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ANTHOCYANINS ALTER ENDOTHELIAL CELL DYNAMICS

by

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Abstract

Cardiovascular disease is the leading cause of death in the United States. Previous studies suggest that a diet high in blueberries correlates with lower rates of heart disease and improved endothelial cell function. Anthocyanins, which are phenolic compounds, are known components of wild blueberry extracts. Studies suggest that cells treated with anthocyanins have increased migration and increased VEGF (vascular endothelial growth factor) production. VEGF activates a signal cascade that ultimate leads to the activation of Hsp27 (heat-shock protein 27) and LIMK (LIM-kinase). Cofilin, which is phosphorylated by LIMK, is an important protein involved in cellular migration, and plays a role in regulating actin dynamics, enabling migration. Activated Hsp27 and deactivated cofilin work in concert to facilitate migration. Paxillin is an important protein involved in focal adhesion structures, which attach to the surrounding extra cellular matrix and allow for cell motility. Based on this knowledge it was postulated that bovine aortic endothelial cells treated with 20μg/mL anthocyanins would have increased concentrations of phosphorylated cofilin and a change in paxillin concentrations. This study found that cells treated with 20μg/mL anthocyanins for 2 and 3 hours had increased perimeters and increased filopodia formation. BAECs treated with anthocyanins for 3 hours were stalled in cytokinesis resulting in multinucleated cells and cells with fragmented nuclei. Changes in concentrations of cofilin, p-cofilin, paxillin, and actin were not significant. This study suggested that anthocyanin treatment affected cellular actin dynamics, but did not affect phosphorylated cofilin concentrations.

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Chapter 1: Introduction

Coronary heart disease is one of the leading causes of death in the United States. It is estimated that by 2030 nearly 40.5% of U.S. population will suffer from some form of cardiovascular disease (Heidenreich et al, 2011). In 2011 coronary heart disease caused 1 out of every 7 deaths in the United States (Mozaffarian et al, 2015). Cardiovascular disease is a widespread and extremely debilitating condition. Even with treatment, the quality of life is reduced immensely. Dysfunction of the endothelial cells that line the blood vessels can contribute to atherosclerosis and increased risk for cardiovascular disease (Stull et al., 2015). Studies have found that a daily increase in anthocyanins has induced endothelial cell function (Zhu et al., 2011). Wild blueberries are rich sources of anthocyanins, and extracts of wild blueberries can be used to test the cellular responses to anthocyanins (Müller et al., 2012). Various studies have linked treatment of anthocyanins to increased endothelial nitric oxide synthase (eNOS) activation and ultimately increased cellular migration (Banaszewski et al., 2012). Cellular migration can help to maintain vascular tone and a healthy endothelium especially after damage, such as from a heart attack (Deanfield et al., 2007). Migration is especially important during angiogenesis, the process of forming new blood vessels from existing ones, which contributes to wound healing and tissue regeneration (Lamalice et al., 2007). The molecular mechanisms that link anthocyanins to increased cellular migration are relatively unknown because of the enormous amount of signaling molecules involved in migration. This study aims to show a connection between anthocyanin treatment and the activation or deactivation of some known proteins involved in endothelial cell dynamics. Determining the mechanisms of anthocyanins in

the cell could potentially lead to the discovery of new preventative and reparative treatments for cardiovascular disease.

1.1 Characteristics of Anthocyanins

Anthocyanins are water soluble pigments found in many different fruits and vegetables. Anthocyanins are classified as members of the flavonoid family. Flavonoids are polyphenolic compounds (containing multiple carbon ring structures with oxygen and hydrogen groups attached), which have a characteristic 15-carbon structure (Chen et al., 2014). Anthocyanins specifically, tend to have a C6-C3-C6 structure, made up of three rings, typically with a minimum of 4 hydroxyl (oxygen and hydrogen) groups (Bunea et al., 2013). Over 600 different types of anthocyanins have been classified, however only six of these are commonly found in fruits and vegetables. The six anthocyanins commonly found in foods; cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin are categorized based on the number and location of their hydroxyl groups (Fang, 2015). These exist mainly with bound sugar groups at different locations on the structure of the molecule. The common sugars include: glucose, arabinose, galactose, rutinose, rhamnose, and xylose. These sugars can be bound as either a mono-, di-, or trisaccharide (Fang, 2014). Foods containing anthocyanins can be classified into three different groups based on their anthocyanin content. The cyanidin/peonidin group, the pelargonidin group, and the multiple anthocyanidins group (anthocyanidins meaning the structure of the anthocyanins without their connected sugar groups). Blueberries are categorized into the multiple anthocyanidins group and contain most commonly cyanidin, delphindin, petunidin, peonidin, and malvidin with multiple variations of each differing in their bound sugar groups (Fang, 2015, Bunea et al., 2013).
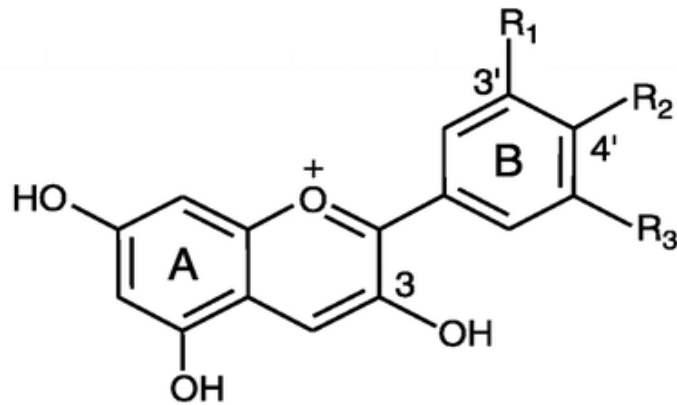
ANTHOCYANIDIN	R_1	R_2	R_3
Pelargonidin (Pg)	H	OH	H
Cyanidin (Cy)	OH	OH	H
Delphinidin (Dp)	OH	OH	OH
Peonidin (Pn)	OMe	OH	H
Petunidin (Pt)	OMe	OH	OH
Malvidin (Mv)	OMe	OH	OMe

Figure 1. **Structure of anthocyanins.** The six most common anthocyanins found in foods all share a relatively characteristic C6-C3-C6 structure and differ based on the number and placement of their hydroxyl groups (Prior and Wu, 2009).

Because of their structure, anthocyanins show significant absorptions at two ranges of wavelengths, 465 to 550nm range and 270 to 280 nm range. In acidic conditions the ring structure containing oxygen carries a positive charge that gives them their characteristic blue-violet to red-orange color (Wallace, 2011). Blueberries have been shown to be one of the richest in anthocyanins for food sources (Müller et al., 2012). Specifically, lowbush (*Vaccinium angustifolium*) wild blueberries ranked consistently higher in concentrations of anthocyanins, antioxidants, and total phenolics than the

comparison cultivated high bush (*Vaccinium corymbosum*) variety (Vendrame et al., 2014, Kalt et al., 2001). The mean amount of total anthocyanins in cultivated high bush blueberries per serving size of 145g was measured to be 509mg in comparison to wild blueberries, which were measured to have a mean of 705mg per 145g serving (Wu et al., 2006).

Studies suggest that anthocyanins mainly exist in circulation in the blood and urine as metabolites in their glucuronidated (containing a glucuronic acid) and methylated forms (containing a methyl, CH_3, group), with the peak concentration in the plasma at 1-3 hours after being consumed (Wallace, 2011). Maximum concentrations of anthocyanins in the plasma varied from 1-100 nmol/L after doses of 0.7-10.9 mg/kg were administered (Prior and Wu, 2009). A study administering 721 mg oral doses of chokeberry anthocyanin extracts to healthy male subjects of varying ages, found that 32% of the anthocyanins detected in serum existed in the parent form with 68% existing as metabolites. Anthocyanin derivatives continue to be methylated (modified with the addition of methyl, CH_3, groups) as a function of time over a period of 6-24 hours, suggesting that bioactivity deceases with time (Mazz and Kay, 2008). Bioactivity is a measure of the affect the anthocyanins have on the cell. This is greatly impacted by the bioavailability of the substance. Bioavailability is defined as the rate of absorption and the amount of the substance available at a site of action. As anthocyanins move through the body, the molecule's structure is subsequently modified allowing for breakdown and excretion, and this modification decreases the ability of the cell to absorb and use the substance, therefore decreasing its bioavailability and bioactivity (Thilakarathna and Rupasinghe, 2013). pH can also affect bioactivity of anthocyanins, eight varying structure

states have been detected in solutions with varying pH. Since the pH can vary throughout the human body, anthocyanins exist in different forms in the stomach than in the blood and other organs in the body. Anthocyanins are thought to exist as a flavyium cation (this is the positively charged derivative of the molecule and the most stable form) in the stomach at a lower pH and in the hemiketal, chalone, or quinonoidal form (varying slightly in number of hydrogen atoms and structure) in the rest of the body due to higher a pH (McGhie and Walton, 2007). Studies done in rats found that anthocyanin metabolites rapidly appeared in the plasma after consumption (Milbury et al., 2002), suggesting that absorption occurs rapidly in the stomach.

1.2 Anticarcinogenic Properties of Anthocyanins

Anthocyanins have been shown to exhibit anticancer effects by inhibiting cell growth and promoting cell cycle arrest. In tumor cells treated with anthocyanins from black rice, specifically the anthocyanins cyanidin and peonidin, showed signs of inhibited cell growth and apoptosis. These treated cells showed characteristics morphologies of apoptosis (Chen et al., 2005). Apoptosis is a term used to describe programmed cell death. This process typically begins with chromosome and cytosol condensation in the cell, called pyknosis. Pyknosis is typically followed by the fragmentation of the nucleus known as karyorrhexis. As the nucleus continues to breakdown the cell membrane begins to bleb creating an unusual protrusion of the cell membrane, and the cell splits and forms multiple apoptotic bodies (Eidet et al., 2014).

In a study using colon cancer cells, cells treated with blueberry extracts showed a 1.8 fold increase of apoptosis compared to controls (Seeram et al., 2006). A seperate study looking closer at the effects of anthocyanins on the cell cycle, treated human colon

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cancer cells with anthocyanins extracted from purple-fleshed sweet potatoes for 48 hours. After this 48 hour period treated cells showed cell cycle arrest at the G1 phase. In the G1 phase of the cell cycle, the cell increases in size and synthesizes an increased amount of protein and RNA, to ready itself for the replication of its DNA in the S phase of the cell cycle. In this case they reported that as the percentage of cells in the G1 phase increased, the percentage of cells in the S phase showed a decrease (Lim et al., 2013). Meaning that cancerous cells are being halted in the cell cycle and prevented from continued growth.

1.3 Anti-inflammatory Effects of Anthocyanins and Endothelial Function

Anthocyanins, besides showing promise as anticancer agents, also have beneficial effects on the cardiovascular system. Studies have shown that consumption of anthocyanins has led to reduced inflammatory markers and improved endothelial function (Kuntz et al., 2005). The endothelium cell layer lines the heart, blood, and lymph vessels and is an important component of vascular function and integrity (Banaszewski et al., 2012). Endothelial dysfunction has been classified as an early marker of atherosclerosis, the condition of plaque building up inside the artery walls (Zhu et al., 2011). Atherosclerosis can lead to many other serious heart conditions, and endothelial cell function is an important part of prevention for many cardiovascular diseases. Atherosclerosis has also been associated with a condition called low-grade inflammation, this is associated with metabolic syndrome and can lead to diseases like heart disease, cancer, and diabetes. In the condition of low grade inflammation, platelets can be induced to bind and aggregate to a non-damaged intact endothelium. Platelet aggregation is associated with coronary artery disease (Luzak et al., 2014). Low grade inflammation and endothelial dysfunction have been postulated to be related to poor

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diet: diets low in fiber, vegetable protein, unsaturated fat, and high in cholesterol (van

Bussel et al., 2013).

A recent study had evidence to suggest that anthocyanins decreased the

inflammatory response of the cell by down regulating certain cytokines, which are

important proteins in cell signaling. Specifically this study documented a decrease in IL-

8 (Interleukin-8), a substance that works as a chemokine and chemically induces

movement of cells toward a particular area. This occurs with neutrophils that are

involved with the process of inflammation (Kuntz et al., 2015). An increase in IL-8 and

other cytokines in the blood is a characteristic of endothelium dysfunction because it

causes chronic activation of the endothelial cells lining the blood vessels. Chronic

activation will eventfully lead to plaque rupture and a heart attack (Kuntz et al., 2015). A

double-blind and placebo-controlled clinical study with 44 adults, found that subjects

with characteristic metabolic syndrome (hypertension, high levels of fat and sugar in the

blood, and central obesity) that were given a blueberry smoothie once a day for six

weeks had increased endothelial function compared to placebo groups. Endothelial

function was based on reactive hyperemia index (RHI) measurements, which compares

the ratio of the average increase of pressure in the arteries measured over 60 seconds,

beginning one minute after deflating a blood pressure cuff on one arm, to the average

pressure in the arteries at the baseline level. A higher RHI correlates to improved

endothelial function. RHI measurements were found to be 0.23 ± 0.14 in the blueberry

smoothie group compared to -0.23 ± 0.13 in the placebo group (Stull et al., 2015).

1.4 Endothelial Function and eNOS Activation

Dysfunction of the endothelial cells that line the blood vessels can contribute to atherosclerosis and increased risk for cardiovascular disease (Stull et al., 2015). Impaired endothelial cell function has reportedly been associated with impaired expression of endothelial nitric oxide synthase (eNOS). eNOS is an enzyme that synthesizes nitric oxide (NO). NO is extremely important in inducing vasodilation and inflammation. Endothelial dysfunction is characterized by reduced production of NO (Zhu et al., 2011). Endothelial dysfunction is often an early marker for cardiovascular diseases and atherosclerosis. In cases where the activation of eNOS is prevented or impaired, other cell processes are reduced including cell migration and the dilation of blood vessels. eNOS is activated by a series of signaling cascade molecules which promote cell migration in endothelial cells. A study using blueberries and other fruit extracts found that eNOS was activated by polyphenolic compounds in berry extracts. Anthocyanins are a known polyphenolic compound in berry extracts. Higher amounts of activated eNOS led to increased endothelial cell migration (Banaszewski et al., 2012). Evidence suggests that NO derived from eNOS is an important mediator in endothelial cell migration and works by stimulating VEGF and other factors involved in cell migration (Zhang et al., 2015). Vascular endothelial growth factor (VEGF) is an important signaling molecule in endothelial cell migration. Endothelial cells contain receptor tyrosine kinases (VEGFR-1 and VEGFR-2) on their surfaces that are activated by VEGF and stimulate various signaling pathways within the cell (Ballestrem et al., 2000). In a study focusing on wound induced migration of human dermal fibroblast and human keratinocyte cells, cells were treated with anthocyanins and cell migration assays

and VEGF immunoassays were performed to determine the levels of VEGF and it's effect on migration. The study found that in both cell lines, migration increased. VEGF production increased more dramatically in fibroblasts, though it was increased slightly at variable times in keratinocytes (Nizamutdinova et al., 2009). VEGF is an important signaling molecule in endothelial cell migration. Activation of the VEGFR-1 and VEGFR-2 receptors by VEGF then leads to signal cascades, which are believed to be important in endothelial cell migration by regulating actin dynamics and organization (Koayashi et al., 2006).

