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THE EFFECT OF REACTIVE OXYGEN SPECIES GENERATION ON THE CROSSTALK BETWEEN BRADYKININ AND SPHINGOSINE-1-PHOSPHATE RECEPTORS IN VASCULAR SMOOTH MUSCLE CELLS

by
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Title: The Effect of Reactive Oxygen Species generation on the Crosstalk Between Bradykinin and Sphingosine-1-Phosphate Receptors in Vascular Smooth Muscle Cells.

Background: Atherosclerosis is a silent chronic inflammatory disease that is a major cause of death worldwide. Due to the injury of endothelial cells aligning the aorta, the blood contents such as white blood cells, cytokines, growth factors and lipids interact directly with the smooth muscle cells of the vessel wall leading to vascular remodeling and lesion formation. Reactive oxygen species (ROS) generation in vascular smooth muscle cells activates signaling pathways, which contribute to vascular remodeling.
Hypothesis: Mechanisms through which atherosclerosis mediate its effect on vessel walls are not well defined; however, there are many factors that contribute to the vascular remodeling like inflammatory mediators, cytokines and growth factors. Hence, we hypothesized that kallikrein-kinin system (KKS) specially bradykinin (BK) has a role in vascular remodeling via ROS generation, crosstalk with Sphingosine-1-Phosphate receptors (S1PR) s, and inducing the signaling pathways.
Results: When BK acts directly on the smooth muscle cells leading to the synthesis of ROS; ROS in turn activate signaling pathways like mitogen-activated protein kinases (MAPK)s and Phosphatidylinositol-3 kinase (PI3K). This was confirmed by using N-acetylcysteine (NAC), a scavenger of ROS that led to a significant decrease in extracellular signal-regulated kinase (ERK1/2) and AKT. We also discovered that there is a crosstalk between bradykinin and S1PRs via sphingosine kinase 1 (SphK1) due to ROS generation by BK. On the other hand, when we used NAC, the ROS generation and the downstream signaling pathways of bradykinin 2 receptor (B2R) were inhibited, also CTFG, Fn and (SphK1) gene expression was decreased.
Besides, we verified that S1P activates MAPK and PI3K pathways by increasing ERK1/2 and AKT respectively, which leads to proliferation and migration of smooth muscle cells. S1P also increases CTGF and Fn production, which leads to the production of extracellular matrix (ECM) particularly by S1PR1.
Conclusion: Conclusion: The results suggest that BK induced ROS generation activates ERK1/2 and AKT to promote vascular remodeling. Moreover, BK initiates a crosstalk between B2R and S1PRs (EDGRs) via activation of SphK1. On the other hand, S1P plays a role in vascular remodeling by activation of MAPK and PI3K pathways and also increases CTGF and Fn production

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LIST OF ABREVEATIONS

BK	Bradykinin
B2R	Bradykinin 2 Receptor
CTGF	Connective Tissue Growth Factor
CVD	Cardiovascular Disease
Fn	Fibronectin
ECM	Extracellular Matrix
ERK1/2	Extracellular Signal Regulated Kinase
KKS	Kallikrein-Kinin System
MAPK	Mitogen-Activated Protein Kinases
NAC	N-acetylcysteine
PI3K	Phosphatidylinositol-3-Kinase
PKC	Protein Kinase C
ROS	Reactive Oxygen Species
S1P	Sphingosine-1-Phosphate
S1PR	Sphingosine-1-Phosphate Receptor
VSMC	Vascular Smooth Muscle Cell

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CHAPTER I

INTRODUCTION

Atherosclerosis, one of the underlying causes of cardiovascular diseases (CVD), is a complicated chronic inflammatory disease, which is still not fully understood and remains the foremost cause of global morbidity and mortality. According to the WHO approximations 17.3 million people died of CVD in 2008 demonstrating almost 30% of global mortality. It is predictable that this number will rise to 23.6 million by 2030, which will be almost 80% of the death arising in low and middle-income countries like Lebanon (1).

Many studies show that atherosclerosis is defined by the environmental factors as well as genetic factors that account more than 50% of the cases (2). Moreover, these studies show that atherosclerosis is the result of unnatural environment, genetic predisposition and increased life span (3).

There are many important risk factors of atherosclerosis that includephysical inactivity, unbalanced diet, smoking, and alcohol abuse. These factors result in elevated blood pressure, increased levels of glucose and lipids in blood, and obesity which create the metabolic disorderand hence increased risk to develop atherosclerosis (4).

Females tend to be somehow protected from atherosclerosis because of estrogen which has many anti-atherogenic effects specially on plasma lipoprotein levels, stimulation of prostacyclin and production of nitric oxide (NO) (5).

A. Vascular Remodeling: The Changes that Accompany Atherosclerosis

Studies have outlined series of changes in the walls of blood vessels that occur during atherogenesis and revealed that the inflammatory cells specially macrophages have a major role (6). During the course of the disease there is accumulation of lipids that result from improper lipid metabolism, overproduction of the extracellular matrix and the chronic inflammatory responses due to the accumulation of macrophages and white blood cells (7).

Atherosclerosis is characterized by the hardening of the blood vessel walls due to the formation of multiple plaques; mostly because of the inappropriate lipid metabolism (8). The atherosclerotic plaques are divided into two categories: stable plaques and unstable plaques (9). Stable plaques have rich extracellular matrix and smooth muscle cells, while unstable plaques are mainly formed of macrophages and foam cells (macrophages that engulfed oxidized LDL) that are separated from the arterial lumen by the extracellular matrix (fibrous cap) (10).

One of the remarkable differences that occur in the vascular wall is the change in the composition of the ECM. In normal conditions, the main constituents of the matrix are basement membrane proteins like collagen type IV and laminin. Also substantial amounts of FN are discovered, but in this case fibronectin lacks the ED-A and ED-B domains (11).

B. Vascular Remodeling: The Role of Endothelial Cells

The highly organized construction of the blood vessel walls and the specific cellular phenotype is disturbed in pathophysiological cases like atherosclerosis. The change in phenotype of the vascular endothelial cells is suggested to play a major role in initiation and advancement of the atherosclerotic lesion (12). In physiological situations, the endothelium performs as a “barrier” to avoid interaction of vascular smoothmuscle cells with circulating inflammatory cells, such as monocytes, but many atherogenic factors such as, dyslipidemia, hypertension, hyperglycemia, and smoking can promote the generation of reactive oxygen species (ROS) in large quantities that injure the endothelial cells and result inendothelial dysfunction and an inflammatory response (13) .

The injured endothelial cells produce and secrete pro-inflammatory factors, especially P-selectin, E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1), which all lead to the attachment of the inflammatory cells to the damaged endothelial cells. Hence, these pro-inflammatory factors play a pivotal role in the formation of atherosclerotic plaques (14).

Endothelin (ET) that originates from vascular endothelial cells (ECs), consists of a 21 amino acid peptide, and has a strong vasoconstrictive role (15). ET peptides are three types (ET-1, ET-2, and ET-3). Each of these peptides plays a specific physiological role according to its distribution and property. There are two types of ET receptor: The ET_A andET_B receptors. ET_A that is primarily located on muscle cells has a high affinity for ET-1 and ET-2, whereas the ET_B, which is found on endothelial, epithelial, endocrine, and nerve cells, has an affinity for all three peptides (16). Of the three peptides, ET1 that is produced by the ECs

as well as the vascular smooth muscle cells and cardiomyocytes, has the most important effect on Cardiovascular diseases (CVD) because of its role in regulating the vascular tone (17).

Studies show that the endothelium plays a major role in mediating inflammation and the buildup of oxidatively modified LDL in the intima that recruit monocytes and form foam cells (18).

For an intact vascular endothelial function, NO is important in inducing blood vessel dilation. However, damaged endothelium induced vessel dilation is one of the main features of the endothelial dysfunction that is the main originator of coronary heart diseases comprising atherosclerosis. As a result, the main cause of vascular endothelial dysfunction is the reduction in the level of NO, in addition to the increase of ROS that induce apoptosis (19). Beside its main role in keeping the homeostasis of the blood vessels, NO blocks major steps in initiation and advancement of atherosclerosis (20, 21). NO, which has many anti-atherogenic properties including vasorelaxation, is inhibited by the oxidized LDL. Elimination of the endothelium results in smooth muscle cells (SMC) migration and proliferation (18).During this stage, vascular smooth muscle cells (VSMC) convert their phenotype from the inactive “contractile” to the active “synthetic” state that facilitates their migration from media to the intima and their proliferation. Cytokines and growth factors that are produced by macrophages and T cells are essential for SMC migration and proliferation and extracellular matrix production. Eventually proliferated smooth muscle cells and their derived matrix build up to form the fibrous cap of the atherosclerotic lesion (22).

C. The role of ROS in Atherosclerosis

ROS are produced by normal cellular metabolism (mitochondrial oxidative phosphorylation) or they may result from bacterial invasion, cytokines and growth factors such as TNF-α, interleukin-1, angiotensin II, platelet-derived-, epidermal- and transforming growth factors, and macrophage inducing factor1(23). Cellular enzymes are major sources of ROS, such as xanthine oxidase, NADPH oxidase, uncoupled endothelial nitric oxide synthase (eNOS), cytochrome P-450 enzymes, cyclooxygenase and lipoxygenase, as well as the mitochondrial respiratory chain (24).

The major source of ROS in atherosclerosis is the macrophages and smooth muscle cells where the mitochondria of these cells generate superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) as a result of metabolic stress; though there are other sources like endothelial and adventitial cells (25).

ROS, like H_2O_2, O_2^-, and hydroxyl radical ($HO_•$), contain a radical and non-radical oxygen species produced by the partial reduction of oxygen (26). Free radicals originated from oxygen signify the most important class of radical species. Their high reactivity comes from their unpaired electrons. The crucial reactive oxygen species $O_2^{•-}$, results in the contraction of vascular smooth muscle by scavenging the NO inside the vascular wall (27). The hydroxyl radical, $^{•}OH$, which is generated from the iron (by the Fenton reaction) that is released by superoxide effect under oxidative stress conditions, has a very short half-life of 10^{-9}s. It can randomly oxidize its adjacent targets. In lipid membranes, $^{•}OH$ undertake fatty acid peroxidation (28).

On the other hand, H_2O_2, which is generated by peroxisomes through metabolic reactions that use oxygen, is a small, uncharged reactive oxygen species that easily diffuses through cellular membranes. H_2O_2contribute to oxidative stress as they are released in the cytosol because the peroxisomes are damaged and the H_2O_2 utilizing enzymes are low in level (29).

ROS can generate either positive or negative effects. The positive effects of ROS arise at low concentrations and include physiological functions in host defense mechanism and in a number of cellular signaling systems. While the negative effects of ROS is whenever "oxidative stress" occurs when the level of ROS inside the cell increases to an extent that exceeds the capacity of cellular antioxidants defense (30). As a result, the oxidative stress causes direct or indirect ROS mediated destruction of proteins, lipids, and nucleic acids. ROS has been also involved in carcinogenesis (31) neurodegeneration (32), atherosclerosis and aging (33).

Although there are many studies that present the general consequences of the oxidative stress on signaling pathways, very little information is present on how ROS affects directly the signaling molecules, or what is called the "oxidative interface". To understand better how ROS affects signaling molecules, we must shed light on how ROS alters the protein function. During the oxidative interface, ROS regulates primarily the redox reactions of cysteine (Cys) residues found on proteins.Oxidation of redox-reactive (Cys) residues, form reactive sulfenic acid (—SOH) that in turn may form disulfide bonds with adjacent cysteines (—S—S—). The reactive sulfenic acid might even undergo further oxidation to sulfinic (—SO_2H) or sulfonic (—SO_3H) acid. When neighboring nitrogen is available, sulfenic acid can also form a sulfenamide (34). As a result, these oxidative changes cause modifications in the structure

as well as the function of proteins. These reactions are irreversible except for sulfonic acid and sulfinic acid, in which redox alterations are reversible by reducing systems like thioredoxin and peroxiredoxin (35).

There is activation of a number of ROS mediated signaling pathways in atherosclerosis that elevate vascular permeability and cell adhesion like cytokine-mediated activation of the JAK-STAT, NF-κB, and MAPK signaling pathways. The downstream effects of these activated signaling pathways can include changes in vasoconstriction and vasodilation mediated by bradykinin, endothelin, angiotensin II, and NO and prostacyclin (36).

Diabetics who have high blood glucose usually suffer from oxidative stress, which results in increased ROS generation that leads to insulin resistance, β-cell dysfunction as well as microvascular and macrovascular complications (37). Increased ROS generation in diabetics also reduces NO formation, one of the characteristics of atherosclerosis (38).

N-acetylcysteine (NAC) is an antioxidant that is rich in cysteine and thiol. It has been in clinical use since 1960s in the curing of various pulmonary diseases as a mucolytic drug. And it has been proven to be the exact medication to treat liver injury caused by acetaminophen poisoning (39). Saritas *et al* have also shown that NAC decreases the inflammation induced in the liver due to Acetaminophen toxicity (40). NAC induces its antioxidant activity by increasing the synthesis of reduced glutathione (GSH), which scavenges ROS generated in oxidative stress (41, 42).

D. The Modulation of Signaling Pathways in Vascular Smooth Muscle Cells

One of the obvious features of the atherosclerosis development that follows the endothelial injury is the vascular smooth muscle cell (VSMC) proliferation. The MAPKs lead

to cellular proliferation or differentiation due to incorporation of multiple signals from various second messengers. The MAPKs are a group of Ser/Thr kinases that are activated via specific extracellular stimuli in mammalian cells. The MAPK family comprises extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2 or p42MAPK and p44 MAPK), p38 MAPK and the c-Jun NH_2-terminal kinases (JNK, also known as stress-activated protein kinase, SAPK) (43). The activated MAPK translocates to the nucleus, where it controls the expression of transcription factors such as c-fos proto-oncogene through the phosphorylation of the transcription factor p62TC (44).

It has been shown by Jaffa *et al* that BK induces the activation and tyrosyl phosphorylation of p42 MAPK and p44 MAPK in rat aortic smooth muscle cells. Moreover, they validated that tyrosine phosphorylation of p42 MAPK and p44 MAPK in VSMC by BK is pertussis toxin (PTx) and cholera toxin CTx insensitive and includes the activation of protein kinase C (PKC) and cytoplasmic tyrosine kinases. These outcomes deliver the first confirmation that BK stimulates early mitogenic signals related with activation of ERK in VSMC (45).

Another important pathway of signal transduction in VSMCs is Phosphoinositide 3-kinase (PI3K)/AKT signaling pathway that plays a key role in the regulation of cellular growth, migration, metabolism and apoptosis (46). The PI3K/AKT signaling is apparently needed for VSMCs migration and proliferation, since the lack of AKT interferes with the VSMCs proliferation and migration. AKT induces cell growth and survival by phosphorylating as well as inactivating apoptosis-inducing proteins. Hence, PI3K/AKT synergistically works with ERK1/2 MAPK pathway to induce cell proliferation and migration in VSMCs (47).

E. The Kalikrein-Kinin System and Bradykinin Receptors

1. *The Kalikrein-Kinin System (KKS)*

The Kinins are known as vasoactive compounds and they are composed of three members: kallidin, methionyl-lysyl-bradykinin and bradykinin from which the first two are quickly changed into bradykinin by aminopeptidases (48). In addition to their vasoactive effect, Kinins play a major role in inducing inflammation by increasing edema and pain. Regarding the function and regulation, two kallikrein-kinin systems (KKS) are found in humans that include the certain subtypes of kallikreins and kininogens that are circulating plasma KKS and tissue-specific KKS.Kallikreins are defined as peptidases that cleave the human nona-peptide bradikinin and deca-peptide Kallidin from Kininogen (49).

The circulating plasma KKS include the “High Molecular Weight” HMW-kininogen and prekalikrein that are produced in the liver and secreted in the form of plasma proteins. Plasma Kallikrein is cleaved by proteases and activated factor XII, in this way it initiates blood coagulation and inflammation and cleaves bradikinin from HMW-kininogen. Besides the plasma KKS, there is the tissue specific system that is either produced in the liver or locally that is the “Low Molecular Weight” LMW-Kininogen and tissue Kallikrein which is a serine protease that is found in many glands and tissues like pancreas, vascular system, kidney tubules, myocardium and central nervous system (50).

2. ***Bradykinin and its Receptors***

Bradykinin is a nine amino acid; low molecular weight (1 kDa) peptide that is quickly metabolized by endogenous metalloproteases like angiotensin-converting enzyme (ACE or kininase II), carboxypeptidase N (CPN or kininase I), aminopeptidase P and neutral endopeptidase (NEP or neprilysin) (51). Bradykinin is a vasodilator hormone, and it exerts its effect via increasing the release of endothelial-derived relaxing factor that is produced through stimulating the B2 receptor (52, 53).

There are two types of bradykinin receptors B1 and B2 that are widely distributed in tissues and organs including the smooth muscle cells of blood vessels. B2 receptors are constitutively expressed while B1 receptors are expressed only due to pathological conditions when exposed to bacterial endotoxins like lipopolysaccharides and cytokines like interlukin-1. B2 receptor can be stimulated by BK and KD while B1 is almost not stimulated by BK (54) .

Stimulation of the B2 receptor which is a GPCR by BK in vascular smooth muscle cells activates phospholipase C that leads to the production of inositol phosphate and diacylglycerol, which consequently leads to an increase in the intracellular calcium and activation of PKC (55).

F. Sphingosine-1-Phosphate and its Synthetic Pathway

The Sphingosine-1-phosphate (S1P) is a sphingolipid metabolite characterized by its potent inflammatory effect (56). Also it is characterized by its ability to induce cell proliferation, invasion and migration (57). S1P originates from the hydrolysis of

sphingomyline, which is abundant in cell membranes, by the actionof thesphingomylinase enzyme which will convert it into ceramide, then the ceramidase enzyme converts the ceramide into sphingosine that will either be phosphorylated and converted into S1P by kinases, such as, sphingosine kinase 1 and 2 or reacylated to ceramide (58). On the other hand, S1P is also formed due to de novo synthesis by the formation of ceramide from condensation of palmitoyl-COA and serine (59). Once formed S1P can be degraded by dephosphorylation by S1P phosphatase (SPP) or by type 2 phosphotidate phosphohydrolases, or by S1P lyase. S1P stimulates MAPK and PI3K pathways leading to ERK1/2 and AKT activation (58-60).

