Finding a Link Between the TRPV4 Ion Channel and Angiogenesis: A potential Therapeutic Target for Ischemic Heart Disease

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A Thesis

in

The Department

of

Exercise Science

Presented in Fulfillment of the Requirements

For the Degree of

Master of Science (Exercise Science) at

Concordia University

Montreal, Quebec, Canada

April 2016

CONCORDIA UNIVERSITY
School of Graduate Studies

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Abstract

Finding a Link Between the TRPV4 Ion Channel and Angiogenesis: A potential Therapeutic Target for Ischemic Heart Disease

Vanessa Salucci

Angiogenesis is the physiological process of creating new blood vessels. It is the body's natural healing response to restore blood flow to damaged tissue. For people dealing with chronic diseases such as ischemic heart disease, the angiogenic response can be compromised. Calcium ions play an important role in several cellular processes, including cell proliferation. Transient Receptor Potential cation channel subfamily Vanilloid member 4 (TRPV4) is moderately permeable to Ca^{2+} and can be found in endothelial cells, which make up the inner lining of blood vessels. This study investigates the link between the TRPV4 channel and angiogenesis using an Aortic Ring Assay (ARA) on a mouse model. By exposing the rings to specific ion channel agonist (GSK1016790) and antagonist (HC067047), measurements of length and number of new blood vessel outgrowth were taken. Significant findings from this study can provide pertinent information on TRPV4 playing an important role as a mediator for intracellular signaling and can potentially be a therapeutic target for vascular remodeling to improve cardiac function and increase perfusion to ischemic areas.

Acknowledgements

I would like to extend my gratitude to my supervisor Dr. Andreas Bergdahl, for all his support and guidance. I am truly grateful for his guidance throughout my entire graduate degree.

I would also like to thank the other members of my thesis committee, Dr. Robert Kilgour and Dr. Nicolas Noiseux, for great feedback and contributions to my project.

Thank you to Aileen Murray and her team at the Concordia University Animal Care Facility for the housekeeping and caregiving they provided throughout this project. A special thank you to Jean-François Trudel for all of his help.

Last but certainly not least, thank you to my family and friends for their constant support throughout this entire process.

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Introduction

Theoretical Context

Angiogenesis

Angiogenesis is the process of creating new blood vessels (Nicosia, 2009). It is a normal and critical process for the growth and development of the body. Vessels are responsible for the transfer of oxygenated blood and nutrients to the cells and the removal of metabolic waste and carbon dioxide from the cells (Vriens et al., 2004). There are two primary mechanisms by which angiogenesis occurs. The first is sprouting, which is the mechanism of interest for the purpose of our research. The second is intussusception, also known as splitting angiogenesis; this method uses an existing blood vessel that will eventually split into two new vessels (Ribatti et al., 2012; Vriens et al., 2004).

Sprouting occurs in many well-defined phases. As depicted in figure 1, endothelial cells need to break through the basement membrane of a preexisting vessel, proliferate, migrate and come together to form a new blood vessel (Spin et al., 2004; Vriens et al., 2004). For this to begin, biological signals, call upon angiogenic growth factors that need to activate receptors on the endothelial cells found in preexisting blood vessels. Pro-angiogenic factors are produced while anti-angiogenic factors are decreased by mechanisms such as high blood glucose, shear force, hypoxia or metabolic stress (Vriens et al., 2004; Zent and Pozzi, 2007). The combination of these factors is what initiates angiogenesis.

Once the cells are activated, they release enzymes called proteases; their purpose is to degrade the basement membrane to allow endothelial cells to escape their vessel of origin (Ribatti et al., 2012). The breakdown causes perivascular cells (pericytes and smooth muscle cells) to detach from escaped endothelial cells (Thibeault et al., 2008). When the cells are released, they proliferate into the surrounding matrix and form sprouts. While extending toward the source of the stimulus, the cells migrate collectively using adhesion molecules called integrins. As they mature, the sprouts eventually form a loop to create the vessel lumen (Spin et al., 2004). Once the structures are created, they recruit perivascular cells for support and stabilization.

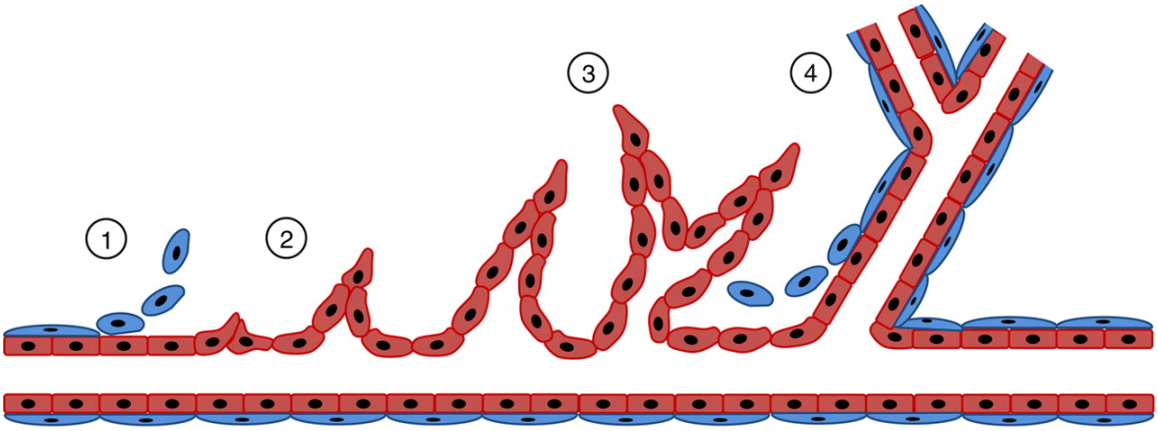

Figure 1: **Stages of Angiogenesis** The red cells represent endothelial cells while the blue ones are perivascular cells. In the early stages, there is a degradation of the basement membrane (1). The endothelial cells then begin to migrate and begin to form sprouts (2). They then further elongate via endothelial cell proliferation and branch out (3). Lastly the mature sprouts recruit the perivascular cells to support and stabilize the vessel (Laschke et al., 2011).

Angiogenesis as a therapeutic target

Angiogenesis is controlled by the production of a precise balance of growth and inhibitory factors in healthy tissue. This process is the body's natural healing response to restore blood flow to the damaged tissue. To initiate activity, various chemical signals such as growth factors, inflammatory and immune proteins are released into the tissue in need and are responsible for stimulating angiogenesis. When this balance is disturbed, the result is either too much or too little angiogenesis, which can lead to important and sometimes fatal conditions (Masson et al., 2002). For people dealing with chronic diseases, the angiogenic response can be compromised for different reasons. Diabetes for example, can cause many secondary conditions such as foot ulcers and nerve damage; they are the result of insufficient angiogenesis (Vriens et al., 2004). Angiogenesis may be a target for therapies dealing with such diseases. Applying specific agents that inhibit or stimulate the creation of new blood vessels in the body may assist in the prevention or control of these diseases.

The absence of blood vessels in a metabolically active tissue may inhibit essential functions. Many diseases, such as ischemic heart disease (IHD) or coronary heart disease, are the result of a lack of sufficient blood vessel formation and thus an insufficient blood supply to the area that can lead to tissue death (Stegmann et al., 1998; Stegmann et al., 2000; Folkman, 1998).