1.5 Actin Dynamics and Cell Migration

Actin is a protein found in the cytoplasm of cells that forms filaments that are a part of many of the migration structures in the cell. Remodeling of actin in the cell is necessary for many morphological changes including migration and replication (Ballestrem et al., 2000). These actin filaments are made up of smaller monomers, which can be assembled and disassembled. Actin (along with the help of another protein called myosin) allows the cell to stretch out, and also pull itself forward during the act of migration. The stretching out of the cell at the front is what forms the structure called the lamellipodium. Adhesive contacts form on the bottom of the cell that keep it bound to the extracellular matrix proteins and allow for the cell to pull itself forward. As the cell migrates, the adhesive contacts at the rear of the cell are released and the rear of the cell retracts (Kardos et al., 2013). Various signaling pathways affect actin remodeling, therefore although the remodeling of actin can be directly linked to cellular migration, various molecules can work through different signaling pathways with a similar result. Actin dynamics are also important in other cellular processes where the cell must

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reorganize its cytoskeleton including the process of mitosis.

1.6 Actin Dynamics and Mitosis

Mitosis is the process of cell replication and division; it begins with one cell and results in two identical daughter cells. The regulation of actin dynamics is important part of this process (Lancaster and Baum, 2014). Mitosis occurs in six distinct phases: prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis, respectively. The basic process involves the chromosomes condensing and being pulled to either end of the cell by microtubules originating at the mitotic spindles. Once these are polarized to two ends of the cell, the outer membrane is essentially split in two to create distinct daughter cells. This is a dynamic process where the cell is changing shape and morphology and must occur in a highly organized and methodical manner (Lancaster and Baum, 2014). Mitosis is driven by changes in the cytoskeleton of the cell, specifically actin and microtubules. Actin cycles through two different states, globular actin (G-actin), the monomeric form, and the filamentous form (F-actin) (Belsky et al., 2016). Actin reorganization is believed to play a key role in various stages of mitosis including cell rounding during the onset of prophase, mitotic spindle orientation and chromosome aggregation during metaphase, and especially in cleavage furrow formation in cytokinesis (Chircop, 2014). Cytokinesis is the actual splitting of the cell to create two daughter cells. This process is driven by a network of actin and non-muscle myosin II, which creates what is known as the contractile ring or the cleavage furrow (Lancaster and Baum, 2014). As the cleavage furrow tightens and the actin and myosin contract, an invagination of the cell membrane occurs and the cell begins to separate into two. Mitosis and migration are processes that are both controlled in part by actin reorganization. Complex molecular

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signaling pathways within the cell induce reorganization of actin structures and allow for theses processes to proceed. Often the cycling of actin monomers is controlled by actin binding proteins (ABP).

1.7 VEGF Affects LIM-kinase Activation and Cofilin Phosphorylation

An important example of an ABP is cofilin. Cofilin is a globular protein that contains a core of four or five beta sheet structures with four or five alpha helices surrounding this core; it works by binding to the actin filaments and severing the bonds between actin monomers in the F-actin creating G-actin monomers and short oligomers of actin filaments. In an alternative model, cofilin is thought to sever and create shorter actin filaments which generates barbed ends (growing end of the F-actin filament) for G-actin to bind to and assemble new filaments. This process occurs only when the protein is activated. When cofilin is phosphorylated (p-cofilin) it is in the inactivated form (Ballestrem et al., 2000). Cofilin preferentially binds to ADP bound actin, which means that it is breaking apart actin filaments at the pointed end loaded with ADP and not the barbed or growing end which is bound to the ATP nucleotide (Ohashi, 2015). Cofilin therefore supplies G-actin monomers for polymerization, and promotes the disassembly and turnover of migratory structures like lamellipodia, stress fibers, and filopodia. Lamellipodia are fan like structures at the leading end of the cell that are rich in F-actin, allowing the cell to pull itself forwards during migration. Filopodia are long, slender projections of the cell that often are the precursors to lamellipodia formation (Johnson et al., 2015). Stress fibers are made of F-actin and stretch across the cell in long filaments, and contribute to migration morphologies.
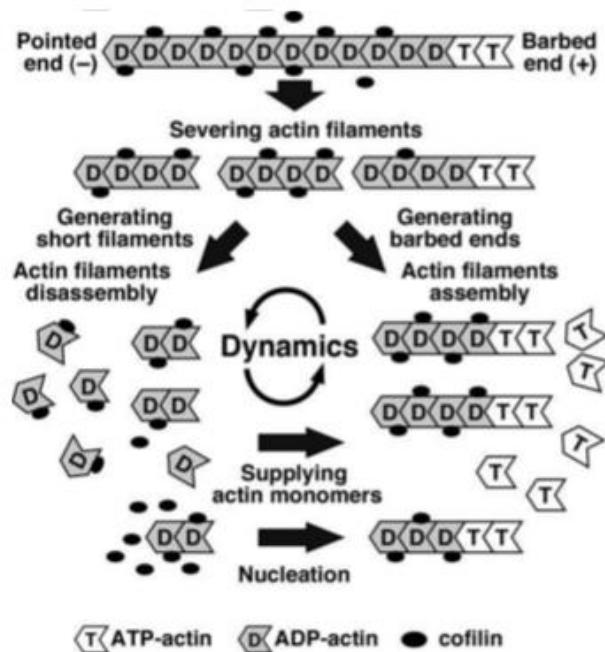
Figure 2. **Actin dynamics are regulated by cofilin binding.** Two different models exist to describe the process of increased actin dynamics by cofilin. One is that cofilin severs actin polymers and increases the concentration of actin monomers for reassembly. The second model is that cofilin creates new barbed ends for which actin can then add to these barbed ends. Both processes increase actin turnover by creating an increased number of short actin monomers (Ohashi, 2015).

Cofilin is inactivated and phosphorylated by regulatory molecules, which are the result of different signal cascades. One such molecule is LIM-kinase (LIMK). LIMK phosphorylates cofilin (p-cofilin) at its Ser-3 residue. Phosphorylation inhibits cofilin's actin binding ability slowing down the process of actin turnover and enabling the assembly of stress fibers (Ohashi, 2015). VEGF has been experimentally shown to increase LIMK activity and in turn increase p-cofilin concentration in human umbilical vein endothelial cells (HUVECs). Therefore, it is proposed that VEGF activates a signal cascade that ultimate leads to the phosphorylation of LIMK at its serine-323 residue and activates it, which leads to phosphorylation of cofilin (Koayashi et al., 2006). This signal

cascade begins with VEGF activating the surface receptor VEGFR-2. The receptor then becomes activated leading various signaling cascades that ultimately activates an enzyme known as MKK6 (mitogen activated protein kinase kinase 6). A kinase is an enzyme that transfers a phosphate group to another molecule phosphorylating it. Activated MKK6 then phosphorylates and activates an enzyme called p38 MAPK (p38 mitogen-activated protein kinase). p38 MAPK will then activate a third enzyme known as MK2 (MAPK-activated protein kinase-2). MK2 will then phosphorylate Hsp27 (heat-shock protein 27) and LIMK. The protein Hsp27 works to cap actin filaments, thereby reducing the number of free barbed ends. When phosphorylated, its actin capping abilities are disabled, thereby generating more barbed ends for actin polymerization. Phosphorylation activates LIMK leading to a conformational change that increases its ability to phosphorylate cofilin (Koayashi et al., 2006). Increased levels of p-cofilin promotes the polymerization of actin monomers and therefore increased stress fiber formation. Stress fibers are extremely important in adhesion and contraction of the cell during migration (German et al., 2014). Berry extracts and anthocyanins have been shown experimentally to increase levels of activated eNOS leading to increased concentrations of NO (Zhang et al., 2015). Knowing that increased levels of NO has been shown to stimulate VEGF activation, it can be postulated that through this pathway p-cofilin levels will increase in a cells treated with anthocyanins.
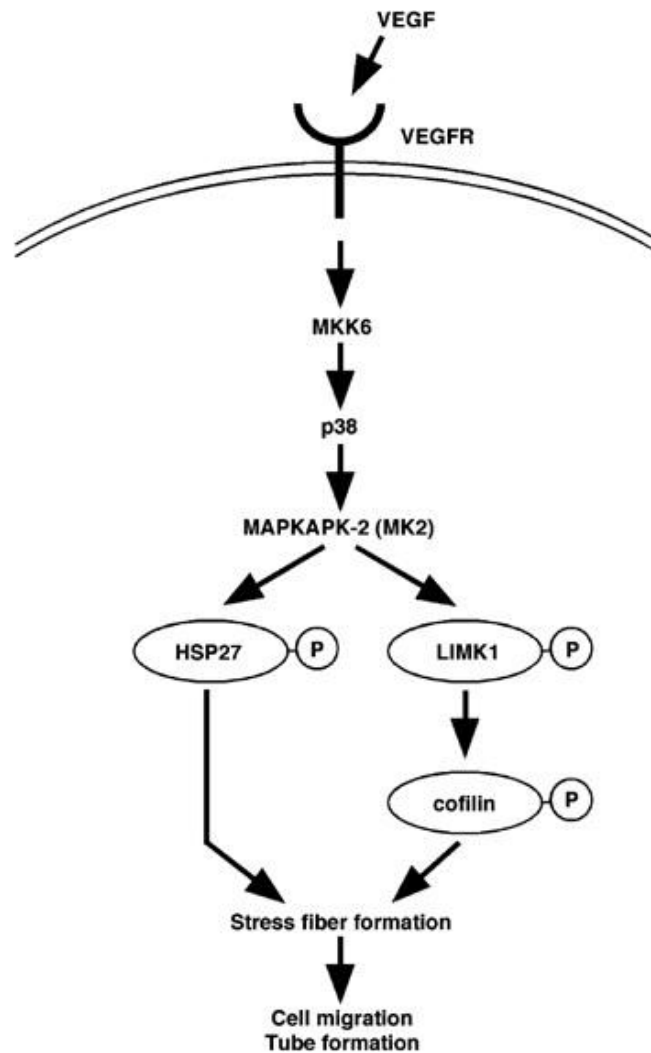
Figure 3. **VEGF induces LIMK and cofilin phosphorylation.** A proposed signal transduction pathway suggests that activation of VEGF stimulates activation of LIMK which results in cofilin inactivation and phosphorylation. When cofilin is phosphorylated its actin binding ability is inhibited, and polymerization of actin monomers into stable fibers occurs as a result. VEGF stimulates a second pathway that phosphorylates the protein Hsp27, leading to the generation of free barbed ends and migratory structures (Kobayashi et al., 2006).

1.8 Rho GTPases and Corresponding Cellular Morphologies

Many cell signaling pathways exist, including the VEGF/MK2/LIMK/pcofiln described above, to regulate migratory morphologies and many of these involve small GTPases. Small GTP (guanosine triphosphate) binding proteins, and more specifically the Rho family of GTPases, are important regulators of actin dynamics and cellular migration. Rho GTPases cycle between the GTP-bound, active state, and the GDP-bound (guanosine diphosphate), inactive state (Chan and Nance, 2013). The action of guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP, effectively activating the protein. The GTP is then catalytically converted to GDP by the GTPase activity of the Rho GTPase protein and by the action of the GTPase activating proteins (Chircop, 2014). Specifically, there are three proteins that contribute to actin dynamics and increase migratory morphologies, these are Cdc42, RhoA and Rac1. Cdc42 (cell division control protein 42), when activated, targets the Wiskott-Aldrich syndrome protein (WASp) family which binds to the actin related protein 2/3 complex (Arp2/3) which promotes the assembly of actin fibers to form filopodia (Lamalice et al., 2007). Rac1 (Ras-related C3 botulinum toxin substrate 1), when activated leads to the activation of phosphatidylinositol 3-kinase (PI3K) and the subsequent activation of phosphoinositol bisphosphate (PIP_2). PIP_2 activation results in the uncapping of the plus end of actin filaments, allowing for increased sites for actin polymerization and the formation of broad lamellipodia (Lamalice et al., 2007). RhoA (Ras homolog, member A), when activated, activates Rho-associate protein kinase (ROCK), which phosphorylates LIMK (discussed early) which subsequently phosphorylates cofilin, leading to the formation of actin stress fibers. Activation of ROCK also inhibits myosin light chain phosphatase,

which leads to the contraction of the myosin-actin fibers and pulls the tail end of the cell forward during migration (Lamalice et al., 2007). Studies indicate that increased concentrations of NO (nitric oxide) produced by eNOS (endothelial nitric oxide synthase) induced cellular migration through the activation of Rho family GTPases (Zhan et al., 2015). As mentioned previously polyphenolic compounds from berry extracts, such as anthocyanins, have led to increased eNOS activation and subsequent increase of NO concentrations (Banaszewski et al., 2012). The Rho family of GTPases is another potential pathway for anthocyanins to alter cellular actin dynamics, and induce migratory structures.

