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CROSSTALK BETWEEN THROMBOXANE AND BRADYKININ RECEPTORS IN VASCULAR SMOOTH MUSCLE CELLS

by
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AN ABSTRACT OF THE THESIS OF

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Title: Crosstalk between Thromboxane and Bradykinin Receptors in Vascular Smooth Muscle Cells

Vascular inflammation and atherosclerosis are currently major causes of morbidity and mortality. Upon vascular injury, Thromboxane (TX) and Bradykinin (BK) contribute to the exacerbation of vascular diseases by inducing proliferation and constriction of vascular smooth muscle cells (VSMCs) through activating their corresponding G-protein coupled receptors (GPCRs): TX receptor (TP) and BK type-2 receptor (B2R) respectively. Cell surface localization of each of B2R and TP on the plasma membrane of VSMCs has been individually demonstrated in previous studies. Other studies have also shed light on the individual capability of each of BK and TX to favor VSMCs proliferation by activating the mitogen activated protein kinase (MAPK) cascade. Although much is known about how stimulation of either B2R or TP could activate MAPK in isolation, to our knowledge, no previous work has been done addressing how MAPK activity is regulated when those receptors are activated simultaneously. Here, we investigated the crosstalk between B2R and TP in VSMCs. Our findings provide pharmacological evidence of cooperative activation of the ERK pathway when both TP and B2R were simultaneously activated. This cooperation was synergistic in BK-dependent modulation of TP mitogenic responses and additive in the inverse direction. We also found that this cooperation could be inhibited when VSMCs were pretreated with the TP antagonist, SQ29548. However, this reciprocal effect between B2R and TP could not be secondary to non-specific binding of IBOP (TP stable agonist) or SQ29548 to B2R as seen in HEK293T cells overexpressing B2R. On the other hand, this synergy could not be reverted by the B2R-selective antagonist, HOE140, which could be exerting a possible biased agonist signaling property on the ERK1/2 pathway during the cooperation between TP and B2R. Therefore, a possible physical interaction could be involved in the cross-modulation between TP and B2R and could thus account to the pharmacologically suggested synergy. Taking into account the previous evidence emphasizing the ability of each of B2R and TPα to form heterodimers with other GPCRs, our findings could be pointing to a possible B2R/TPα heterodimerization that might be affecting the downstream signaling and trafficking properties of individual receptors. Thus, additional work should be conducted to study receptor co-internalization and dimerization between TP and B2R and its implications on vascular injury. These findings would be of substantial importance in the advancement of the field of cardiovascular research, and would help provide a new platform for more effective therapies.

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ILLUSTRATIONS

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ABBREVIATIONS

α-SM…….....Alpha smooth muscle

ANOVA……..….Analysis of variance

AT1…….….Angiotensin type1 receptor

bFGF……....Basic fibroblast growth

BK……..…..…Bradykinin

B1R……..…….....Bradykinin receptor type 1

B2R…….....Bradykinin receptor type 2

BRKRB1……..……gene for BK B1 receptor

BRKRB2……..……gene for BK B2 receptor

B2R-YFP……YFP-cpnjugated-human B2R factor

BSA…….....Bovine Serum Albumin

Ca^{2+}…….…….....Calcium

$CaCl_2$…….....Calcium chloride

cAMP…….…….Cyclic adenosine monophosphate

cGMP…….……Cyclic guanosine monophosphate

COX……...…....Cyclooxygenase

$CaPO_4$…….....Calcium phosphate

CTx……..Cholera toxin

DAPI…….....4',6-diamidino-2-phenylindole

DMEM…….……...Dulbecco's modified eagle's medium

DMSO…….…...Dimethyl sulfoxide

DNA…….....Deoxyribonucleic acid

E……..…….……..Exon

EC_{50}…….....Median effective concentration

ECL……..Enhanced Chemiluminescence

EDHF……..……Endothelium-derived hyperpolarizing factor

EDTA…….…….....Ethylene diamine tetraacetate

EGF…….….Epidermal growth factor

EGFR……..….Epidermal growth factor receptor

eNOS……...…Endothelial nitric oxide synthase

ERK…….…..…..Extracellular signal-regulated kinase

FBS…….…....Fetal bovine serum

FITC…….....Fluorescein Isothiocyanate

GPCRs…….……...G-protein coupled receptors

GRK…….……G protein-coupled receptor kinase

GTP…….….Guanosine triphosphate

HA…….....Human influenza hemagglutinin

HA-hTPα……..……HA-tagged-human TPα

HASMCs……..……Human aortic smooth muscle cells

HEK293-T……...…….Human embryonic kidney-T cells

HOE140……...…….B2R selective antagonist

hr…….…..……Hour(s)

IBOP……...….TP agonist

IgG……..….….Immunoglobulin G

IP……..….PGI2 receptor

IP_3…….....Inositol triphosphate

JNK1/2/3…….…..….Jun NH2 terminal kinases

MAPK……..……...Mitogen activated protein kinase

Mg^{2+}……..…....Magnesium

min……...…..minute(s)

MKK…….….....MAPK kinase

MKKK……...MAPK kinase kinase

MMPs……Matrix metalloproteinases

mRNA…….……...messenger RNA

NADPH……..Nicotinamide adenine dinucleotide phosphate

Na_2HPO_4…….…...Disodiumphosphate

NGF……....Nerve growth factor

NO…….....Nitric oxide

NS…….....Unstimulated basal

$O_2^{\cdot -}$ ……..…...Superoxide

$ONOO^-$……..Peroxynitrite

$P125^{FAK}$…….….Focal adhesion kinase

PBS…….....Phosphate buffered saline

pERK1/2…….Phospho ERK1/2

PGI_2…….…...Prostacycline

PI_3K……Phosphoinositide-3-kinase

PKA…….…...Protein kinase A

PKC……....Protein kinase C

PLC……..…..Phospholipase C

PTx…….Pertussis toxin

RASMCs…….…...Rat aortic smooth muscle cells

RNA…….....Ribonucleic acid

ROS…….…...Reactive oxygen species

RTK……..……..Receptor tyrosine kinase

SDS……..……..Sodium dodecyl sulfate

SDS-PAGE…….SDS polyacrylamide gel electrophoresis

Ser/Thr ……...……Serine/Threonine

siRNA…….....small interfering RNA

SQ29548……TP antagonist

TBST-T……..Tris-buffered saline containing Tween 20

TEY motif……..…Threonine-Glutamic acid-Tyrosine motif

TGF……Tumor growth factor

TL…….....Transmission light

TP……...…Thromboxane prostanoid receptor

TX…….…..…Thromboxane

U46619……...…...TP agonist

VSMCs…….…..…Vascular smooth muscle cells

YFP…….Yellow fluorescent protein

YFP-B2R-HEK-Ts…….HEK293-Ts overexpressing B2R-YFP

CHAPTER I

LITERATURE OVERVIEW

A- Endothelial Dysfunction and Atherosclerosis:

In normal homeostatic conditions, the healthy endothelium regulates vascular tone by preferentially shifting the balance between various vasoactive molecules towards a dominant vasoprotective outcome [**1**]. Damage to the endothelium impairs its protective function and places blood contents of leukocytes, platelets, macrophages, growth factors, lipids and cytokines in the vicinity of vascular smooth muscle cells (VSMCs). This reverses the balance within the vasculature shifting it towards vasoconstriction, pro-inflammation, thrombogenesis, and increased VSMC proliferation and migration; thus initiating and exacerbating atherosclerosis (Figure 1) [**2, 3**].

Atherosclerosis is a multi-stage inflammatory disease of the medium and large-sized arteries that leads to serious clinical consequences on the heart, brain, or extremities. Aggravating risk factors include elevated and modified LDL, reactive oxygen species (ROS), hypertension, diabetes mellitus, genetic alterations, and elevated plasma homocysteine concentrations, or their combinations [**3**]. As endothelial injury endures, proliferative and migratory VSMCs become characteristic determinants in the accumulation and progression of atherogenesis (Figure 2) [**4, 5, 6**].