G. Sphingosine Kinase 1 and 2 Enzymes

1. *Structure and localization*

There are two mammalian sphingosine kinase isoforms, coded by two *Sphk* genes, *SphK1* and *SphK2*;these two genes have different tissue distribution, cellular localization, and expression during physiological or pathophysiological conditions (61). In humans, the SphK1 gene is found on chromosome 17 (17q25.2) whereas the SphK2 gene is on chromosome 19 (19q13.2). The two isoforms are highly homologous with 80% amino acid similarity. Both contain five conserved domains, including the conserved diacylglycerol kinase ATP binding domain (62). Unlike Sphk1, which is mainly located in the cytosol, the Sphk2 subcellular localization differs depending on the cell type (63). For instance, in HEK293 cells SphK2 is localized in the plasma membrane, mitochondria, endoplasmic reticulum, golgy and in the

cytosol. However, in COS7, HeLa, MCF7 and NIH3T3 cells, SphK2 is mainly localized in the nucleus (64, 65).

2. *SphK1/2 Activation and Function*

There are many activators of SphK1 like growth factors and hormones, some of them are platelet-derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor beta (TGFβ), and insulin-like growth factor-1 (IGF-1), cytokines such as TNF-α and interleukins, and hormones (estradiol and prolactin) (66). It is important to mention that those activators of SphK1 can activate it in a biphasic manner, where the first phase is rapid and happens in minutes due to post translational modifications which increase the enzyme activation and cause its translocation to the membrane, while the second phase occurs after 24 hours due to upregulation of the transcriptional activity (8).

Sphingosine kinase 1/ 2 catalyzes ATP-dependent phosphorylation of sphingosine converting it into S1P that penetrates the plasma membrane and acts as a ligand either in a paracrine or an autocrine fashion on five G- protein coupled receptors (GPCR) S1PR1-5 (67, 68).

H. S1P Receptors (S1PR)

S1P receptors were previously named endothelial differentiation gene receptors (EDGR) s; S1PR1 (Edg1), S1PR2 (Edg5), S1PR3 (Edg3), S1PR4 (Edg6) and S1PR5 (Edg8) (69).

When activated, these receptors couple to different heterotrimeric G proteins, which in turn stimulate different downstream signaling pathways (63). Each receptor signals through different G proteins. For example, S1PR1 signals mainly through Gi, while S1PR2, S1PR3, S1PR4 and S1PR5 signals through Gi, Gq and G12/13 (70).

Both human and mouse S1PR1 are composed of 382 aa and have a molecular weight of 43kD. On the other hand S1PR2 is composed of 352 aa with a molecular weight of 39kD in mouse. S1PR3 is 378 aa long and has a molecular weight of 42kD both in human and mouse. Each one of these three receptors is encoded on a single exon (71).

S1PR1, 2 and 3 are expressed differently in different cell lines. S1P2 and S1P3 are expressed on adult medial VSMC while S1PR1, S1PR2 and S1PR3 are expressed in the pup intimal VSMCs (59) and are considered the most important receptors that contribute to vascular development. It has been reported that the role of S1PR1 in angiogenesis is major because knocking it down led to embryonic death in mice around the day E12.5 (61, 62), while S1PR2 and S1PR3 null mice showed defective vasculature leading to bleeding due to thin defective vessel structure and consequently to death in the 50% of the cases (61). S1P1 receptor that is coupled to Gi-linked P70S6 kinase pathway induces proliferation and is overexpressed in human atherosclerotic lesions (72).

I. Connective Tissue Growth Factor and Atherosclerosis

CTGF, which has many functions related to vascular endothelial cells, chondrocytes and osteoblasts, is a member of the CCN cysteine-rich protein (Cyr61), CTGF and nephroblastoma overexpressed gene (nov) family of proteins (73).

CTGF is a cysteine rich, heparin-binding protein that enhances extracellular matrix production (74). CTGF intracellular protein is upregulated in atherosclerotic vascular diseases and it is an indicator of fibrotic disease. High levels of mRNA and protein of CTGF were detected in vascular smooth muscle cells in advanced atherosclerosis.Moreover, the vascular smooth muscle cells that express CTGF protein reside where extracellular matrix is produced specially around the fibrous cap suggesting that CTGF regulates matrix protein production (75).

Studies conducted by Jaffa *et al* show that LDL could enhance the expression of CTGF in human aortic endothelial cells and mesangial cells under the effect of autocrine activation of TGF-β and c-Jun NH2-terminal kinase (76). It has been shown also that the CTGF levels of LDL receptor knockout mice ($LDLR^{-/-}$) fed with high fat diet have higher levels of CTGF than the ($LDLR^{-/-}$) knockout mice with normal fat diet (77). These findings suggest a novel mechanism through which CTGF is related to lipoproteins promotion of vascular sclerosis.

A high risk factor for progressive renal failure and cardiovascular disease, which is also a marker of diabetic nephropathy, is microalbuminuria. Microalbuminuria is related to high cardiovascular mortality in diabetic patients (78). In one of the studies by Jaffa *et al*, they showed that CTGF levels with microalbuminuria patients is 2 folds higher than the macroalbuminuria and normal albumin excreting cohort suggesting that CTGF might be involved in one of the pathways leading to vascular disease (79).

J. Fibronectin (Fn) in Atherosclerosis

Another key role player in Atherosclerosis is Fn that is an adhesive protein that exists in two forms: plasma soluble and insoluble forms. Fn has the ability to react with receptors found on cell surfaces in its insoluble state while it is not interactive in its soluble state. Fn isoforms ED-A and ED-B expression that is not normally seen in the normal adult vessel is strictly linked to the development of atherosclerosis (80).

Inhibition of expression of Fn is related to a decrease in the whole intimal thickening. The VSMC in intimal thickening of atherosclerotic lesions, convert from the contractile to a synthesizing or proliferative phenotype due to increased expression of FN. In atherosclerotic lesions, there is an obvious increase in Fn that localizes with collagen type III, which is also increased (81).

Furthermore, it has been found that Fn is rich in the ECM of regenerating or injured tissues, even though it is found in most ECMs, as well as in the basement membranes. Soluble Fn is a dimer, each subunit is composed of a chain of repeating modules: 12 type I modules, 2 type II, 15-17 (depending on splicing) type III, and a variable (V) sequence that is not homologous to other parts of Fn (82).

Liver hepatocytes are responsible for the synthesis of plasma Fn that is devoid of ED-A and ED-B; however, cellular Fn that is synthesized in tissues contains variable amounts of either or both ED-A and ED-B. Fn binds to α5β1 and IIbβ3 integrins via a cell adhesive site that includes modules III8-III10 where the most important site is the Arg-Gly-Asp (RGD) sequence in III10 that blocks integrin-mediated cell adhesion to Fn (83).

K. Hypothesis

Due to the malfunctioning of the endothelial cells and their destruction, bradykinin acts directly on the rat aortic smooth muscle cells (RASMC) and generates ROS that leads to the activation of ERK1/2 (MAPK)that activates SphK1 by phosphorylating it. Activated SphK1 in turn phosphorylates sphingosine (SP) that is abundant intracellularly rendering it the active ligand (S1P). S1P interacts with the S1PR1, S1PR2 and S1PR3 that are ubiquitously distributed on RASMC leading to the activation of ERK1/2 MAPKs and PI3K-AKT, signaling pathways which result in cell proliferation, and migration. Moreover, there will be production of CTGF and fibronectin that enhance the extracellular matrix production contributing to atherosclerosis.

On the other hand, treating the RASMC with NAC, a reducing agent and a scavenger of ROS, inhibits ROS generation and downregulates ERK1/2, AKT as well as the downstream pathway of B2 receptor. This outcome results in the disruption of the crosstalk between B2R and S1PRs, since ERK1/2 activates SphK1 by phosphorylating it.

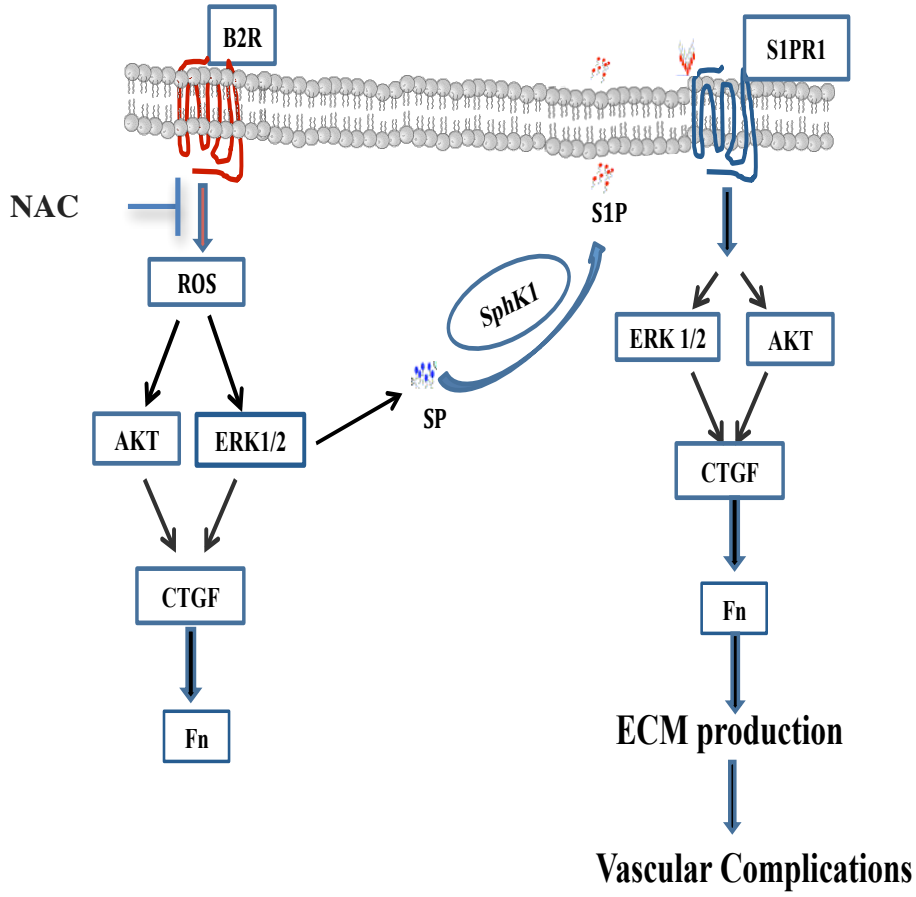


Figure 1: Proposed Pathway for the Effect of ROS on the Crosstalk Between Bradykinin and S1P Receptor.

CHAPTER II

MATERIALS AND METHODS

A. Primary Cells Preparation

Rat aortic smooth muscle cells (RASMC) were extracted from male Sprague Dawley rats weighing 100-150g. The aorta was cleaned from connective tissue and fat layers, and then it was cut and washed with PBS with Ca^{2+}/Mg^{2+} to remove blood. Under Bio-cabinet hood, aorta was longitudinally opened, the endothelium is scrubbed with the bent-head forceps, and the aorta was incubated with Collagenase A (1 mg/ml) for 1 hour at 37^{o}C. Afterwards, the Adventitia was sloughed with bent-head forceps, and the aorta was cut into 6 to 8 pieces that were transferred into T25 cell culture flask with appropriate amount of complete media Dulbeccos Minimal Essential Medium (DMEM, 1g/L Glucose 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 20 mM HEPES) and incubated in humid 5% CO2 incubator at 37^{o}C for 7-12 days to allow the cells to migrate out of the aorta and adhere to the flask. The adhered cells were considered at passage zero and upon subculturing, the passage was counted. At 60-80 % confluence, cells were collected by the action of Trypsin and subcultured into new cell culture flasks. Cells were identified as RASMC by their characteristic morphology and in culture acquired a proliferative state with the typical "hill and valley" growth pattern. Cells between passages 2 and 7 were used in all experiments. Cells were incubated in serum free medium for 24 hours prior to experimentation.

B. Treatment of Cells

For treatment, cells were incubated in serum free medium in absence or presence of different concentrations of BK, SEW2871 or S1P, or for different time points of BK 0.1μM, SEW2871 1nM or S1P 1nM. In some experiments specific inhibitors were used. HOE 140 (1μM)-potent bradykinin B2 antagonist- or W146 (10μM)-selective S1P receptor 1 antagonist- were incubated for 30 minutes prior to the addition of BK or S1P, respectively. Also NAC 10^{-4} was used as a scavenger of ROS.

Antagonists were maintained in the medium for the whole incubation period.

C. Western Blot Analysis

After incubation, cells ($1.5x10^5$/well, 12- well plates) were washed with PBS containing Ca^{2+}/Mg^{2+} and lysed on ice in 100 μl of lysis buffer (20 mM Tris/HCl pH 7.5 containing 1mM EDTA, 1% Triton X-100, 1mM PMSF, 0.33mM Benzamidine, Aprotinin, Sodium Floride, Sodium Orthovanadate). Cell lysates were centrifuged at 15000 g at 4^oC. Total protein concentration was determined using Bradford assay (Bio-Rad) with BSA as standard.

1. SDS-PAGE

20-30 μg of total cell proteins were heated to 95^o C for 5 minutes in Lammeli buffer solution (0.1 mM Tris pH 6.8, 10% glycerol, 0.01% bromophenol blue dye, 5% beta-mercaptoethanol, and 2% SDS) and submitted to SDS-PAGE using 10% polyacrylamide gel (29:1 acrylamide:bis). Migration was allowed to proceed in 1X migration buffer (final concentrations 0.05 M Tris, 0.384 M glycine, and 2% SDS). The gels were then transferred to

a nitrocellulose membrane (Bio-Rad, porosity 0.22 μm) using a Trans-Blot Turbo (Bio-Rad). Membranes were stained with Ponceau red.

2. Western blot analysis

Membranes were incubated for 1 hour in Tris buffer saline tween (TBST) (25 mM Tris pH 7.4, 150mM NaCl, 2mM KCL and 0.1% Tween-20) -5% non-fat dry milk, at 37^{o} C. Immunoblot analysis was performed using polyclonal rabbit anti-rat phospho(ERK and AKT)peptide and donkey anti-rabbit IgG coupled to horse-raddish peroxidases, both at a dilution of 1:2000 in TBST-5% milk were used. Membranes were stripped off their antibodies by washing two times with stripping buffer pH 2.2 (0.2 M glycine, 0.1% SDS, and 1% Tween-20) for 30 min and redeveloped with monoclonal mouse anti- β-actin (Sigma Aldrich) at a dilution of 1:10000 or total ERK and AKT at a dilution of 1:5000, and donkey anti-mouse IgG coupled to horse-raddish peroxidase. Protein band signals were developed on Agfa autoradiography films using enhanced chemiluminescence (ECL kit Roche) according to manufacturer instructions, and scanned using HP scanner. Bands were quantified by Image J software.

D. RNA extraction

Total RNA was extracted from incubated RASMC (3x10^{5} cells/ 6-well plate) using 500 μl of RiboZol (Amresco) according to the manufacturer's instructions. Briefly, 100 μl chloroform was added, mixed and the mixture was centrifuged at 12,000 g for 15 min at 4^{o}C. 250 μl of isopropanol was added to the collected aqueous phase followed by centrifugation at 12000 g for 10 min at 4^{o} C. The RNA pellet was washed with 500 μl 75% ethanol followed

by centrifugation at 12, 000 g for 5 min at 4^{o} C and left 10-20 minutes to dry. The pellet was dissolved in 30 µl of RNase free water, incubated for 5 min at 60^{o} Cand then quenched. RNA concentration was measured at 260/230 nm, and 260/280 ratios.

E. qRT-PCR

1 µg of total RNA were reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad), according to manufacturer's instructions. cDNA amplification reaction was done using the iQ SYBR green mix kit (Bio-Rad), according to the manufacturer's instructions. Primer sequences for the genes of interest are shown in table1.

Table1:Oligonucleotide primer sequences and conditions used for qRT-PCR.

Gene	Direction	Sequence	Annealing Temperature	Fragment Size
CTGF	rCTGF F	CCCCCGCCAACCGCAAGATT	55	131
	rCTGF R	CGGCCCCATCCAGGCAAGTG		
Fibronectin	rFn F	CCACAGCCATTCCTGCGCCA	55	149
	rFn R	TCACCCGCACTCGGTAGCCA		
SphK1	rSphK1F	GCTAGGGTCAGGGGACGCCA	55	122
	rSphK1R	CTTCGGGCACGCGTGGTTCT		
SphK2	rSphK2F	GCCGCACTTCTACGAATTTTG	55	145
	rSphK2R	CTAATTCCCCATCTACAGTGAC		
S1PR1	rS1PR1F	CGCAGCTTCGTCCCGCTTGA	55	133
	rS1PR1R	GGCGAGGTTGAGCGAGCCTT		
S1PR2	rS1PR2F	GTCCGGGCCTGTCCTGTCCT	55	104
	rS1PR2R	AGGTCCCGGCTACGCCATGT		
S1PR3	rS1PRF	GGCAACTTGGCTCTCTGCGA	55	143
	rS1PR3R	GTGGATGCGCCCAGGGCTAC		
GAPDH	rGAPDHF	GGGGCTCTCTGCTCCTCCCTG	55	74
	rGAPDHR	CGGCCAAATCCGTTCACACCG		

PCR was performed using the iCycler iQ (BioRad) programmed for a 1 min initialization at 98^{o} C (1 cycle) followed by 40 cycles for: 9 sec denaturation at 95^{o} C, 12 sec annealing at 55^{o} C, 9 sec extension at 72^{o} C and finally was followed by melt curve analysis. Quantification of the genes was calculated by the $\Delta\Delta C_t$. After amplification, samples were undergone melt curve analysis to check the purity and integrity of amplified samples. The annealing temperature for each gene was optimized in the lab.

F. ROS GENERATION MEASURMENT

RASMC (1x10^4/well, 96- well plates) were serum starved for 24 hours. Cells were incubated with 10 μM DCF for 30 min in dark humid 5% CO2 incubator. Cells were washed with warm PBS with Ca^{2+}/Mg^{2+} and cells were incubated in serum free media for 2 hours in order for the endogenous esterase activity to cleave the 2,7-dichloroflorescene (DCF) and render it fluorescent upon reacting with ROS. Cells are then activated by BK 0.1μM in the absence or presence of HOE140 1 μM and NAC 10^2 μM. Fluorescence was measure by Spectramax Gemini at Exciting with 480 nm and emission at 530 nm for 1 hour with 5 min as an increment.