1.9 Paxillin and Focal Adhesions

Filopodia are actin structures that allow the cytoskeleton of the cell to stretch out and bind to the extracellular matrix (ECM) proteins that surround it. The strength of the filopodia's contacts with the environment determines lamellipodia formation and directs cell movement (Hu et al., 2014). Paxillin is a focal adhesion associated adaptor protein. It exists primarily in the cell's focal adhesion complexes that allow the cell to bind to its external environment. Paxilllin acts as a scaffold and recruits other proteins to form the focal adhesion complex, which can be made up of a multitude of proteins that enable the interaction between the ECM and the cytoskeletal structures inside the cell (Brown et al., 2002). Interactions with integrins (the transmembrane protein of the focal adhesion complex that binds to the ECM) located in these complexes and the ECM can affect the direction of migratory structures like lamellopodia and filopodia (German et al., 2014). The consequential assembly and disassembly of these focal adhesions allow for the cell

to migrate effectively (Nayal et al., 2006). Paxillin is typically phosphorylated on tyrosine residues 31 and 118 by focal adhesion kinases (FAK) or by Src family kinases (SFK) and it is associated with many other signaling molecules and adhesion proteins (German et al., 2014). The p21-activated kinase (PAK) is a signaling molecule that has been shown experimentally when active to increase the depolymerization and turnover of focal adhesion complexes. PAK is activated by Cdc42, a small GTPase that has also been linked with filopodia formation (Zhoa et al., 2000). Phosphorylation of paxillin has been linked with increased migration and adhesion complex turnover (Nayal et al., 2006). Depending on where the focal adhesions are located on the cell, it can either inhibit or encourage cellular migration. Focal adhesions located at the tail end of the cell will inhibit migration, whereas adhesions at the leading edge of the cell will encourage mobility (German et al., 2014).

Based on the knowledge that concentrations of VEGF have increased in cell lines treated with anthocyanins and increased concentrations of VEGF activate a signal cascade leading to cofilin phosphorylation (Nizamutdinova et al., 2009); this study hypothesized that concentrations of p-cofilin will increase in BAECs (Bovine aortic endothelial cells) treated with anthocyanins. This study also hypothesized that cells treated with anthocyanins for short exposures (1-3 hours) will show increased migratory morphologies based on the knowledge that previous studies have seen increased migration in anthocyanin treated cells (Banaszewski et al., 2012). Paxillin concentrations are also hypothesized to increase because of the important role they play in migration (Nayal et al., 2006). Time course studies were performed to determine if migratory morphologies were altered in BAECs treated with anthocyanins. Confocal images were

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taken to compare actin, paxillin, and nuclear morphologies of cells treated with anthocyanins to control cells. Western blot analysis was performed to determine cellular concentrations of paxillin, p-cofilin, cofilin, and actin to determine which cellular signaling pathways were affected by the anthocyanin treatment.

Chapter 2: Methods

2.1 Dulbecco's Modified Eagle Medium (DMEM)/Ham's Nutrient Mixture F12 media

To make media for cell culture, 6g of Dulbecco's Modified Eagle Medium

(DMEM)/Ham's Nutrient Mixture F12 media was added to 1.22g of $NaHCO_3$ and

500mL of double distilled water (ddH_2O). The solution was mixed thoroughly and

brought to a pH of 7.0 using NaOH or HCl depending on the pH of the solution. After the

solution was mixed consistently for 30 minutes, 2mM of L-glutamine powder was added.

The pH was then adjusted again to pH 7.0. The media was filter sterilized using 0.22µm

500mL bottle top filters (Corning; Corning, NY) and was supplemented with 10% fetal

bovine serum, 2mM L-glutamine and 1% penicillin/streptomycin antibiotics.

2.2 Cell Culture

Bovine Aortic Endothelial Cells (BAECs) (NIGMS Human Genetic Cell Repository

GMO7373) were cultured with Dulbecco's Modified Eagle Medium (DMEM)/Ham's

Nutrient Mixture F12 media (Sigma-Aldrich; St. Louis, MO) supplemented with 10%

fetal bovine serum, 2mM L-glutamine and 1% penicillin/streptomycin antibiotics. These

cells were grown in a 37ºC, 5% CO_2 incubator. Cells were passed into either 60mm petri

dishes or 35mm MatTek glass bottom dishes for treatment with anthocyanins. BAECs

were grown to sub-confluency and treated with 20µg/mL anthocyanins in above media

for 3 hours in a 37ºC, 5% CO_2 incubator. Controls were also passed and grown to sub-

confluency and incubated for 3 hours with changed media and no anthocyanin treatment.

2.3 Anthocyanin Extraction

Wild blueberries used for the anthocyanin extract were provided by Wyman's (Cherryfield, Maine) and processed following standard procedures to obtain a freeze-dried powder according to Vendrame et al. (2014) (FutureCeuticals; Momence, IL). Anthocyanin extracts were provided by the Klimis-Zacas laboratory and were extracted based on procedures described by Del Bo' et al. (2015).

2.4 Cell Extraction

BAECs grown in three separate 60mm petri dishes were placed on a slant of ice and media was removed. 2 mL of phosphate buffered saline+ (PBS; 8.5g/L NaCl, 0.18g/L NaH$_2$PO$_4$, and 1.24g Na$_2$PO$_4$) (1mM MgCl$_2$ and 0.5mM CaCl$_2$ added for PBS+) was added to each plate, mixed, and removed. This was repeated two more consecutive times. All PBS+ was then removed. 100μL of cold extraction buffer was added to each plate. Extraction buffer (60mM PIPES, 25mM HEPES, 10mM EGTA, 2mM MgSO$_4$, 10μg/mL CLAP (Chymostatin leupeptin, aprotin and pepstatin-A.) 0.1% TritonX-100, 0.5mM phenylmethanesulfonyl fluoride, and 0.1 mM dithiothreitol) was mixed on each plate for 5 minutes while plates remained on ice slant. A cell scraper was used to scrape the cells off the plate in both vertical and horizontal directions. Cells were removed from plates and transferred into three separate pre-cooled centrifuge tubes, and then the cells were homogenized with eppendorf homogenizers. Cells were centrifuged for 3 minutes at 5,000 rpm at 4˚C. The supernatants were recovered and 60μL from each sample was added to three different centrifuge tubes containing 15μL of 2x Laemmeli Buffer each.

These samples were used to separate proteins on SDS page gels. 40µL from each of the remaining samples was recovered in centrifuge tubes for Bradford protein quantification.

2.5 Bradford Protein Quantification

40 µL of cell extracts were thawed and kept on ice. 10µL of the cellular extracts was added 990µL of ddH$_2$O and mixed. 96 well plates were used for protein quantification. 50µL of Bovine Serum Albumin Standards (5µg/mL to 40µg/mL in succession of increments of 5µg/mL) were added in triplicate to the plate. 50µL of diluted cellular extracts were also added in triplicate to the 96 well plate. 50µL of Quick Start Bradford Dye Reagent (Bio-Rad Laboratories; Hercules, CA) was added to each loaded well. Protein concentrations were determined on an ELISA Bio-Tek (Bio-Tek Instruments Inc.; Winooski, VT) plate reader at 595nm wavelength. The amount of protein to be loaded in µL was determined based on the absorbance reading. Each well was loaded with 10µg of protein.

2.6 SDS-PAGE Analysis of Proteins (All reagents used in SDS-PAGE and Western blot analysis are products of Bio-Rad Laboratories; Hercules, CA)

Bio-Rad casting stands were assembled. To make the lower gel, 3.4 mL of ddH$_2$O, 2.5mL of 4x Lower gel buffer (1.5M TRIS-HCl pH8.8, 0.4% w/v SDS), and 4.1mL of 30:1 Acrylamide was mixed. To polymerize the gel, 34µL of fresh 10% Ammonium persulfate (APS) and 5µL TEMED was added to this mixture. After being mixed well the lower gel solution was added into the 1MM gel plates set up on the casting stand and overlayed with ddH$_2$O and allowed to set for 45 minutes to polymerize. The water was then

removed and the 3% stacking gel was prepared by mixing 3.25mL of ddH$_2$O, 1.75mL of Upper gel buffer (0.5M TRIS-HCl pH 6.5, 0.4% w/v SDS), and 1mL Acrylamide. This solution was mixed and 23µL of APS and 7.5 µL of TEMED was added. After being mixed for a second time this solution was added to the top of the lower gel in the 1MM gel plates. Fifteen well combs were inserted and the gel was left to polymerize for another 30 minutes. The Bio-Rad chamber was then assembled and filled with 1X Running Buffer made from a 5X stock (72g Glycine, 15g Tris-base, 5g SDS) diluted to 1L with ddH$_2$O. Gels were then loaded with 5µg/mL of each protein sample. Proteins were electrophoresed at 120V for approximately 1.5 hours or until the bromophenol blue dye reached the bottom of the gel.

2.7 Western Blot Analysis

Polyvinylidene Difluoride (PVDF) membrane was wet with methanol (MEOH) for 5 minutes in a plastic container on a rocker. The PVDF membrane was then washed with ddH$_2$O until the membrane was able to float. A cassette sandwich was assembled in the following order in a dish containing 1X Transfer Buffer (700mL ddH$_2$O, 100mL 10X Transfer Buffer (1000mL ddH$_2$O, 30g Tris Base, 144g Glycine), 200mL methanol, 20mL 5% SDS.): The black side of the cassette containing two Scotchbrite pads, Whatman 3mm filter paper, the acrylamide gel notched at left side corner containing control sample wells, the PVDF membrane, Whatman 3mm filter paper, and two Scotchbrite pads. Each layer was run over with a wheeling tool to remove bubbles. The sandwich cassette was then loaded into the chamber containing 1X transfer buffer and the proteins were electrophoresed and transferred from the gel to the PVDF membrane for 1 hour and 15

minutes at 65V. The cassette was removed and the PVDF membrane put in blocking

solution (10% new born calf serum in Tris buffered saline (TBS; 50mM Tris-Cl, 150mM

NaCl with 0.5% Tween 20(TBST)) for 45 minutes at room temperature on a rocker. The

blocking solution was discarded and the specific primary antibody was diluted in the

blocking solution; either rabbit anti-cofilin polyclonal primary antibody (Cytoskeleton

Inc.; Denver, CO), rabbit p-cofilin polyclonal primary antibody (Santa Cruz

Biotechnology Inc.; Dallas, TX), mouse anti-actin, clone C4 monoclonal primary

antibody (Millipore; Billerica, MA), or mouse anti-paxillin monoclonal primary antibody

(BD BioSciences; Franklin Lakes, NJ) (1:5,000 dilution). The PVDF membrane was put

on a rocker for 2 hours at room temperature. The PVDF membrane was then washed in

TBS for 5 minutes, TBST for 5 minutes, and TBS for another 5 minutes. The secondary

antibody, either HRP (horseradish peroxidase) labeled goat anti rabbit secondary

antibody or HRP labeled goat anti mouse secondary antibody, was diluted diluted in

blocking solution (1:60,000). The PVDF membrane was put in secondary antibody for 2

hours on a rocker at room temperature. PVDF was then washed with TBS for 10 minutes,

TBST for 10 minutes, TBS for another 10 minutes, and TBST for another 10 minutes.

The PIERCE Chemiluminenscent kit was used to detect the horseradish peroxidase

(HRP) labeled secondary antibody (1:1 ratio of SuperSignal West Dura Stable Peroxide

Buffer to SuperSignal West Dura Luminol/Enhancer Solution). The PVDF membrane

was placed in the solution for approximately 1-2 minutes. The blot was developed using

the ImageQuant LAS4000 imager (GE Healthcare Bio-Sciences; Pittsburgh, PA).

Calculations were made using Fiji/ImageJ imaging software. A rectangle was generated

and moved from one band to the next to keep a consistent size. The gray scale value of each band was then recorded and averaged for each sample group.

2.8 Fixation for Confocal Microscopy

BAECS were cultured until sub-confluent in 35mm MatTek glass bottom dishes. Cell media was removed and the cells were washed with 2mL 1xPBS+ three consecutive times. To fix the cells, 2mL of 4% paraformaldehyde was added for 15 minutes. Paraformaldehyde was then removed and the cells were washed with 2mL 1xPBS+ three consecutive times. To permealize the cells, 0.1% Triton in 1xPBS+ was added for 15 minutes and removed. The cells were then blocked with 2mL of 2% bovine serum albumin, BSA (1g in 50mL of 1xPBS+) for 30 minutes at room temperature. Cells were probed with 1:200 dilution of mouse-anti-paxillin primary antibody (BD BioSciences; Franklin Lakes, NJ) in 2% BSA blocking buffer for 60 minutes at room temperature. Cells were then washed three times with 2mL of PBS+. Cells were stained with 1mL of a 1:100 dilution of fluorescein isothiocyanate (FITC) conjugated goat-anti-mouse secondary antibody (Jackson ImmunoResearch Laboraories Inc.; West Grove, PA), a 1:200 dilution of 4',6-diamidino-2-phenylindole (DAPI), and a 1:200 dilution of rhodamine phalloidin (Invitrogen; Carlsbad, CA) in 2% BSA blocking buffer. Cells were then washed three times with 2mL of PBS+. Cells were stored in PBS+ and viewed using the Olympus Fluoview 1000 Laser Scanning Confocal Microscope (Olympus America Inc.; Center Valley, PA).

2.9 Timed Study Using Nikon Eclipse TS100 Microscope

BAECs were cultured in 60mm petri dishes until sub-confluent. Media was changed and the cells in the treated petri dish were treated with 20 µg/mL anthocyanins. Control BAECs had media changed but were not treated. Cells were then incubated for one hour in a 37˚C/5% CO_2 incubator and observed under a Nikon Eclipse TS100 microscope (Nikon Instruments Inc.; Melville, NY) and pictures were captured of three frames at 20X for both the treated and the control cells. This procedure was repeated for 2, 3, and 24 hours. Pictures were then analyzed using Adobe Photoshop imaging software. 50 cells from each the treated and control sample groups and measured for area and perimeter in each time group. The average and standard deviation of each of these groups was determined, as well as the significance using a student t-test on Microsoft Excel software. Differences were determined significant if t-test value was below 0.05. Standard error was calculated using the equation $\frac{\sigma}{\sqrt{n}}$.

Chapter 3: Results

Time course studies using the Nikon Eclipse TS100 microscope were used to determine the treatment time that resulted in the greatest change in cellular morphologies. Time course studies found that cells treated with 20µg/mL anthocyanins for 2 and 3 hours had increased perimeters and decreased area at 24 hours compared to control cells. Confocal images of BAECs were used to determine changes in actin, paxillin, and nuclear morphologies in cells treated with anthocyanins for 3 hours. Confocal images revealed BAECs treated with 20µg/mL anthocyanins for 3 hours were stalled in cytokinesis which resulted in increased numbers of cells with multiple and fragmented nuclei. BAECs were also observed in confocal images to have thick stress fibers, and areas of concentrated paxillin. Western blot analysis was performed to determine if there were any significant changes in concentrations of proteins known to be involved in migration and actin dynamics. Changes in concentrations of cofilin, p-cofilin, paxillin, and actin in cells treated with 20µg/mL anthocyanins for 3 hours were not significant.