Pro-angiogenic therapies are being investigated as an option to treat and prevent cardiovascular disease, the primary cause of death in the world (World Health Organization, 2013). These studies include using fibroblast growth factor 1 (FGF-1) for the treatment of coronary heart disease (Stegmann et al., 1998; Stegmann et al., 2000; Folkman, 1998). Also, many studies have looked at vascular endothelial growth factor (VEGF) (Nicosia, 2009; Ribatti et al., 2012).

Angiogenesis is an excellent potential therapeutic target for the treatment of cardiovascular disease. It is an effective, physiological process that triggers the natural way our bodies respond to a decreased blood supply to a vital organ. It may even help regenerate damaged tissue in ways that were previously considered impossible (Spin et al., 2004; Woudenberg-Vrenken et al., 2009).

Transient Receptor Potential (TRP)

The transient receptor potential (TRP) superfamily consists of numerous cation channels that are expressed in the majority of tissues and cell types. They can be found in the neuronal, renal and smooth muscle tissue among others, thus playing an important role in the regulation of these different cells. There are 28 mammalian TRP channels that can be divided into six subfamilies: the TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystic), TRPML (mucolipin) and TRPA (ankyrin) groups (Dietrich et al., 2006; Nilius et al., 2007).

All TRP channels are composed of 6 transmembrane domains, which present themselves in tetramers to form the pore responsible for cation entry into the cell. In these structures, it is the fifth and sixth transmembrane domains from each unit that form this pore and the sixth domain actually controls the intracellular gating, as seen in figure 3 (Clapham, 2003; Dietrich et al., 2006; Nilius et al., 2013). Once the opening is formed, it allows cations such as Ca^{2+} into the cell, which in this case plays an important role in cellular homeostasis and proliferation.

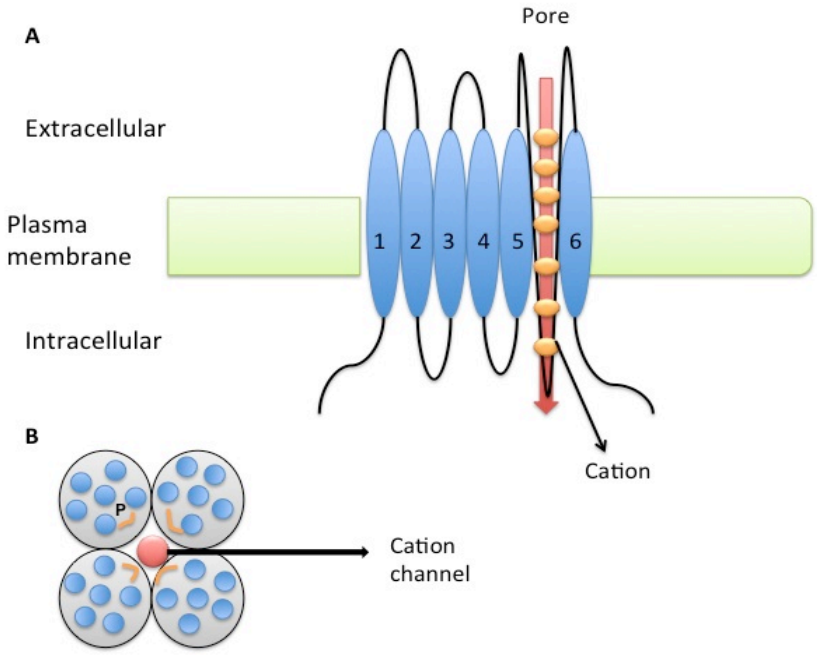

Figure 2: TRP Cation Channel Structure A schematic depiction of TRP cation channel structure to show all 6 transmembrane domains (A), and in tetrameric formation to show the cation pore (B) (Adapted from Berridge et al., 2000).

Most TRP channels are permeable to Ca^{2+}, however other ions such as Na^{+} undergo cellular influx as well (Berridge et al., 2000; Christensen and Correy, 2007). The majority of these channels are poorly selective for calcium ions, with permeability ratio (P_{Ca}/P_{Na}) in the range of 0.3 to 10. TRP channels are gated by different stimuli that include intracellular and extracellular messenger binding, temperature changes, chemical or mechanical stress (Nilius et al., 2007).

Several of these channels are involved in the control of cell proliferation and maturation. Alterations in this function may lead to growth disruptions. Disturbances in physiological functions controlled by ion channels can contribute to the start of certain diseases. Changes in the number of channels activated or channel sensitization will result in exaggerated or decreased responses to stimuli. Many members of the TRP superfamily are prone to being targets for these factors. They are mostly polymodal meaning they are activated by many different types of gating stimuli and act like mediators for internal and/or external signals. Abnormal regulation of these

ion channel functions can be linked to many forms of inflammation and systemic diseases, such as neurodegenerative, cardiovascular and respiratory diseases (Nilius et al., 2007).

Unfortunately, the mechanisms of TRP channel functions are not completely understood yet. Many mechanisms have been suggested for the dysregulation of TRP channel function leading to disease. One of the mechanisms states that, since most TRP channels play a role in Ca^{2+} signaling, changes in signaling due to disrupted TRP channel function can have severe effects on many cellular and systemic processes (Nilius et al., 2007).

Transient Receptor Potential Cation Channel, Vanilloid subfamily Member 4 (TRPV4)

The TRPV (vanilloid) subfamily is comprised of six members (TRPV1-6). TRPV1-TRPV4 channels are all heat-activated channels that are nonselective for cations and modestly permeable to Ca^{2+} (Vriens, 2004). The channel of interest for the purpose of our research is the non-selective cation channel, TRPV4. It is an important factor in many physical and chemical stimuli and is involved in multiple physiological functions (Verma et al., 2010). This specific channel is activated in the presence of cell swelling, moderate heat, shear stress and many protein kinase enzymes (Nilius et al., 2007; Plant et al., 2007). Although it allows both calcium and sodium cations into the cell, it has a P_{Ca}/P_{Na} equal to 6. This means that it is six times more selective to Ca^{2+} than Na^{+} (Christensen et al., 2007; Clapham, 2003; Dietrich et al., 2006).

There are other ways to activate TRPV4 such as agonistic compounds (Berridge et al., 2000; Christensen et al., 2007; Nilius et al., 2007; Swapnil et al., 2012). Among these activating compounds, GSK1016790A is considered one of the strongest and most specific agonists (Jin et al., 2011; Rerup et al., 1970; Schnedl et al., 1994).

Calcium signaling and endothelial cell proliferation

Calcium ions play an important role in several cellular processes, which include muscle contraction, cell proliferation, gene transcription and cell death (Berridge et al., 2000). Under normal circumstances, proliferation of capillary endothelial cells is particularly limited within an adult, taking into consideration their 1000-day half-life (Engerman et al., 1967). However proliferation is triggered if the cells are exposed to angiogenic stimuli (Haas et al., 2012).