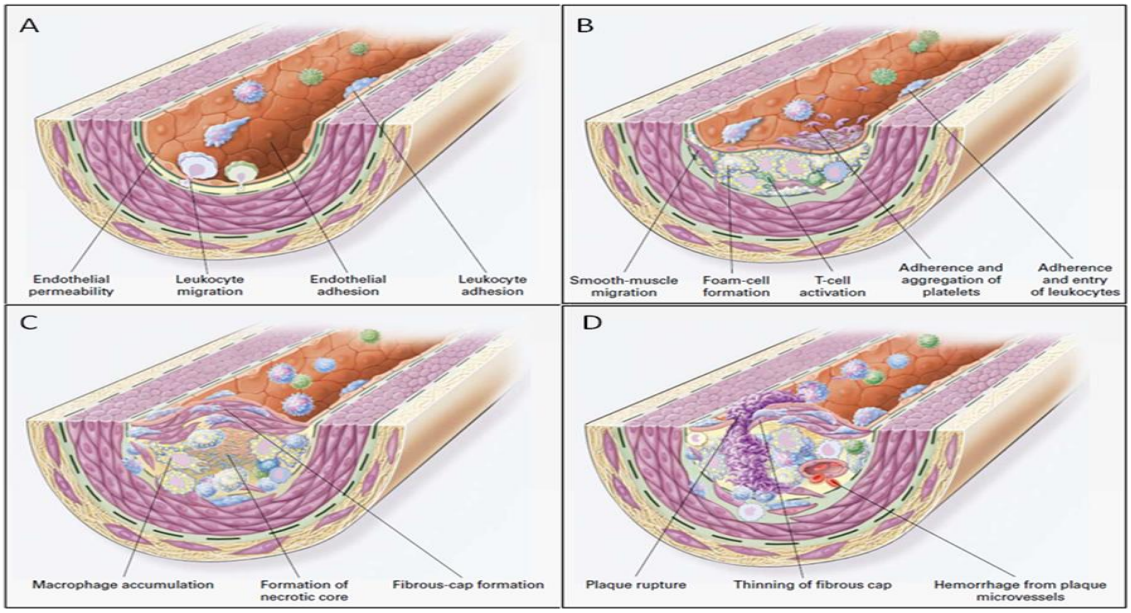

Figure 1: Steps of Formation and Progression of the Atherosclerotic Lesion
A: Endothelial Dysfunction. This includes increased endothelial permeability to lipoproteins and other plasma constituents; up-regulation of leukocyte adhesion molecules; up-regulation of endothelial adhesion molecules; and migration of leukocytes into the arterial wall.
B: Fatty Streak Formation: Initially lipid-loaded macrophages and monocytes, together with T cells constitute the early fatty streaks. With progress, migratory smooth muscle cells become involved. Steps entailed are foam cell formation; T-cell activation; adherence and migration of leukocytes; platelet adherence and aggregation; and VSMCs migration.
C: Fibrous Cap Formation. With the intermediate and advanced complication of fatty streaks, a fibrous cap is formed, as a type of healing of the continuous injury. The fibrous cap forms a wall between the arterial lumen and the growing lesion. The mixture of leukocytes, lipid, and debris constituting the lesion may form a necrotic core which results from cellular apoptosis and necrosis, increased proteolytic activity, lipid accumulation, and continued macrophage accumulation.
D: Formation of Unstable Fibrous Plaques. The continuous influx and activation of macrophages in the advanced lesion, which release metalloproteinases and other proteolytic enzymes, result in the thinning of the fibrous cap, degradation of the matrix, and subsequent hemorrhage from the arterial lumen. The resulting thin fibrous cap becomes prone to rupture and ulceration, hence contributing to thrombus formation and occlusion of the artery [**3**].

B- Vascular Homeostasis and Vascular Mediators:

The vasoprotective profile of the intact endothelium is mainly mediated by nitric oxide (NO), the most potent endogenous vasodilator. Besides vasodilation, NO

inhibits platelet adherence and aggregation, leukocyte adhesion and infiltration, oxidative modification of low-density lipoprotein, and VSMC proliferation; thus affecting the major characteristic events underlying the formation of atherosclerotic lesions [**1, 7**]. Furthermore, decreased production or activity of NO was shown to be a major contributor to vascular complications of endothelial dysfunction and atherosclerosis [**1, 8**].

In this work, we are mainly interested in studying the crosstalk between two prominent vascular mediators, Bradykinin (BK) and Thromboxane (TX), which normally contribute to vascular homeostasis by exerting divergent effects on NO synthesis and release; the former resulting in vasodilation, while the latter causes vasoconstriction [**9, 10**]. However, in cases of vascular injury, each of BK and TX is now known to induce vasoconstriction and enhanced proliferation of VSMCs.

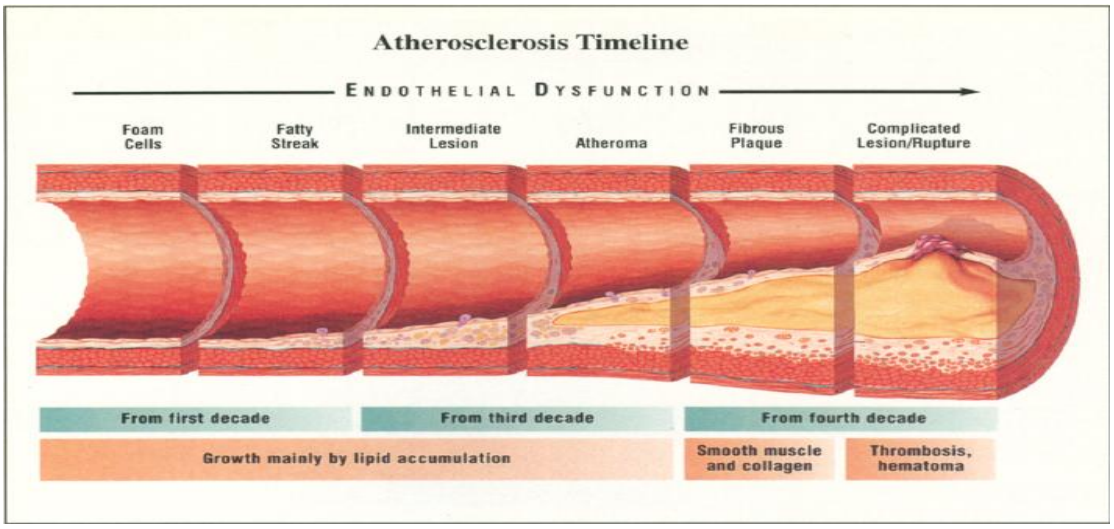

Figure 2: Progression of Atherosclerosis with time
The figure illustrates the pathways of initiation and exacerbation of atherosclerotic lesion with time. Both increased collagen and increased VSMCs proliferation and migration play pivotal roles in the formation of the fibrous cap and its complication [**5, 6**].

1. Kallikrien Kinin System and BK:

BK is a vasoactive peptide that acts in an autocrine or paracrine fashion to impact vascular tone and inflammation [**11**]. It can be generated both systemically and locally within the vascular wall, following the cleavage of its precursor, high molecular weight kininogen, by kallikriens [**12, 13, 14, 15**].

In normal physiological conditions, endothelium-derived BK favors vasodilation and inhibition of platelet aggregation by stimulating the release of second messengers such as NO, prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF) (

Figure 3) from the endothelium [**9, 16, 17**]. However, upon endothelial injury, BK directly increases intracellular calcium influx within VSMCs, thus initiating vasoconstriction [**18**].

Two types of receptors, B1R and B2R kinin receptors, mediate the action of kinins at the cellular level. Cloning studies have revealed the colocalization of human B1R and B2R encoding genes on chromosome 14q32, clustered in tandem in a compact locus, where B2R gene is only 12 kb upstream of B1R gene (Figure 4; [**19**]). Both receptors belong to the family of G-protein coupled receptors (GPCRs), mainly coupled to Gαq and Gαi (Figure 5; [**20, 21**]). While B2R is constitutively active with high affinity to BK, B1R is induced upon pathological conditions having des-Arg9-BK and des- Arg10-kallidin as its preferential agonists [**22, 23, 24, 25**].

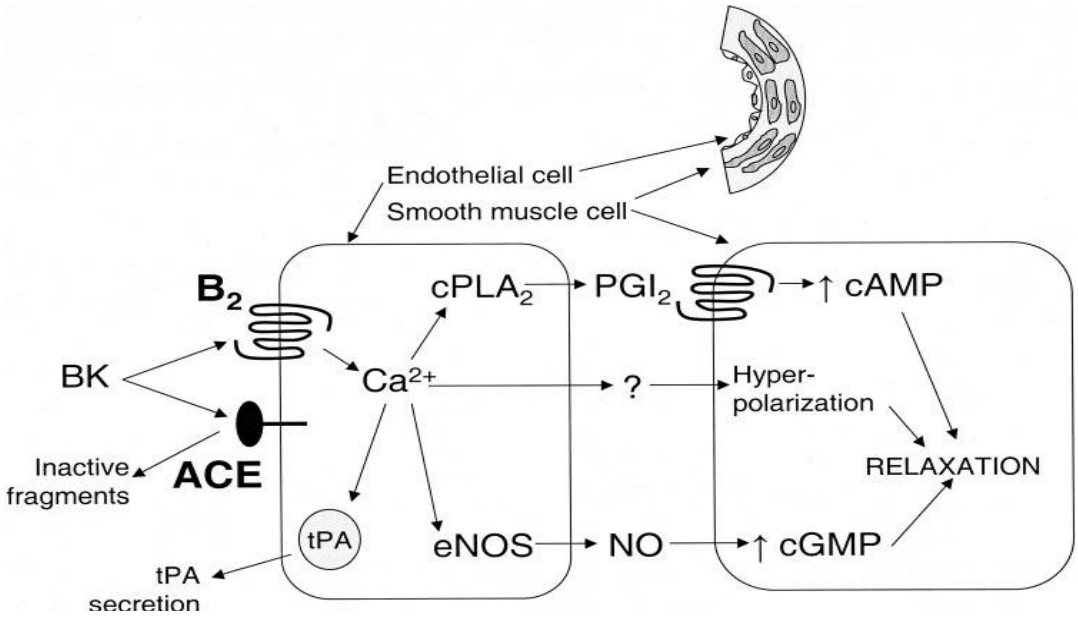

Figure 3: Mechanisms of endothelium-dependent relaxation of VSMCs in response to BK

Vascular responses to BK involve the stimulation of the endothelium to release secondary mediators such as NO and PGI2 in a Ca^{2+}-sensitive mechanism. Endothelium-derived NO and PGI2 diffuse to VSMCs leading to the subsequent activation of cGMP and cAMP respectively, thus resulting in smooth muscle relaxation. cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate [**17**].

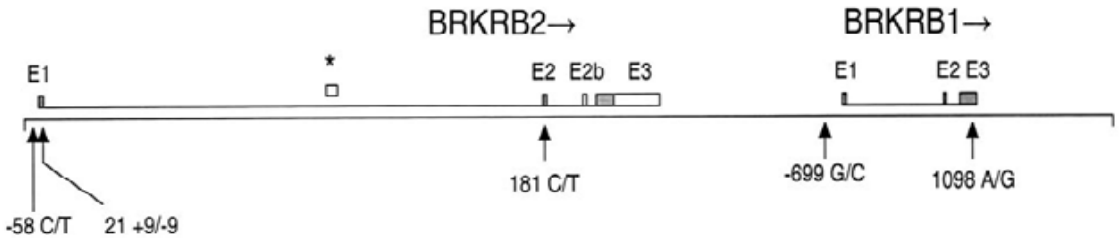

Figure 4: loci of genes encoding for human B1 and B2 receptors on chromosome 14q32.

A 70kb total area between positions 94.66 and 94.73 Mb of chromosome 14q32 is represented. This encompasses B1R and B2R genes that are shown clustered in tandem, with B2R gene locus only 12kb upstream of B1R gene. The three major exons of both genes are shown, with the alternatively spliced E2b of B2R. BRKRB1 and BRKRB2: genes for B1 and B2 receptors respectively; E: exon [**19**].