G. Membrane-Bound Protein Extraction

RASMC (1x10^6/well, 100mm dish) were serum starved for 24 hours. Cells were incubated by BK 0.1μM for short time points (1, 5, 15, 30 and 60 min). Excess media was aspirated and the cells were washed twice with cold PBS 1X with Ca^{2+}/Mg^{2+}. Cells were scraped and collected in 300 μL Cytosolic Lysis Buffer (10mM Tris-HCl, pH 7.5, 5mM $MgCl_2$, 2mM EDTA, and 250mM Sucrose), and lysed by shearing by syringes 1ml 27 G1/2

needle. Lysates were centrifuged at 100000 x g, 4^{o} C for 30 min. The supernatant layer represented the cytosolic fraction. The pellet was resuspended in 100 µL Membrane Lysis Buffer(10mM Tris-HCl, PH 7.5, 5mM MgCl2, 2mM EDTA, 250mM sucrose, 1% Triton-X100, 1% Sodium Cholate), sonicated, and incubated for 30 min at room temperature. Samples were centrifuged at 800 x g for 15 min 4^{o} C, and the supernatant layer represents the membrane fraction while the pellet was resuspended by 100 µL laemelli 1X to check the cytoskeleton and/or nuclear proteins. Samples were prepared for SDS-PAGE as described earlier.

H. Statistical Analysis

Results are expressed as mean ± SE from 3 to 6 independent experiments. Statistical significance is assessed by student's unpaired t-test. Significance is determined as probability (p) <0.05.

CHAPTER III

RESULTS

A. ROS Generation Assessment

First, we checked ROS generation in RASMC due to bradykinin. Figures 2 and 4 show that bradykinin induced ROS generation in RASMC over time. Moreover, Figure 3 and 5 show that bradykinin induced the Vmax of ROS generation compared to the basal level(4115.7 ± 720.3 mU/min, * $p<0.05$) and (23233.8 ± 1615.3 mU/min, *** $p<0.001$), respectively. HOE140 (specific B2 receptor antagonist) decreased bradykinin-induced ROS generation and its rate (Vmax) in RASMC (516.1 ± 110.0mU/min, ** *$p<0.001$).

N-acetylcycteine (NAC), which is a scavenger of ROS, also decreased bradykinin-induced ROS generation and its rate (Vmax) in RASMC (3266.1 ± 981.2 mU/min, *** $p<0.001$).

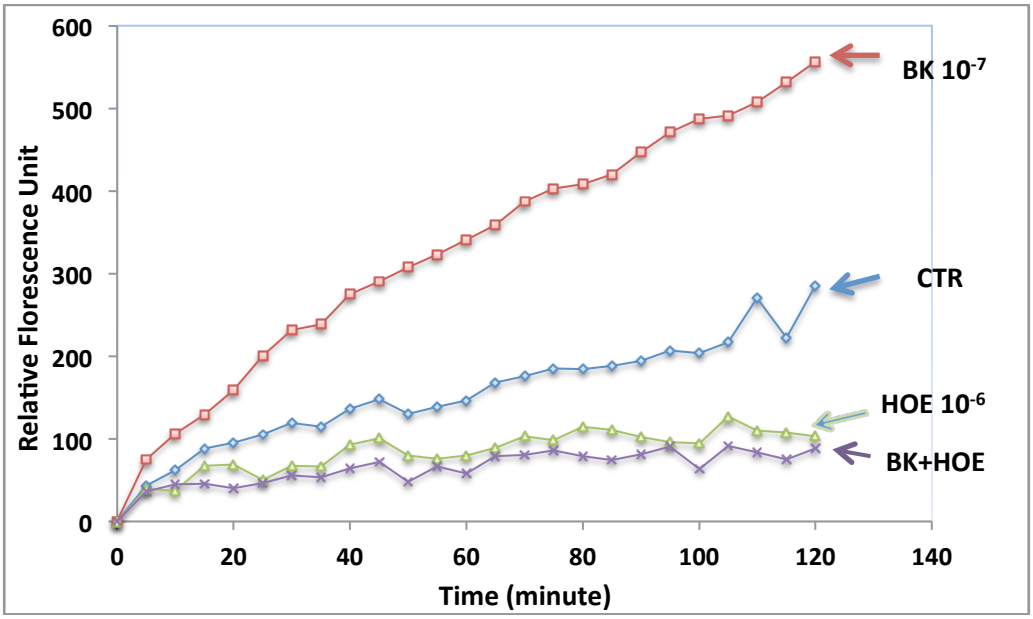


Figure 2: Effect of HOE140 10^{-6} on Bradykinin-Induced ROS Generation. ROS generation was traced by DCF dye over 120 minutes. Bradykinin induced the generation of ROS in RASMC compared to the basal ROS levels, and it reached its maximum after 2 hours of bradykinin incubation. HOE-140 diminished ROS generation, even lower than the basal level. The data is mean ± SE of 6 similar experiments.

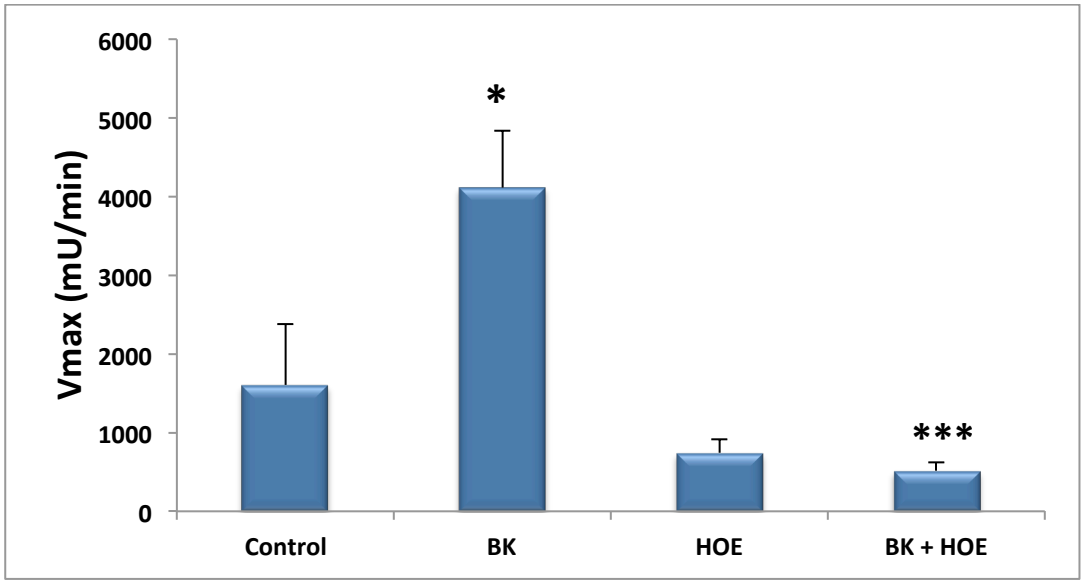


Figure 3: Effect of Bradykinin on the Rate of Enzymatic ROS Generation. Vmax of ROS generation was calculated by tracing ROS generation over 120 minutes. Bradykinin induced the Vmax of ROS generation in RASMC compared to the basal ROS levels (*BK vs.Control, $p<0.05$). HOE-140 diminished the Vmax of ROS generation (** *BK + HOE vs BK, $p<0.001$), even lower than the basal level. The data is mean ± SE of 6 similar experiments.

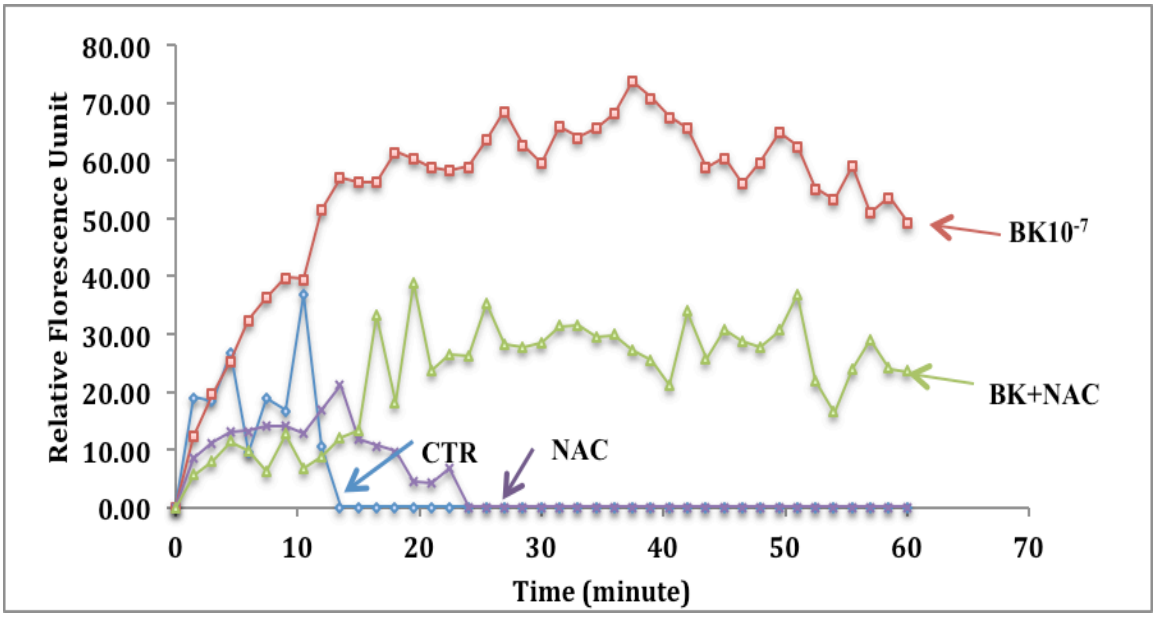


Figure 4: Effect of NAC on Bradykinin-Induced ROS Generation. ROS generation was traced by DCF dye over 60 minutes. Bradykinin induced the generation of ROS in RASMC compared to the basal ROS levels. NAC diminished bradykinin-induced ROS generation compared to the basal levels. The data is mean ± SE of 6 similar experiments.

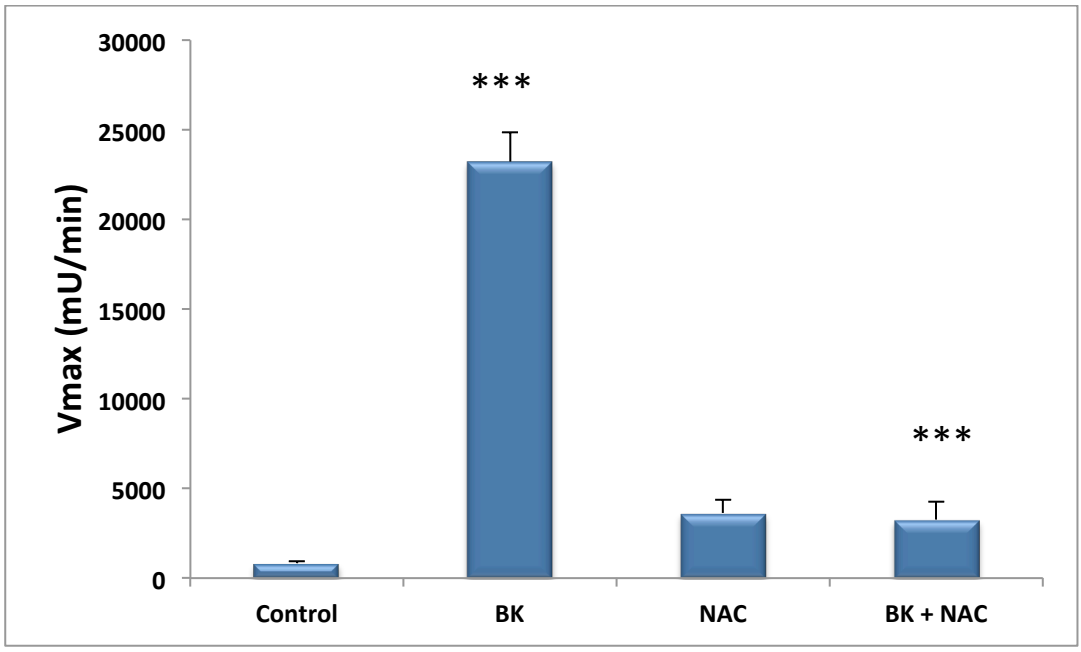


Figure 5: Effect of NAC on Bradykinin-Induced Rate of Enzymatic ROS Generation. Vmax of ROS generation was calculated by tracing ROS generation over 60 minutes. Bradykinin induced the Vmax of ROS generation in RASMC compared to the basal ROS levels (***BK vs. Control $p<0.001$). NAC diminished the Vmax of ROS generation (*** BK + NAC vs. BK, $p<0.001$), even lower than the basal level. The data is mean ± SE of 6 similar experiment.

B. Activation of Signaling Pathways

We evaluated the activation of ERK1/2 and AKT to assess the effect of bradykinin on intracellular signaling pathways. Figures 6 and 7 show that bradykinin induced the phosphorylation of ERK 1/2 (Thr 202/Tyr 204) (2.14 ± 0.24 folds, * $p<0.05$) and AKT (Thr 308) (5.1 ± 0.33 folds, * $p<0.05$) after 10 minutes of bradykinin incubation, respectively.

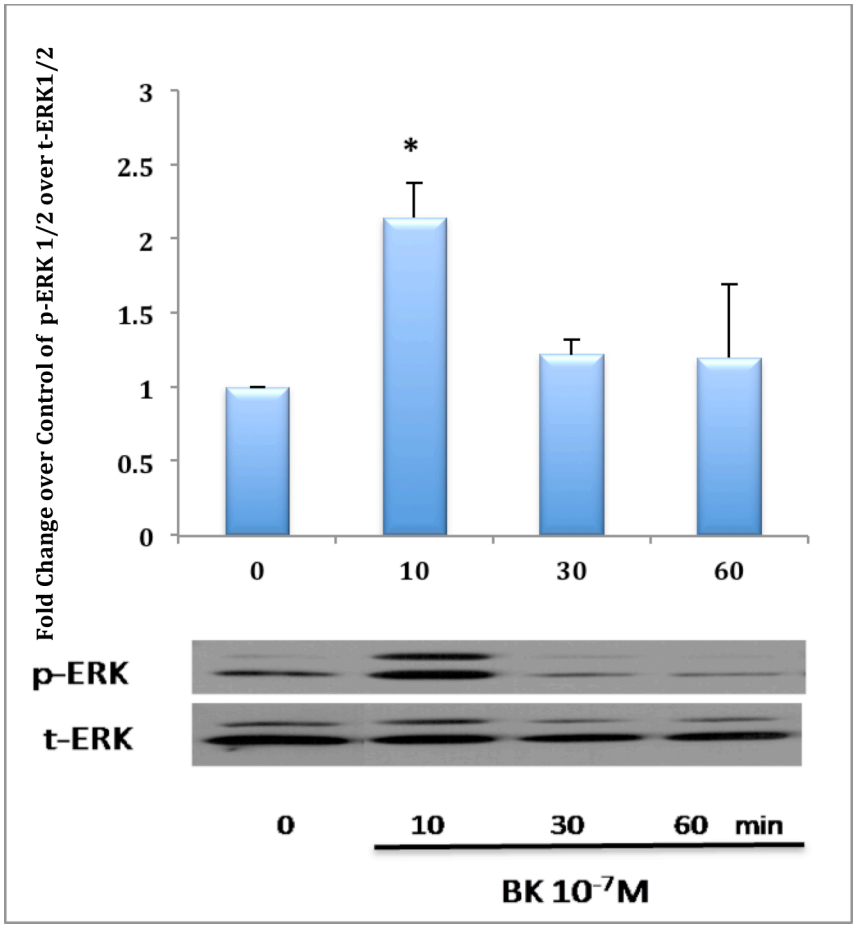


Figure 6.Bradykinin Activates ERK 1/2. Bradykinin induced the phosphorylation of ERK1/2 after 10 minutes (* BK vs. Control, $p<0.05$), and returned to its basal level after 30 minutes. Bar-Graph is the value of the densitometric analysis of the ratios of phospho ERK to total ERK of the western blots. The data is mean ± SE of 4 separate experiments.