Resting Ca^{2+} concentration in the cytoplasm can be found anywhere between 10 and 100nM. To maintain this constant concentration, Ca^{2+} is pumped into the extracellular area and in the endoplasmic reticulum (ER) (Clapham, 2007). TRP channels contribute to changes in the cytosolic free-Ca^{2+} concentration. They do so by either acting as calcium ion entry pathways or via changes in membrane polarization, ultimately modifying the driving force for calcium ion entry mediated by alternative pathways (Garcia-Elias et al., 2008; Nilius et al., 2007; Strotmann et al., 2003). This means that intracellular Ca^{2+} increases in endothelial cells due to Ca^{2+} influx, but can also arise from Ca^{2+} release from internal calcium ion stores such as the Golgi apparatus, the endoplasmic reticulum (ER), or in the case of muscle cells, the sarcoplasmic reticulum (SR) (Nilius et al., 2007). In the case of members of the TRP family, concentrations of intracellular Ca^{2+} are increased by influx and by release from intra-cellular stores in the ER (Swapnil et al., 2012).

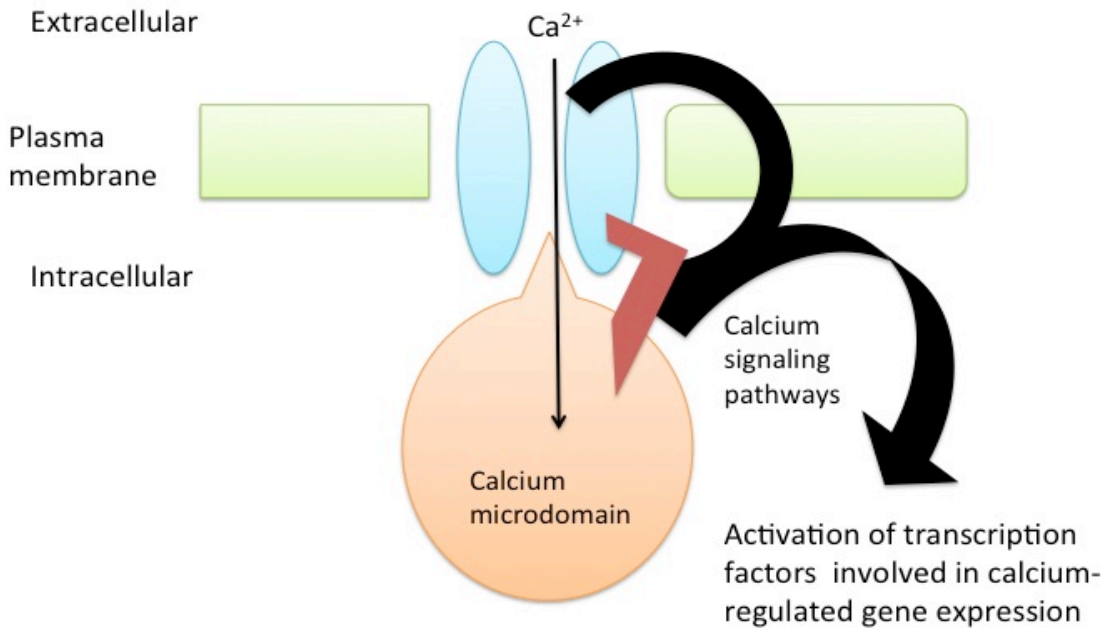

Figure 2: **Cellular Ca^{2+} Signaling** Specific channels mediate Ca^{2+} signaling through certain pathways. These pathways are activated by the entry of calcium ions and control gene transcription (Adapted from Senatore et al., 2009)

Inositol triphosphate (IP_3) and Calmodulin (CaM) are two proteins that have been found to interact with TRPV4, serving as messengers (Verma et al., 2010). In figure 2, we can see that CaM is bound to the channel (represented in red) and is activated by the Ca^{2+} influx. The ions then travel across the plasma membrane and form intracellular Ca^{2+} stores. The accumulation of calcium ions in the cytosol is quickly buffered into microdomains: calcium reservoirs, which can be found close to the opening of the channels (Senatore et al., 2009). IP_3 on the other hand, will bind to ligand-gated Ca^{2+} channels found on the surface of the ER and trigger the release of Ca^{2+} into the cytoplasm, increasing the intracellular Ca^{2+} concentration (Clapham, 2007). These mechanisms are what trigger transcriptional factors through various pathways, which bring on the start of endothelial cell proliferation.

Aortic Ring Assay

The study of angiogenesis as a therapeutic target is highly present in the literature. Scientists are using various methods to elicit and control the growth of the new blood vessels and determine the mechanisms involving angiogenic growth. Few however, have addressed the link between TRPV4 channel and angiogenesis. In an attempt to find the potential link between these two, we will be employing the aortic ring assay (ARA) from a mouse model. Originally based on the rat aorta, this method has been used to study angiogenic growth since its initial development in 1990, by Nicosia and Ottinetti (Nicosia and Ottinetti, 1990). Since then, it has become one of the leading assays for angiogenesis simulation (Aplin et al., 2008; Masson et al., 2002; Nicosia, 2009).

The ARA is an organ culture model where vessels grow from a segment of an aorta. In this kind of assay, the thoracic aorta is removed from the mouse, the adventitia is cleared away and the segment is cut into approximately 1mm rings, which are then embedded into a collagen gel (Nicosia and Ottinetti, 1990). The rings are then ready to be cultured in a serum-free medium. In our case, we will also be adding the specific ion channel agonist (GSK1016790A) and antagonist (HC067047) to certain rings. The angiogenic growth can be seen quite rapidly, after 2-3 days of culture (Nicosia, 2009), through microscopy (Goodwin, 2007).

The vessels that grow from the aortic rings are anatomically similar to new agiogenic growth that can be observed in vivo, meaning that they recruit smooth muscle cells and pericytes to come together with the endothelial cell tube (Nicosia et al., 1992; Nicosia and Villaschi,

1995). There are many limitations, however, to this assay. The handling of the rings and the removal of adventitia can greatly vary depending of the laboratory technician and can influence vessel growth. The use of different aortas from different mice, different ages and strain can lead to variability when measuring angiogenic growth (Zhu et al., 2003). Also, vessel outgrowth normally occurs in three dimensions, this can complicate photography and quantification of the sprouts. Neovessels usually grow from microvessels such as capillaries, not from the aorta, which is a major vessel. However, for the purpose of our study, the aorta will be the vessel of choice. It is the only artery large enough to use from a mouse approximately 4 days old. Another limitation would be the fact that when compared with in vivo models, an ARA lacks blood flow. This drawback is shared with other methods such as in vitro and ex vivo models of angiogenesis. As a consequence, the ARA model lacks haemodynamic factors and mechanochemical forces that therefore cannot be studied in this particular environment (Shiu et al., 2005). When all factors are taken into consideration, with enough internal control and with multiple repeats, this assay is fairly simple, inexpensive, reproducible, greatly correlates with in vivo studies, highly informative and significantly superior when compared to endothelial cultures in terms of its relevance and biological complexity (Bellacen and Lewis, 2009; Nicosia, 2009).

Objectives and Rationale

Objective 1

By applying the aortic ring assay method, we determined the effect and degree that the specific ion channel agonist (GSK10161790A) has on TRPV4, when it comes to lengthening and multiplying new sprouts during angiogenesis.