Studies conducted on rat and human vascular systems located B2R both in the endothelium and tunica media of the aorta and other VSMCs [**26**]. B2R-mediated BK response in VSMCs is Gαq-dependent, and involves the activation of Phospholipase C (PLC) which leads to the formation of inositol triphosphate (IP_3) and Diacylglycerol, thus leading to intracellular calcium mobilization and activation of protein kinase C (PKC) [**27, 18**]. Intracellular calcium influx promotes vasoconstriction and mitogenesis, whereas PKC is involved in BK-induced proliferation and migration of VSMCs [**28, 18, 29**].

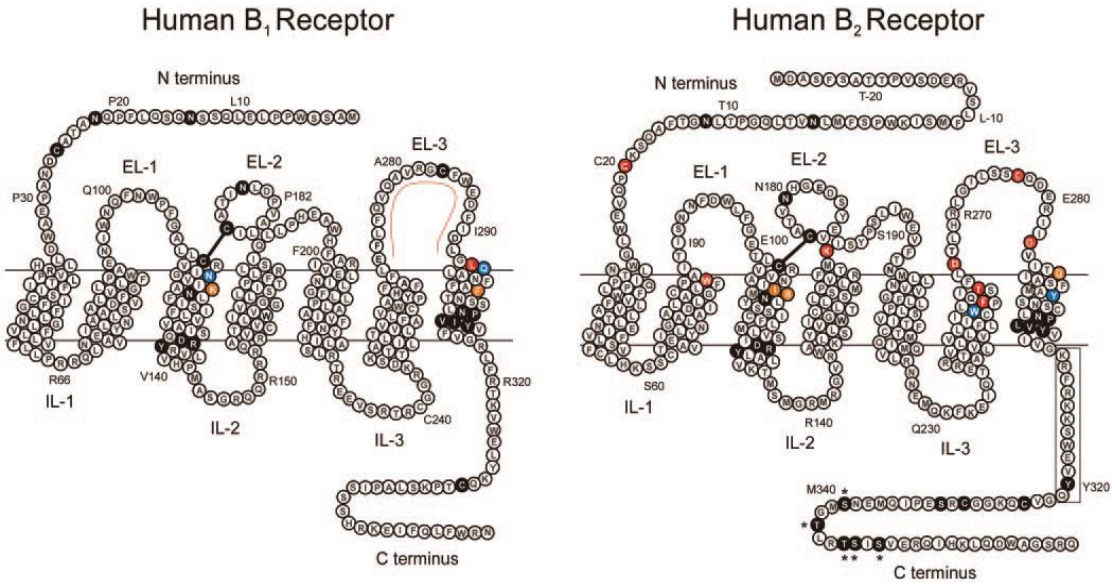

Figure 5: Schematic representations of the amino acid sequences of human B1R and B2R.
For both B1R and B2R, potential amino acid residues facing specifically the binding pockets are filled with red or red line (agonist), blue (antagonist), and orange (agonist and antagonist). In the B2 receptor, the box includes the putative helix 8, and the stars indicate the cluster of serine and threonine residues important for B2R desensitization. EL, extracellular loops; IL, intracellular loops [**17**].

It is hypothesized that the initial inflammatory response in vascular endothelial cells occurs through the constitutive B2Rs, followed by the induction of B1Rs whose

actions mainly comprise the chronic inflammatory phase [**30, 31, 32, 33**]. This is consistent with the rapid and pronounced phosphorylation, desensitization, and internalization of agonist-stimulated B2R, as compared to the considerably less agonist-desensitization of B1R [**34, 35, 36, 37**].

2. Thromboxane and Thromboxane Receptors:

Thromboxane A2 (TXA2) is an unstable endogenous autacoid generated from arachidonic acid by the sequential enzymatic actions of Cyclooxygenase (COX) and TXA2 Synthase. Although mainly released from platelets, other cell types such as macrophages are also capable of releasing TXA2 [**38**].

Initially known for its role in platelet aggregation, TXA2 elicits a range of biological and pathophysiological functions related to homeostasis-thrombosis, inflammatory and immune responses, atherogenesis, and asthma [**39**]. TXA2 cellular functions are mediated by the activation of its receptors, termed thromboxane prostanoid (TP) receptors [**40**].

Recently, lots of studies addressed the role of TP activation in the initiation and exacerbation of endothelial dysfunction and atheroscelrosis. This was proved through pharmacological inhibition or genetic deletion of TP in non-diabetic apolipoprotein E knockout mice, where either case resulted in blocked progression of atherosclerosis [**41, 42**]. Subsequent studies have also demonstrated the ability of TXA2 *via* TP to induce ROS formation both in VSMCs and endothelial cells [**43, 44, 45**]. Lately in 2011, Zhang and his colleagues proposed that increased endothelial production of ROS such as superoxide ($O_2^{\cdot -}$) and peroxynitrite ($ONOO^-$) is NADPH oxidase-dependant, and leads to uncoupling of endothelial nitric oxide synthase (eNOS), and thus decreased

production of NO [**45**]. This results in oxidative stress that impairs the endothelium and exacerbates atherosclerosis.

In humans, splicing of TP gene results in two isoforms, TPα (originally cloned from placenta) and TPβ (originally cloned from endothelial cells), with an extended C-terminal cytoplasmic domain, specific for TPβ, that aids in its internalization [**46, 47, 48, 49, 50**]. Both receptors belong to the family of 7-heterotrimeric transmembrane GPCRs, and initiate downstream signaling cascades by coupling to at least four different G-proteins, the most prominent being $G_{\alpha q}$ and $G_{\alpha 12/13}$ (

Figure 6) [**47, 39, 51, 52, 53**].

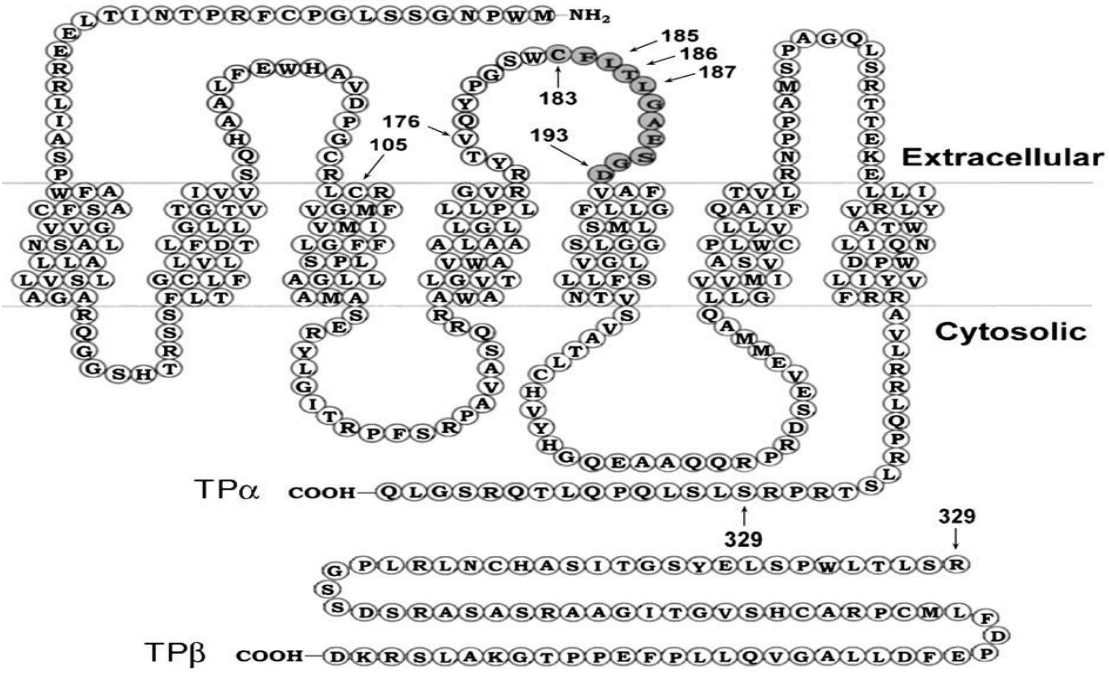

Figure 6: Schematic representing the protein structures of TPα and TPβ [**39**]

Since TXA2 has a short half-life of 30 seconds [**54**], several stable analogues have been synthesized and utilized to study its mechanisms of action. Ligand binding

studies utilizing IBOP and U46619 as TP agonists provided pharmacological evidence of the localization of TP receptors both in platelets and VSMCs [**55, 56, 40, 57**]. In 1990, Morinelli and his colleagues described IBOP as the most potent agonist of TP having a very high receptor affinity (2 nM) as compared to other agonists (100 nM) [**55**]. Other studies have shown that stimulation of cultured smooth muscle cells with U44619, another TXA2 analogue, resulted in smooth muscle contraction. This was due to TX-induced increase in IP_3, mobilization of intracellular Ca^{2+}, and phosphorylation of myosin light chain kinase [**58, 59, 60**].

C- Signaling Cascades Involved in Vascular Proliferation:

Among the various signaling cascades involved in VSMC proliferation, the mitogen activated protein kinase (MAPK) pathway plays a major role. Both BK and TX are capable of inducing its activation, thus exerting their mitogenic effects on VSMCs [**61, 62**].

1. MAPKs and their Signaling Cascades:

MAPKs are ubiquitous "Proline-directed" Serine/Threonine (Ser/Thr) protein kinases capable of transducing signals downstream of multiple extracellular stimuli including growth factors, hormones, cytokines, and stress. Thus, MAPK pathways could be coupled to different families of receptors such as, receptor tyrosine kinases (RTKs), GPCRs, cytokine receptors, and Ser/Thr kinase receptors [**51**].