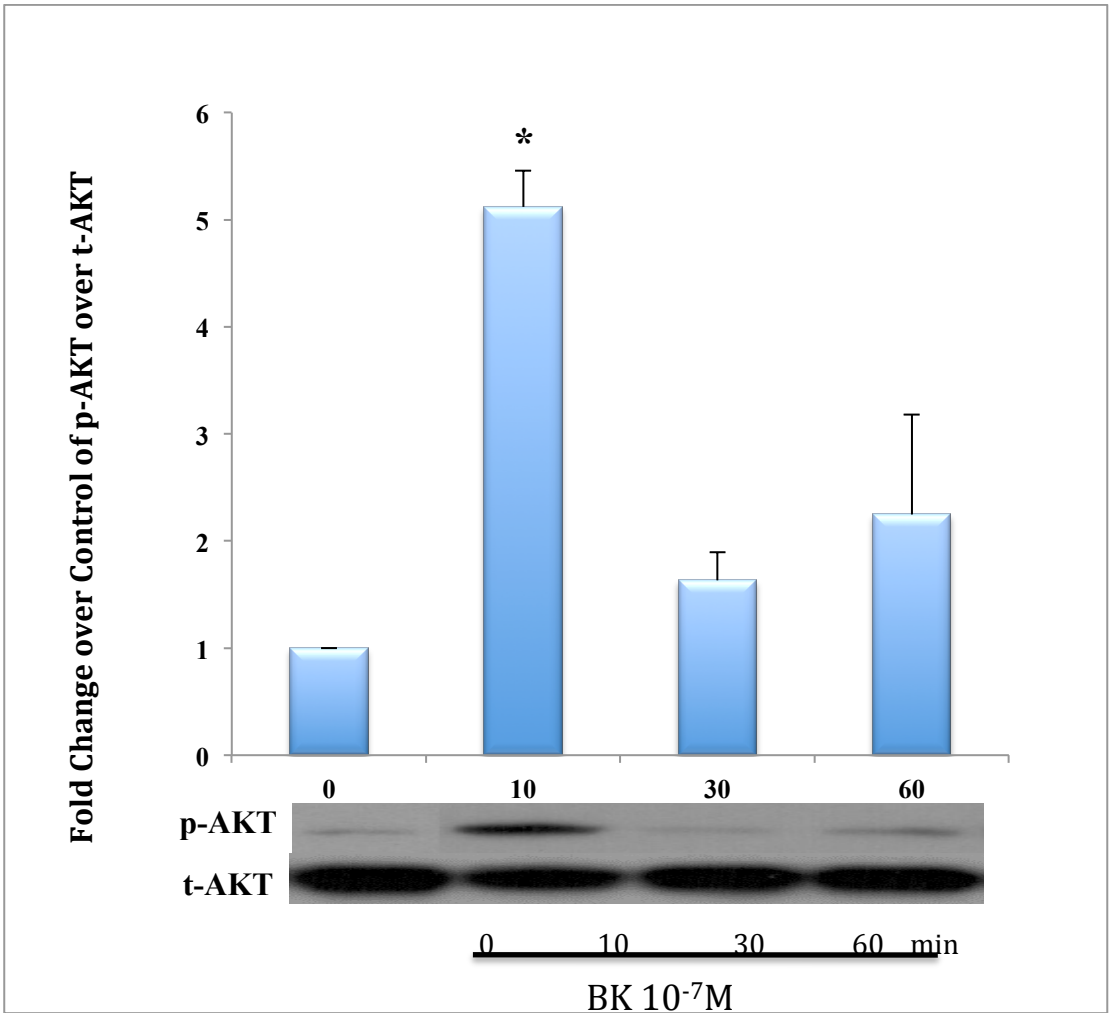


Figure 7.Bradykinin Activates AKT. Bradykinin induced the phosphorylation of AKT (Thr 308) after 10 minutes (* BK vs. control, $p<0.05$), and returned to its basal level after 30 minutes. Bar-graph is the value of the densitometric analysis of the ratios of phosphoAKT to total AKT of the western blots. The data is mean ± SE of 4 separate experiments.

C. The Effect of NAC on ERK 1/2 and AKT Activation

Next we tried to find the effect of NAC on bradykinin-induced ERK1/2 activation since NAC is a reducing agent that chelates ROS. Because NAC inhibited ROS activation in our previous experiments, through this experiment we tried to determine whether ERK1/2 is downstream ROS and ROS is activating ERK1/2 in the downstream pathway of bradykinin.

As we see in figure 8 bradykinin activates ERK1/2 after 10 minutes (3.85±0.82 folds, *p<0.05) and NAC inhibits the ERK1/2 activation after 10 minutes of bradykinin treatment (2.30±0.57 folds, *p<0.05).

On the other hand, figure 9 shows that bradykinin activates AKT at 5 and 10 minutes while NAC inhibits it after 5 minutes (0.99±0.34, *p<0.05).

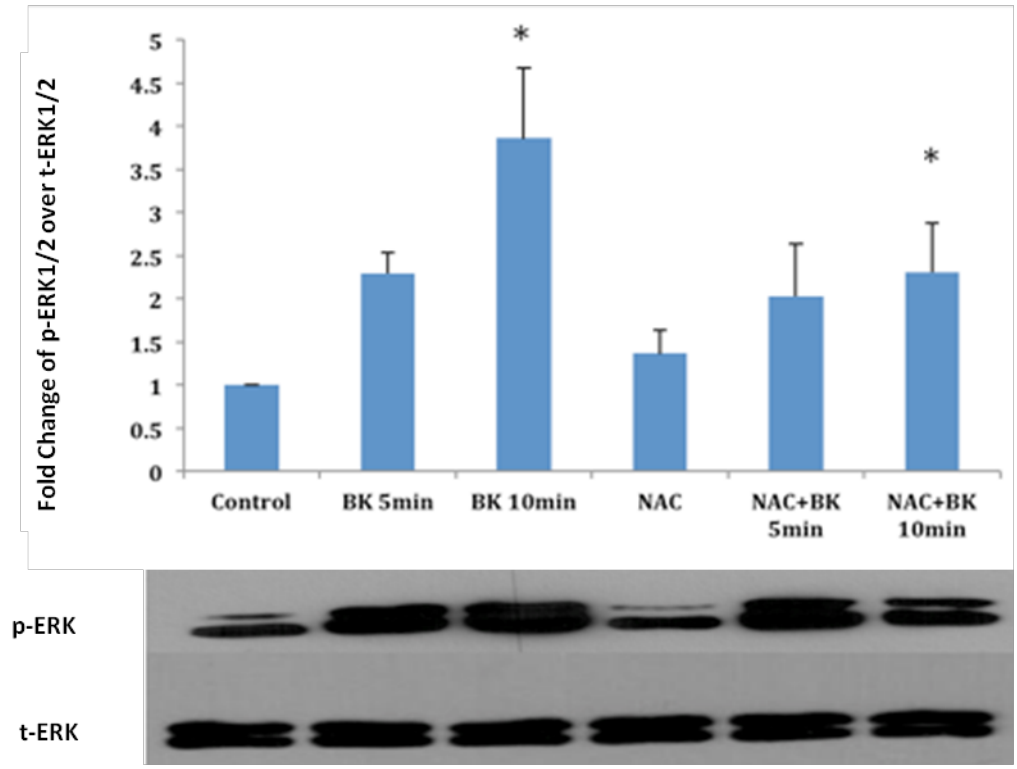


Figure 8. Bradykinin Activates ERK1/2 while NAC Inhibits It. Bradykinin induces the phosphorylation of ERK1/2 after 5 and 10 minutes (* BK vs. Control, p<0.05), while NAC inhibits its activation after 5 and 10 minutes (*NAC+ BK vs. BK, p<0.05). Bar-Graph is the values of the densitometric analysis of the ratios of phospho ERK1/2 to total ERK1/2 of the western blots. The data is mean ± SE of 3 separate experiments.

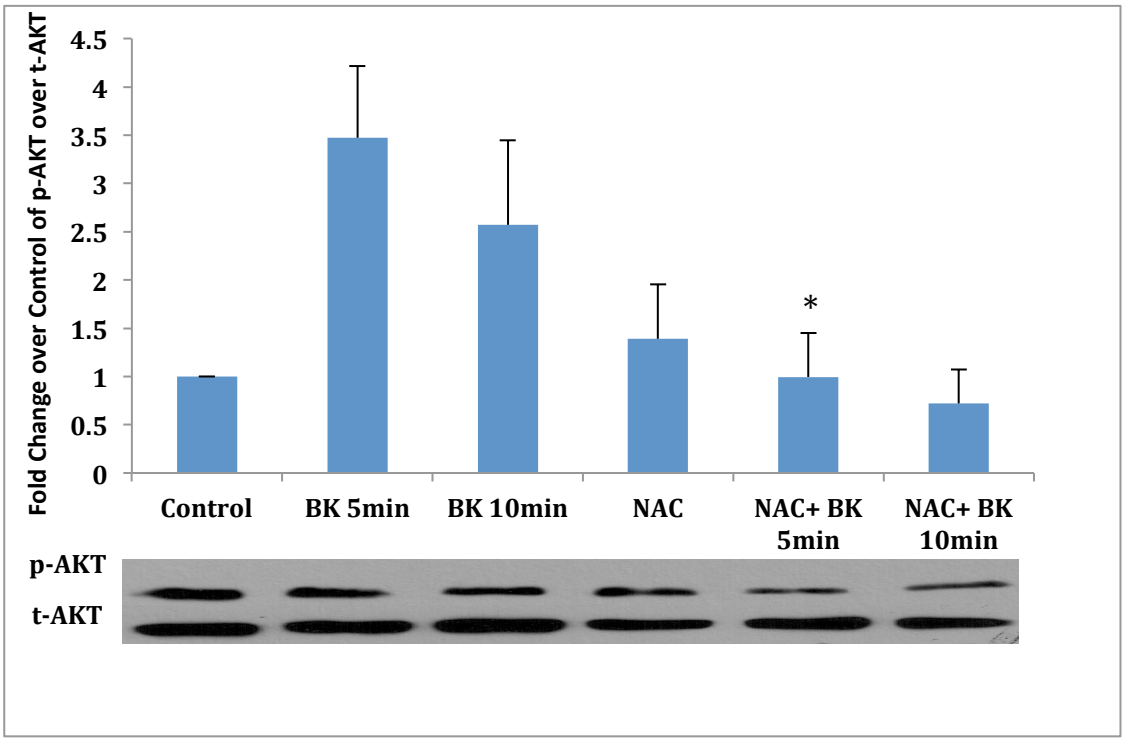


Figure 9. Bradykinin Activates AKT While NAC Inhibits it. Bradykinin induces the phosphorylation of AKT after 5 and 10 minutes while NAC inhibits its activation after 5 minutes (* NAC +BK vs. BK, $p<0.05$). Bar-Graph is the values of the densitometric analysis of the ratios of phospho AKT to total AKT of the western blots. The data is mean ± SE of 3 separate experiments.

D. Regulation of CTGF Gene Expression by Bradykinin

Since CTGF expression is involved in fibrotic diseases, we tested whether bradykinin affects CTGF gene expression. Figure 10 shows bradykinin inducing the transcript level of CTGF after 2 hours (2.36 ± 0.33 folds, * $p<0.05$).

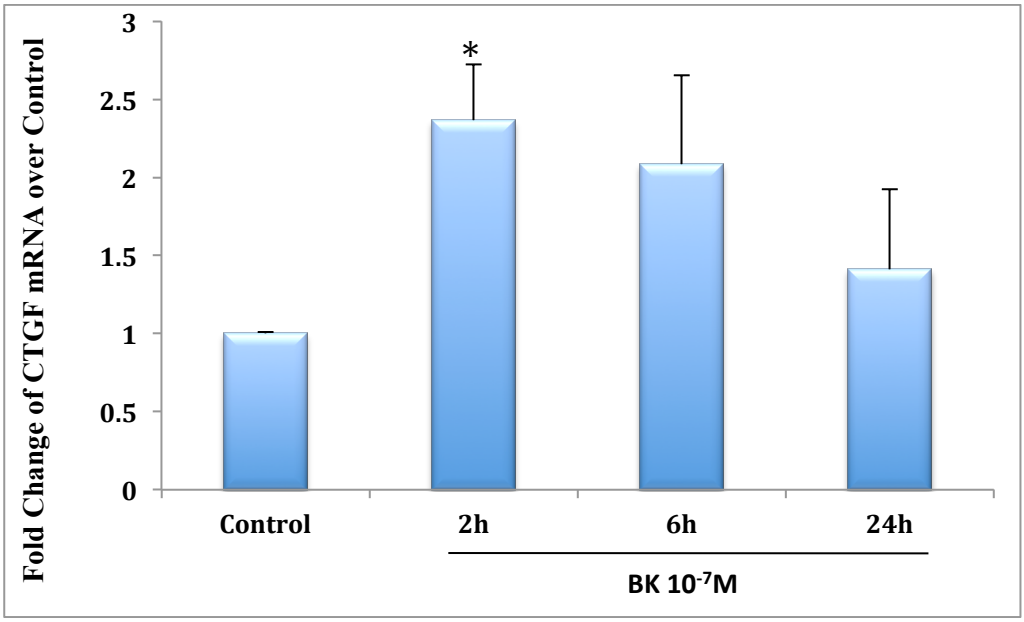


Figure 10. Induction of CTGF Transcript by Bradykinin. VSMCs were treated with bradykinin 0.1 μM for 2, 6 and 24 hours. RNA was extracted, and qRT-PCR was performed using rat CTGF-specific primers (n=3). Bar graph. Mean ± SE. *P<0.05 vs control.

E. Regulation of Fibronectin Gene Expression by Bradykinin

We first tested the effect of bradykinin on the mRNA expression of fibronectin after 2, 6 and 24 hours of stimulation. Figure 11 shows that bradykinin induces the gene expression of Fibronectin (3.4 ± 0.1 folds after 24 hrs, **p< 0.01).

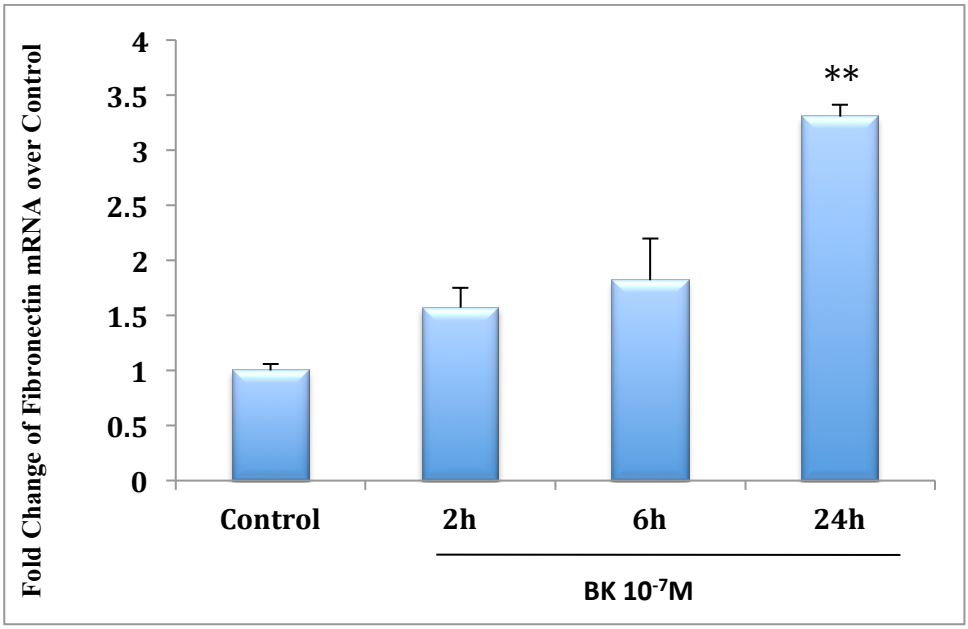


Figure 11. Induction of Fibronectin Transcript by Bradykinin. VSMCs were treated with Bradykinin 0.1 µM for 2, 6 and 24 hours. RNA was extracted, and qRT-PCR was performed using rat Fibronectin-specific primers (n=3). Bar graph. Mean ± SE. **P<0.01 vs control.

F. Regulation of Sphk1 Gene Expression by Bradykinin

It has been shown by Jaffa *et al* that Sphk1 is one of the activated enzymes by the agonist of bradykinin receptor in the mesengeal cells of the kidney. We next assessed whether BK can modulate the mRNA expression of SPK1 gene in the rat aortic smooth muscle cells. Figure 12 shows that bradykinin induces the expression of Sphk1 after 2 hours (3.1 ± 0.5 folds, ** p<0.01), 6 hours (3 ± 0.23 folds, ** p<0.01), and 24 hours (1.8 ± 0.4 folds, * p<0.05). This is quite novel finding in vascular smooth muscle cells under the effect of bradykinin and it suggests that there is a link between bradykinin and S1P receptors via increased activation of SphK1.

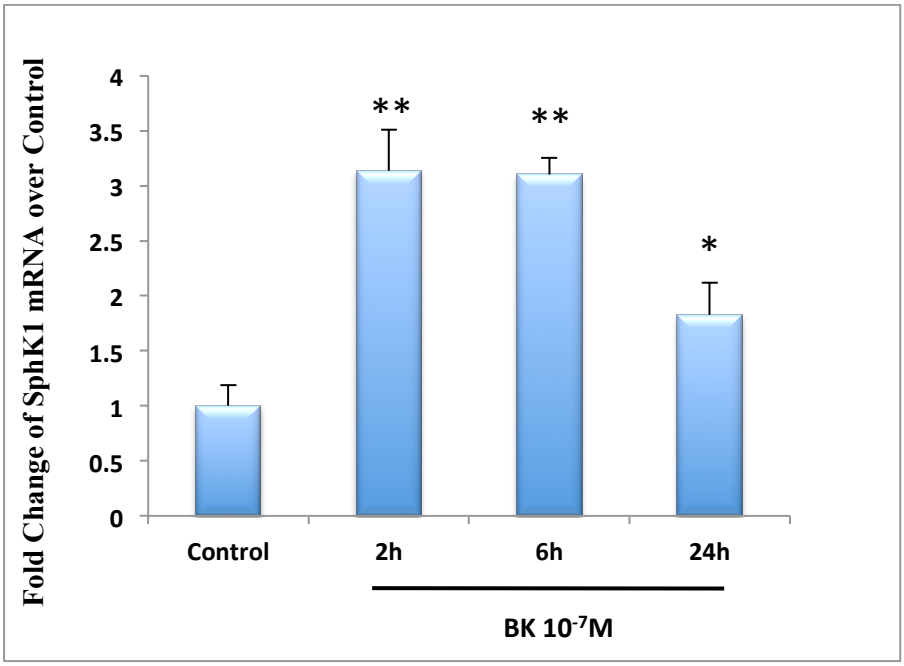


Figure 12. Regulation of SphK1 Gene Expression by Bradykinin. VSMCs were treated with bradykinin 10^{-7}M for 2, 6 and 24 hours. RNA was extracted, and qRT-PCR was performed using rat SphK1-specific primers (n=3). Bar graph, Mean ± SE. **P<0.01 vs control, **P<0.01 vs control, *P<0.05 vs control.

G. Effect of NAC on Bradykinin-induced Gene Expression of CTGF, Fibronectin and SphK1

Next we tested whether generation of ROS plays a role in bradykinin-induced expression of CTGF, fibronectin and SphK1. In this regard, we examined whether inhibition of ROS by NAC will module the bradykinin response as shown in Figure 13, 14 and 15.

In Figure 13, CTGF transcripts were induced significantly by bradykinin after 24 hours (1.73 ± 0.11, *p=0.05) while were not by NAC. A similar pattern was observed in figure 14 and 15 for fibronectin and SphK1 enzyme after 6 hours (3.40 ± 0.21, *p<0.05), respectively. This is a novel finding and suggests that ROS has a direct or indirect effect on mRNA expression of CTGF, fibronectin and SphK1 by bradykinin.