Objective 2

By applying the aortic ring assay method, we determined the effect and degree that the specific ion channel antagonist (HC067047) has on TRPV4, when it comes to inhibiting angiogenesis.

Rationale

Insufficient amounts of blood vessels contribute to the start of many diseases. Stimulating angiogenesis can improve and increase blood flow in patients with ischemic diseases (Goodwin,

2007). Pro-angiogenic agents have been used to treat diseases like peripheral vascular disease and coronary artery disease, however many of their mechanisms are unknown (Khan et al., 2003; Zhou et al., 2007).

The influx of calcium ions via the TRPV4 ion channel is important for cellular homeostasis seeing as how it maintains basal Ca^{2+} concentrations and allows Ca^{2+} dependent events to occur, such as signaling (Clapham et al., 2003; Dietrich et al., 2006; Nilius and Owsianik, 2010). Since TRPV4 can be found in many different types of tissue such as endothelial cells, smooth muscle cells, the bladder, the brain, the kidney, neurons and cartilage (Dietrich et al., 2006; Nilius et al., 2007), maintaining homeostasis through the TRPV4 ion channel may have a great impact.

Exposing the surface of endothelial cells to the selective TRPV4 channel agonist GSK1016790A (GSK; 10nM) prompted an increase in Ca^{2+} signals [30.4 ±2.5 (SEM) –fold, n=5], creating an increase in calcium ion influx and stimulated intracellular receptor activity responsible for calcium signaling (Swapnil et al., 2012). It is 300 fold more potent than 4α-PDD, a strong TRPV4 agonist. As for HC067047, it is a potent and highly selective TRPV4 antagonist (Sigma-Aldrich, 2014). It has been shown to reduce the number of active sites, as well as the activity per site (Swapnil et al., 2012). In our study, we will be using the TRPV4 channel to determine if its effect on cellular homeostasis and proliferation will influence angiogenesis.

Hypotheses

- We hypothesized that when the TRPV4 agonist, GSK10161790A, and growth media are introduced to an aortic ring of a mouse while performing an ARA, the ion channel will increase angiogenic activity by increasing the length and number of sprouts when compared to an aortic ring that is introduced solely to growth medium.

- We hypothesized that when the TRPV4 antagonist, HC067047, and growth media are introduced to an aortic ring of a mouse while performing an ARA, the ion channel will decrease angiogenic activity by decreasing the length and number of sprouts when compared to an aortic ring that is introduced solely to growth medium.

II

Effect of TRPV4 Ion Channel on Angiogenesis Using an Aortic Ring Assay

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Contribution of authors

Vanessa Salucci: Animal handling, surgical extraction of aorta, aortic ring assay, statistical analysis, and preparation of manuscript.

Andreas Bergdahl: Concept development, euthanized animals, preparation of manuscript, provided animals, laboratory space and equipment.

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Abstract

Angiogenesis is the physiological process of creating new blood vessels. It is the body's natural healing response to restore blood flow to damaged tissue. In the case of ischemic heart disease, the angiogenic response can be compromised. Calcium ions play an important role in several cellular processes, including cell proliferation and gene transcription. Transient Receptor Potential cation channel subfamily Vanilloid member 4 (TRPV4) is moderately permeable to Ca^{2+} and can be found in endothelial cells, which make up the inner lining of blood vessels. This study investigated the potential link between the TRPV4 channel and angiogenesis using an Aortic Ring Assay (ARA) on a mouse model. 4-day-old C57Bl/6 pups were euthanized prior to the removal of the aortas. The vessels were cleaned and cut into ±1mm wide rings and were kept viable in a collagen growth matrix. By exposing the rings to specific ion channel agonist (GSK1016790) and antagonist (HC067047), we measured the length and number of new sprouts. The results revealed a significant decrease in number and length of sprouts when rings were exposed to the antagonistic compound. As well as a significant increase in sprout length when exposed to the antagonistic compound. This study can provide pertinent information on TRPV4 playing an important role as a mediator for intracellular signaling and can potentially be a therapeutic target for vascular remodeling.

Keywords: Angiogenesis, endothelial cells, TRPV4 ion channel, Aortic Ring Assay, Ischemia.

Introduction

Angiogenesis is the process of creating new blood vessels (Nicosia, 2009). It is a normal physiological function and is a critical process for the growth, development and healing process of the body. Vessels are responsible for the transfer of oxygenated blood and nutrients to the cells and the removal of metabolic waste and carbon dioxide from the cells (Vriens et al., 2004). The endothelial cells (EC), which make up the inner lining of blood vessels, are the key players in the angiogenic process. In order for sprouting to occur, ECs need to break through the basement membrane of a preexisting vessel. They then need to proliferate to provide enough cells before they can migrate and come together to form a new blood vessel (Spin et al., 2004; Vriens et al., 2004).

Calcium ions play an important role in several cellular processes, which include muscle contraction, cell proliferation and gene transcription (Berridge et al., 2000). Calcium influx through gated calcium channels activates gene transcription. The calcium signaling depends on a cascade of events, which ultimately open these calcium channels. The ions are then able to travel across the plasma membrane and form intracellular Ca^{2+} stores through specific receptors. The accumulation of calcium ions in the cytosol is quickly buffered into microdomains: calcium reservoirs. They are responsible for triggering transcriptional factors through several different pathways, which is the start of endothelial cell proliferation (Senatore et al., 2009).

Transient Receptor Potential Cation Channel, Vanilloid subfamily Member 4 (TRPV4) is modestly permeable to Ca^{2+} (Vriens, 2004). It is an important factor in many physical and chemical stimuli and is involved in multiple physiological functions (Verma et al., 2010). This specific channel is activated in the presence of cell swelling, moderate heat, shear stress and many protein kinase enzymes (Nilius et al., 2007; Plant et al., 2007). Among these activating

compounds; GSK1016790A is considered one of the strongest and most specific agonists (Jin et al., 2011; Rerup et al., 1970; Schnedl et al., 1994).

Originally based on the rat aorta, the aortic ring assay has been used to study angiogenic growth since its initial development in 1990, by Nicosia and Ottinetti and has become one of the leading assays for angiogenesis simulation (Aplin et al., 2008; Masson et al., 2002; Nicosia, 2009). The vessels that grow from the aortic rings are anatomically similar to angiogenic growth that can be observed in vivo, meaning that they recruit smooth muscle cells and pericytes to come together with the endothelial cells to for the tube structure (Nicosia et al., 1992; Nicosia and Villaschi, 1995).

In an attempt to find the potential link between TRPV4 and angiogenesis, we will be employing an aortic ring assay. We hypothesized that TRPV4 agonist GSK1016790A and antagonist HC067047 could directly affect the formation of new blood vessels created by sprouting angiogenesis. The purpose of this study is to investigate whether these two compounds would affect angiogenic growth in terms of number and length of the new microvessels.

Materials and Methods

Tissue preparation

Four-day-old C57BL/6 mice were euthanized by decapitation according to the protocol approved by the Animal Ethics Committee of Concordia University (#30000259). Before any ventral incisions, the surface was sterilized using 70% ethanol. Once the abdominal cavity was exposed, the aortic arch was located and was followed down the anterior of the spine. After separating it from the connective tissue, the aorta was placed in iced, sterile Ca^{2+}-free Krebs solution containing (in mmol/L) 122 NaCl, 15.5 $NaHCO_3$, 4.7 KCL, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 1.5

glucose, 100 U/mL penicillin, and 100Ig/mL streptomycin. Using a dissection microscope, all excess adipose tissue was carefully removed and vessels were cut into ~1mm long rings.