Among the six distinct groups of MAPKs that have been characterized in mammals, p44 and p42 extracellular regulated kinases (ERK1 and ERK2, respectively),

Jun NH2 terminal kinases (JNK1/2/3), and p38 (p38 α/β/γ/σ) MAPKs are the most prominent [**51, 63, 64**]. In this thesis, we are mainly interested in the signaling through the ERK pathway.

Mammalian ERK1 and ERK2, usually referred to as ERK1/2, are co-regulated isoforms that have 83% amino acid homology, but might have distinct functions. Regarded as the prototype of MAPKs, the ERK pathway was the first identified Ras-effector pathway and the best studied pathway regarding nuclear translocation [**65, 66**].

The MAPK pathways are conserved among eukaryotes, featuring activation of a three-kinase sequential module; MAPK kinase kinase (MKKK)/ MAPK kinase (MKK)/ MAPK [**51**]. For ERK1/2 pathway, these are Raf/MEK/ERK, respectively, downstream of Ras (a small G-protein with a GTPase activity) (

Figure 7; [**67, 51**]). Evidence has been shown linking the Ras/Raf/MEK/ERK pathway to the regulation of a multiplicity of cellular functions including cell proliferation, survival, differentiation, migration, apoptosis, metabolism, and cytoskeletal rearrangement [**68, 69, 70**]. Recent *in vivo* studies, as well, have linked ERK pathway to an increase in neointimal proliferation following vascular injury [**71**].

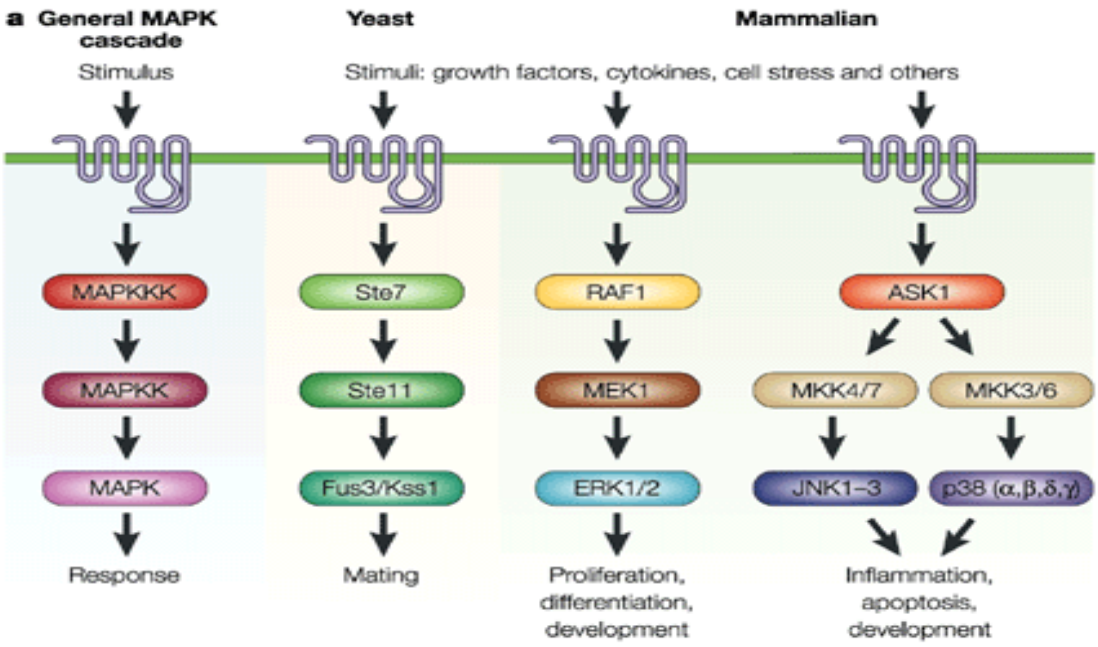

Figure 7: MAPK signaling cascade
GPCR activation of MAPK cascades comprises the consecutive activation of MAPKKK, MAPKK, and MAPK. In yeast, the G -dependent activation of the MAPK cascade is downstream of the pheromone receptors. In case of mammals, ERK activation is both Gα - and Gβγ –dependent. More studies need to be done for the determination of the early intermediates in the activation of the JNK and p38 MAPK cascades [**67**].

a- <u>Signaling through the ERK1/2 Pathway</u>

Various extracellular and intracellular stimuli are capable of activating the ERK1/2 pathway [**51**]. While growth factors, phorbol esters and serum are considered the most potent activators, other stimuli including GPCR ligands, cytokines, osmotic pressure, and microtubule disorganization can also activate this pathway but to a lesser extent [**72**].

Signaling through this pathway is initiated by the activation of GPCRs or transmembrane receptors such as RTKs [**73, 74**]. The signal is subsequently transduced to GTP-bound Ras, which anchors and recruits Raf kinase to the plasma membrane, inducing its activation. Activated Raf initiates a triple-kinase cascade where it

phosphorylates and activates MEK, which in turn dually phosphorylates ERK on its regulatory Threonine and Tyrosine residues located in the TEY (Threonine-Glutamic acid-Tyrosine) motif of its activation loop, thus leading to its activation [**65, 51**].

More than 150 substrates for activated ERK have been identified and localized at different cellular compartments including the plasma membrane, cytosol, cytoskeleton, and nucleus (

Figure 8; [**75, 76**]). Examples of these substrates include nuclear transcription factors, cytoskeletal proteins, signaling proteins and receptors. It is now thought that a significant portion of activated ERK tends to dissociate from its anchoring proteins in the cytoplasm, followed by its translocation to the nucleus, few minutes later, to regulate gene transcription (

Figure 9) [**77, 78, 79**].

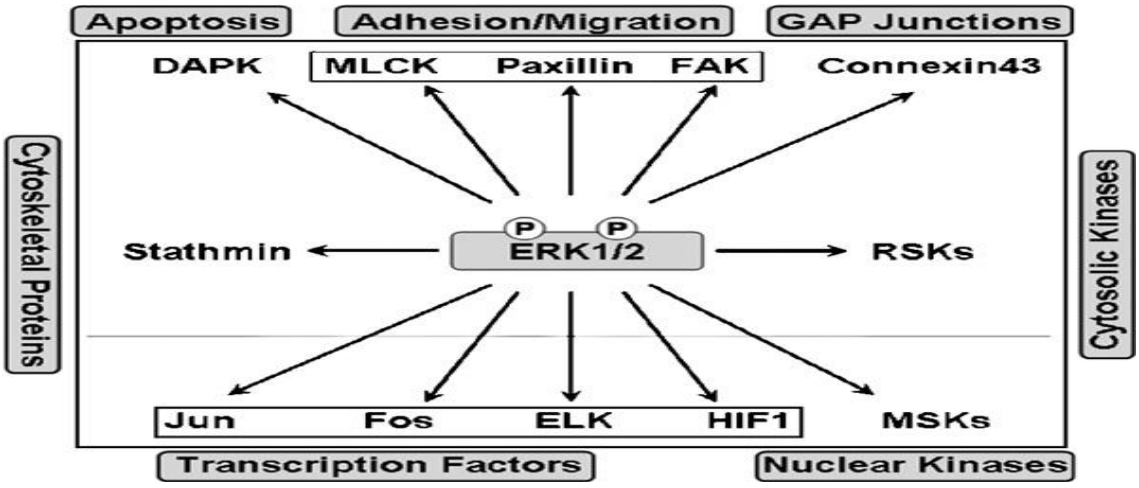

Figure 8: Subcellular Localization of ERK1/2 substrates
ERK targets and phosphorylates various substrates located in the nucleus, cytosol, cytoskeleton, and plasma membrane. These include nuclear kinases and transcription factors, cytosolic kinases and structural proteins, cytoskeletal proteins, and plasma membrane proteins that regulate cell adhesion, GAP junctions, and cell survival and apoptosis [**75**].

Several factors could affect the biological outcome resulting from the activation of the ERK pathway. For instance, previous studies done on both PC12 cells and fibroblasts have shown that the subcellular compartmentalization of ERK is a key determinant in its induction of mitogenesis or cellular differentiation, and varies according to the specific cell types [**80, 81**]. However, later studies conducted on PC12 cells indicated that the duration of ERK activation dictates its subsequent biological responses. While increased cellular proliferation is mainly attributed to a transiently induced- ERK activity, sustained ERK activation is thought to lead to increased cellular differentiation (

Figure 10; [**68**]). Thus, ERK's biological responses are cell type specific, and are affected by its localization and duration of activity.

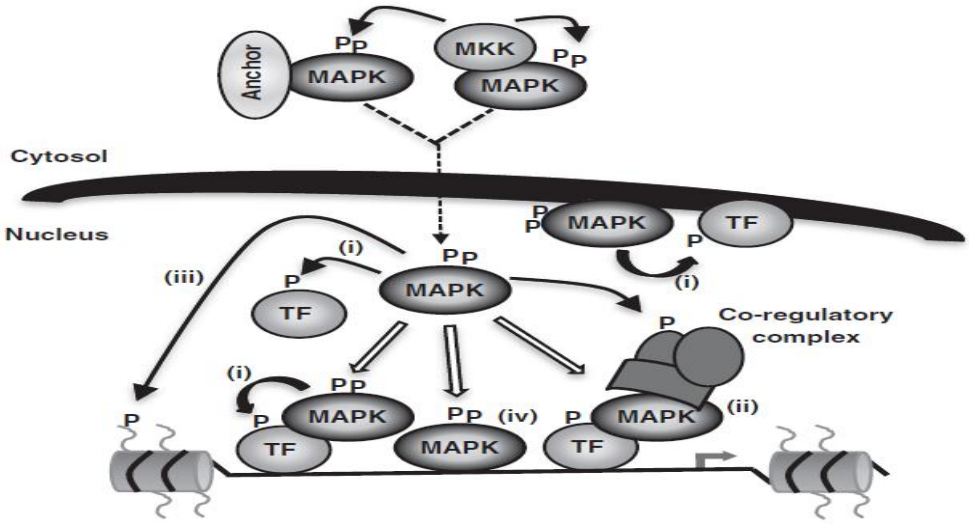

Figure 9: Mechanisms of Nuclear Translocation and Transcriptional Regulation by Activated MAPK
In the nucleus, MAPKs can be anchored to the nuclear periphery by interacting with lamins or they can be recruited onto chromatin. Once on the chromatin, MAPKs can regulate transcription via many mechanisms, the integration of which will determine the transcriptional output. MAPKs transcriptional regulation includes: (i) phosphorylation of transcription factors; (ii) recruitment of co-regulators to DNA; (iii) alteration of

chromatin structure by inducing phosphorylation of histones; and (iv) direct binding to specific DNA sites. (TF = transcription factor; P = phosphorylation site) [**79**].