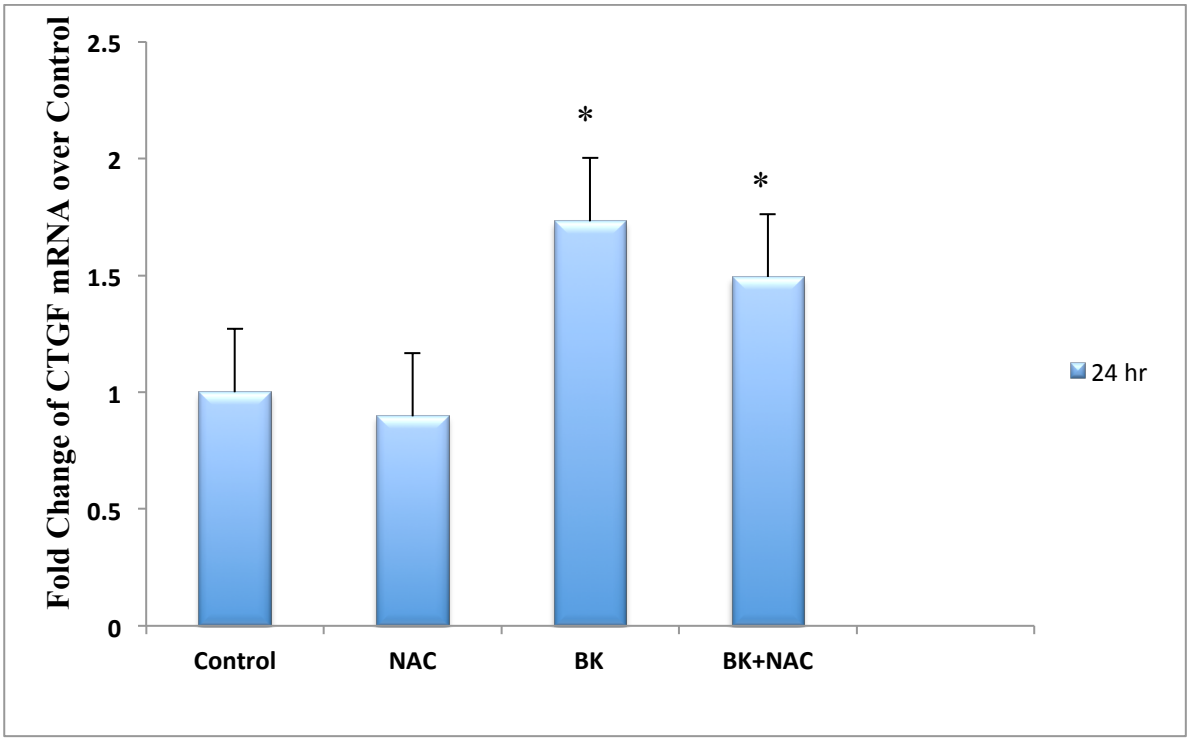


Figure 13. The Effect of NAC on CTGF Transcript Expression by Bradykinin. RASMCs were treated with BK 10^{-7}M for 24 (*BK vs. Control, $p<0.05$) hours and inhibited by NAC for 30 minutes prior stimulation with bradykinin (*BK+ NAC vs. Control, $p<0.05$). RNA was extracted, and qRT-PCR was performed using rat CTGF-specific primers (n=3). Bar graph, Mean ± SE.

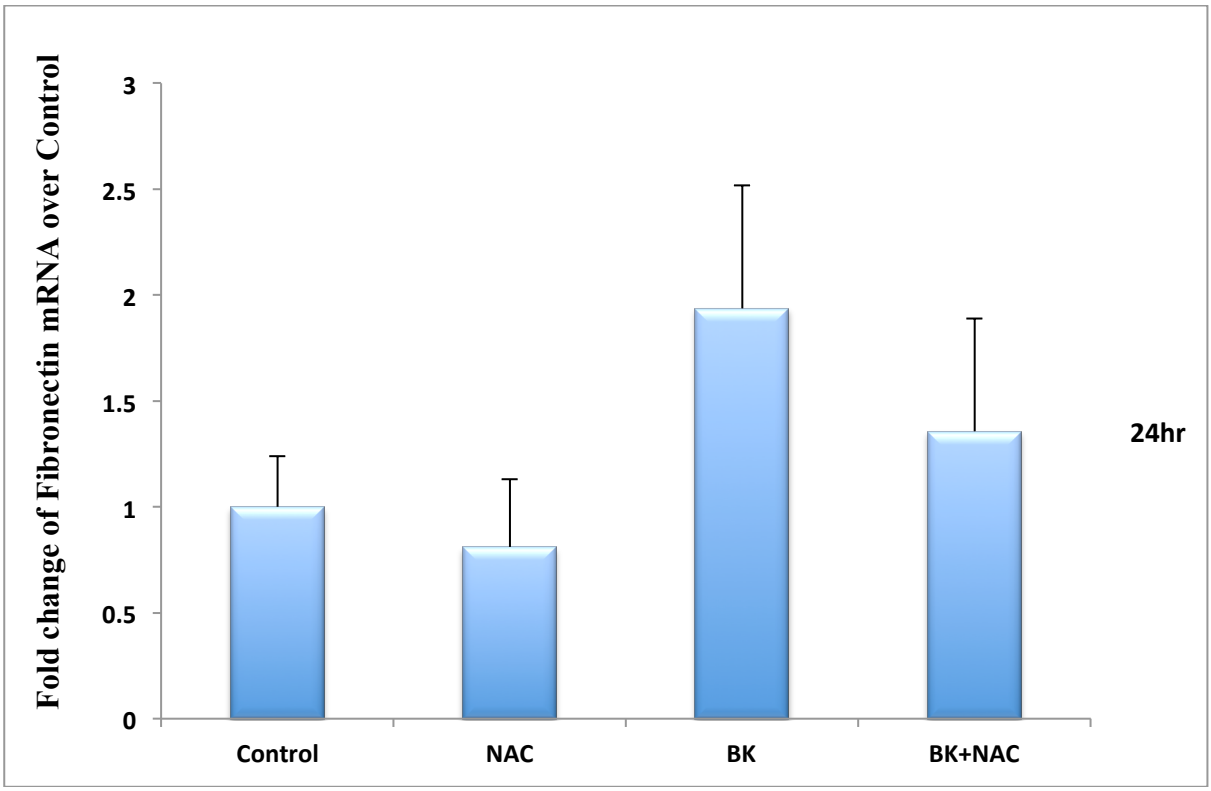


Figure 14. The Effect of NAC on Fibronectin Transcript Expression by Bradykinin. RASMCs were treated with $BK10^{-7}M$ for 24 hours and inhibited by NAC for 30 minutes prior to stimulation with bradykinin. RNA was extracted, and qRT-PCR was performed using rat fibronectin-specific primers (n=3). Bar graph, Mean ± SE.

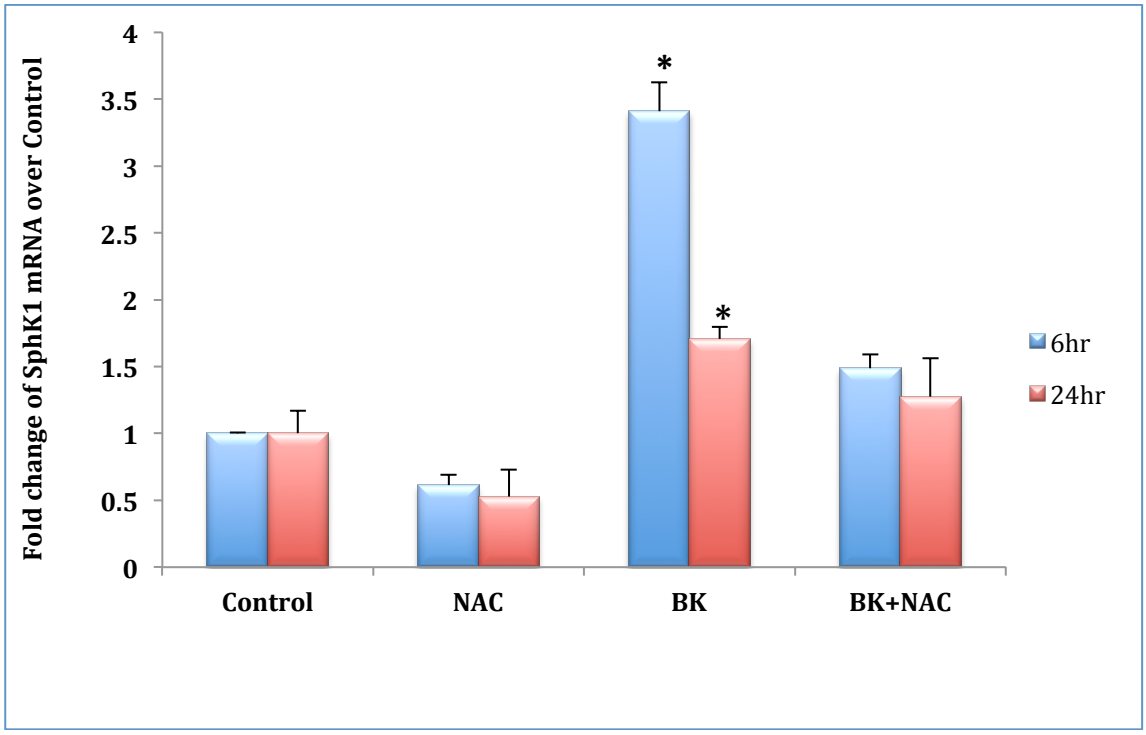
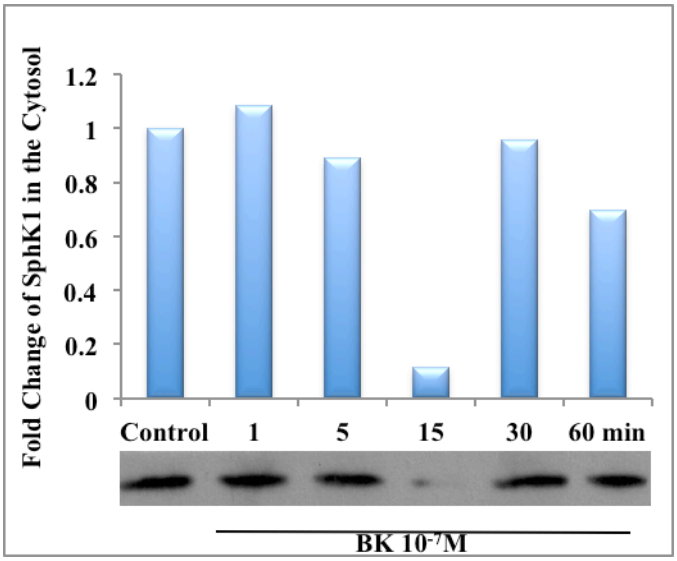


Figure 15. The Effect of NAC on SphK1 Transcript Expression by Bradykinin. RASMCs were treated with bradykinin 10^{-7}M for 6 and 24 (*BK vs Control, $p<0.05$) hours and inhibited by NAC for 30 minutes prior stimulation with bradykinin. RNA was extracted, and qRT-PCR was performed using rat SphK1-specific primers (n=3). Bar graph. Mean ± SE.

H. Bradykinin and S1PRs Crosstalk: Activation of Sphk1.

We then checked the activity of Sphk1 due to bradykinin. SphK1 translocates from the cytosol to the membrane of cells when it is activated by phosphorylation. Membrane bound proteins were extracted and we compared the localization of Sphk1 in the cytosol and membrane as sign of activation of this protein. Figure 16 shows that SphK1 protein is expressed in constant amounts in the cytosol where it is localized naturally, while it translocated to the membrane after 15 minutes of stimulation by bradykinin.



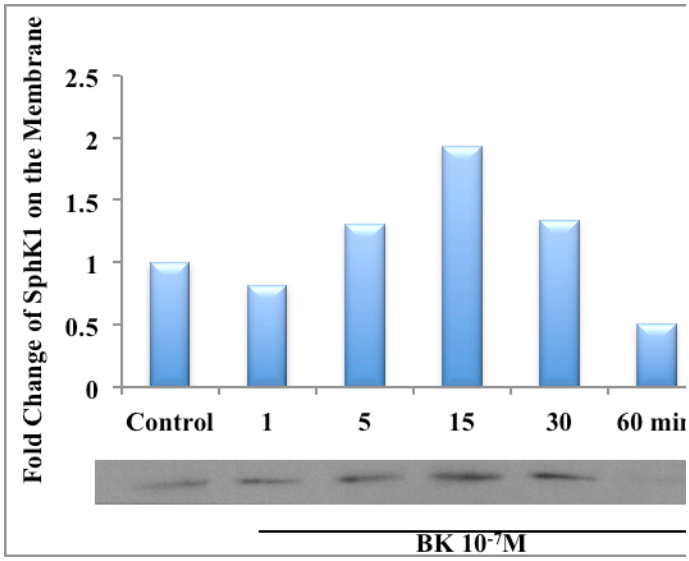


Figure 16. Translocation of Sphk1 Protein from the Cytosol to the Membrane. RASMCswere stimulated by bradykinin 0.1 µM for different time points (1, 5, 15, 30 and 60 minutes). Cells were lysed and cytosolic and membrane-bound proteins were fractioned by ultracentrifugation. Bradykinin activates sphk1 that translocate from the cytosol to the membrane. Bar-graphs are the values of the densitometric analysis of the ratios of total SphK1of the western blots. Results are representative of 1experiment.

I. Regulation of Sphk2 Gene Expression by Bradykinin.

To check the effect of bradykinin on the gene expression of Sphk2, we assessed the transcript levels after 2, 6 and 24 hours stimulation by the agonist. Figure 17 shows that bradykinin induces the expression of Sphk2 (2.2 ± 0.3 folds after 2 hrs, * $p<0.05$, and 2 ± 0.28 folds after 6 hrs, * $p<0.05$).

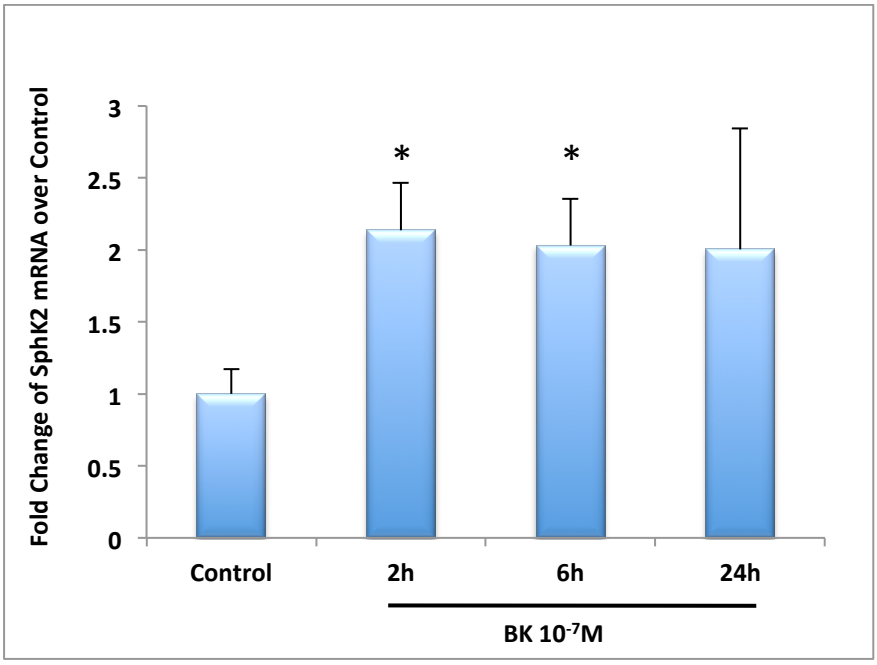


Figure 17. Regulation of SphK2 Gene Expression by Bradykinin. RASMCs were treated with bradykinin 0.1 μM for 2, 6 and 24 hours. RNA was extracted, and qRT-PCR was performed using rat SphK2-specific primers (n=3). Bar graph, Mean ± SE. *P<0.05 vs control.

J. Basal Expression of S1P Receptors

First, we investigated the expression of S1P receptors in the RASMC to check which are the dominating ones. Figure 18 shows that the three isoforms (S1PR 1, 2, and 3) are evenly expressed as assessed by qRT-PCR.