Matrix preparation

Gel matrices were prepared under sterile conditions in a biological safety cabinet (Lanconco Class II, type A2) by mixing 1.5ml of DMEM:F12 and 30μL rat collagen. Further mixtures were made with the TRPV4 channel agonist (GSK1016790A) and antagonist (HC067047) each having 2 concentrations of 3 and 10nM, all with 2% collagen concentration. Solutions were kept on ice to prevent early polymerization of the collagen.

DMEM/F12 contains 0.1545g/L calcium chloride and is frequently used as a base media for the development of cells. Calcium is essential for the life of all cells. It acts as an important second messenger in the majority of cellular functions (Voccoli et al., 2014). Since calcium affects cell signaling and differentiation, media developed for specific cell types may require lower calcium levels for cell viability (Huang et al., 2006).

Embedding

Mixtures were transferred to a 96-well plate using a 0.45μm syringe. The aortic rings were placed in the center of each well with the luminal axis perpendicular to the bottom to facilitate sprout visualization. The plate was then placed in an incubator (VWR Symphony) at 37 °C for about 15 – 20 minutes to accelerate the solidification of the collagen before filling the rest of the well with its respective growth media. The plate was then put back into the incubator.

Visualization

Every second day, the aortic rings were imaged using a Leica inverted microscope (Leica DMIRE2) with a 10x objective connected to a camera, for up to 12 days. To maintain consistency and allow for accurate quantification and analysis, images were taken at roughly the same focal distance. ImageJ software was used to measure maximum sprout length and total number of sprouts.

Statistical Analysis

Experiments included a sample size of 12 rings per condition and were conducted on 3 separate 96-well plates. The number and length of sprouts were determined, as well as growth ratios and number of ECs. The following statistical parameters were calculated to describe the distribution of microvessels: the mean distribution and standard deviation. Summarized data are presented as mean ± standard error (SEM) in all figures. Statistical comparisons were done using an ANOVA test. For all statistical assessments a p value of <0.05 was considered significant.

Results

In order to avoid masking the angiogenic effects of GSK1016790A and HC067047, aortic rings were deprived of all exogenous growth factors during the 12-day, sterile incubation. In figure 1A we observed that under control conditions, the aortic ring generated new blood vessels and that the density of these vessels increased over time. Fig.1B demonstrates that with the addition of 3nM of the agonist, GSK1016790A, the vessels grew at a faster rate and sprouts grew longer over time. Conversely, in Fig.1C there is an inversely proportional trend, adding 3nM of antagonist HC067047 shows a clear reduction in microvessel formation.

The number of new sprouts developing from a segment of aortic tissue is a common method for measuring angiogenic stimulation or inhibition. As seen in Fig.2, a dose-dependent response for both the agonist and antagonist can be seen. We can see that the number of sprouts increases as the agonist concentration increases as well as a decrease in number when the antagonist concentration increases. The greatest amount of microvessels can be seen at day 10 for all treatments, right before it slightly decreases at the end of the study.

The capacity in which a capillary can penetrate into surrounding tissue was also taken into consideration. The maximum length of sprouts is depicted in Fig.3. The longest microvessels can be seen in the group containing the highest concentration of the agonistic compound (10nM), an average of 1.51 ± 0.059 mm. Meanwhile, the shortest outgrowths can be seen in the most concentrated antagonist group (10nM), an average of 0.83 ± 0.05 mm. The growth ratio of each compound's concentration is depicted in Fig.4, where the control condition was set at 1. Both agonist groups showed similar trends as of day 6, both revealed at least 20% longer microvessels. Looking at the antagonist groups, the higher concentration (10nM) indicated a greater decrease in sprout length, at least 20%. In Fig.6, the logarithmic plot of the same growth ratios is depicted. This view allows seeing the exponential growth of the vessels. A visible difference can be seen between the various compounds and their different concentrations at the very beginning. Day 2 shows the largest gap in growth between the agonist and antagonists. We can hypothesize that the compounds affect the ECs at the start of the angiogenic growth.

Cell proliferation, which is the first stage of angiogenesis, was measured at the beginning of the experiment, at day 2 and 4 (Fig.5). The addition of agonistic and antagonistic compounds does not seem to significantly influence this stage of angiogenesis.

Discussion

The absence of blood vessels in a metabolically active tissue may inhibit essential functions. Many diseases, such as ischemic heart disease, are the result of a lack of sufficient blood vessel formation and thus an insufficient blood supply to the area that can lead to tissue death (Stegmann et al., 1998; Stegmann et al., 2000; Folkman, 1998). Stimulating angiogenesis can improve and increase blood flow in patients with such diseases (Goodwin, 2007). Cell proliferation is the first step of angiogenesis, in which calcium plays a large role in (Berridge et al., 2000). Intracellular Ca^{2+} increases in endothelial cells due to increased Ca^{2+} influx but can also arise from Ca^{2+} release from internal calcium ion stores such as the endoplasmic reticulum (ER)(Nilius et al., 2007, Swapnil et al., 2012). We are reporting a novel finding in that the TRPV4 ion channel can act as a mediator in intracellular signaling and can potentially be a therapeutic target for vascular remodeling.

The TRPV4 agonist, GSK1016790 is considered one of the strongest and most specific agonists (Jin et al., 2011; Rerup et al., 1970; Schnedl et al., 1994). Our results show us that the aortic rings treated with 3nM of this agonist do not show a significant increase in the number of sprouts grown when compared to the control condition. However, the group treated with 10nM demonstrates a significant difference as of day 8. This indicates that the concentration of GSK1016790 could perhaps show significance throughout the entire process at an increased concentration. As for the antagonist, HC067047, it is a potent and highly selective TRPV4 antagonist (Sigma-Aldrich, 2014). We can observe significant inversely proportionate relationship between its concentration and the number of microvessel outgrowth. This can be explained by the inhibition of Ca^{2+} influx and thus a decrease of intercellular Ca^{2+}.

The degree of capillary penetration into the surrounding tissue is as important as the number of sprout growth. In all conditions, the maximum length can be seen at day 10 right before a visible regression. The longest vessels are found in the 10nM treatment of agonist compound and all values are significant as of day 8. The regression can be explained by a cessation of growth after approximately 1 week of sprouting, branching and network formation. This occurrence can be seen especially in serum-free collagen gel cultures (Nicosia, 2009). As the new vessels are reabsorbed, the gel inside of the lumen and around the aortic ring starts to deconstruct, creating the periaortic halo seen in the Fig.1 (day 12) (Zhu et al., 2000).

We wanted to investigate what stage of angiogenesis TRPV4 actually influences. Using the images taken during the ARA, the ECs present in the images were counted to see if the ion channel plays on the proliferation stage. There is no significant difference between the numbers of cells of each treatment group which would lead to believe that TRPV4 actually influences the sprout formation phase. This assumption is based on the fact that, among the control and agonist conditions, the least amount of free ECs can be found in the highest concentration of agonist compound (10nM), the same condition with the highest number of sprouts.