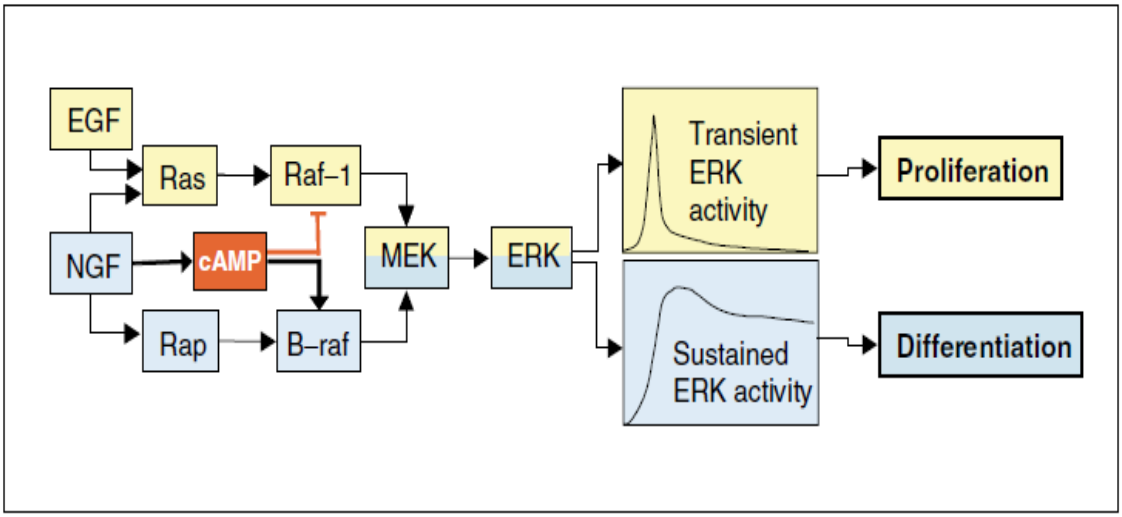

Figure 10: Role of Transient and Sustained ERK Activity in PC12 proliferation and differentiation
PC12 cells differentiate in response to the nerve growth factor (NGF) that induces sustained ERK activation. Activation of cAMP further enhances their differentiation. However, epidermal growth factor (EGF)-induced transient activation of ERK results in increased proliferation of PC12 cells [**68**].

b- <u>GPCR and ERK1/2 Pathway in VSMCs:</u>

GPCRs are ubiquitous receptors for extracellular signals and cytokines with seven transmembrane domains. Once activated by ligand binding, their c-terminal couples to a hetrotrimeric G-protein Gαβγ, and initiates a cascade of downstream signaling that affects various cellular processes. Several mechanisms of GPCR-induced ERK1/2 activation have been demonstrated, including generation of ROS, transactivation of RTKs (such as Epidermal Growth Factor Receptor, EGFR), scaffolding of β-arrestins, activation of protein kinases (PK)-A and -C, or Src kinases [**29, 82, 83, 84, 67, 85**]. Among these GPCRs, each of B2R and TP receptors is known to individually induce ERK activation in VSMCs [**28, 85**].

i. BK and ERK1/2 Activation in VSMCs:

Earlier studies revealed that BK leads to enhanced proliferation of rat mesangial cells through the activation of its B2R. This mitogenic effect was mediated *via* the activation and nuclear translocation of ERK1/2, thus leading to increased expression of c-fos and c-jun mRNA levels and the assembly of the AP-1 complex [**86**].

In1999, Velarde *et al.* provided evidence that BK, through its B2R, is also capable of inducing ERK1/2 activation in rat aortic smooth muscle cells (RASMCs). This early mitogenic effect of BK on VSMCs involved PKC, P60-Src,Grb2 and focal adhesion kinase ($P125^{FAK}$)-mediated pathways, but was independent on phosphoinositide-3-kinase (PI_3K), pertussis toxin (PTx), or cholera toxin (CTx) [**28**]. Relevant work has also shown that BK-induced activation of MAPK and subsequent nuclear c-fos mRNA expression was mediated by Ca^{2+}-calmodulin and ROS generation [**28, 87**]. While BK-induced ROS generation was leading to ERK activation, inhibition of ERK pathway resulted in reduced ROS production, suggestive of a reciprocal activation of ROS production induced by ERK [**29, 87**] .

Furthermore, a study conducted by Douillet *et al.*, 2001 demonstrated the involvement of MAPK pathway in BK-induced fibrosis of VSMCs. Their findings suggest that this fibrotic effect is due to an increased production of collagen type I, which results from both the activation of ERK1/2 pathway and the autocrine activation of tumor growth factor (TGF) β1 in response to BK-stimulation of VSMCs [**88**].

ii. TX and ERK1/2 Activation in VSMCs:

On the other hand, studies employing TP stable agonists have demonstrated its ability to promote contraction and proliferation of VSMCs [**56, 89**]. The mitogenic effect was shown to be mediated *via* the activation of MAPK, leading to increased DNA synthesis and shift in the cell cycle from S to G2/M phase [**90, 62, 91, 89**].

In 2001, Miggin and Kinsella, showed that ERK activation by TX in human uterine smooth muscle cells involved PKC, PKA, and PI_3K-dependent mechanisms besides the transactivation of EGFR, but was PTx-independent [**92**]. Later in 2003, Gallet and his colleagues, provided evidence supporting that TP-induced ERK activation, in human aortic smooth muscle cells, involves both Gαq and Gαi-proteins, Src kinase and PKC and is mediated by transactivation of matrix metalloproteinases (MMPs) and EGFR [**85**].

While most conducted studies provide evidence of a mitogenic role of TX on VSMCs, it is worth noting that some studies hypothesized this effect to be hypertrophic rather than mitogenic. This hypertrophic effect was attributed to a TX-induced increase in the synthesis and release of endogenous basic fibroblast growth factor (bFGF) [**58**]. Such discrepancies in the findings could be partly explained by differences in species, and/or experimental culture conditions [**39**].

D- Receptor Heterodimerization and Co-internalization:

1. GPCR Oligomerization:

Recently, lots of studies have demonstrated the ability of the cell surface GPCRs to heterodimerize, thus affecting the pharmacological activity, signal transduction, and internalization of individual parent receptors (reviewed in [**93**]).

a- Oligomerization of kinin B2-Receptors (B2R):

Studies conducted by AbdAlla *et al*., 2000, described an angiotensin AT1/bradykinin B2R heterodimer in both A10 VSMCs and HEK293-transfected cells [**94**]. The formation of this complex was agonist-independent; however, it led to enhanced Angiotensin II responses but decreased potency and efficacy of those of BK. Moreover, this heterodimerization shifted the mode of receptor trafficking from dynamin I-independent internalization for the individual receptors to dynamin I-dependent sequestration of the complex. Similarly, Kang et al., 2004 provided evidence of spontaneous heterodimerization between human B1R and B2R co-expressed in HEK293 cells undergoing persistent insult [**95**]. This cell-specific regulatory mechanism resulted in enhanced agonist-dependent and -independent signaling of the heterodimer and the conversion of the kinin signal from B2R to B1R types.

b- Oligomerization of Thromboxane A2 Receptors:

Studies conducted on TP receptors have also shown the ability of each of human TPα and TPβ to form homo-or hetero-oligomers, which would affect the trafficking and signaling properties of these receptors. For instance, Laroche and

coworkers demonstrated that TPα, which does not internalize individually even after ligand binding, can be co-internalized with TPβ secondary to the formation of a TPα/TPβ heterodimer [**96**]. This leads to reduction in the availability of TP subtypes on the plasma membrane, and thus reduction in TP-mediated responses and signaling [**97**]. TPα/TPβ heterodimerization might also result in conformational changes in ligand binding sites, as suggested by Wilson *et al.*, 2007 whose findings have revealed that the formation of this complex enhanced isoprostane-mediated signaling, but not IBOP- or U46619-induced signaling [**98**]. Another example of TPα heterodimerization is that reported with the PGI2 receptor (IP) that resulted in enhanced TP-mediated cAMP formation [**99**]. Later in 2007, the same group of researchers provided evidence of an IP-mediated internalization of TPα once the TPα/IP heterodimer was formed [**100**].

2. Receptor Internalization:

a- Internalization of TP:

In 1999, Parent and his colleagues demonstrated that in HEK293 cells expressing TPα and TPβ, only TPβ undergoes U46619-induced G protein-coupled receptor kinase (GRK)-dependent phosphorylation [**50**]. This is followed by binding of β-arrestins to the GRK phosphorylation site and results in the uncoupling of G proteins from TPβ and its subsequent internalization [**101**].However, this could not occur with TPα, whose C-terminal could not be phosphorylated by GRK5 or GRK6 [**102**].

b- Internalization of B2R**:**

Studies tracking the cell surface expression and trafficking of B2R have identified a rapid (within minutes) BK-promoted endocytosis of B2R that resulted in desensitization and later recycling of the receptor (reviewed in [**17**]).

This was dynamin and β-arrestin independent and specifically localized in caveolae-like lipid rafts [**36, 103**]. Moreover, both BK-induced human B2R internalization and disensitization are dependent on the phosphorylation of a cluster of serines and threonines located in the receptor C-terminal tail [**104**]. Studies tracking BK-B2R internalized complex in cultured smooth muscle cells located it at the subcellular microsomal and plasma membrane-associated compartments [**105**]. This was followed by redistribution of B2R to caveolae and its rapid recycling to the cell surface of the plasma membrane [**103**].