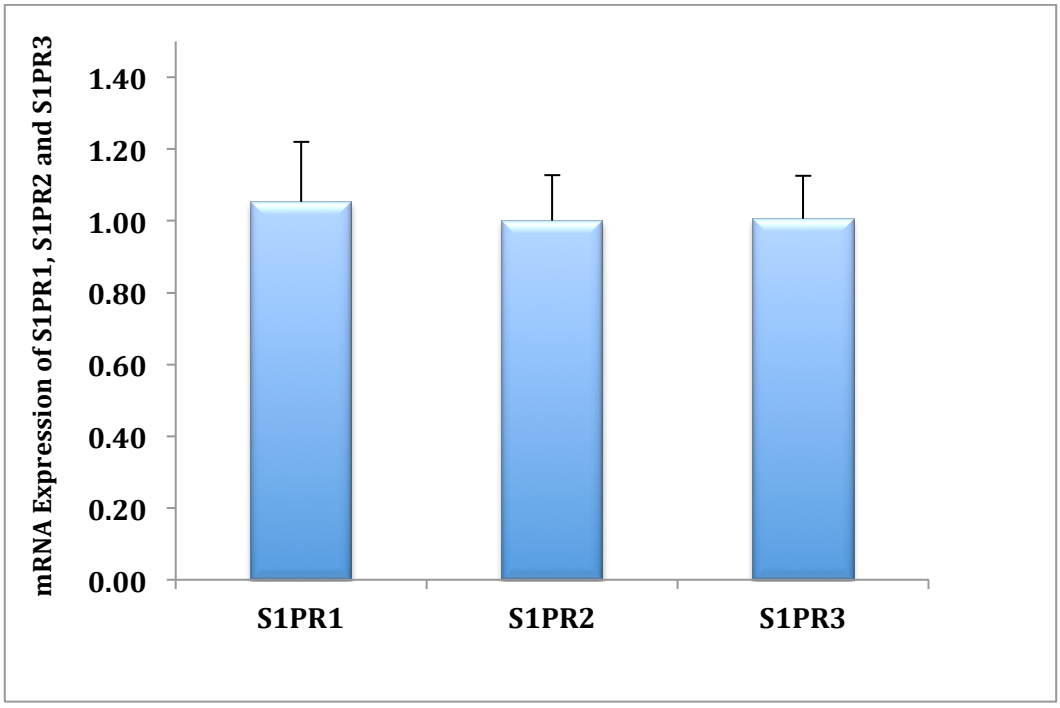
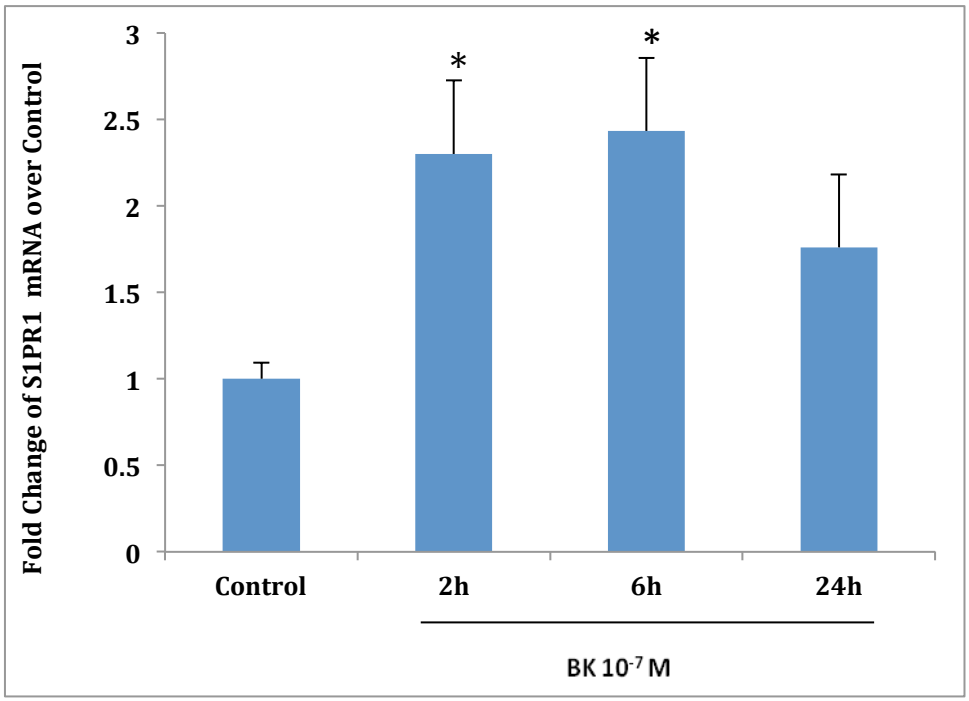


Figure 18. Basal Expression of the S1P Receptors in RASMC. RNA from untreated cells was extracted and qRT-PCR was performed for the basal expression of the three receptors. n=4

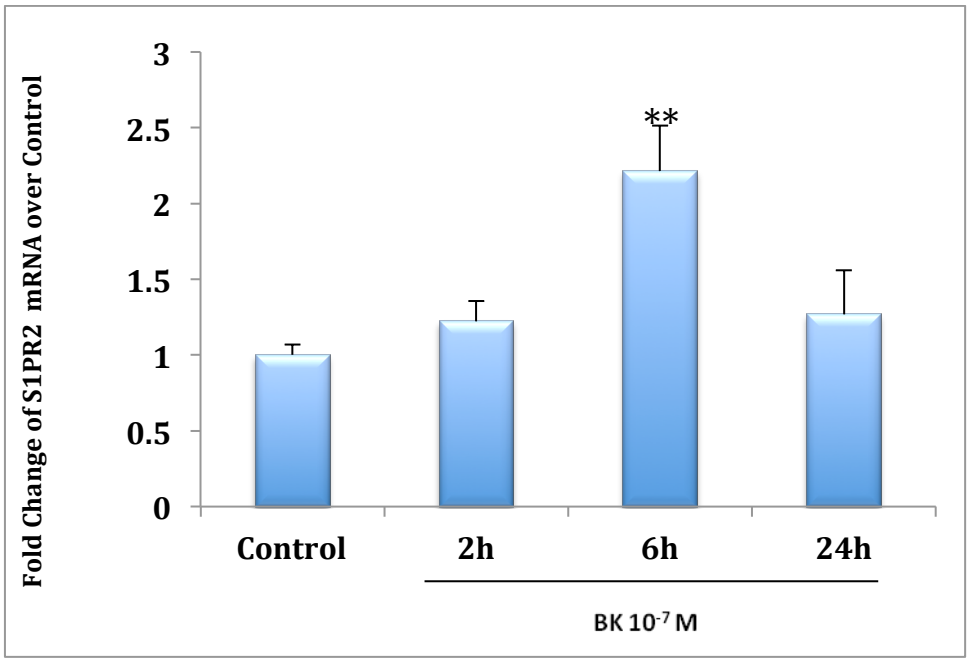
K. S1PR1, S1PR2 and S1PR3 Gene Expression Due to Bradykinin

Next we assessed the effect of bradykinin on the expression of the three S1P receptor genes for 2, 6 and 24 hours. Figure 19 (A, B and C) shows that bradykinin induced the mRNA expression of the three receptors: S1PR1 (2.3 ± 0.4 folds after 2 hrs, * $p<0.05$), and (2.4 ± 0.4 folds after 6 hrs, *$p<0.05$), and (1.7 ± 0.2) folds after 24 hrs. S1PR2 (1.22 ± 0.1) folds after 2 hrs, and (2.2 ± 0.2,** $p<0.01$) after 6 hours and (1.2 ± 0.2) folds after 24 hrs. S1PR3 (1.83 ± 0.2 folds after 2 hrs, * $p<0.05$), (2.19 ± 0.2 folds after 6 hrs, **$p<0.01$), and (1.69 ± 0.4) folds after 24 hours. This new finding indicates that the genes of the S1PR1, S1PR2 and S1PR3 are significantly upregulated by bradykinin stimulation.

A.


Fold Change of S1PR1 mRNA over Control
3
2.5
2
1.5
1
0.5
0
*
*
Control
2h
6h
24h
BK 10-7 M

B.


Fold Change of S1PR2 mRNA over Control
3
2.5
2
1.5
1
0.5
0
**
Control
2h
6h
24h
BK 10-7 M

C.

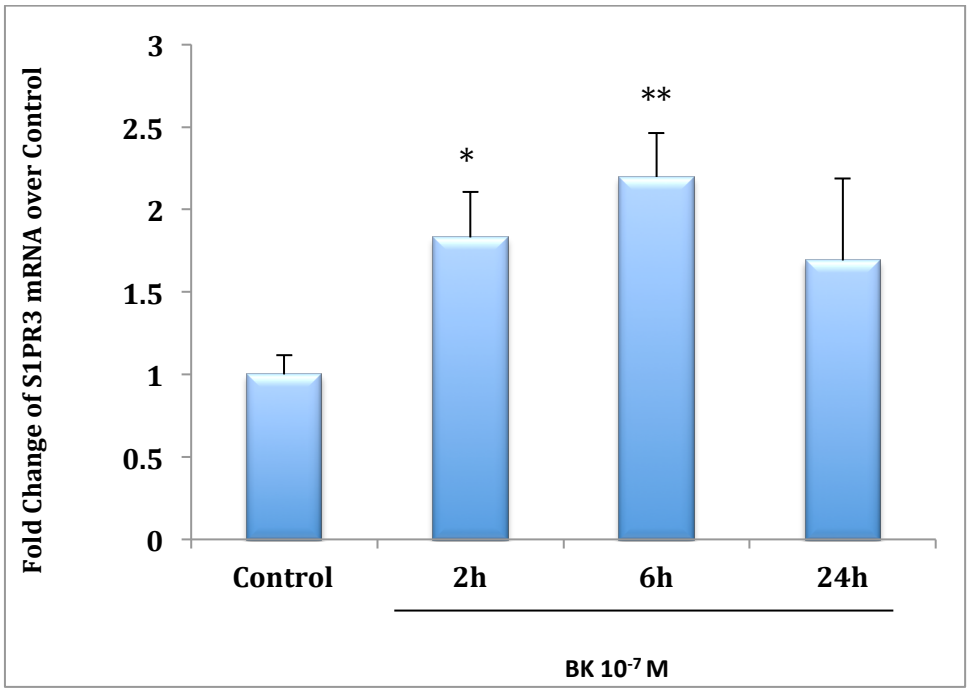


Figure 19. Bradykinin Induces the Expression of the different S1PRs on RASMC. A, Gene Expression of S1PR1 by Bradykinin. RASMCs were treated with bradykinin 0.1uM for 2, 6 and 24 hours. RNA was extracted, and qRT-PCR was performed using rat S1PR1-specific primers (n=4). Bar graph, mean ± SE. *P<0.05 vs. control. **B, Gene Expression of S1PR2 by Bradykinin.** RASMCs were treated with bradykinin 0.1 uM for 2, 6 and 24 hours. RNA was extracted, and RT-PCR was performed using rat S1PR2-specific primers (n=4). Bar graph, mean ± SE. **P<0.01 vs control. **C, Gene Expression of S1PR3 by Bradykinin.** RASMCs were treated with bradykinin 0.1uM for 2, 6 and 24 hours. RNA was extracted, and RT-PCR was performed using rat S1PR3-specific primers (n=4). Bar graph, mean ± SE. *P<0.05, **P<0.01 vs control.

L. Signaling Pathway Activation by S1P

Evaluating the activation of ERK1/2 and AKT assessed the effect of S1P on intracellular signaling pathways. Figures 20 and 21 show that S1P induces the phosphorylation of ERK1/2

(Thr 202/Tyr 204) (5.72 ± 0.71, *p< 0.05) and AKT (Thr 308) (2.87 ± 0.21, *p< 0.05) after 3 minutes of S1P incubation.

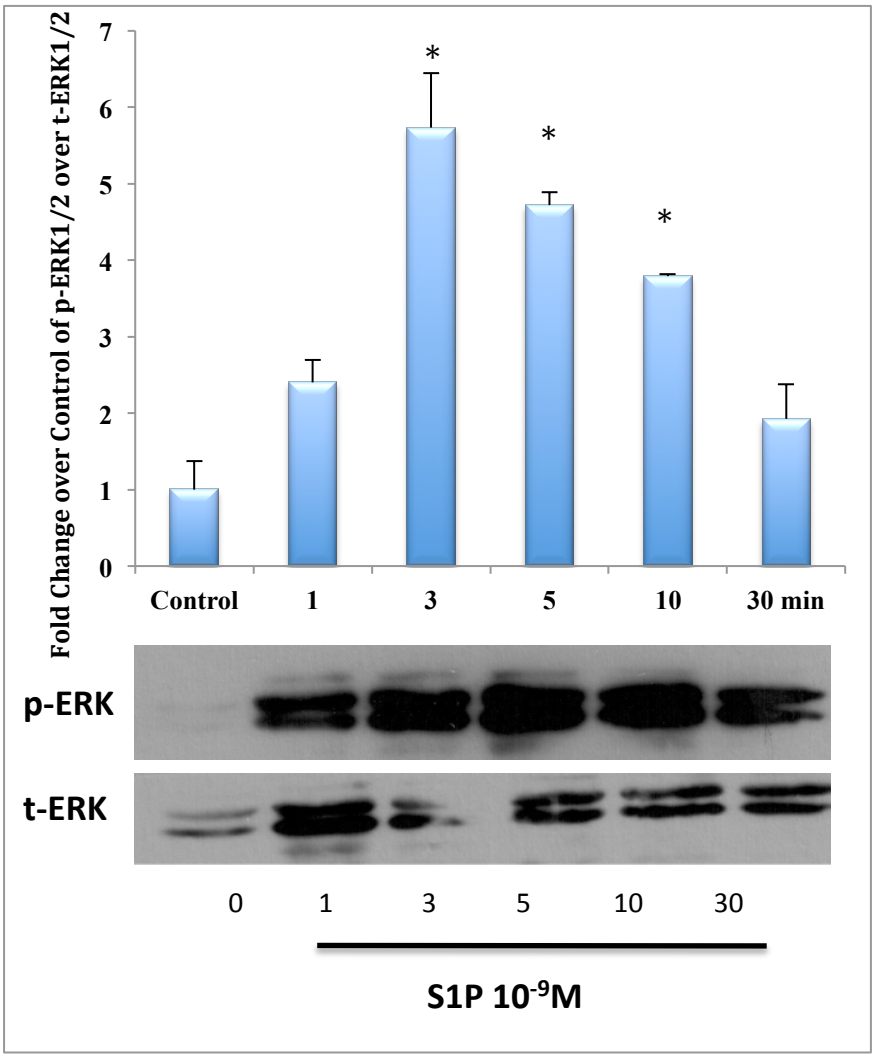


Figure 20. S1P Activates ERK 1/2. S1P 10^{-9} M induces the phosphorylation of ERK1/2 after 3 minutes (* S1P vs. control, $p<0.05$), and returned to its basal level after 30 minutes. Bar-graph is the value of the densitometric analysis of the ratios of phospho ERK to total ERK of the western blots. The data is mean ± SE of 3 separate experiments.

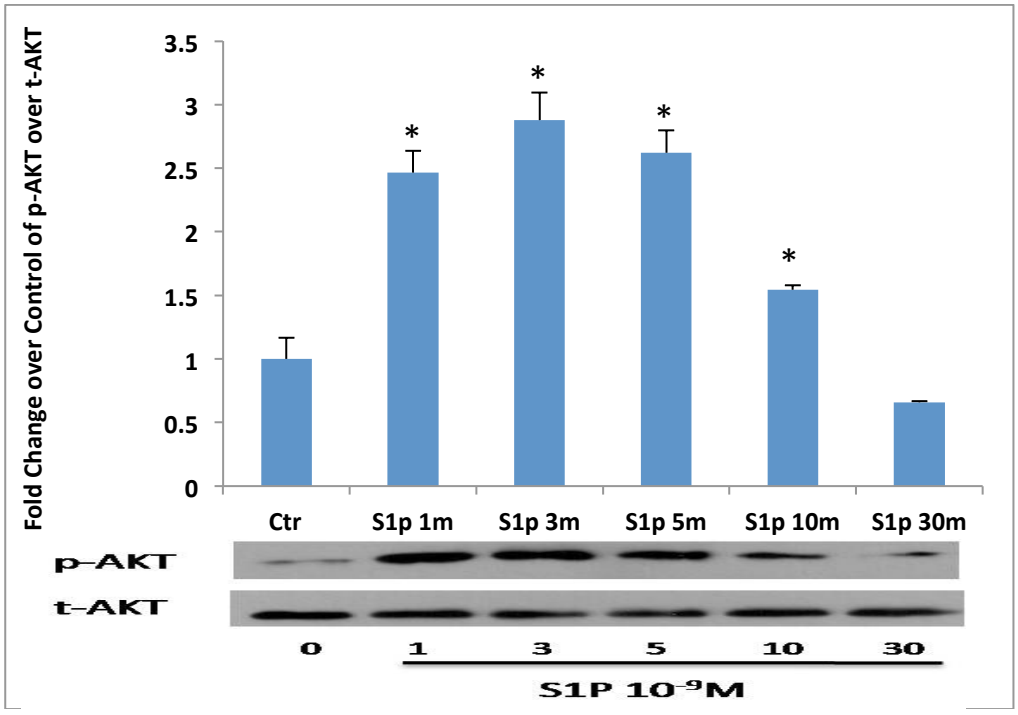


Figure 21. S1P Activates AKT. S1P 10^{-9}M induced the phosphorylation of AKT as early as 1 minute (* S1P vs. control, $p<0.05$), and returned to its basal level after 30 minutes. Bar-graph is the value of the densitometric analysis of the ratios of phospho AKT to total AKT of the western blots. The data is mean ± SE of 3 separate experiments.

M. ROS Generation by SEW2871

We also assessed the effect of SEW2871, which is the specific agonist of S1PR1. Figure 22 shows that SEW2871 did not contribute to ROS generation. Moreover, SEW2871 decreased the rate of ROS generation below the basal level (Figure 23).

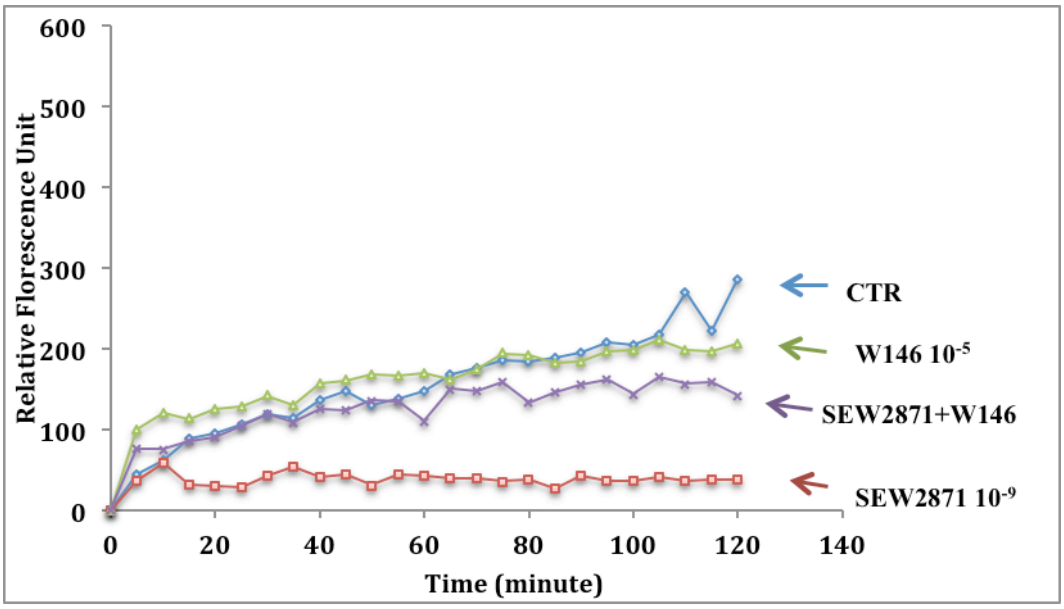


Figure 22: Effect of SEW2871 on ROS Generation.SEW2871 10^{-9}M, W146 10^{-5}M and their combination did not generate ROS in RASMC compared to the basal ROS levels.