Our findings indicate that TRPV4 can act as a potential mediator for intracellular signaling and can affect angiogenesis. We hypothesize that this channel can used as a potential therapeutic target for vascular remodeling to improve cardiac function and increase perfusion to ischemic areas.

Acknowledgments

We are very grateful to the Concordia University Animal Care Facility Manager Aileen Murray and her staff for the housekeeping and caregiving they provided throughout this project. Concordia University provided the funds for this project.

Fig. 1. Aortic rings under control condition (A), agonist [3nM] (B) and antagonist [3nM] (C) at day 2, 6 and 12, respectively. Arrows are pointing to examples of microvessel outgrowths.

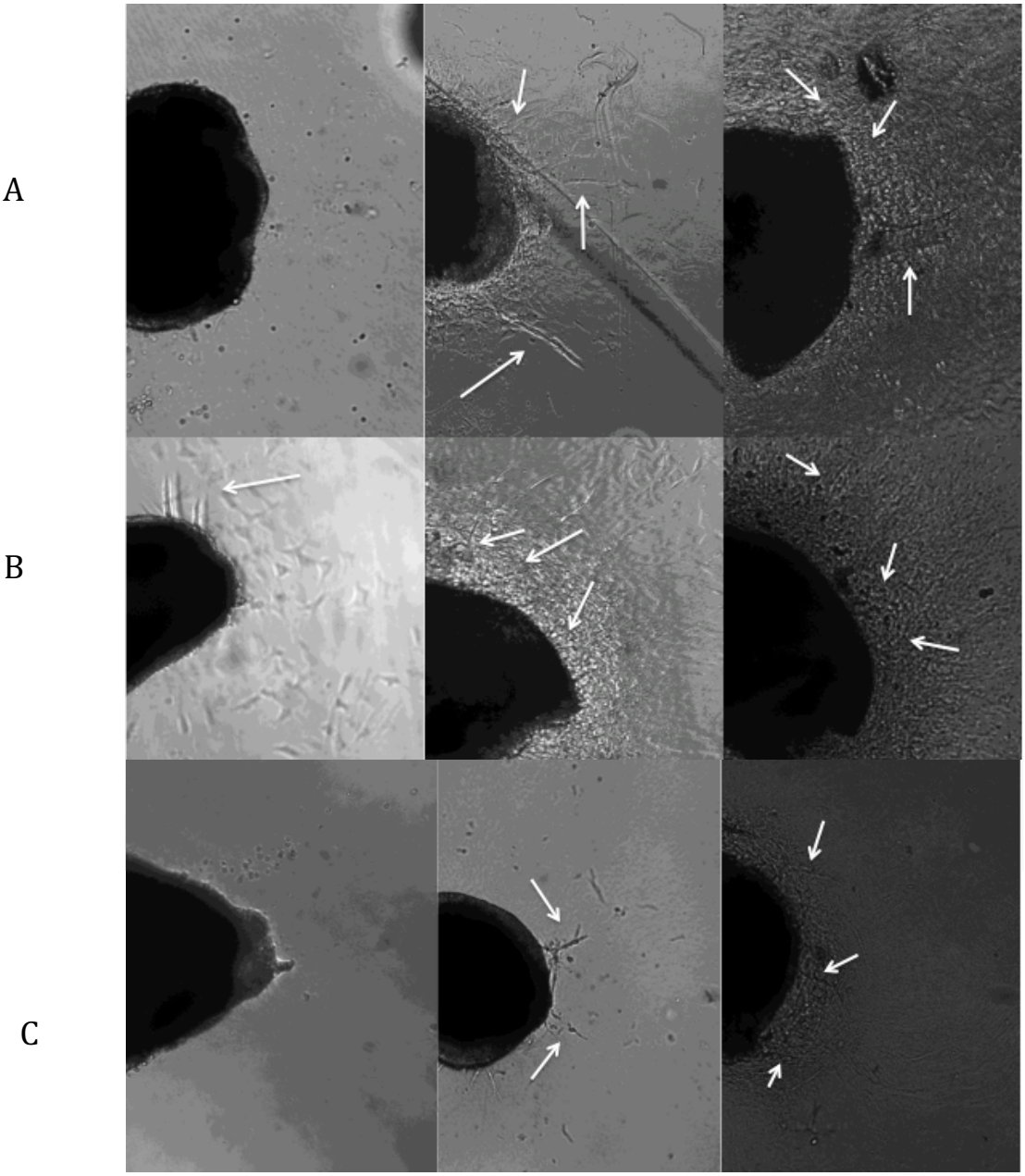


Fig.2. Number of microvessel outgrowths as a function of time for various agonist (GSK1016790A) and antagonist (HC067047) concentrations compared with control condition (means ± SE). Significant ($p<0.05$) findings in antagonist group [3nM] and [10nM] and agonist [10nM] only as of day 8 when compared to control.

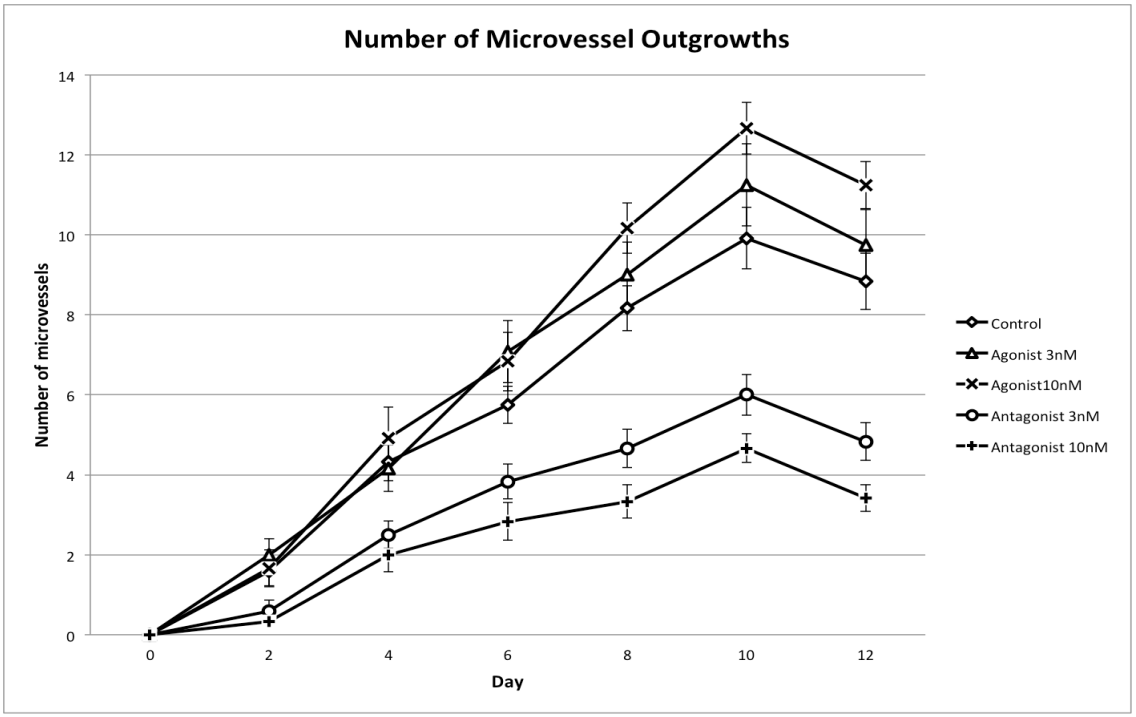


Fig.3 Mean maximum length of microvessels for each treatment condition (means ± SE), representing the capacity in which the sprouts can penetrate surrounding tissue. Findings are significant when $p<0.01$.