CHAPTER II

THESIS HYPOTHESIS, AIM, SIGNIFICANCE, AND LIMITATIONS

A- Thesis Hypothesis, Aim, and Objectives

1. Thesis Hypothesis:

Cell surface localization of each of B2R and TP receptors, both Gαq-coupled, on the plasma membrane of VSMCs has been individually demonstrated in previous studies. Likewise, evidence has been provided regarding the individual capability of BK or TX to favor VSMCs proliferation by activating the MAPK cascade. Although much is known about how stimulation of either B2R or TP receptors could activate MAPK in isolation, to our knowledge, no previous work has been done addressing how MAPK activity is regulated when those receptors are activated simultaneously. Here, we investigated the crosstalk between B2R and TP in VSMCs. Our results suggest that a combination of BK and TX synergistically potentiates signaling through the ERK1/2 pathway in VSMCs, thus implicating ERK1/2 as a point of convergence downstream of B2R and TP. We also found that the potentiating effects of BK on IBOP-induced ERK1/2 phosphorylation could be completely inhibited when RASMCs were pretreated with the TP antagonist, SQ29548. However, this reciprocal effect between B2R and TP could not be secondary to non-specific binding of IBOP or SQ29548 to B2R as seen in HEK293T cells overexpressing B2R. On the other hand, this synergy could not be reverted by the B2R-selective antagonist, HOE140, which could be indicative of a possible biased agonist signaling mechanism exerted by HOE140 on the ERK1/2 pathway. Therefore, a possible physical interaction could be involved in the cross

modulation between TP and B2R and could thus account to the pharmacologically suggested synergy. Hence, additional work should be done to investigate the likelihood of heterodimerization between both receptors and its subsequent impact on the pathogenesis of vascular injury and atherosclerosis.

2. Thesis Aim, Objectives, and Design:

The aim of this work was to examine the crosstalk between B2R and TP in VSMCs, and the possible pharmacological and physiological mechanisms that could be involved.

The design of our work was customized to achieve several experimental objectives:

- To inspect the likelihood of synergy in the effect exerted by BK and TX on the ERK1/2 pathway in VSMCs, RASMCs were treated with BK (10^{-11} to10^{-7} M) or IBOP -TP agonist- (10^{-11} to 10^{-7} M) or combinations thereof. Fold over basal change in ERK1/2 phosphorylation was then assessed, as an indication of changes in ERK1/2 activity.
- Pretreatment of RASMCs with a TP antagonist, SQ29548 10^{-5} M, or B2R selective antagonist, HOE140 10^{-6} M [**106**], followed by treatment with BK, IBOP, or their combination was also examined to determine the type of receptors involved and further evaluate the extent of coordination between both receptors.
- To rule out any possible effect of IBOP or SQ29548 on the binding of B2R to BK, HEK293-T cells were individually transfected with YFP-conjugated-human-B2R (B2R-YFP). Subsequently, cells were either kept unstimulated, or treated with 10^{-7} M of BK, 10^{-7} M of IBOP, or their combination for 30 min. Or,

cells were pretreated with 10^{-6} M of HOE140 or 10^{-5} M of SQ29548 for 30 min, prior to stimulation with 10^{-7} M of BK for additional 30 min. B2R internalization was then evaluated as an indication of B2R activity using confocal microscopy.

- Finally, to determine whether a possible physical interaction is taking place between both receptors, B2R-YFP and HA-tagged-human-TPα (HA-hTPα) receptors were overexpressed in HEK293-T cells. The aim was to assess receptor trafficking upon stimulation with BK, IBOP, or combination using confocal microscopy. However, some technical problems impeded this assessment at the timing being.

B- Thesis Significance:

This is the first study, to our knowledge, that provides pharmacological evidence of a synergistic cooperation between BK and TX in VSMCs. Furthermore, our results demonstrate altered modulation of the downstream signaling of individual receptors, once both receptors were activated simultaneously. This could be suggestive of a physically-possible interaction between B2R and TP that could account for the crosstalk observed between both receptors. Thus, taking into account the previous evidence emphasizing the ability of each of B2R and TPα to form heterodimers with other GPCRs, our findings could be pointing to a possible B2R/TPα heterodimerization that might be affecting the downstream signaling and receptor trafficking properties of the individual receptors.

On the other hand, the inability of HOE140 to normalize the cooperative activation of ERK1/2 induced by BK and TX in RASMCs unveils a possible biased agonist property of HOE140, when both B2R and TP are simultaneously activated.

Recently, researchers in Jerini AG have been conducting clinical phase trials to investigate the efficacy of using HOE140 for the management of angioedema [**17**]. Hence, our study emphasizes the need for caution when prescribing HOE140, Icatibant®, for the management of pathological cases including angioedema, and suggests further investigation of the biased agonist property that might arise.

Moreover, to our knowledge, this is the first study to demonstrate a partial agonist effect for SQ29548 on B2R-mediated effects in VSMCs. Such finding requires further investigation to determine its implications on the vasculature.

With these findings, we hope we could contribute to the novel and emerging lines of research addressing GPCR oligomerization and biased agonist signaling. We seek that our results could add to the existing knowledge of the cooperative mechanisms underlying mitogenesis in VSMCs. Ultimately, this would help in a better understanding of the physiological and pathophysiological impact of such cooperation on the vasculature.

Finally, several approaches have been made to pharmacologically inhibit the individual effects of BK and TX on the injured vasculature. However, a complex cooperation seems to exist between BK and TX, which could account for their roles in the exacerbation of vascular diseases. Hence, taking this synergy into consideration would provide a new platform for more effective therapies.

C- Thesis Content and Scope:

This research starts with an introductory chapter which provides a concise literature overview that serves as a background introducing the reader to the main concepts covered in our work. Then, the second chapter sheds light on the aim,

objectives, significance, limitations, and organization of this research project. In the third chapter, the adopted experimental methodologies are explained, with a list of the materials used, and the statistical analysis methods applied.

The body of the research is comprised in chapters -four to six-, which state the results obtained after applying the diverse experimental methodologies that serve to attain our thesis objectives. Chapter four includes the preliminary data that pave the way for our research work, while chapters five and six provide a thorough explanation of our attained results. Then, the seventh chapter is dedicated for the critical analysis and discussion of the aforementioned collected data.

Finally, this work ends with a conclusion that sums up the final hypotheses deduced and provides recommendations for future work.

D- Thesis Limitations:

As in any experimental research work, there are some potential limitations that would affect our research output.

One limitation of our work is that it was conducted on cultured RASMCs which are known to be prone to lots of variability. These primary cells might also lack some environmental signals involved in VSMC proliferation and differentiation that are usually found *in vivo*. Hence, a complementary approach could be by conducting parallel experiments on *in vivo* models and matching the outcome of the *in vitro* and *in vivo* counterparts.

Furthermore, tracking receptor trafficking was conducted by overexpressing B2R-YFP in HEK293T cells. HEK293T cells have been recently shown to have intrinsically functional B2R [**107**], but lack any functional endogenous TP receptors. A

better representative outcome would have been gained, had these experiments been conducted on cells endogenously expressing both receptors such as cultured VSMCs. However, our lacking of selective antibodies for B2R ruled out the possibility of conducting such work on RASMCs for the time being.

The limitation of using pharmacological inhibitors to shut down the signaling through receptors lies in the partial inhibition of the pharmacological activity of the receptors, or biased agonist signaling that might arise. Thus, as supporting evidence, targeted deletion of B2R gene using siRNA or in knockout mice should be performed, as better representatives of total blockade of receptor activity.

Moreover, data collected for time- and concentration-response curves were results of densitometric quantification of protein bands revealed by Western blotting. However, with our knowledge of the subjectivity of densitometry, and of the semi-quantitative aspect of Western blotting technique, such results would be indicative of the fold-change in ERK1/2 phosphorylation in response to different treatment combinations, but could not account to the exact change in protein levels inside the cells.

Finally, in this work, we only studied the crosstalk between TP and B2R in VSMCs on the ERK1/2 pathway. However, additional work should be done addressing other MAPK pathways, like p38 and JNK pathways.

CHAPTER III

MATERIALS AND METHODOLOGIES

A- Materials:

IBOP and SQ29548 were from Cayman Chemical Co. (Ann Arbor, MI, USA). Bradykinin, B-3259 (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and HOE140, H157 were from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Minimum Essential Medium (DMEM), Penicillin, Streptomycin, L-Glutamine, and Trypsin were obtained from Cambrex (Rockland, ME, USA). Collagenase A was from Roche Diagnostic (Indianapolis, USA). Acrylamide, Agarose, Fetal bovine serum (FBS), Bovine Serum Albumin (BSA), TritonX, Ethidium bromide, Poly-D-lysine and Kanamycin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), Hepes, Sodium Chloride, and Tween 20 were obtained from Ameresco (Solon, Ohio, USA). Supported nitrocellulose membranes, Sodium dodecyl sulfate (SDS), TEMED, Glycine, and Braddford reagent were obtained from BioRad (Hercules, CA, USA). Tris, LB medium, and LB agar were obtained from MP-Q-Biogene (Carlsbad, CA, USA). Anti- pERK1/2 rat monoclonal antibody was obtained from Cell Signaling (USA). Anti- total ERK2 IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Donkey anti-rabbit IgG was from Jackson ImmunoResearch (Baltimore, PA, USA). XhoI, HindIII, Mini and Midi Prep kits for plasmid extraction, and Enhanced Chemiluminesence (ECL) kit were obtained from Roche (Mannheim, Germany). Autoradiograph films were obtained from Fuji (Tokyo, Japan). FluorSave TM mounting reagent was from Calbiochem (San Diego, CA, USA). DH5α bacteria were obtained from Invitrogen (Carlsbad, CA, USA). Formaldehyde and ethylene diamine tetraacetate

(EDTA) were obtained from Fisher Scientific (London, UK). Anti- alpha-smooth muscele (anti-α-SM) actin mouse monoclonal antibody (MC-clone 1A4 ascites fluid) was obtained from Sigma-Aldrich (St. Louis, MO, USA). HEK293-T cells were a gift from Dr. Raya Saab's lab.