3.1 Anthocyanin treatment of BAECs for 2 and 3 hours increased filopodia formation

BAECs treated with anthocyanins had exaggerated and increased numbers of filopodia per cell at the 2 and 3 hour time periods (Figure 4). Lamellipodia were also observed in treated cells, but cells with exclusively broad lamellipodia were not as common. Control cells had lamellipodia that were broad and typically present at all edges of the cell, while long, outstretched filopodia was observed less frequently. Figure 4A1 and 4B1 are representative images demonstrating the prominent cellular morphologies observed at these time periods. Figure 4A1 and 4B1 are images taken from the 3 hour

time period however they are representative of both the 3 hour and 2 hour time periods. The differences in migration morphologies were primarily at the 2 and 3 hour time intervals. At the 24 hour time interval cell numbers had increased and were condensed together with less migratory morphologies. At the 24 hour time period, the cells began to become confluent. As cell contacts increased filopodia formation decreased in both control and treated sample groups. Figure 4A2 and 4B2 are representative pictures of control and treated cellular morphologies at the 24 hour time period. At the 24 hour time period control and treated cells began to spread out considerably and were nearly indistinguishable from each other. Cells at this time period were more ovoid and oblong in shape.
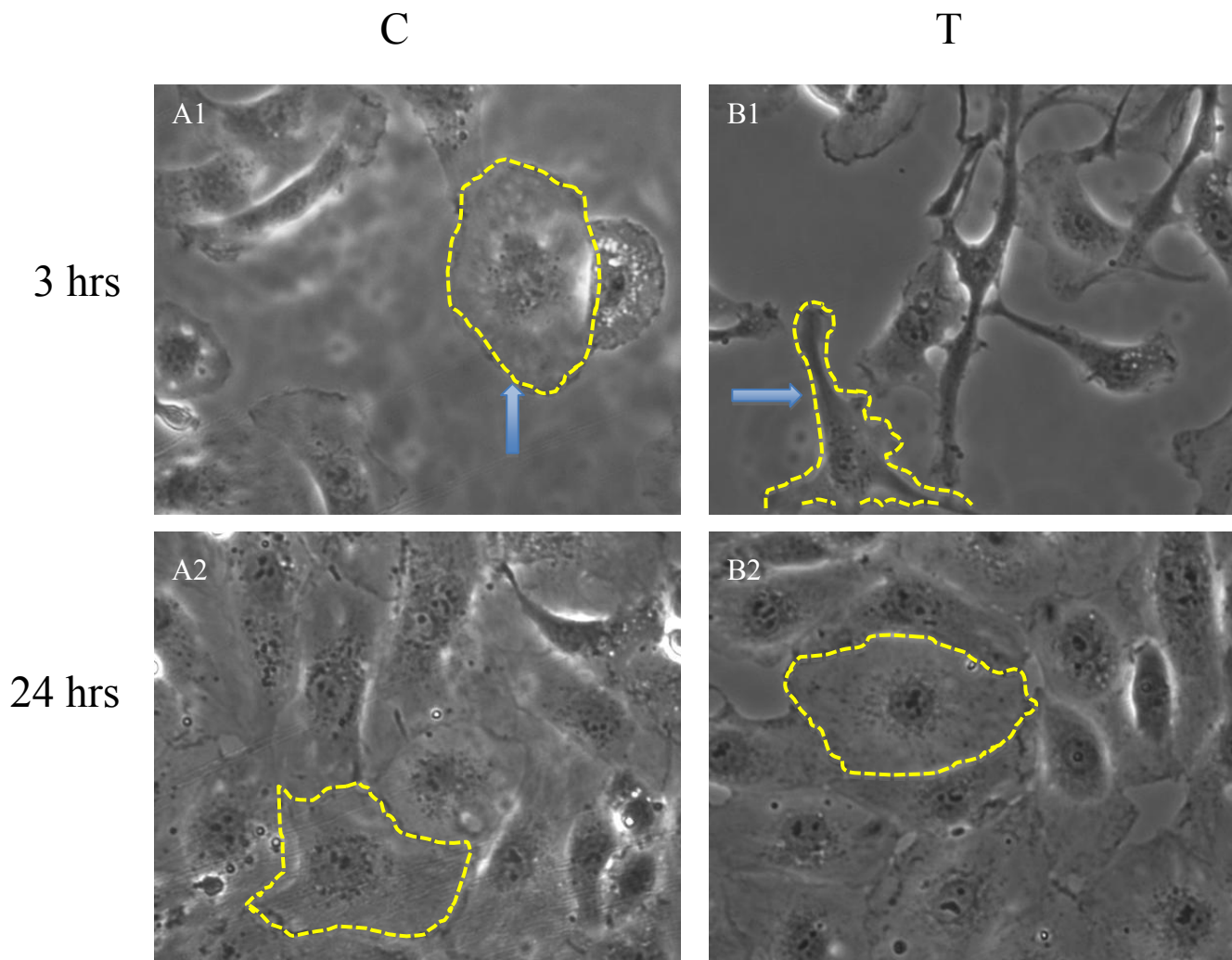
Figure 4. **BAECs treated with anthocyanins for 3 hours had increased filopodia formation. BAECs treated with anthocyanins for 24 hours had similar morphologies to control cells.** Images were taken with a Nikon Eclipse TS100 microscope at 20x magnification. Control cells at 3 hours formed large broad ruffles of lamellipodia (A1, arrow). Cells treated with anthocyanins for 3 hours formed outstretched long filopodia (B1, arrow). Control cells at 24 hours were reaching confluency and were adjacent to one another, similar to anthocyanin treated cells (A2). Cells treated with anthocyanins for 24 hours did not have distinct morphologies compared to control cells (B2).

3.2 Anthocyanin Treatment of BAECs for 24 Hours Decreased Cell Area

Area measurements were taken using pixels^2. Approximately 2700 pixels were equal to 1mm at 20x magnification. Significance was established for the data sets with a p value of less than 0.05. Figure 5 gives the averages of the control and treated cell areas analyzed at each time interval; significance is marked with an asterisk. Differences in area between control and treated cells were not significant at the 1 hour (p=0.342), 2 hour (p=0.735), or 3 hour (p=0.235) exposure times. The difference in area at the 24 hour exposure time was significant (p=0.0293). The difference in area at the 24 hour time interval was 3,090.64 pixels^2, this was a decrease of about 13.8% from the average control cell area. Average area increased for both the control and the treated cells from 3 hour to 24 hours. This difference was significant for both the control (p=1.24E-10) and the treated (1.90E-05) sample groups. Average area increased in control cells by 9,799.81 pixels^2, which is a 77.3% increase in area from 3 hours to 24 hours. Average area increased in treated cells by 5,352.31 pixels^2, which is a 38.1% increase in area from 3 hours to 24 hours. The area of the control cells was about 13.8% larger than the average treated cell, and it had a 39.2% greater increase from the 3 hour time period to the 24 hour time period compared to the treated cells. This decrease in area in cells treated with anthocyanins for 24 hours was not observed by the eye.
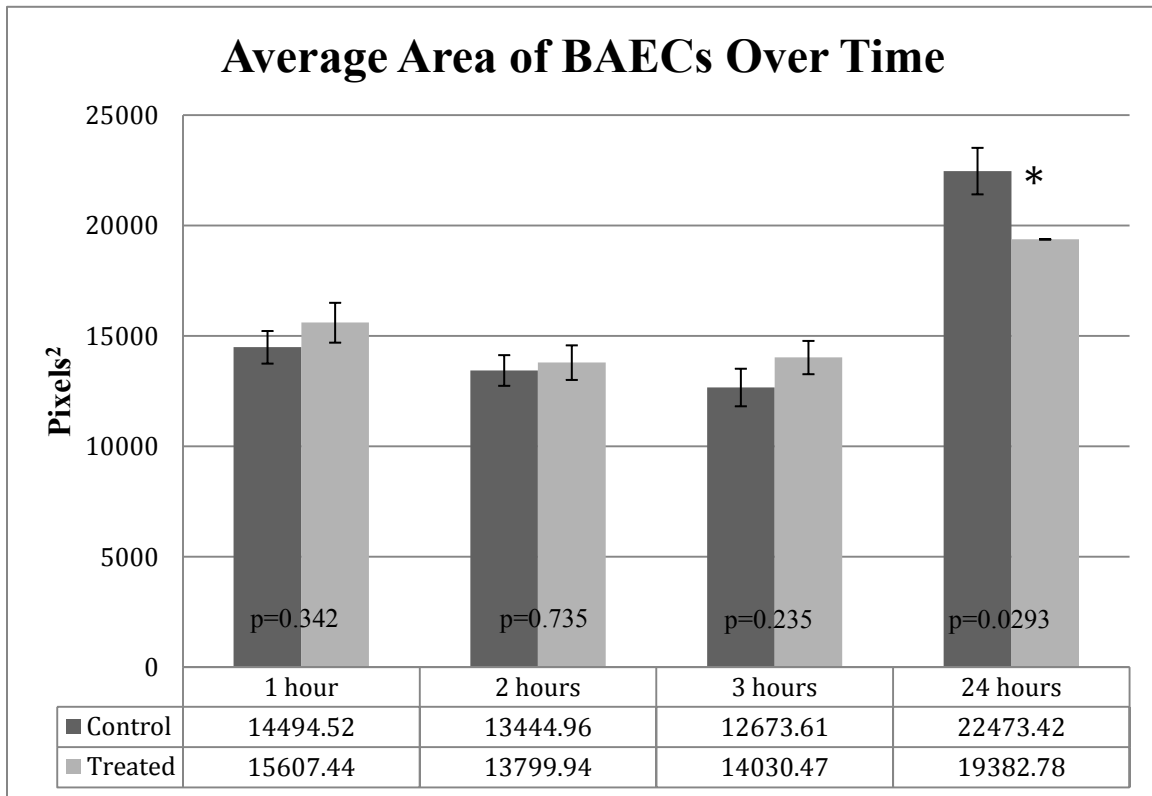
Figure 5. **BAECs treated with anthocyanins only had a significant change in average area after being treated for 24 hours.** Area was measured in Adobe Photoshop and the averages were calculated. Bars show standard error calculated by the equation: $\frac{\sigma}{\sqrt{n}}$. n=50 cells were observed for each the control and the treated sample groups. Significance was established for the data sets with a p value of less than 0.05(*). Cells treated with anthocyanins had a significant decrease in area after being treated for 24 hours (p=0.0293). Differences in area at other time periods were not significant. BAECs treated with anthocyanins for 24 hours had a 13.8% decrease in area from control cells which was significant.

3.3 Anthocyanin Treatment of BAECs for 2 and 3 Hours Increased Average Cell

Perimeter

Perimeter measurements were taken using pixels. Approximately 2700 pixels were equal to 1mm at 20x magnification. Significance was established for the data sets with a p value of less than 0.05. Figure 6 gives the averages of the control and treated cell perimeter analyzed at each time interval; significance is marked with an asterisk. Differences in perimeter between control and treated cells were not significant for the 1 hour (p=0.643), and the 24 hour (p=0.875) time intervals. The differences in perimeter between control and treated cells were significant for the 2 hour (p=9.30E-6), and the 3 hour (p=1.05E-8) time intervals. Difference in average perimeter between control and treated cells at 2 hours was 128.877 pixels, this was a 22% increase in perimeter in treated cells. Difference in average perimeter between control and treated cells at 3 hours was 247.79 pixels, this was a 43.5% increase in perimeter in treated cells. These measurements correspond with the observations that treated cells at the 2 and 3 hour time periods displayed higher numbers of exaggerated filopodia than control cells.

Average Perimeter of BAECs Over Time

|	1 hour	2 hours	3 hours	24 hours
■ Control	653.8119181	586.302328	569.2759	684.0926
■ Treated	669.7757	715.1798	817.0681	688.1586
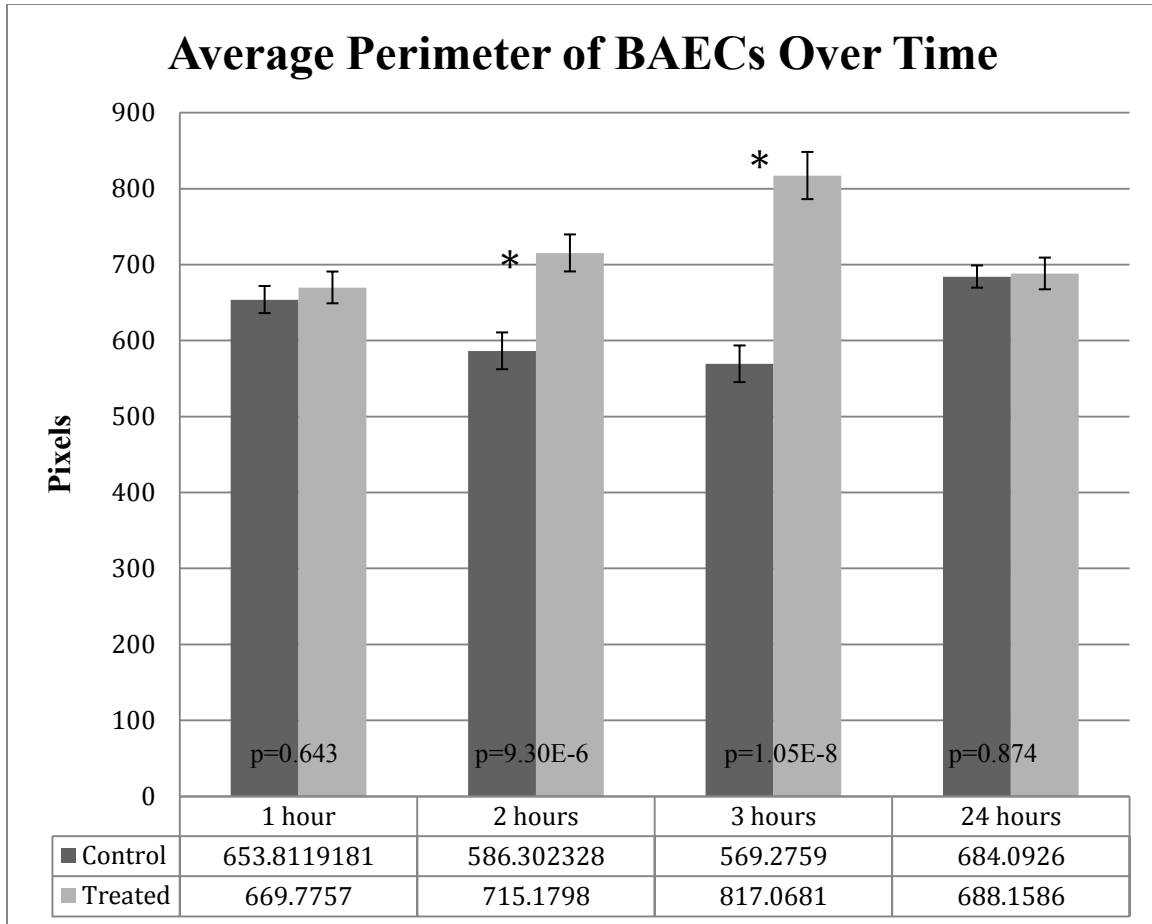
Figure 6. **BAECs treated with anthocyanins had a significant change in perimeter after being treated for 2 and 3 hours.** Perimeter was measured in Adobe Photoshop and the averages were calculated. Bars show standard error calculated by the equation: $\frac{\sigma}{\sqrt{n}}$. n=50 cells were observed for each the control and the treated. Significance was established for the data sets with a p value of less than 0.05(*). BAECs treated with anthocyanins for 2 hours had a 22% increase in perimeter from control cells which was significant (p=9.30E-6). BAECs treated with anthocyanins for 3 hours had a 43.5% increase in perimeter from control cells which was significant (p=1.05E-8). One millimeter is approximately equal to 2,700 pixels.