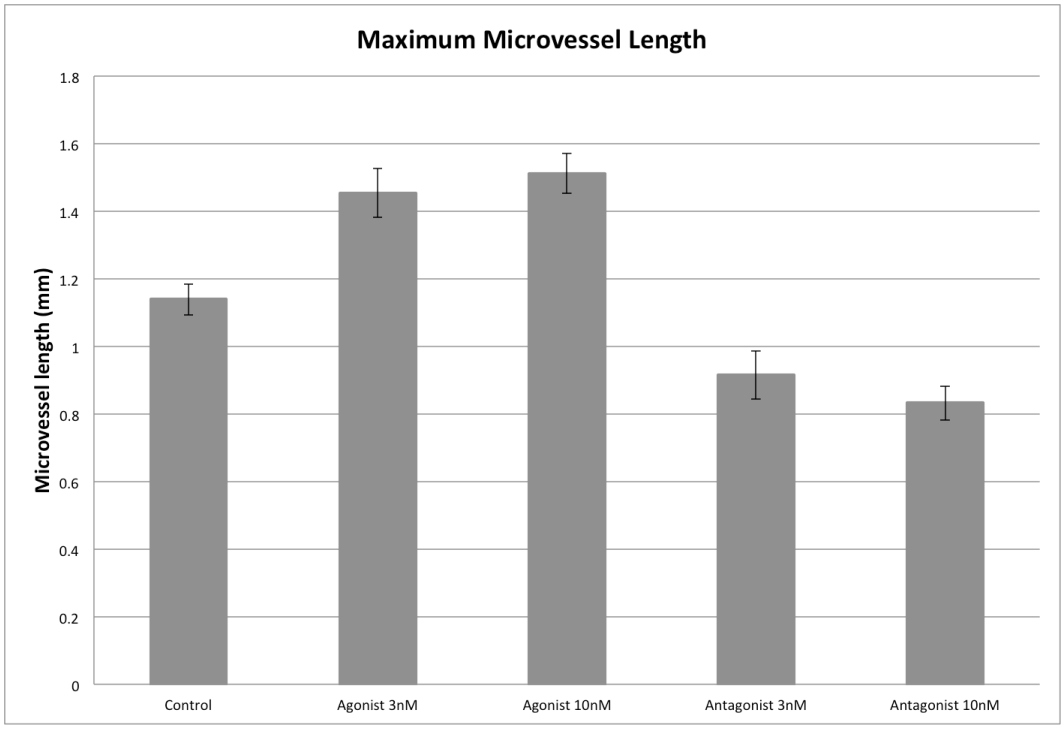


Fig.4. Growth ratio of each agonist and antagonist treatment, where control condition is equal to 1.

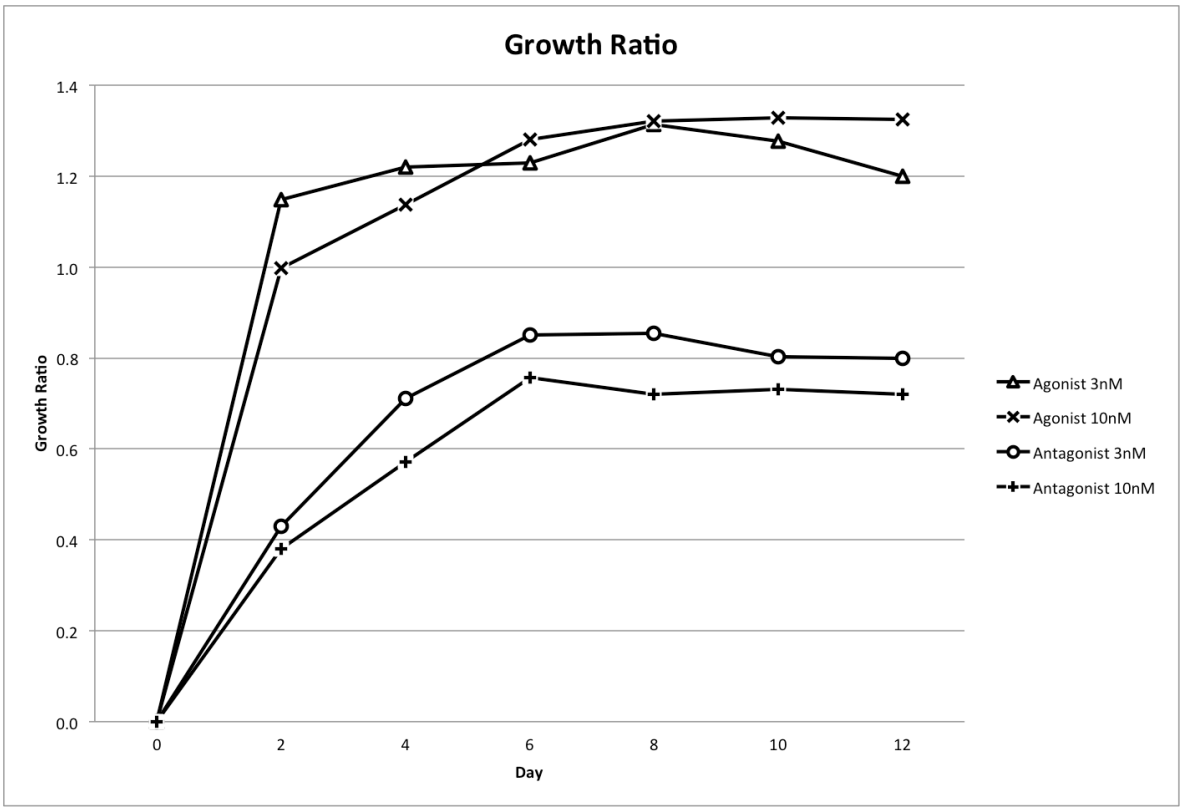


Fig.5. Endothelial cell proliferation as a function of time for various agonist (GSK1016790A) and antagonist (HC067047) concentrations compared with control condition (means ± SE)