B- Experimental Methodologies:

1. Cell Culture:

100-150 g male Sprague Dawley rats were anesthetized using carbon dioxide prior to their dissection. Rat aorta was then extracted and placed into warm sterile phosphate buffered saline (PBS). Under a cell culture biosafety cabinet, adventitia was stripped off using a forceps. The aorta was then cut off longitudinally, and the inner endothelial cells scraped off gently with the forceps. Then, aorta was transferred to 15 ml-tubes containing collagenase A solution (dilution1:20) contained in 5 ml DMEM-FBS free media- for 1 hr at 37ºC in a water bath for further digestion. This was followed by scraping of the aorta, which was then grinded into fine aortic rings using a forceps. Finally, rings of each aorta were kept in a T25-flask of DMEM containing 1 g/L glucose, 10% FBS, 1% penicillin, 20 mM HEPES (pH 7) and 1% L-Glutamine. The flask was then placed in a 37ºC incubator with 5% CO_2 to allow adherence and growth of cells. Cells were used between passages 2 and 6 inclusive. Our work on RASMCs was done by using low glucose media (1 g/L = 5 mM), due to the fact that B2R expression was upregulated in specific experimental systems when culture medium with high-glucose was used [**108**]. Moreover, MAPK pathway is known to be activated when high glucose media is used which might interfere with our aimed results.

2. Western Blotting:

RASMCs were plated on six-well plates (150,000/well) and allowed to grow in 10% FBS-containing media (low glucose DMEM). Subconfluent cells were serum-deprived for 48-72 hrs and starvation medium (low glucose DMEM + BSA 0.1%) was changed 2 hrs prior to stimulation. After incubation with the proper treatments, cells were washed with ice-cold PBS containing Ca^{2+} and Mg^{2+}, pH 7.4, and lysed by incubating in ice-cold lysis buffer [25 mM Tris, pH 7.4, containing 1% (v/v) NP40, 150 mM NaCl, 1 mM EDTA, 5% (v/v) Glycerol, 1mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM sodium orthovanadate,1mM PMSF, 2μg/ml leupeptin, 2μg/ml aprotinin, and 1 mM Benzamidine] for 10 min. Lysates were then centrifuged at 15000 G (12000 rpm) for 15 min at 4°C. Clear supernatants were collected for protein quantification (Braddford assay) and Western blotting. 10 μg of the protein samples were prepared in Laemmli4x sample buffer, then separated by SDS-PAGE (10% gels), and transferred on to supported nitrocellulose membranes (pore size of 0.45 μm). Blots were then blocked for 1 hr in Tris-buffered saline containing Tween 20 (TBS-T) [50mM Tris, pH 7.5, 250mM NaCl and 0.1% (v/v) Tween 20], containing 5% (w/v) fat free milk powder. Blocked membranes were subsequently incubated for 1hr at 25°C with anti-phospho- ERK1/2 (Cell Signaling® - dilution: 1;8000 in TBS-T) or anti-total ERK2 (Santa Cruz® - dilution: 1:8000 in TBS-T). Membranes were then washed five times, 10min each, with TBS-T followed by incubation for 1hr at 25°C with donkey anti-rabbit antibody coupled to horseradish peroxidase (Jackson ImmunoResearsh® - dilution 1:6000 in TBS-T containing 5% nonfat dry milk). Washes were repeated for three times, 10 min each and bands revealed by an enzymatic chemiluminescence detection solution using ECL (Roche)® according to the manufacturer's protocol.

Signals were quantified by densitometry using ImageJ® and plotted as fold over basal phosphorylation - (results were rectified to the basal according to the ImageJ® user guide). Finally, experimental data were fitted to an EC_{50} model using GraFit 7 Erithacus Software®.

3. Plasmid Preparation:

BDKRB2 pcDNA3.1 was obtained from UMR cDNA Resource Center, University of Missouri-Rolla and further conjugated to YFP at Dr. Jaffa's lab, Medical University of South Carolina. The plasmid was transformed in DH5α bacteria, whereby 10 µl of B2R-YFP was added to 50 µl of the bacteria and incubated on ice for 30 min. The bacteria were then heat-shocked at 42°C for 45 sec followed by another rapid incubation on ice for 2 min. 0.95 ml LB broth was then added to the mixture followed by incubation in a 200 rpm shaker at 37°C for 1 hr. Colonies of transfected bacterial cells were selected on LB agar plates containing 25 µg/ml of Kanamycin. Roche® midi plasmid preparation kit was used to obtain sufficient amounts of the purified plasmid whose DNA quantification was determined by reading absorbance at 260 and 280 nm using the NanoDrop ®. Identity of the DNA plasmid was detected by digestion with specific restriction enzymes (XhoI + HindIII) according to the respective map of B2R-YFP followed by gel electrophoresis on 1% agarose gel containing 1% Ethidium Bromide. Identity was approved by visualizing the gel with a UV light and a gel imaging device.

4. Calcium Phosphate Transfection:

150,000 HEK293T cells/well were seeded 48 hrs before the start of transfection onto glass coverslips coated with 0.1 mg/mL poly-D-lysine in 12-well plates. Cells were allowed to grow in DMEM containing 10%FBS. Cells were transiently transfected with 1 μg B2R-YFP plasmid for 48 hrs. Plasmid DNA was mixed with 4X $CaCl_2$ (2 mM Tris-HCl pH 7.9, 0.2 mM EDTA, 500 mM $CaCl_2$.2H2O), and then added drop by drop to equal volume of 2X Hepes-Buffered Saline pH 7.1 (280 mM NaCl, 1.5 mM Na2HPO4 .7H2O, 50 mM Hepes). Bubbling through was applied to ensure the formation of calcium phosphate precipitates containing the desired plasmid. The transfection mixture was kept for 25 min at room temperature whereby a fine opalescent precipitate was obtained. Subsequently, 100 μl of DNA-$CaPO_4$ co-precipitate was added dropwise to each well followed by gentle mixing. 24 hr post-transfection, the medium was replaced with fresh DMEM containing 10% FBS. Transfection was stopped after 48 hr followed by the application of the desired treatments.

5. Subcellular Localization of B2R-YFP and Confocal Microscopy:

Forty-eight hr post-transfection of HEK293-T cells with 1μg of B2R-YFP, different treatment combinations were applied. Treatment was then stopped by washing cells with PBS containing 0.1% sodium azide. Cells were then fixed with 2% formaldehyde in PBS for 20 min at room temperature, followed by another wash with PBS. Finally, cells were stained with 1% DAPI in PBS for 5min, and coverslips were mounted using FluorSave TM mounting reagent. Confocal microscopy was performed using a 63xoil immersion objective of Zeiss LSM scanning confocal microscope. DAPI

was excited at 405 nm. YFP fluorescence was excited using the Argon 510 nm laser. Fluorescence confocal images were later processed with Zen 2011 software.

6. Immunofluorescence and Detection of α-smooth Muscle (SM) Actin:

100,000 RASMCs were plated on 12-well plates onto glass coverslips coated with 0.1 mg/mL poly-D-lysine and maintained in low glucose-DMEM containing 10% FBS. Subconfluent cells were then washed with PBS containing 0.1% sodium azide and subsequently fixed with 2% formaldehyde for 20 min. Cells were washed again with PBS, permeabilized 20 min with 0.1% Triton-X-100 in PBS and blocked with 0.1% Triton-X-100 in PBS containing 2% BSA for 30 min at room temperature. Cells were then incubated with mouse mononclonal antibody against α-SM actin (dilution 1:1000 in blocking solution) for 1 hr at room temperature. Subsequently, cells were washed twice with PBS, and incubated with goat anti-mouse secondary antibody conjugated to Fluorescein Isothiocyanate (FITC) (dilution 1:1000) in blocking solution for 1 hr at room temperature. Cells were washed twice with PBS, and coverslips were mounted using using FluorSave TM mounting reagent. FITC fluorescence was excited using the Argon 488-nm laser. Cells were visualized under the confocal microscopy.

7. Statistical Analysis:

Results of Western blot experiments were expressed as mean ± SE of at least three independent experiments. Unpaired t-test was used for EC_{50} statistical comparison. One Way ANOVA or Two Way ANOVA were used followed by Bonferroni procedure

as *post hoc* analysis for comparing data within the same treatment group or between different treatment combinations, respectively. Differences in compared data of $P < 0.05$ were considered as statistically significant.

CHAPTER IV

PRELIMINARY DATA

A- Extraction and Identification of RASMCs:

To confirm that the protocol for RASMC extraction was valid and yielding VSMCs, we visualized cells under the transmission light (TL)-phase microscopy. The "Hill and Valley" growth pattern of the cells, specific for VSMCs, was obvious (Figure 11).