Figure 7 is a representation of the averages of the control and treated cell perimeters in a scatter plot. The difference in average perimeter between the control and treated cells was not significant at the 1 hour time interval (p=0.643). The differences in perimeter between the control and treated cells increased at 2 hours (p=9.30E-6) and at 3 hours (p=1.05E-8) was significant. The difference in perimeter then decreased back to a similar average perimeter as control at the 24 hour time interval (p=0.874). The change in average perimeter from 1 to 2 hours was significant in control cells (p=0.0273), this was a decrease of 67.5 pixels, a 10.3% decrease. As treatment time increased the difference in perimeter between control and treated cells increased from 1 to 3 hours then decreased to no difference at 24 hours. The change in average perimeter from 1 to 2 hours was not significant in treated cells (p=0.160). The difference in perimeter for control cells between 2 and 3 hours was not significant (p=0.572). The increase in average perimeter from 2 hours to 3 hours was significant in treated cells (p=0.00789), this was a increase of 101.9 pixels, a 14.2% increase. The change in perimeter from the 3 hour to the 24 hour time interval was significant for both the control (p=0.00011) and the treated (p=0.00089) sample groups. The control group increased in perimeter by 114.8 pixels, which was a 20.2% increase in perimeter. The treated group decreased in perimeter by 128.9 pixels, this was a 15.8% decrease in average perimeter. As treatment time increased for cells treated with anthocyanins from 1 to 3 hours the perimeter increased and then decreased from 3 to 24 hours. As time increased for control cells from 1 to 3 hours, average perimeter decreased and then increased from 3 hours to 24 hours.
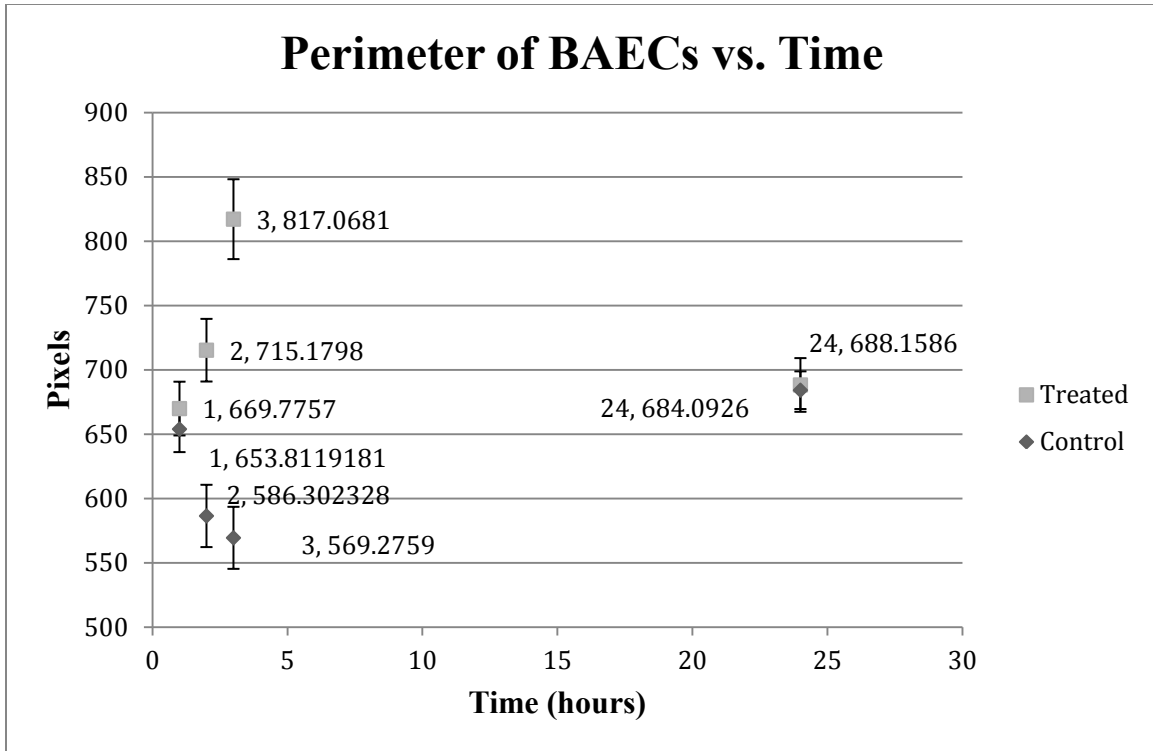
Figure 7. **Perimeter of BAECs treated with anthocyanins varied from control increasingly until 3 hours.** One millimeter is approximately equal to 2,700 pixels. Perimeters of BAECs treated with anthocyanins increased with time up to 3 hours. At 1 hour after treatment treated BAECs have an average area that is not significantly different than control cells (p=0.643). 2 hours after treatment BAECs had a 22% increase in perimeter from control cells which was significant (p=9.30E-6). BAECs treated with anthocyanins for 3 hours had a 43.5% increase in perimeter from control cells which was significant (p=1.05E-8). At 24 hours after treatment the treated BAECs have an average area that is not significantly greater than the control cells (p=0.874). Perimeter of treated BAECs increased with time and began to decrease back to a similar perimeter as controls at 24 hours after being treated.

3.4 Anthocyanin Treatment of BAECs for 3 Hours Altered Filamentous Actin Stress Fibers and Localization of Paxillin

Control and anthocyanin treated BAECs for 3 hours had no obvious differences between confluent and sub-confluent cultures when probed with rhodamine phalloidin, DAPI and antibodies to the focal adhesion protein, Paxillin and imaged with the Olympus Fluoview 1000 Laser Scanning Confocal Microscope. Figure 8 is a representative image of control BAECs and BAECs treated with anthocyanins observed with the Olympus Fluoview 1000 Laser Scanning Confocal Microscope at 40x magnification and 2.5x zoom. Control BAECs had increased areas of bundled filamentous actin oligomers (short filamentous actin) and had thin less defined stress fibers. Control cells did form actin stress fibers although they had noticeably more bundles of actin oligomers. BAECs treated with 20µg/mL anthocyanins for 3 hours had increased thick well defined stress fiber formation that stretched across the cell, and noticeably less actin oligomers. Paxillin was not localized with actin oligomers in control cells, but was associated with some actin stress fibers in other control cells. Paxillin was associated at either end of the actin stress fibers in BAECs treated with anthocyanins for 3 hours, this localization was easily observed in an overlay image (Figure 8, B4, arrow). Areas of more defined and concentrated regions of paxillin were observed in treated cells (Figure 8, B2, arrow). Control cells had diffuse paxillin throughout the cell (Figure 8, A2).
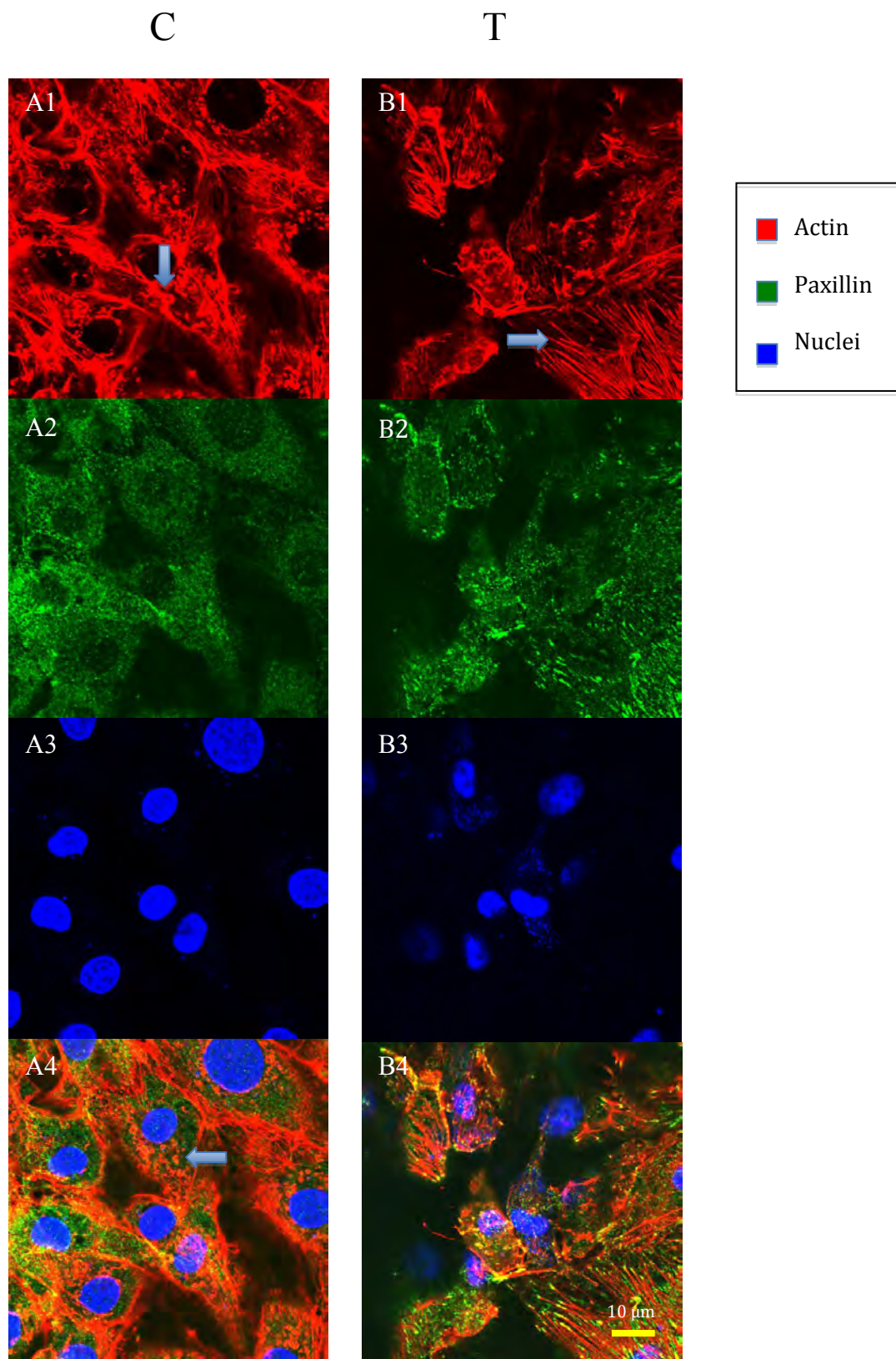
C   T

Actin
Paxillin
Nuclei

Figure 8. **BAECs treated with anthocyanins displayed thicker and more defined stress fibers.** Control cells had clumps of filamentous actin fixed with rhodamine

phalloidin and less defined stress fibers (A1). Anthocyanin treated BAECs had long filamentous actin stress fibers across the cell body (B1, arrow). Control cells had freely associated diffuse paxillin (A2). Treated cells displayed defined paxillin containing structures (B2). Overlay of control cells demonstrated little paxillin associated with actin structures (A4, arrow). Overlay of BAECs treated with anthocyanin have filamentous actin stress fibers with associated paxillin complexes at either end of these fibers (B4).

3.5 Anthocyanin Treated Endothelial Cells Were Delayed in Mitosis Resulting in Multinucleated and Nuclear Fragmented Cells