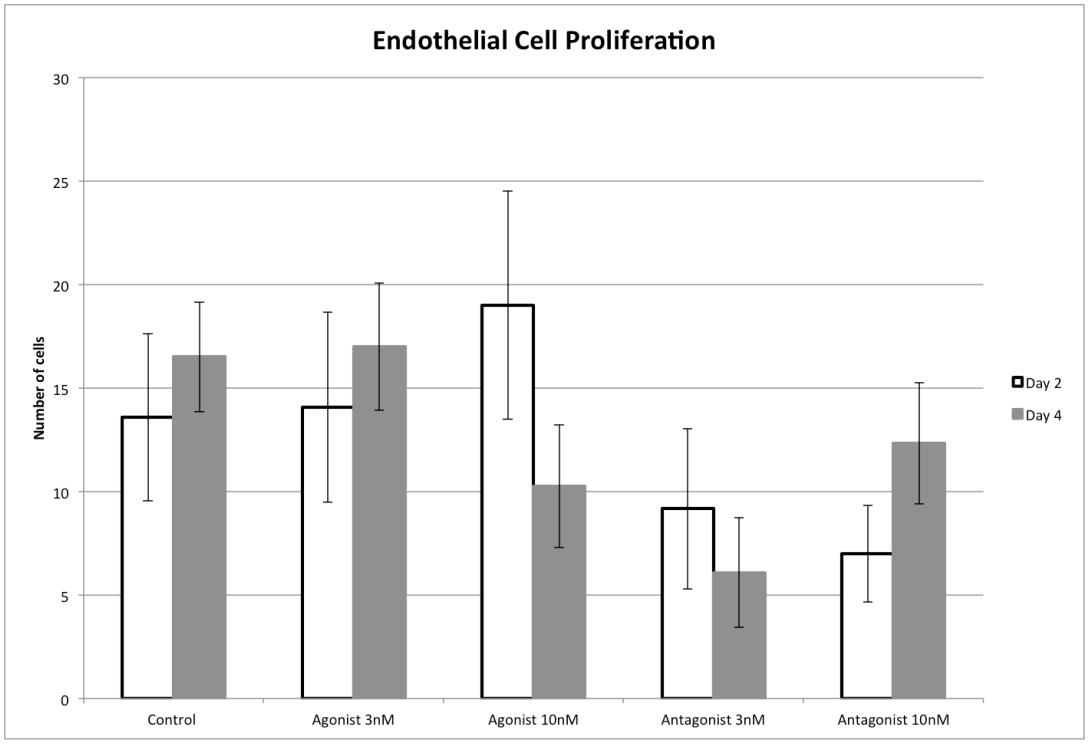
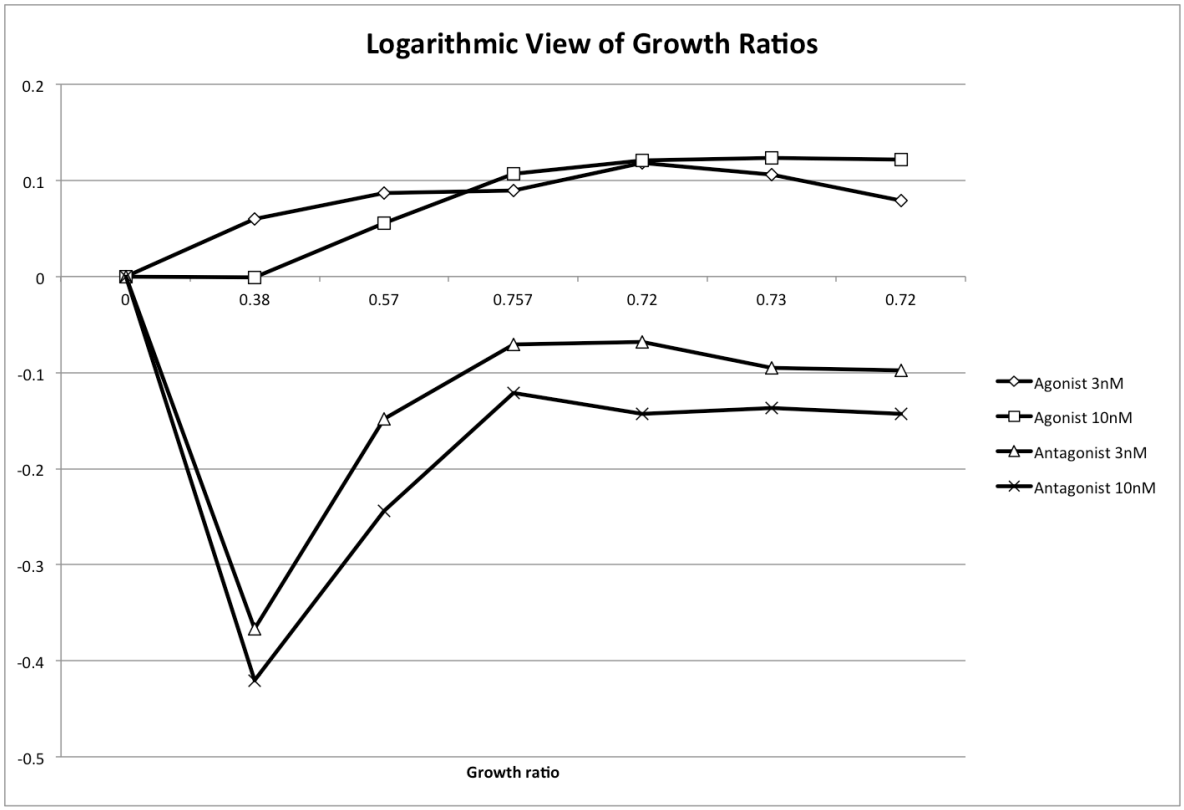


Fig.6. Logarithmic plot of growth ratios of each agonist and antagonist treatment.



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III

Closing Remarks

Closing Remarks

The aim of this thesis was to find a correlation between the activation and inhibition of the TRPV4 ion channel and angiogenesis. The development of new blood vessels is primordial for the vitality of organisms; vessels allow a transfer of oxygenated blood and nutrients to cells and remove metabolic waste and carbon dioxide from the cells and surrounding tissues. Stimulating this growth to accelerate the rehabilitation process of a patient who has just suffered a myocardial infarction or perhaps try to prevent the ischemia from occurring in the first place has significant clinical relevance.

Our findings show that the inhibition of the TRPV4 ion channel shows significant decrease in the number and length of vessels sprouting from the aortic rings. As for the agonist stimulating the ion channel, significant increase in the length of the sprouts can be seen as of day 8. Increasing the length of the vessels can play an important role in the penetration of neighboring tissue. By increasing perfusion to an ischemic region, coronary artery disease symptoms can be alleviated and cardiac function can improve.

Future work should include an *in vivo* model where the agonist and antagonist compounds are given orally to the mice. The rodents should then follow a high intensity, aerobic exercise regiment. This type of exercise has been associated with angiogenesis. Capillaries deliver oxygen and nutrients to the muscles and angiogenesis allows the growth of this capillary bed in order to keep up with the demand of the working muscles (Barry et al., 2004). Also, taking into consideration the results found in this study, seeing similar effects translated in human ECs would be great for future research dealing with IHD patients.

After investigating the change in number and length of the microvessel outgrowths, we needed to determine what stage of angiogenesis was being affected. We decided to count the amount of ECs directly from the images being captured from the assay. By doing this, results from all rings were taken into consideration and no external factors would influence the results. The changes in number of ECs present in each condition were not significant. More experiments would need to executed with a larger population in order to investigate what phase of angiogenesis is most affected by these compounds.

This thesis supports our hypothesis; TRPV4 might in fact act as a mediator of intracellular signaling for angiogenesis. It can be considered as a target for future research on treatment and prevention of diseases dealing with abnormal angiogenic growth.

IV

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