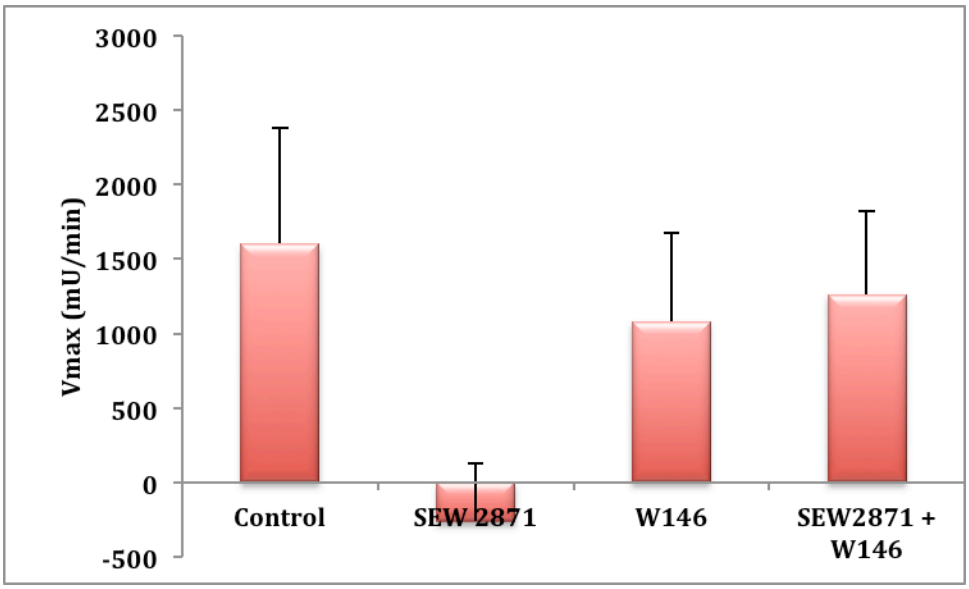


Figure 23.Effect of SEW2871 on the Rate of Enzymatic ROS Generation.SEW2871 did not induce the Vmax of ROS generation in RASMC compared to the basal ROS levels. W146 10^{-5}M also did not have any effect on the Vmax of ROS generation compared to the basal level. This result is representative of 6 similar experiments.

N. Regulation of CTGF Gene Expression by SEW2871

To discover if S1P receptor is involved in fibrotic diseases, we tested S1P receptor agonist effect in CTGF gene expression. As we see in Figure 24, SEW2871 did not affect the mRNA levels of CTGF gene.

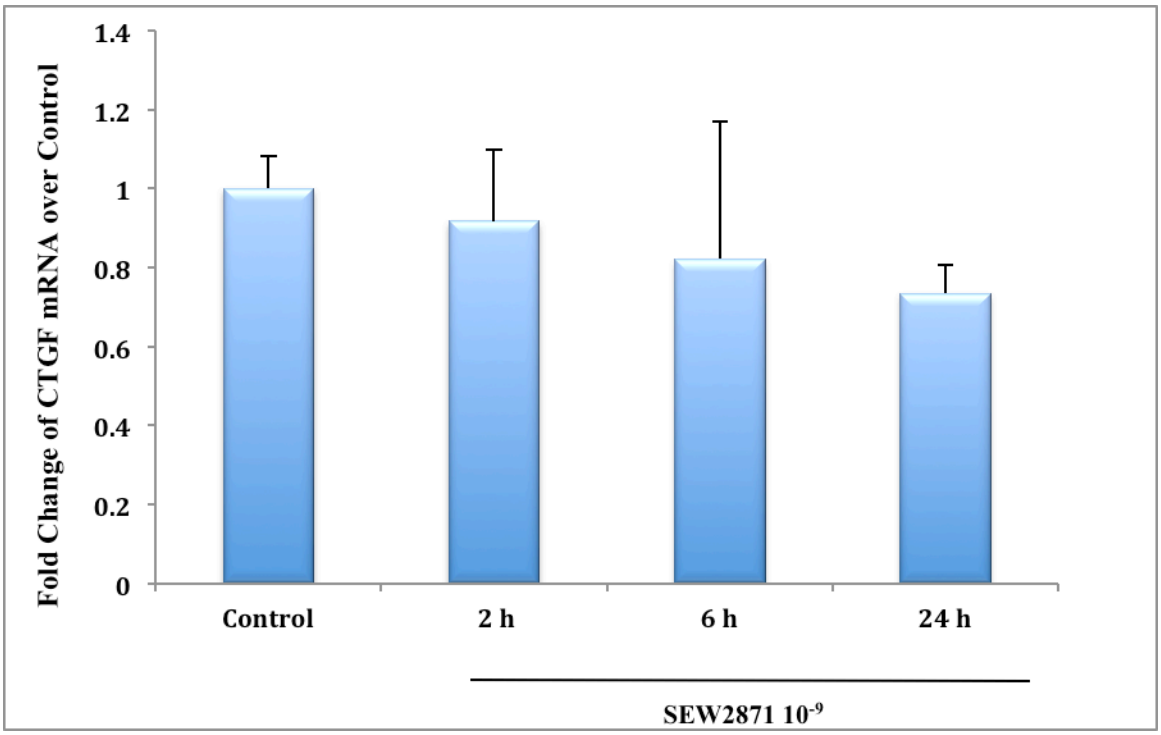


Figure 24. Regulation of CTGF Gene Expression by SEW2871. RASMCs were treated with SEW2871 1nM for 2, 6 and 24 hours. RNA was extracted, and RT-PCR was performed using rat CTGF-specific primers (n=3). Bar graph, mean ± SE.

O. Regulation of Fibronectin Gene Expression by SEW2871

Fibronectin is one of the ECM proteins that is upregulated in fibrotic diseases. To detect if fibronectin gene transcript was modulated by SEW2871, we performed qRT-PCR. As we see in Figure 25, SEW2871 did not affect the transcript level of fibronectin.

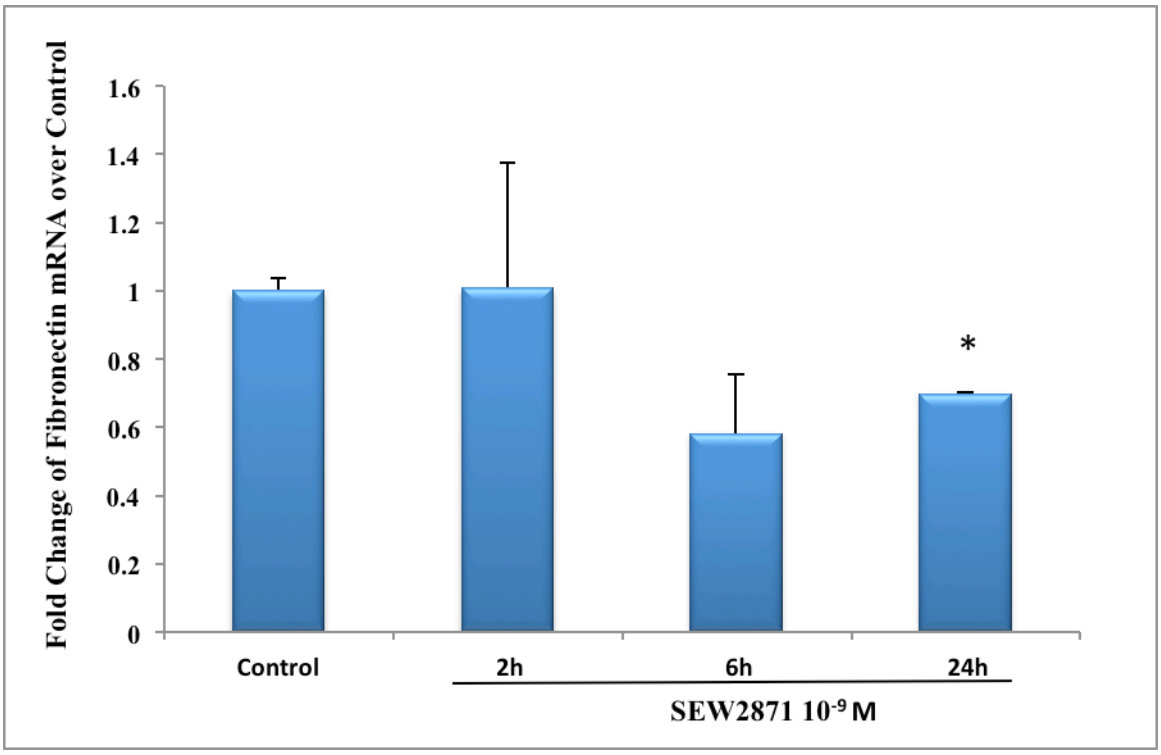


Figure 25. Regulation of Fibronectin Gene Expression by SEW2871. RASMCs were treated with SEW2871 1nM for 2, 6 and 24 hours. RNA was extracted, and RT-PCR was performed using rat fibronectin-specific primers (n=3). Bar graph, mean ± SE.*p<0.05 vs control.

P. Regulation of Fibronectin Protein Expression by S1P and SEW2871

Then we tested the effect of S1P or S1PR1 agonist, SEW2871, on the protein expression of fibronectin after 6 and 24 hours of stimulation. Figure 26 shows that S1P induced fibronectn protein expression (2 ± 0.0837 folds, ** p<0.01), and remained for 24 hours (1.754 ± 0.395 folds, * p<0.05) above the basal levels. Moreover, Figure 27 shows that SEW2871 induced fibronectin protein expression (2.39 ± 0.24 folds after 6 hrs, *p<0.05 and 1.423 ± 0.0315 folds after 24 hrs, ** p<0.01) above the basal levels.

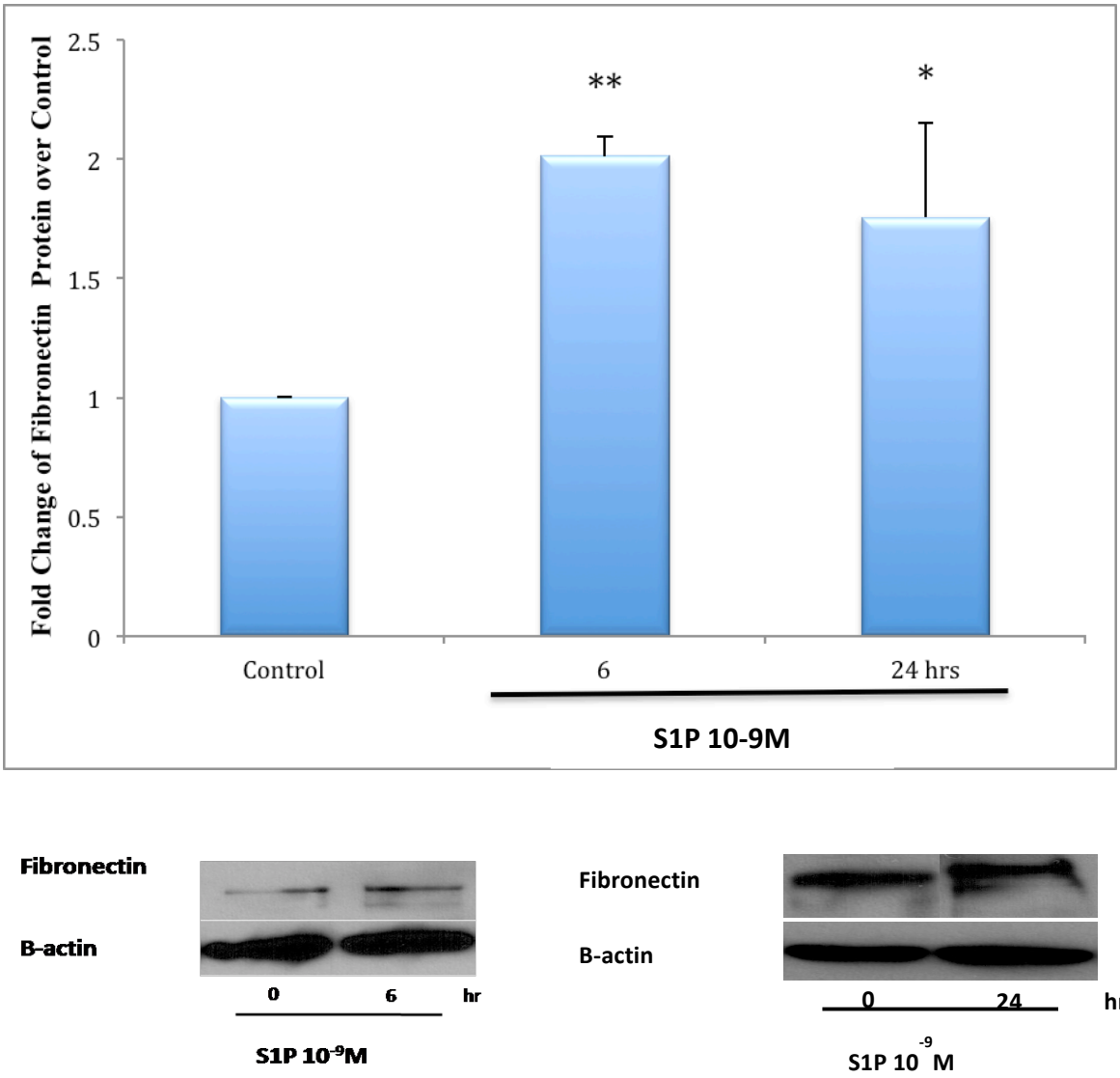


Figure 26. S1P Induces Fibronectin Protein Expression After 6 and 24 Hours.RASMCs were treated with S1P 1nM for 6 and 24 hours. Fibronectin protein was measured using western blot analysis (n=3). Bar graph is the value of the densitometric analysis of the intensities of bands of western blots. Mean± SE *p<0.05 vs control,**p<0.01 vs control.

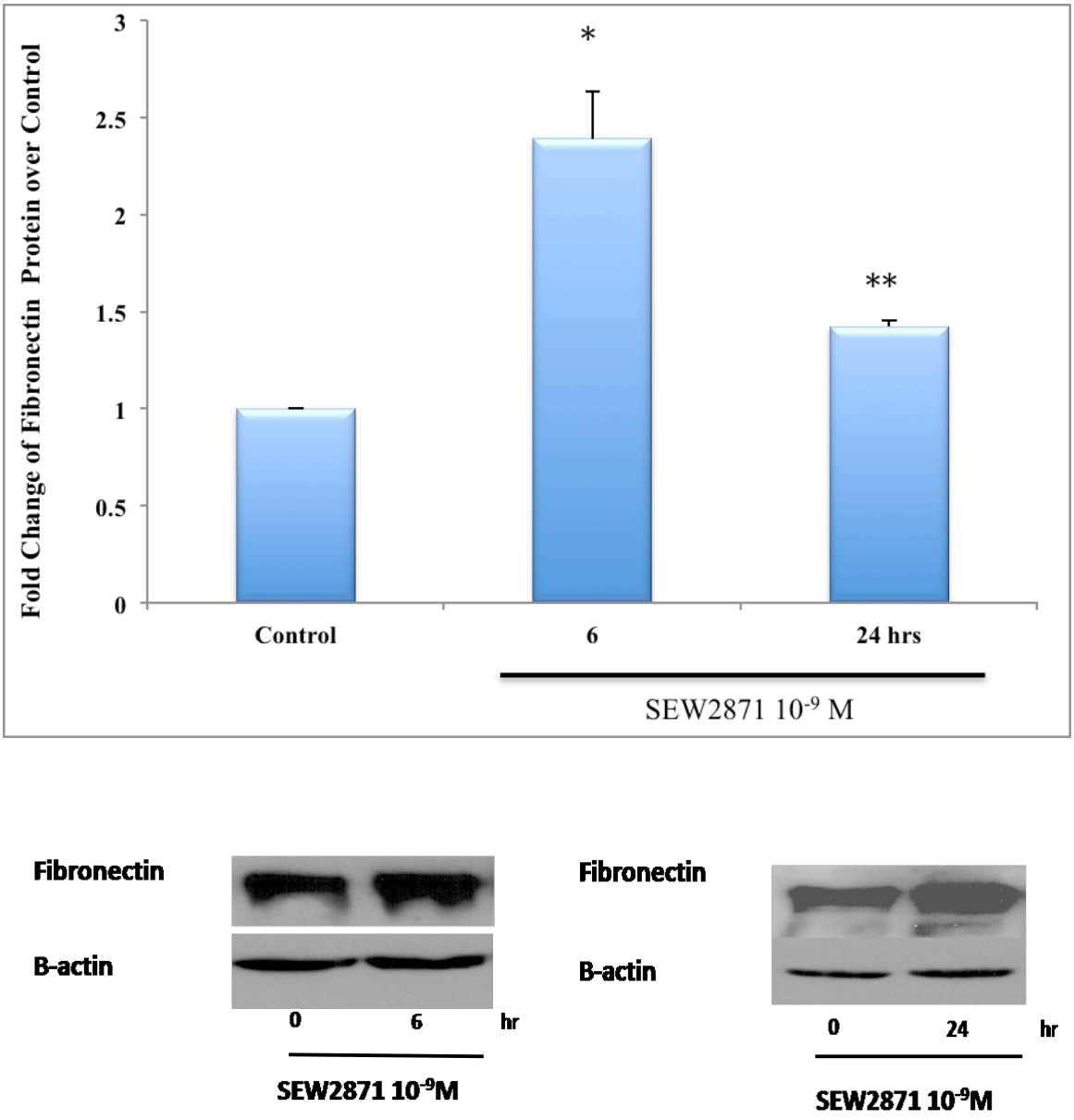


Figure 27. SEW2871 Induces Fibronectin Protein Expression After 6 and 24 Hours. RASMCs were stimulated with SEW2871 1nM for 6 and 24 hours. Fibronectin protein was measured using western blot analysis (n=3). Bar graph is the value of the densitometric analysis of the intensities of bands of western blots. Mean± SE. *P<0.05 vs control, **P<0.01 vs control.

CHAPTER IV

DISCUSSION

Although there are many role players in atherosclerosis, the factors and mechanisms that cause vascular remodeling leading to lesion formation are not yet completely defined. Bradykinin has a vasodilator effect on the blood vessel walls in normal physiological conditions; however, during pathophysiological conditions when the endothelium of the blood vessels is damaged, it could act directly on the smooth muscle cells leading to oxidative stress.