Cells in mitosis were characterized as having condensed nuclei. Cells in metaphase had condensed chromosomes in the center of the cell on the metaphase plate. Cells in anaphase had two condensed nuclei separated to opposite poles of the cell. Cells in telophase were characterized as having two condensed nuclei separated to opposite poles of the cell and contained a ring of actin that was beginning to form the cleavage furrow. The cleavage furrow is an area thick with filamentous actin laterally down the center of the cell. Cell nuclei were stained with DAPI. BAECs treated with anthocyanins for 3 hours had increased numbers of cells in mitosis. The majority of cells in mitosis were observed to be in metaphase. BAECs treated with 20μg/mL anthocyanins had increased numbers of multinucleated cells. Multinucleated cells had nuclei that were not condensed and were not separated by actin filaments. These nuclei were not localized to opposite poles of the cell but instead in close proximity to each other in the center of the cell. BAECs with multiple nuclei were not established to be in mitosis because their nuclei were not condensed or at opposite poles of the cell. Figure 9 is a representative image containing two cells in the metaphase stage of mitosis (Figure 9C, arrows) and a cell containing multiple nuclei (Figure 9C, *). Multinucleated cells did not have actin structures separating their nuclei (Figure 9A).
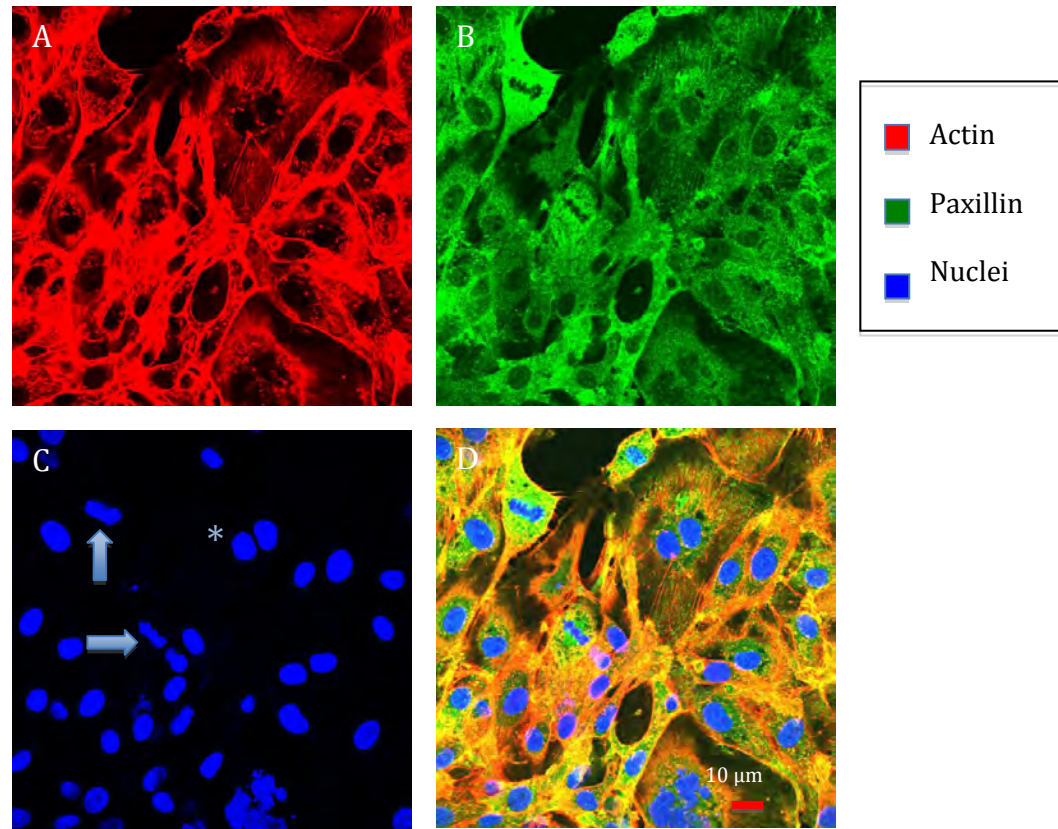
Figure 9. **Anthocyanin treated cells had increased numbers of cells in mitosis and more multinucleated cells than control cells.** BAECs treated with anthocyanins had increased numbers of cells in mitosis. Dividing cells had condensed chromosomes that were stained with DAPI and aligned on the metaphase plate. This indicated that cells were in the metaphase stage of mitosis (C, arrows). Cells containing multiple nuclei were observed more frequently in BAECs treated with anthocyanins, cell nuclei were not condensed in these cells and not observed to be in mitosis (C, *).

BAECs treated with anthocyanins for 3 hours had increased numbers of fragmented nuclei. BAECs with fragmented nuclei in some cases also contained one nucleus that was fully intact (Figure 10B1, *). In some instances BAECs had fragmented nuclei that were broken down even further into small DAPI stained nuclear material and no longer in large fragments (Figure 10B1, arrow). Cells treated with anthocyanins had increased numbers of cells with unusually shaped nuclei, with condensed chromosomes. Condensing of chromosomes is a typical symptom of pyknosis, a beginning stage of apoptosis or programmed cell death. Fragmented nuclei or karyorrhexis is usually the next step in apoptosis. Control cells typically had fully intact and spherical shaped nuclei, very few exhibited karyorrhexis.
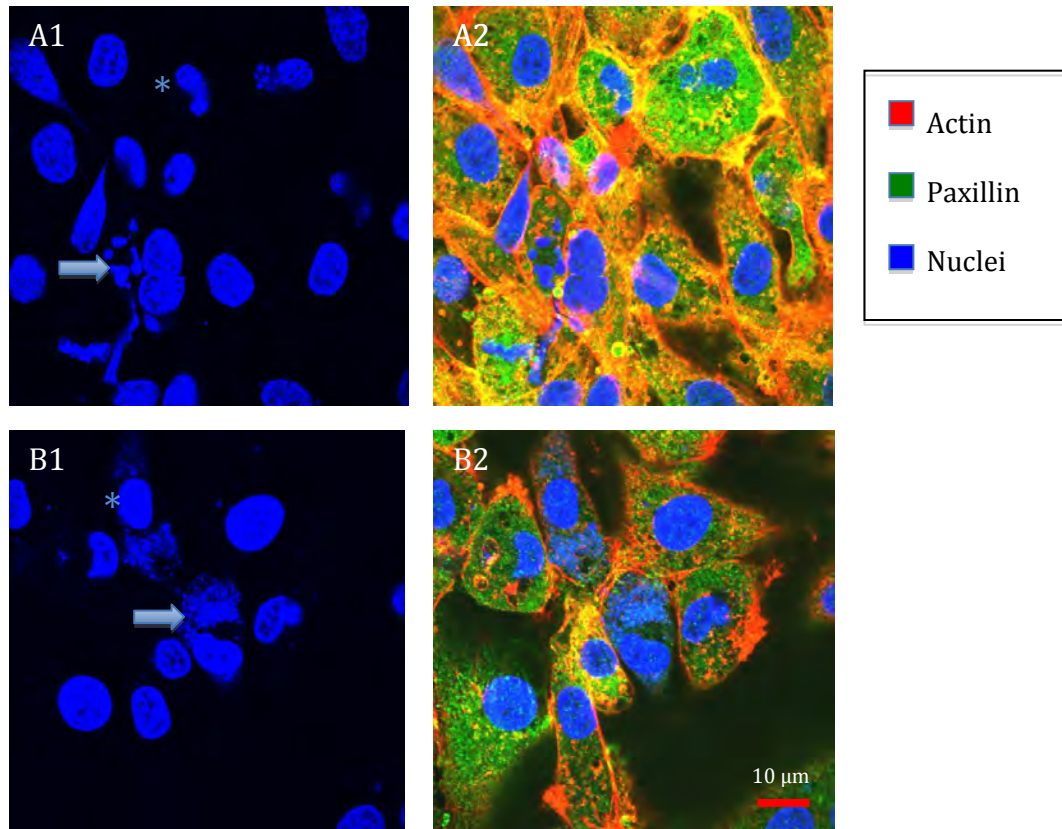
Figure 10. **BAECs treated with anthocyanin had multiple cases of deteriorating nuclei.** Nuclei were stained with DAPI. Nuclei of treated cells were fragmented into multiple components (A1, arrow). Cells treated with anthocyanins had increased numbers of cells with unusually shaped nuclei (A1, *). Breakdown of the nuclei was difficult to distinguish in overlays (A2). Cell with fragmented nuclei also contained a separate intact nucleus in some cases (B1, *). Nuclei were broken down even further; multiple examples of this were observed in treated cells in six separate cultures (B1, arrow).

3.6 BAECs Treated with Anthocyanins for 3 Hours Were Unable to Form a Cleavage Furrow

Cells in mitosis were characterized as having condensed nuclei stained with DAPI. Figure 11 is a representational image of a cell in telophase in a cell treated with anthocyanins for 3 hours. Treated BAECs were unable to form a cleavage furrow, which is characterized by a thick ring of filamentous actin, fixed with rhodamine phalloidin, in the center of the cell. A thick ring of actin was not observed in treated cells undergoing mitosis (Figure 11A). BAECs treated with anthocyanins had increased numbers of multinucleated cells. Multinucleated cells had at least two daughter nuclei that were not separated by actin filaments and were not localized to opposite poles of the dividing cell. Control BAECs in telophase of mitosis had two condensed daughter nuclei located on opposite poles of the cell with a region of thick filamentous actin separating the two daughter nuclei (Figure 12A). The region of thick filamentous actin is the cleavage furrow that separates the daughter nuclei during cytokinesis. The thick filamentous actin tightened and separated the control cell daughter nuclei creating an invagination in the cell (Figure 12A, arrow). Control BAECs had regions of thick actin filaments surrounding the cell (Figure 12A).
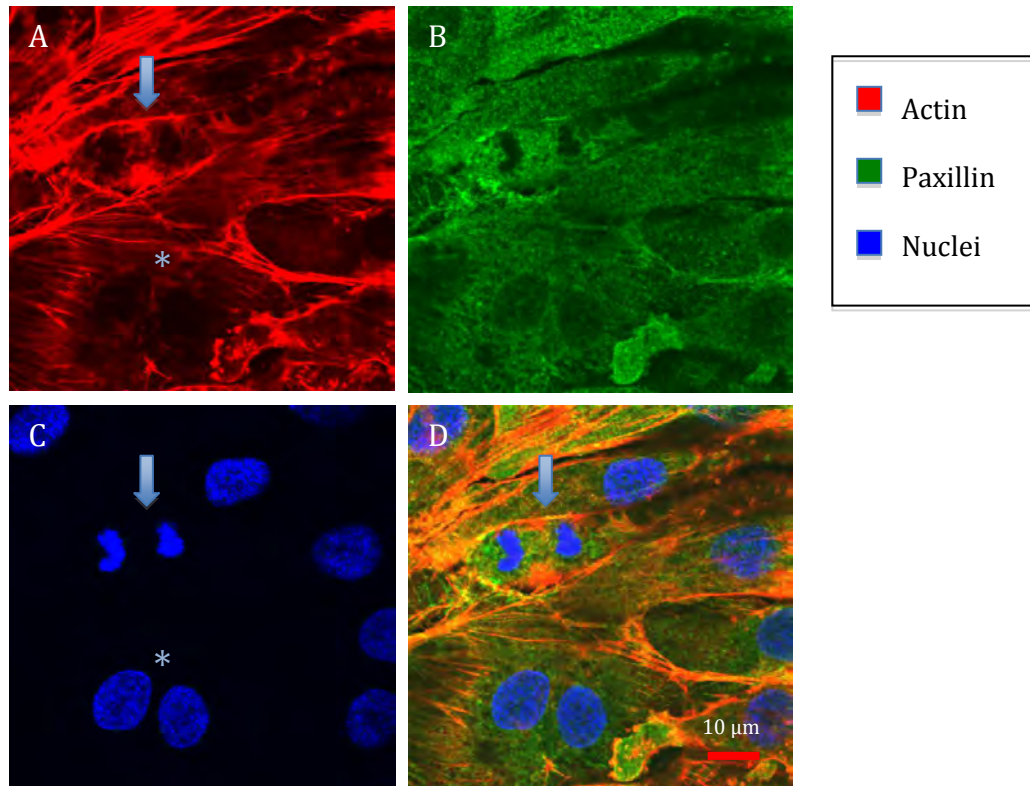
Figure 11. **BAECs treated with anthocyanins exhibited increased numbers of multinucleated cells and were not able to create a cleavage furrow.** Actin fibers did not separate nuclei in multinucleated cells (A, *). Cells were stained with rhodamine phalloidin which stained filamentous actin structures. Anthocyanin treated BAECs were unable to form a cleavage furrow which are made of a thick ring of actin splitting the cell laterally in two (arrow, A). Nuclei were stained with DAPI. Nuclei during mitosis were condensed and at opposite poles of the cell during anaphase (arrow, C). Multinucleated cells contained multiple nuclei in noncondensed condition (C, *). Overlay represents actin structures, paxillin structures, and nuclei. A cleavage furrow to separate daughter nuceli was not observed (D, arrow).
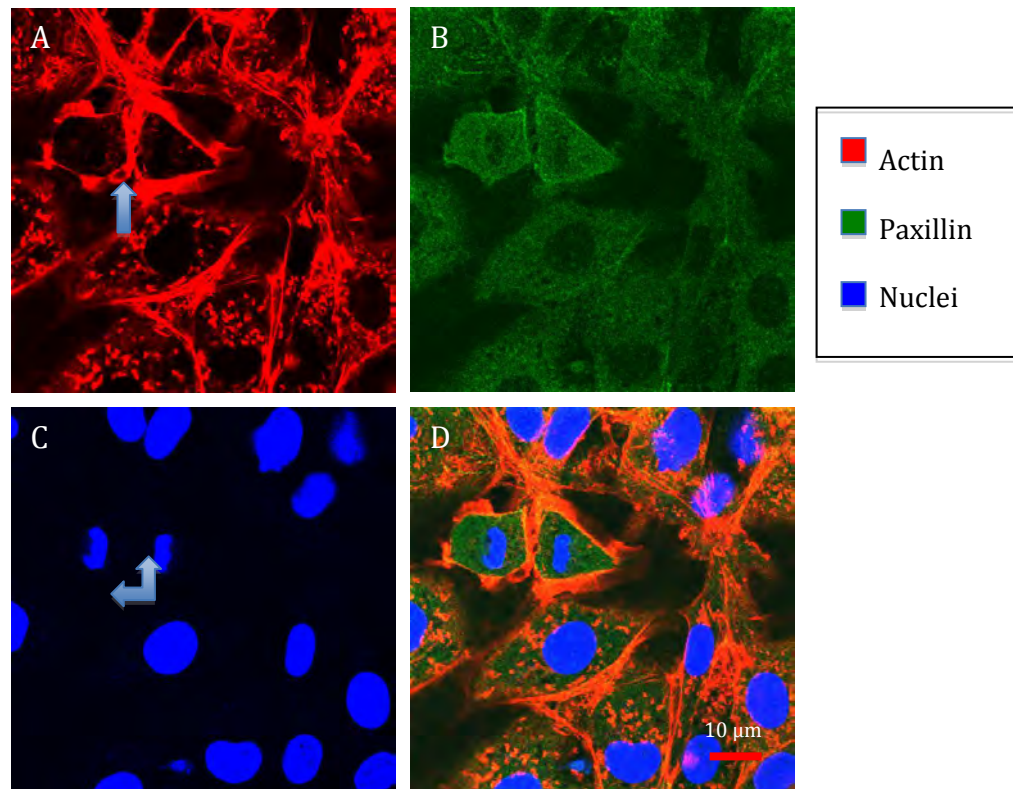
Figure 12. **Control BAECs not treated with anthocyanins had thick regions of actin separating daughter nuclei during mitosis.** Filamentous actin was tagged with rhodamine phalloidin. Thick actin filaments separated dividing cell nuclei, this is the cleavage furrow that creates an invagination in the cell (arrow, A). Thick actin filaments were at the outer edges of the cell (A). Nuclei were stained with DAPI. Cells that were dividing had condensed nuclei separated to opposite poles of the dividing cell in telophase of mitosis (C).

