increased cross sectional area, decreased lumen diameter, and an increased wall:lumen ratio, all characteristic of hypertrophic or eutrophic inward remodelling (Baumbach, and Heistad, 1989; Jarajapu, et al., 2001; Jarajapu, et al., 2001). These morphological adaptations often result in modifications of the functional properties of the affected vessels (Coats, & Hillier, 2001; Wong, & 1996). Antioxidant treatments in hypertensive rats that were able to restore blood pressure to normal (i.e. NAC), could reverse structural adaptations occurring during hypertension (Deng, and Schiffirin, 1991). A few studies also investigated the in vivo effects of low blood pressure (hypotension) and low blood flow on resistance vascular structures (Gibbons, & Dzau 1996; Den, et al 1991 and Arribas, et al., 1997). Low blood flow resulted in outward hypotrophic remodeling (Deng, & Achiffrin 1991), whereas low blood pressure resulted in a reduced media:lumen ratio in the arteries (Arribas, et al., 1997 & Gibbons, et al., 1994).

The goal of our study was to assess the effects of altering smooth muscle contractility that is due to changes in actin polymerization on vascular function and cardiovascular diseases. In order to achieve our goal we have overexpressed transgenically either the cDNA of human profilin I WT or the dominant negative
mutant "88R/L" in blood vessels of FVBIN mice using mouse vascular smooth muscle cell α-actin promoter. Immunohistochemistry analyses of aortic tissues from profilin I or 88R/L transgenic mice showed clear sign of vascular hypertrophy in the profilin I transgenic mice as compared to non-transgenic controls or 88R/L mice. This was confirmed by the activation of the hypertrophic pathway (i.e. ERK 1, 2) as it will be discussed later. As we anticipated, the vascular hypertrophy in profilin I transgenic mice resulted in increase the media: lumen ratio and led to increase the mean arterial pressure. The blood pressure in the profilin I mice was elevated about 25-30 mmHg as compared to non-transgenic controls. On the other hand, the blood pressure in 88R/L mice was below the control littermates. In other words, the transgenic profilin I mouse is a model of vascular hypertrophy that lead to hypertension while, the 88R/L is a model for hypertension.

It has been postulated that left ventricle hypertrophy (LVH) is secondary to the increased workload created by the excess systolic blood pressure. We investigated whether the systolic increase in blood pressure would be sufficient to induce LVH in profilin I transgenic model. Our echocardiography results showed a clear evidence of cardiac hypertrophy in profilin I transgenic mice, as weights of profilin I WT hearts were increased relative to the hearts of aged matched controls and 88R/L mice (data not shown).
Since profilin I WT was not detected in the myocardium, we conclude that the observed cardiac hypertrophy in profilin I WT mice might be the direct consequence of chronic hypertension in these animals.

Our results also showed that the overexpression of profilin I WT in vascular smooth muscle cells led to increase actin polymerization in these cells as compared to controls, however, the level of actin polymerization in the 88R/L vascular smooth muscle cells was below the non-transgenic controls. The increased actin polymerization was evident by increase stress fiber formation as was as ruffling in primary vascular smooth muscle cells derived from profilin I aortic tissues. Studies have shown that profilin binds to most proteins with prolin reach sequence including WASP protein (Henry N. Higgs and Thomas D. Pollard, 2001, Thomas D. Pollard, et al., 2000). WASP is protein kinase that activate Arp 2/3 complex and then activating nucleation and net filament formation. Once Profilin binds to WASP, it enhances the ability of WASP to induce the stress fibers formation (Shiro Suetsugu et al., 1999). It is possible to speculate that the increase of actin polymerization, specially stress fibers formation will affect the relaxation/contraction process of the smooth muscles which make it less responsive to vasodilators such nitric oxide (NO). That could be an important factor that contributes to hypertension besides the vascular hypertrophy in the profilin I WT transgenic mice.
In addition, our results also showed that migration of profilin I vascular smooth muscle cells was much faster as compared to non-transgenic controls or 88R/L vascular smooth muscle cells. These results are in agreements with the published reports that showed that decreasing actin dynamic net filaments formation prevented efficient migration, reduced motility because of the limitation of available barbed ends and limitation an monomer actin binding to ATP. Moreover, ERK ½ that have been activated in vascular smooth muscle cells or profilin I transgenic mice, known to be involved in regulation of cell motility in endothelial cells (Herve Chaulet et al., 2001). Based on this data we anticipate that profilin I transgenic mice under high fat diet to develop arteriosclerosis lesions much faster than the control non-transgenic mice. We have already initiated this study in our laboratory, however, that is out of the scope of this study.