Since S1P receptors are ubiquitously distributed on different types of tissues including vascular smooth muscle cells, and because S1P is known as an atherogenic sphingolipid, we hypothesized that it could have a role in atherosclerosis via crosstalk withB2R.

Before going further in our experiments we made sure that the three receptors S1PR1, S1PR2, and S1PR3 are evenly expressed on the rat aortic smooth muscle cells (RASMC) via qRT-PCR (Figure 18).

In this study we shed light on many areas related to the two receptors of interest B2R and S1PRs specifically S1PR1. In pathological conditions like atherosclerosis, BK induces ROS generation. Han *et. al.* stated that ROS generated inhibits the Nitric Oxide that is important for vascular integrity (84). Through DCF technique we confirmed that ROS generation is induced significantly by BK above basal levels and ROS generation is inhibited by NAC, which is a reducing agent and a scavenger of ROS, and by the specific B2R antagonist HOE140. The role of the SEW2871, a specific agonist of S1PR1, was also tested on ROS

generation and there was a confirmation that neither SEW2871 nor its specific antagonist W146 contribute in ROS generation. This suggests that S1P can have a negative feedback on ROS generation by BK and it can open a new window for further investigation. It has been stated by Gong *et. al.* that ROS activates ERK1/2 **(85)**. Western blot analysis of proteins extracted from bradykinin-treated cells at different time points show that BK significantly activates ERK1/2 and AKT at 10 minutes after stimulation. To verify that ROS is upstream of ERK1/2, we treated the cells with NAC 30 minutes prior to stimulating them with BK and we used western blot analysis to check the expression of ERK1/2 and AKT. As a result, we have seen a significant decrease in the expression of ERK1/2 and AKT after 5 minutes suggesting that ROS activates the MAPK and PI3K by elevating their end products ERK1/2 and AKT. Activated ERK1/2 in turn phosphorylates, and subsequently activates SphK1 that translocates from the cytosol to the membrane upon activation **(86)**. Through membrane and cytosol protein extraction via ultracentrifugation, we checked the levels of SphK1 in the cytosol and membrane at different time points using western blot analysis after treating cells with BK. What we found is that SphK1 expression was constant in the cytosol at different time points except at 15 minutes when there was a decrease in SphK1 levels in the cytosol and an increase in the membrane.

It has been also shown by qRT-PCR that BK induces SphK1and 2, CTGF and Fn gene expression after 2, 6 and 24 of BK treatment. Cells treated with NAC prior to treating with BK decreased the gene expression of SphK1, CTGF and Fn suggesting that their expression is originally related to ROS generation and probably activation of ERK1/2. This novel data that was shown in this research can give way to further studies to show the ROS effect on the crosstalk between B2R and S1PRs.

In this study, we also checked the gene expression modification of the three S1P receptors by BK. Our results show that S1PR1, S1PR2, and S1PR3 transcript expression was increased by two folds and reached the peak at 6 hours when stimulated by BK (Figure 19).

It has been stated that when SphK1 is activated, it translocates to the membrane where it phosphorylates sphingosine molecules and produces S1P.In turn, S1P acts in a paracrine or autocrine fashion on various S1PRs and activates downstream signaling pathways **(87)**.

Our results show that S1P activates MAPK and PI3K pathways by increasing their end products ERK1/2 and AKT after 3 minutes of S1P stimulation and then they go back to the basal levels.Whether SphK1 and SphK2 genes were expressed by SEW2871 stimulation was another question that was answered in this project. What we found was that SEW2871 induces SphK1 gene expression almost two folds comparing to their basal levels while it does not affect the SphK2 gene expression.

Due to the literature, CTGF intracellular and Fn matrix mRNA and proteins elevated levels were correlated with fibrotic diseases. To find the role of S1P in atherosclerosis, we tried to see its effect on the mRNA levels of CTGF and Fn. Jaffa *et al* stated thatCTGF is increased in pathological conditions that contribute to extracellular matrix production and fibrosis. CTGF also controls the level of other ECM proteins like Fn, collagen I and Laminin **(88)**. Because CTGF and Fn are increased in fibrotic diseases, we assessed CTGF gene expression by qRT-PCR and we found that there is no gene expression modification of CTGF and Fn by SEW2871 stimulation. However, this was not in parallel to the protein expression, since S1P induced the protein expression of Fn (Figure 26). Furthermore, we tried to find out if S1PR1 contributes to the Fn production, and we found that there was a significant increase in its production when cells were treated with the S1PR1 specific agonist SEW2871 (Figure 27).

Based on these results, we proposed that S1PR1 contributes in Fn protein expression modification in RASMC. Because Fn induces extra cellular matrix production and its detection was associated with fibrotic diseases like atherosclerosis, hence we verified that S1P contributes to atherosclerosis by inducing Fn production especially via S1PR1.

The results of this study show that ROS generation by BK has its effect on VSMC remodeling in atherosclerosis via generation of ROS and activation of ERK1/2-MAPK. Besides, it is obvious that there is a crosstalk between BK receptor and S1PRs in RASMC model, which leads to vascular remodeling by activation of signaling pathways and increasing CTGF and Fn production.

Limitations

As any other research, many limitations have faced this research. The cell type that is used in this study is an *in vitro* model of atherosclerosis with RASMCs. The major limitation *in vitro* model is that it is not true representative of tissues and organs in humans. Most of the *in vitro* models are two-dimensional compared to real three-dimensional architecture of tissues and organs. Plus almost all *in vitro* models lack interactions with blood vessel contents which is one of the main interacting tissue with most of the cells.

In this project, we were limited to the usage of pharmacologically available antagonists that should be replaced by siRNA.

Since there are three S1P receptors expressed in RASMCs, there is a further diversity of the effects of these receptors, which is limited to the lack of specific agonist or antagonist to the S1PR2 and 3, and limited us to the use of the available S1PR1 agonist and antagonist.

Moreover, one of the available primary antibodies, total SphK1 was not that sensitive to detect the SphK1 protein that confined us to only n=1 of experiments.

Perspectives of the study

The results generated in this study open windows for many experiments to be further done. Downstream or cross inhibition of BK or S1P receptors would allow us to generate a detailed pathway of activation and cross-talk between these receptors. On the other hand, generating a Fluorescent-protein-tagged Sphk1 protein and overexpressing this fusion protein in RASMC will ease tracing the localization of this protein intracellularly, since Sphk1 antibodies present, and from different companies, did not give a good western blot results.

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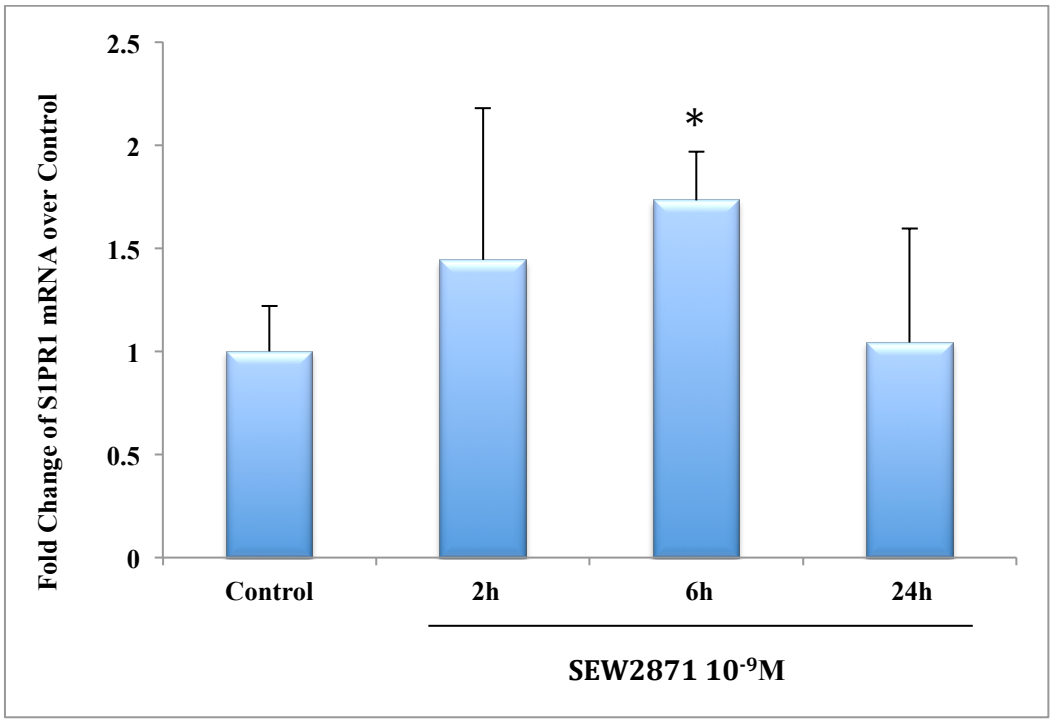
APPENDIX

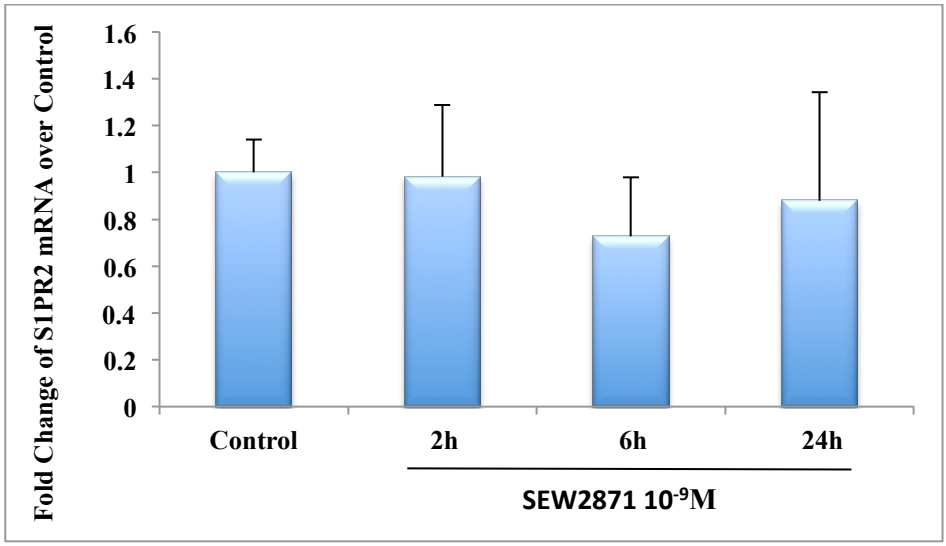
A. S1PR1, S1PR 2 and S1PR3 Gene Expression by SEW2871

We also assessed the induction of the three receptors due to SEW2871 treatment for 2, 6 and 24 hours. Since SEW2871 is the specific agonist of S1PR1, then normally it should induce only the expression of S1PR1 as it was detected by qRT-PCR. The other receptors S1PR2 and S1PR3 were not affected by SEW2871.

A.



B.



C.

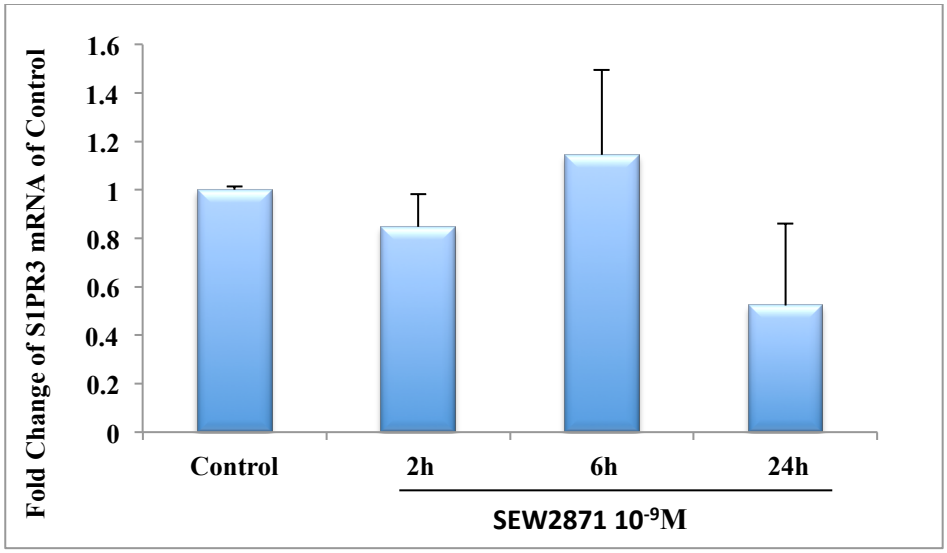


Figure 28. The Effect of SEW2871 on the Gene Expression of S1PRs on RASMC. A, Gene Expression of S1PR1 by SEW2871 1nM.RASMCs were treated with SEW2871 1nM for 2, 6 and 24 hours. RNA was extracted, and RT-PCR was performed using rat S1PR1-specific primers (n=3). Bar graph, mean ± SE.*P<0.05 vs control. **B, Gene Expression of S1PR2 by SEW2871 1nM**. RASMCs were treated with SEW2871 1nm for 2, 6 and 24 hours. RNA was extracted, and RT-PCR was performed using rat S1PR2-specific primers (n=3). Bar graph, mean ± SE.**C, Gene Expression of S1PR3 by SEW2871 1nM.** RASMCs were treated with SEW2871 1nM for 2, 6 and 24 hours. RNA was extracted, and RT-PCR was performed using rat S1PR3-specific primers (n=3). Bar graph, mean ± SE.

B. Sphk1 Gene Expression by SEW2871

Sphingosine kinase 1 (Sphk1) is one of the activated proteins by the effect of bradykinin receptor as indicated earlier. So we checked the gene expression modulation of SphK1 by S1P receptor 1 agonist (SEW2871). Figure 29 show that SEW2871 inducesthe expression of Sphk1 after 6 hours treatment with SEW2871.

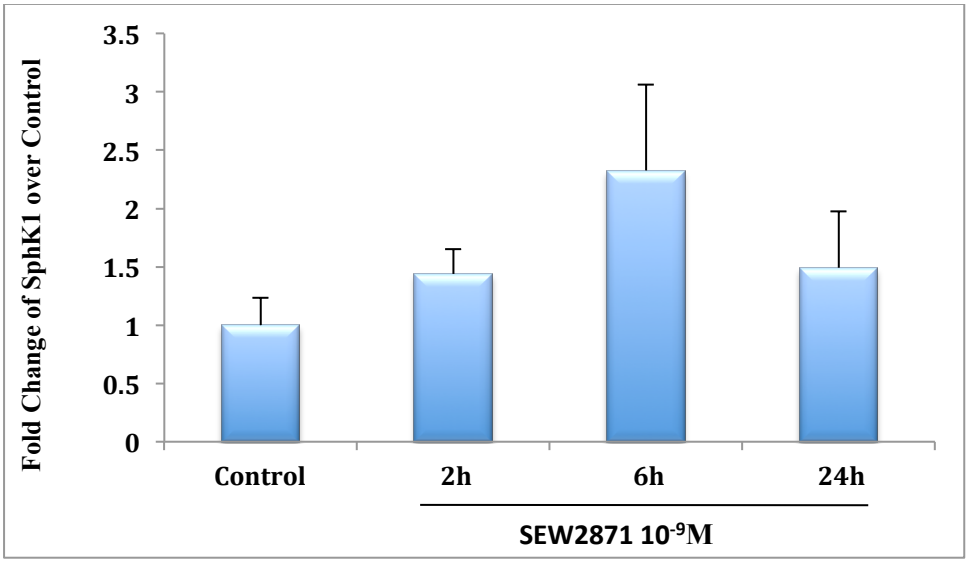


Figure 29. Regulation of Sphk1 Gene Expression by SEW2871. RASMCs were treated with SEW2871 1nm for 2, 6 and 24 hours. RNA was extracted, and qRT-PCR was performed using rat sphk1-specific primers (n=3). Bar graph, mean ± se.

C. SphK2 Gene Expression by SEW2871.

To check whether SphK2 is activated like SphK1 by SEW2871, we tested the SphK2 gene expression modulation by SEW2871 1nM. However, we found that SEW2871 doesn't have any significant modulation on the expression of Sphk2 as indicated in figure 30.

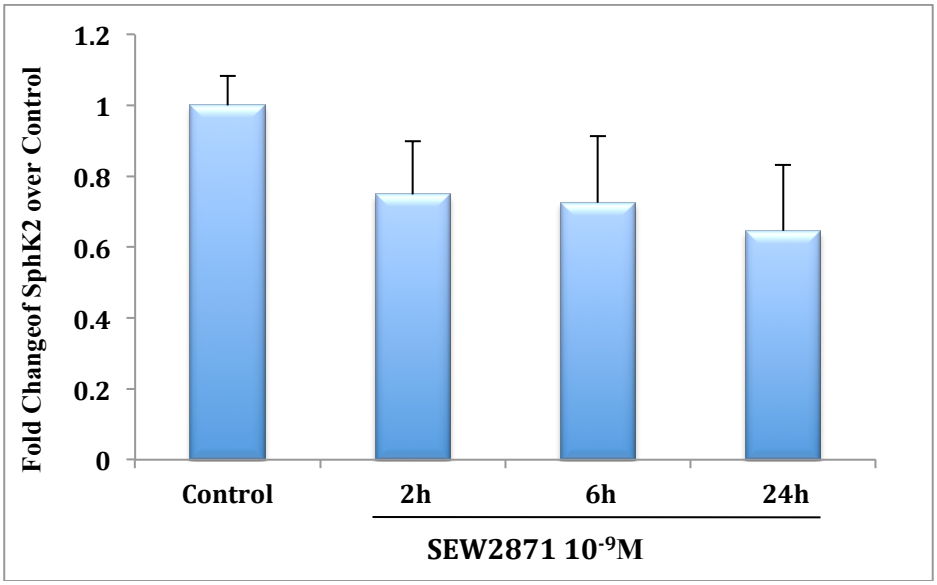


Figure 30. Regulation of Sphk2 Gene Expression by SEW2871. RASMCs were treated with SEW2871 1nm for 2, 6 and 24 hours. RNA was extracted, and qRT-PCR was performed using rat sphk2-specific primers (n=3). Bar graph, mean ± SE.