3.7 Protein Levels of Actin, pcofilin, Cofilin and Paxillin Were Not Significantly Affected by Treatment of Endothelial Cells with Anthocyanins

The higher gray scale value was correlated with an increased fluorescent signal and increased concentration of a protein. Significance was established for the data sets with a p value of less than 0.05. Average gray value for C4 actin in control BAECs was 28,518.17 and the average gray scale value for C4 actin in BAECs treated with anthocyanins for 3 hours was 27,085.16 (Figure 13). This was a difference of 1,433.01. The difference between average gray scale values for control and treated cells was not significant (p=0.1416). Actin is seen on SDS-PAGE at 42kDa. The blot in Figure 13 demonstrates that there is no significant change in actin concentration in BAECs treated with anthocyanins for 3 hours. This does not indicate filamentous actin morphologies because the process of SDS page and Western blot analysis denatures and breaks up actin filaments into globular actin. Therefore this blot only establishes that there is not a significant change in the concentration of actin protein between cells treated with anthocyanins for 3 hours and control cells. Figure 13 is an image of this blot and a graph representing the average gray scale values of the control and treated cells. Standard error was calculated to be very small in relation to the values, meaning that the measurements were relatively accurate and that the standard deviation was low.
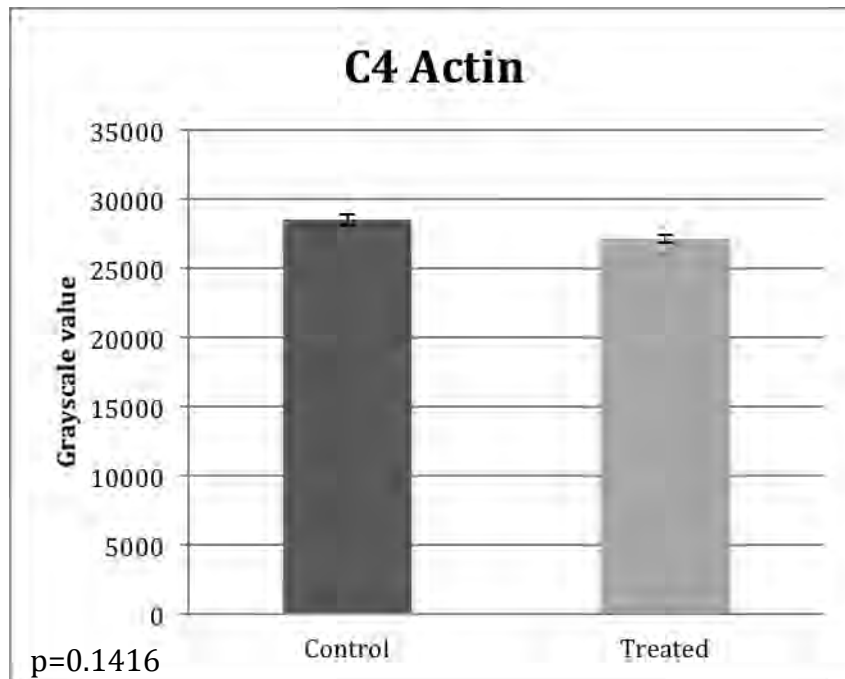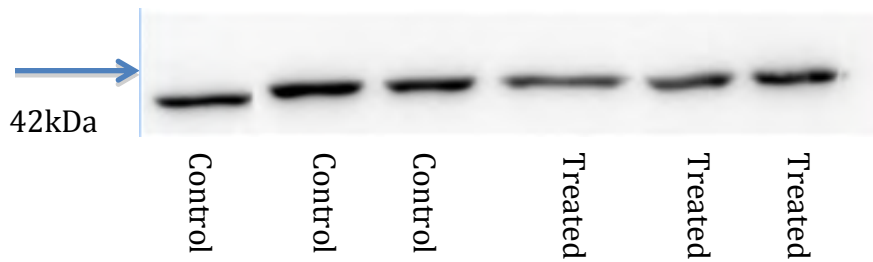
Figure 13. **BAECs treated with anthocyanins did not have a significant change in actin concentrations compared to control cells.** Western blot analysis was performed and gray scale values were interpreted using Fiji/ImageJ imaging software. Graph shows gray scale values measuring the strength of the signal for each sample group. n=3 for both control and treated groups. Average gray scale value for control cell bands was 28,518.17, and the average gray scale value for treated cell bands was 27,085.16. Change in actin concentration in cells treated with anthocyanins was not significant (p= 0.1416). Significance was established if p value was less than 0.05. n=3 cell cultures for both the control and the treated groups. The bars represent standard deviation. Actin is seen on the SDS-PAGE gel at 42kDa.

Phosphorylated cofilin1 and phosphorylated cofilin2 were observed on the PVDF membrane as two separate bands (Figure 14). These were analyzed together as one gray scale value. Average gray value for phosphorylated cofilin (p-cofilin) in control BAECs was 116,422.501, and the average gray scale value for p-cofilin in BAECs treated with anthocyanins for 3 hours was 106,731.47. This was a difference of 9691.031. The difference between average gray scale values for control and treated cells was not significant (p=0.1663). This means that p-cofilin concentrations were not significantly different between cells treated with anthocyanins and control cells. p-cofilin is the deactivated form of the actin binding protein cofilin. p-cofilin1 is seen on SDS-PAGE at 19kDa. Figure 14 is a representational image of three blots and a graph representing the average gray scale values of the control and treated cells.
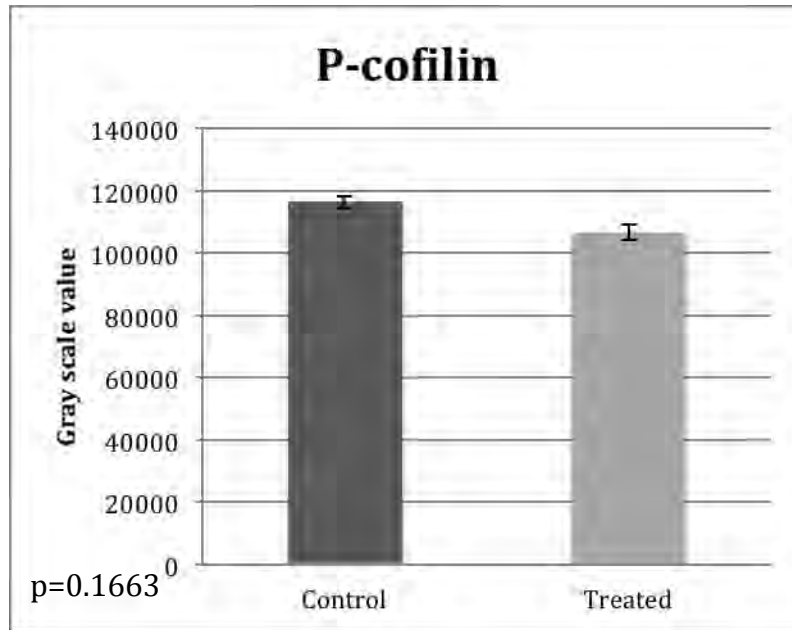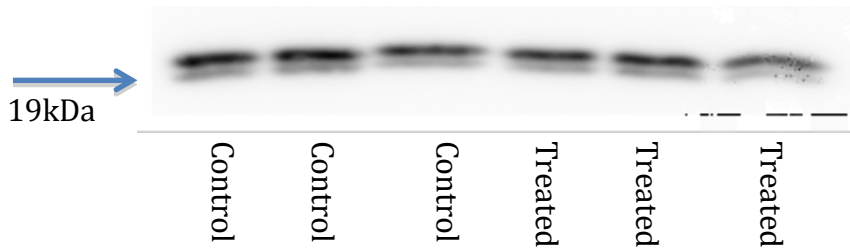
Figure 14. **BAECs treated with anthocyanins did not have a significant change in pcofilin concentrations.** The blot above is a representational blot of three blots performed. Gray scale values were interpreted using Fiji/ImageJ imaging software. Graph shows gray scale values measuring the strength of the signal for each sample group. Both phosphorylated cofilin-1 and phosphorylated cofilin-2 (represented as two separate bands on the PVDF membrane) were used in gray scale value. n=3 for both control and treated groups. Average gray scale value for control cell bands was 116,422.501, average gray scale value for treated cell bands was 106,731.47. BAECs treated with anthocyanins had no significant change in p-cofilin in treated cells (p=0.1663). Significance was established if p value was less than 0.05. The bars represent standard deviation. p-cofilin1 is seen on the SDS-PAGE gel at 19kDa.

Average gray value for non-phosphorylated cofilin in control BAECs was 85,219.17, and the average gray scale value for treated cell bands was 98,251.12. This was a difference of 13,032.04. The difference between average gray scale values for control and treated cells was not significant (p=0.1678). Cofilin is seen on SDS-PAGE at 18kDa. Figure 15 is an image of this blot and a graph representing the average gray scale values of the control and treated cells. This western blot represents the concentrations of activated cofilin in both the cells treated with anthocyanins and the control cells.
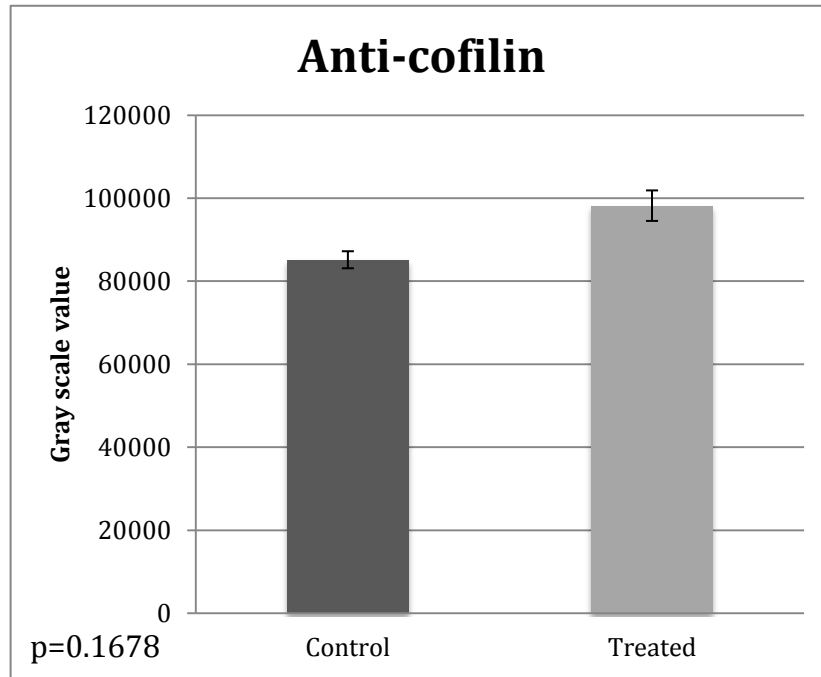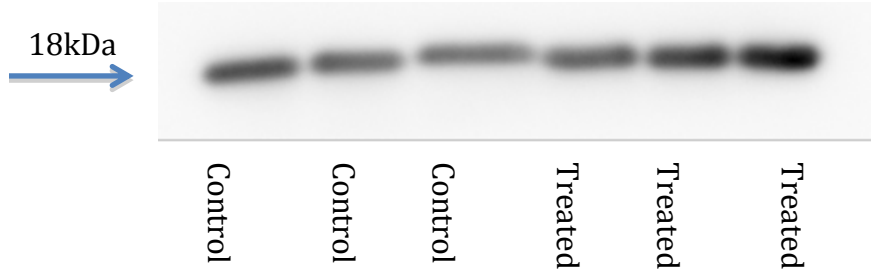
Figure 15. **BAECs treated with anthocyanins had no significant change in cofilin concentration compared to control cells.** Western blot analysis was performed and gray scale values were interpreted using Fiji/ImageJ imaging software. Graph shows gray scale values measuring the strength of the signal for each sample group. n=3 for both control and treated groups. Average gray scale value for control cell bands was 85,219.17, and the average gray scale value for treated cell bands was 98,251.12. BAECs treated with anthocyanins did not have a significant change in cofilin concentration (p=0.1678). Significance was established if p value was less than 0.05. The bars represent standard deviation. Cofilin is observed on the SDS PAGE gel at 18kDa.

Average gray value for paxillin in control BAECs was 40,156.694, and the

average gray scale value for treated cell bands was 37,980.63 (Figure 16). This was a

difference of 2,176.064. The difference between average gray scale values for control and

treated cells was not significant (p=0.4291). Paxillin is seen on SDS-PAGE at 68kDa.

Western blot analysis of paxillin was performed once. Figure 16 is an image of this blot

and a graph representing the average gray scale values of the control and treated cells.
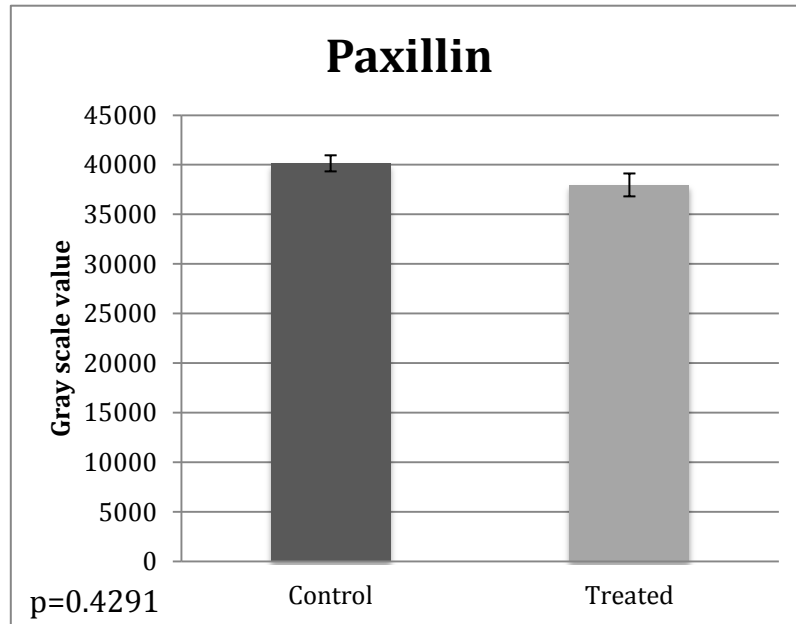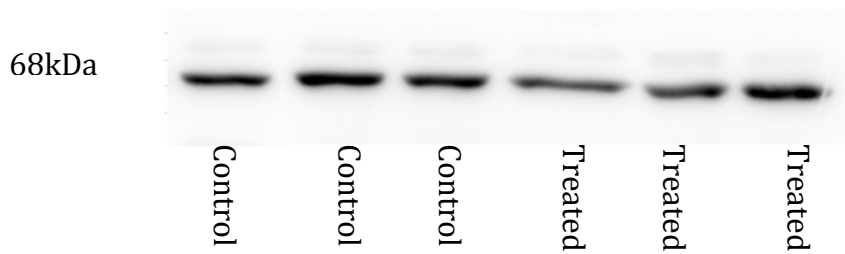
Figure 16. **BAECs treated with anthocyanins did not have a significant change in paxillin concentration compared to control cells.** Western blot analysis was performed and gray scale values were interpreted using Fiji/ImageJ imaging software. Graph shows gray scale values measuring the strength of the signal for each sample group. n=3 for both control and treated groups. The average gray scale value for control cell bands was 40,156.694, and the average gray scale value for treated bands was 37,980.63. Difference in average gray scale value was not significant between control and treated cells (p=0.4291). Significance was established if p value was less than 0.05. The bars represent standard deviation. Paxillin is observed on the SDS PAGE gel at 68kDa.