Furthermore, we have confirmed the activation of the hypertrophy pathway in aorta of profilin I hypertensive mice as compared to 88R/L hypotensive mice and non-transgenic controls. Up-regulation of the hypertrophy pathway was evident by the activation of ERK 1,2 and JNK kinases. This pathway is an important one in the pathogenesis of cardiovascular diseases that is activated by Angiotensin II (Matrougui et al., 2001).

Angiotensin II is the main peptide hormone of the renin-angiotensin system, plays an important role in the cardiovascular diseases, including atherosclerosis.
myocardial infarction, and hypertension (Vekshtein, 1989). Ang II exerts hypertrophic and hyperplastic effects by activating a number of intracellular signal transduction pathways through a 7-transmembrane heterotrimeric G protein–coupled receptor called the Ang II type 1 (AT1) receptor (Alexander, 1991). Ang II can trigger the activation of downstream signals such as Akt and MAPK (Deora, 1998). The MAPKs are a family of serine/threonine kinases that control cellular responses to growth, apoptosis, and stress signals. There are 4 main MAPKs, including extracellular signal–regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNKs, also termed SAPKs), p38 MAPKs, and big MAPK-1. Moreover, JNK-activated c-Jun is one of the major components of the transcription factor AP-1, which regulates the expression of many genes involved in cellular growth, transformation, and differentiation (Clarke, 1998). Taking together, activation of hypertrophy pathway results vascular remodeling as results of in increase the size of vascular smooth muscle cells. This vascular hypertrophy will increase the resistance to the blood flow and results in hypertension and cardiac hypertrophy.

Furthermore, new data indicate that Ang II plays an important role in the generation of reactive oxygen species (ROS) by activation of NADPH oxidase, a plasma membrane–bound protein (Merrill, et al., 1996; Ushio-Fukai, et al., 1998). In the pathway activated by Ang-II, as well as for any putative pathway that requires the production of \( \cdot \text{O}_2^- \) by NADPH oxidase to induce hypertension (Yamazaki, et al., 1996), the final common step is the switching on of the small guanyl binding protein Rac. Numerous reports showed that profilin I can activate Rac I gene that will directly link
profilin to Ang II signaling pathway. If profilin I can activate Rac gene, that could lead to increase in the levels of superoxide as results of the activation of NADPH oxidase pathway. The heightened production of superoxide, in turn, consumes NO, results in peroxynitrite formation with consequent acceleration of protein nitration. Moreover, the loss of NO resulting from its consumption would lead to excessive blood pressure.
CHAPTER 6

CONCLUSION

It has become clear that actin plays an important role in cardiovascular hypertrophy. One of the important upstream signaling molecules that mediate hypertrophic responses is the Ras-like small GTPase Rho. Rho plays a critical role in a variety of cytoskeletal-dependent cell functions including actin polymerization, F-actin bundling, myosin-based contractility, focal adhesion formation and cytokinesis in other cell types (Masahko et al., 1998). In cardiac myocytes, activated Rho has profound effects on myofibrillar organization, and stimulates c-fos and arterial natriuretic factor expression (Moldovan, 1999; Moldovan, 2000). Dominant negative Rho attenuates the Goq- and Ras-induced transcriptional activation of the arterial natriuretic factor gene (Hiata et al., 1992; Marcouz et al., 2000). These results demonstrate that Rho plays an important role in the modulation of cardiac gene expression during hypertrophy that induced by signaling through Goq. Recently, ROCK II (Rho kinase) has been identified and the Rho/ROCK pathway has been suggested to mediate hypertrophic signals in cardiac myocytes (Moldovan, 1999), the relationship between the Rho/ROCK
pathway and downstream ERKs/GATA-4 is unknown. In the recent studies found a
linkage between Rho/ROCK and ERKs/GATA-4 pathways during myocardial cell
hypertrophy.