Chapter 4: Discussion

The American Heart Association reported that approximately 85.6 million adults in the U.S. have at least one type of cardiovascular disease (Mozaffarian et al., 2014). In addition, the increase in child obesity worldwide over the past three decades has contributed an increase risk for cardiovascular disease. Between the periods of 1990 and 2020, deaths from cardiovascular disease is predicted to increase from 28.9% to 36.3% worldwide (Hennekens, 1998). Low-grade inflammation is typically one of the first steps in development of atherosclerosis, the build up of plaque in blood vessels. Inevitably this condition can lead to much more serious diseases like coronary heart disease and carotid artery disease that can lead to a stroke, heart attack, and sometimes death (Kuntz et al., 2015). Anthocyanins, phenolic compounds in blueberry extracts, have been shown to reduce the conditions of low-grade inflammation and to improve endothelial cell function. A healthy endothelium is one that can actively react to stimuli during vasodilation, wound healing, and angiogenesis (Deanfield et al., 2007). These functions are reliant on the cells ability to migrate. These studies have shown that treatment with anthocyanins has increased filopodia formation in bovine aortic endothelial cells (BAECs), which could be related to the cells ability to migrate. The molecular signaling pathway that anthocyanins utilize to regulate cellular function has not been deduced. This present study is a step in the direction of better understanding of the mechanisms of anthocyanins that allow for its positive affects on the heart.

4.1 Differential Actin Morphologies and Paxillin Localization in BAECs Treated with

Anthocyanins Related to Increased Filopoda Formation

Specific cellular morphologies can be linked to migration. In particular lamellipodia and filopodia, which are protrusions of the cell membrane that allow for the cell to pull itself forward during the act of migration. Filopodia are important actin based structures that stretch out as long protrusions from the cell's membrane. They allow the cell to probe their physical and chemical surroundings (Hu et al., 2014). BAECs treated with 20 µg/mL anthocyanins for 3 hours had the greatest increase in perimeter compared to control cells, a 43.5% increase in perimeter and corresponding increased filopodia formation (Figure 5 and Figure 4, B1, respectively). Cells treated with anthocyanins for 3 hours had increased stress fiber formation as well as increased concentration of paxillin that was localized to the either end of these stress fibers (Figure 8). Filopodia formation has been demonstrated in previous studies to be regulated by Cdc42 (Cell division control protein 42), a protein that is a member of the rho family of small-GTPases (Chan et al., 2013). NO (Nitric oxide) has been linked to the activation and increased expression of small-GTPases including Cdc42 (Zhan et al., 2015). Previous studies have observed increased eNOS (endothelial nitric oxide synthase) production in endothelial cells treated with polyphenolic compounds, which has also led to increased NO synthesis (Banaszewski, 2012). These studies suggest that BAECs treated with anthocyanins may have had increased NO synthesis which lead to the activation of Cdc42 and ultimately increased filopodia formation. This increase in filopodia formation then translated into a significant increase in average perimeter, which was shown in the graph of average perimeter of BAECs over time (Figure 6). Based on the western blot analysis it can be

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suggested that anthocyanins may not work through the LIMK/cofilin pathway. There was

no significant change in p-cofilin (inactivated) or cofilin (activated) in cells treated with

anthocyanins, seen in Figure 14 and Figure 15, respectively. Although, based on my

results, cofilin activity may not be affected by treatment with 20μg/mL of anthocyanins

for 3 hours, filamentous actin structures were affected by this treatment based on the

increase in filopodia and stress fiber formation in treated cells (Figure 4 and Figure 8,

respectively). This could be due to the complex signaling pathways that are involved in

regulating actin dynamics.

In BAECs treated with anthocyanins for 3 hours, paxillin was

concentrated in distinct areas compared to control cells that had paxillin diffused

throughout the cell (Figure 8). Studies have postulated that typically where focal

adhesions are established, the cell will develop lamellipodia and will consequently move

in the direction of the adhesions. In cases where the filopodia do not establish stable

adhesions with the extracellular matrix, the filopodia structure then moves along the cell

edge and combines with other filopodia that have established stable adhesions (Hu et al.,

2014). In this case the BAECs treated with anthocyanins for 2 and 3 hours had filopodia

that appeared to establish strong adhesions in all directions of the cell. Increased focal

adhesion complexes could mean that the cells were not able to establish directionality and

possibly retained their filopodia formation longer than a control cell because of the lack

of directionality. The western blot analysis of paxillin (Figure 16) did not show a

significant difference in paxillin concentrations between control cells and cells treated

with anthocyanins for 3 hours. Western blot analysis does not give any information about

localization of focal adhesion complexes but instead gives the concentrations. Focal

adhesion turnover is an important part of establishing directionality and allowing for migration. As the cell migrates the focal adhesion complexes will form and disassemble synchronously. This process is regulated by a complicated signaling pathway that involves numerous signaling molecules and is not fully understood. Studies indicate however that a protein call p21-activated kinase (PAK) may promote the disassembly of focal adhesion complexes and therefore enable turnover. Cells that express kinase-deficient PAK (unable to phosphorylate other molecules in a signal cascade) have more stable focal adhesion complexes. Cells that express constituently active PAK display increased depolymerization of focal adhesions and actin stress fibers (Zhoa et al., 2000). PAK may be activated by Cdc42, which potentially causes a conformational change in PAK allowing for auto-phosphorylation. PAK is thought to be recruited to focal adhesion complexes by multiple proteins, however the mechanism of how it interacts with paxillin and focal adhesions complexes is somewhat unclear (Brown et al., 2002). PAK inactivation is a possible cellular molecular pathway that would lead to increased strength of focal adhesion complexes observed in Figure 8B2. Studies have observed that over expression of either Rac or Cdc42 in cells that are depleted of Rho have increased formation of focal adhesion complexes. This links actin dynamics and focal adhesion formation and could be a potential mechanism of action of anthocyanins since concentrated areas of paxillin were observed in BAECs treated for 3 hours (Hu at al., 2014).

4.2 Differential Cell Area in BAECs Treated with Anthocyanins for 24 Hours is related to Strength of Focal Adhesions

Studies suggest that the formation of lamellipodia relates to the strength of the filopodia shaft adhesions. The shaft adhesions in the leading edge of the cell will increase in length and induce the formation of the lamellipodium. The force of the lamellipodium growing and pulling the cell forward at the leading edge will then over power the filopodia growing at the tail end of the cell. As the lamellipodium advances the cell adhesion contacts continue to grow in width and strengthen (Hu at al., 2014). As the lamellipodia advance the cell spreads, this contributes to increased cell area. BAECs treated with anthocyanins for 2 and 3 hours had increased filopodia formation extending from the cell in many different directions. BAECs treated with anthocyanins at the 3 hour time period exhibited concentrated regions of focal adhesion complexes. This could contribute to decreased lamellipodia formation, and decreased cell spreading. Figure 5 shows a graph of average areas of BAECs. The area of the control cells was about 13.8% larger than the average treated cell, and had a 39.2% greater increase from the 3 hour time period to the 24 hour time period compared to the treated cells. This is potentially a result of decreased lamellipodia formation as a result of increased filopodia formation and increased strength of shaft adhesion complexes. Overall, the signal cascade pathways that induce formation of filopodia and lamellipodia are incredibly complex, and are affected by a multitude of signaling molecules.

4.3 Inability of BAECs Treated with Anthocyanins to Form a Cleavage Furrow

Resulted in Multinucleated Cells and Apoptosis

Cytokinesis is the last step in the process of cellular replication and involves the actual splitting of the cell to create two daughter cells. This process is driven by a network of actin and non-muscle myosin II, which creates what is known as the contractile ring or the cleavage furrow. This actomyosin network is what contracts and allows the invagination and dividing of the two daughter cells (Lancaster and Baum, 2014). RhoA is a key player in triggering the assembly of the actomyosin network. RhoA is localized to the midline of the cell at the onset of anaphase. Disruption of localization of RhoA also corresponded with inhibition of membrane invagination during cytokinesis (Chircop, 2014). RhoA is a small GTPase that works through other signaling proteins, activating the protein ROCK, which ultimately activates LIMK and phosphorylates cofilin contributing to increased stress fiber formation (Lamalice et al., 2007). Knowing that there are many other signaling molecules that contribute to cleavage furrow formation, and that prominent stress fibers were observed in BAECs that were treated with anthocyanins for 3 hours (Figure 8), this would imply that RhoA activation was not ibhibited in anthocyanin treated cells.

Studies have also linked Rac1, another small GTPase, with the process of cytokinesis. The inactivation of Rac1 at the center of the cell has been linked with the formation of the cleavage furrow. Inactivation of Rac1 has been postulated to allow for the localization of RhoA and actin filaments promoting invagination of the plasma membrane to separate the daughter nuclei. Constitutively active Rac1 in RatA and HeLa cell lines has lead to an inability of the cell to perform cytokinesis and subsequently lead

to multinucleation (Chircop, 2014). Activation of Rac1 also leads to activation of phosphatidylinositol 3-kinase (PI3K) which activates phosphoinositol bisphosphate (PIP$_2$). Phophorylation of PIP$_2$ results in uncapping of actin, and formation of lamellipodia (Lamalice et al., 2007). BAECs treated with anthocyanins for 2 and 3 hours were observed to have decreased lamellipodia formation (Figure 4). Many molecules can act as effectors to stimulate this pathways. Therefore, although it is difficult to pinpoint the exact pathway, knowing that Rac1 regulates both cytokinesis and lamellipodia formation, and both were affected in these experiments, Rac1 activation could be affected as a result of anthocyanin treatment.

Apoptosis, programmed cell death, is regulated by various signaling cascades, and can be characterized by particular nuclear morphologies. In particular condensation of the nuclei, or pyknosis, followed by fragmentation of the nucleus, or karyorrhexis (Eidet et al., 2014). BAECs treated with anthocyanins for 3 hours had increased condensation of their nuclei, resulting in abnormally shaped nuclei, and an increased number of cells with fragmented nuclei (Figure 10). Apoptosis can be triggered by stress or cell damage by both external and internal stimuli. Procaspases exist in the cell as inactive forms of the proteases (an enzyme that breaks apart proteins) called caspases, and when activated lead to an irreversible self-amplifying cascade ultimately leading to nuclear breakdown and cell death (Cho and Choi, 2002). This cascade is regulated by various adaptor proteins, which initiate the cascade, and other proteins that inhibit the process. Extracellular signals, which act on cell surface receptors, called tumor necrosis factor receptor 1 (TNFR1) and the Fas receptor. When these are activated by extracellular signals this leads to the recruitment of adaptor proteins that aggregate

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procaspases and induce cleavage and activation of the caspase signal cascade (Cho and Choi, 2002). The mitochondria, an organelle involved in cellular respiration, is another site of induction of apoptosis. In response to apoptotic signals, such as cellular damage, the mitochondrion will release a protein embedded in its membrane called cytochrome c, which will bind to an adaptor protein called Apaf-1 (apoptotic protease activating factor 1), leading to aggregation of procaspases, and therefore activation of the caspase signal cascade (Cho and Choi, 2002). Specific anthocyanins (cyanidin and peonidin) from black rice have been observed to induce apoptosis in breast cancer HS578T cells, which has corresponded with increased caspase-3 activation, one of the caspase enzymes involved in the caspase signal cascade (Chen et al., 2005). In a separate study HT-29 colon cancer cells treated with blueberry extracts had a 1.8 fold increase in apoptosis, which was determined based on immunoassays measuring the amount of DNA degradation in the cells (Seeram et al., 2006). Although cancer cells were not used in these studies it could be likely that the concentration of anthocyanins used were of a high enough concentration that they induced an apoptotic stress response. Multinucleation due to the inability to form a cleavage furrow could potentially lead to an intracellular response and induction of the caspase signal cascade, which would induce nuclei fragmentation and apoptosis.

4.4 Concluding Remarks

Further experiments should be performed to confirm the findings that 20μg/mL anthocyanins from wild blueberry extracts induced increased filopodia and stress fiber formation, and apoptosis. Future experiments should include knockdowns or overexpression of small GTPases such as RhoA, Cdc42, and Rac1, to determine if anthocyanins induce cellular responses through activation or inactivation of these

proteins. Varying concentrations of anthocyanins would also be important to study and used in cytotoxic assays to test at what concentrations anthocyanins become cytotoxic. Peak plasma concentrations for anthocyanins from other berry sources were closer to the 100ths of µg/mL and smaller, so the high concentration may have induced cellular responses that would not be likely in a biological system (McGhie and Walton, 2007). Studies addressing both the cytotoxic and the anti-inflammatory effects of anthocyanins should be continued to increase understanding and potentially allow for new treatments of cardiac diseases and cancer in the future. Increasing studies in vivo have observed positive affects, however cellular mechanisms must also be a focus of future research to ensure safety and effectiveness for any potential anthocyanin supplement or pharmaceutical.

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Biography of Author

Katrina E. Ventura was born in Portland, Maine on September 13th, 1994. She was raised in Falmouth, Maine and left to attend the University of Maine in the fall of 2012. She had a passion for nature and the biological sciences at a young age. She thoroughly enjoys hiking, skiing, and birding among many other outdoor activities. Katrina will be receiving a B.S. in Biology. She is a member of the National Biological Honors society and a president of the Student Chapter of Water for ME. She has received an Honors Thesis Fellowship, a Charlie Slavin Research Fund Grant, and recently attended the 2016 CUGR Student Research Symposium.

Upon graduation Katrina will be taking a year off to realize her passions and will be working at a local alpaca farm. Katrina hopes to gain some experience working with both small and large animals before applying to veterinary school. Her future goals are to become a large animal veterinarian working in rural areas and traveling to large livestock farms.