Furthermore, mechanical stress in non-vascular smooth muscle cells induces
changes in actin cytoskeleton that is mediated by integrin dependent activation of other
members of Rho subfamily: Rho, Cdc42 and Rac. In non-VSMCs as well as in
VSMCs, Rho controls actin-myosin contractile function through the phosphorylation of
myosin light chains (Hassanain, and Goldschmidt-Clermont, 2000a, 26,27). The
myosin II in non-muscle cells is homologous to that of muscle cells, cell contraction in
each case is initiated by reversible phosphorylation of the myosin (Azafari, 1988;
Komuro, 1998). The contractile stimuli trigger an elevation in sarcoplasmic free Ca$^{2+}$
which binds to calmodulin and activates the myosin II light chain kinase (MLCK).
MLCK phosphorylate and activate the myosin head that can interact with actin filament
and thereby cause contraction. The small GTP-binding protein Rho regulates actin-
myosin contractility by inhibiting myosin phosphatase which, which dephosphorylates
the myosin light chain and cause relaxation. This effect of Rho does not depend on
change in Ca$^{2+}$ concentration (Somlyo, and Somlyo 1998; Komuro, L, 1998). Thus,
contractile function in non-muscle cells, which results in stress fiber formation and
focal adhesions, and in vascular smooth muscle cells, that leads to vasoconstriction,
could have similar mechanisms.
Profilin I can mediate hypertrophic responses in two ways: directly by induce actin polymerization including stress fiber formation, ruffling and focal adhesions by facilitating nucleotide exchange and/or by activating some other mediators such as Cdc42 and WASP proteins (Suetsugu, et al., 1999). Indirectly, profilin I activates Rac I gene that can induce the expression of Rho and thus activates the hypertrophyic pathway (Amy, et al., 2001; Murasawa, et al., 2000). On the other hand, Rac gene can activate NADPH oxidase and increase the levels of reactive oxygen species (ROS). Current studies in our laboratory showed that there are increase levels of Rac gene expression and ROS production in the microvesseles and that levels of ROS increased by elevating the transmural pressure in microvessels. Thus, it is plausible to speculate that in the normal situation only the arterioles produce free radical that constantly regulates the blood pressure. This regulation could be through polymerization and depolimerization of the actin (actin dynamics) which result in constant vasodilatation of the microvessels. The increased of reactive oxygen species (ROS) production in small vessels, however, was completely abolished in transgenic mice expressing the dominant negative mutant of Rac I gene (Rac DN) in vascular smooth muscle cells or by using infusing antioxidants such as N-acetyl cystein (NAC) in microvessels. This provides strong evidence that actin dynamics play an important role in the myogenic response.

In our future studies we will assess the activation of ROCKII/Rho pathway in the aortas of transgenic mice that overexpressed the profilin I gene and compared that with the expression in mice that express the dominant negative mutant of profilin
(88R/L). In addition, we study the vascular and functional remodeling in the resistance arteries i.e. mesenteric arteries of profilin I transgenic mice as well as the 88R/L transgenic mice. We anticipate a number of morphological changes in profilin I mice compared to 88R/L and non-transgenic controls. There will be increased wall thickness, increased cross-sectional area, decreased lumen diameter and an increased wall: lumen ratio as compared to the non-transgenic controls. The number and density of cells is expected to increase in the adventitial, medial and intimal layers in profiling mice as well as an increase in size of smooth muscle cell layers (hypertrophy), but significantly decrease in the mesenteric arteries of 88R/L mice as compared to the non-transgenic controls. We also anticipate that the activation of the transcription machinery i.e. NF-kB via the activation of Rac gene and increased ROS production. The activation of the hypertrophy pathway (i.e. ERK1, 2 and JNK) may result in the activation of AP1 transcription factor. Activation of NF-kB and AP1 stimulates cell proliferation and increases the size of smooth muscle cells. In contrast, we anticipate that in 88R/L mice, in which there is inhibition of NADPH oxidase activation, ERK1, 2 and JNK, the structural remodeling will lead to a reduction in the size and numbers of cells in the vessel wall, and a reduction in smooth muscle layers.
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