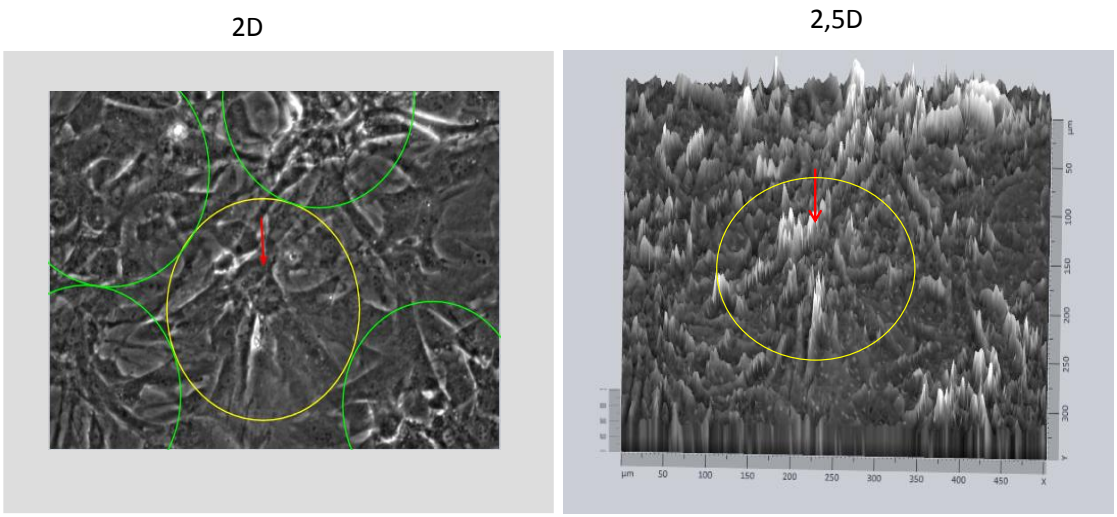

Figure 11: 2- and 2,5- Dimensional representations of RASMCs:
The "Hill and Valley" characteristic of VSMCs was obvious in both the 2-D and 2,5-D demonstrations. Circles enclose several "hill- like" assemblies of cells. The red arrow points to the tip of a hill, which is more obvious in the 2,5D display. The "hill" appearance is due to the overlay of multiple layers of cells in a certain position, and the "valley" represents a monolayer cellular circumscription of the enclosed "hill". Cells were visualized using the 10 x magnification objective and TL- phase of Zeiss Fluorescent Inverted Microscope.

We also checked the presence of α- SM actin, specific for VSMCs. This was done by immunofluorescence analysis, using anti-α-SM actin and FITC-labeled goat

anti-mouse antibodies as primary and secondary antibodies, respectively. Cells were visualized using confocal microscopy, which revealed the presence of green fluorescent α-SM actin filaments (Figure 12).

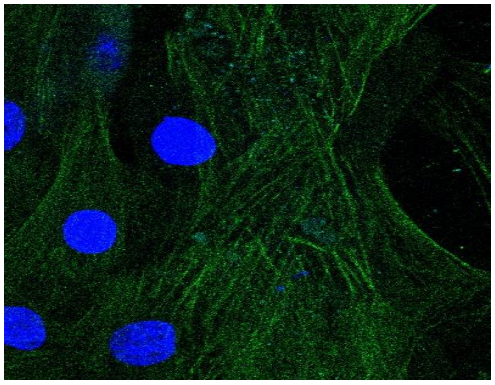

Figure 12: Representative fluorescence microscopy image of α-SM actin in RASMCs.
RASMCs were seeded on glass coverslips in 12-well plates and brought to about 80% confluency. α-SM actin was stained with mouse mononclonal anti- α-SM actin antibody, and visualized with FITC-labeled goat anti-mouse secondary antibody (green). Nuclei were stained with DAPI (blue).

B- IBOP or BK can Individually Induce ERK1/2 Phosphorylation in RASMCs and Enhance their Proliferation:

The ERK1/2 MAPK is implicated in various physiological processes involved in cellular proliferation, migration, differentiation, and survival [**68, 69, 70**]. In this regard, several previous studies have shed light on the individual capability of each of BK and TX to favor VSMCs proliferation by activating the MAPK cascade. However, none of these studies addressed the possibility of crosstalk between both receptors at the level of ERK1/2 signaling pathway. Consistent with these previous data, and to initiate our own study system, we demonstrated the ability of IBOP and BK to individually induce ERK1/2 phosphorylation and, thus, activation in a time- and concentration-dependent manner. Therefore, in this study, we assume that any increase in ERK1/2

phosphorylation induced by BK, IBOP, or their combinations would be leading to enhanced mitogenesis and proliferation of VSMCs. Also in these experiments, the negative control represented un-stimulated cells and accounted for the basal level. Accordingly, fold over basal phosphorylation of ERK1/2 was rectified to this basal.

1. IBOP Induces ERK1/2 Phosphorylation in RASMCs in a Time- and Concentration-dependent Manner:

To determine the time at which IBOP induced optimal ERK1/2 activation, RASMCs were stimulated with 2.5 x 10^{-7} M of IBOP for various times (between 0 and 30 min). ERK1/2 phosphorylation was detectable within 1 min (2.7 ± 1.18 fold/basal phosphorylation) of IBOP addition, significantly peaked around 15 min (10.15 ± 2.46 fold /basal phosphorylation, $p < 0.001$ as compared to basal and $p < 0.05$ Vs. stimulation with IBOP for 1min), and then started to decline within 30 min (7.77 ± 1.49, $p < 0.01$ as compared to basal) (Figure 13A).

Later, RASMCs were stimulated with increasing concentrations of IBOP (10^{-11} to 10^{-7} M) for 10 min to determine the concentration eliciting the maximal activation. Results revealed that IBOP stimulated ERK1/2 activation in a concentration-dependent manner, with maximal activation at 10^{-7} M (11.3 ± 4.3 fold/basal activation, $p < 0.01$ as compared to basal). Treatment with 10^{-8} M of IBOP for 10 min also resulted in a significant increase in tyrosine phosphorylation of ERK1/2 which accounted for (7.11 ± 2.61) folds ($p < 0.05$ as compared to basal). The 50% effective concentration (EC_{50}) value was estimated after fitting the data points to a non-linear regression model using GRAFIT 7 Erithacus® software and accounted for (9.84 x 10^{-9} ± 2.76 x 10^{-9}) M of IBOP (Figure 13B).

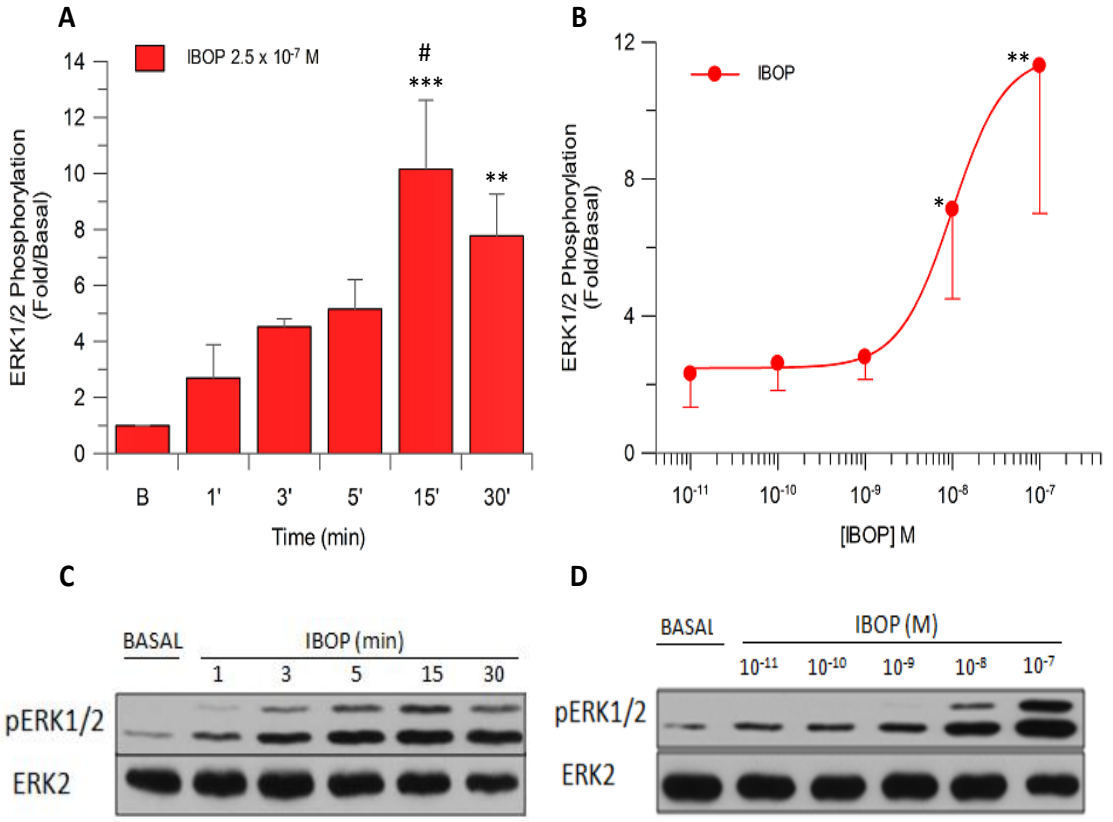

Figure 13: IBOP-Induced Activation of ERK1/2
Densitometric analysis and Western blots of RASMCs treated with (**A and C):** 2.5 x 10^{-7} M of IBOP for the indicated times or (**B and D):** increasing concentrations of IBOP (10^{-11} to 10^{-7} M) for 10 min. **C and D**: Western blots showing protein bands of pERK1/2 and total ERK2. Results are mean ± SE of four independent experiments.
(A): ** $p < 0.01$, *** $p < 0.001$ as compared to basal. # $p<0.05$ as compared to IBOP treatment for 1 min (One Way ANOVA, *post hoc* Bonferroni)
(B): * $p < 0.05$, ** $p < 0.01$ as compared to basal (One Way ANOVA, *post hoc* Bonferroni)

2. BK Induces ERK1/2 Phosphorylation in RASMCs in a Time- and Concentration-dependent Manner:

Likewise, treatment with BK led to enhanced phosphorylation of ERK1/2. For instance, BK-induced ERK1/2 phosphorylation was maximal after stimulating RASMCs with 10^{-7} M of BK for around 15 min (7.5 ± 2.78 fold/basal phosphorylation),

and subsequently decreased to 4.8 ± 2.02 fold/basal phosphorylation after 30 min of stimulation (Figure 14A).

Moreover, concentration-response experiments conducted on RASMCs treated with increasing concentrations of BK (10^{-11} M to 10^{-7} M) elicited an optimal stimulation when cells were stimulated with 10^{-7} M of BK for 10 min (3.23 ± 1.14 fold/basal phosphorylation) (Figure 14B).The estimated EC_{50} was ($1.85 \times 10^{-9} \pm 4.22 \times 10^{-9}$) M of BK.

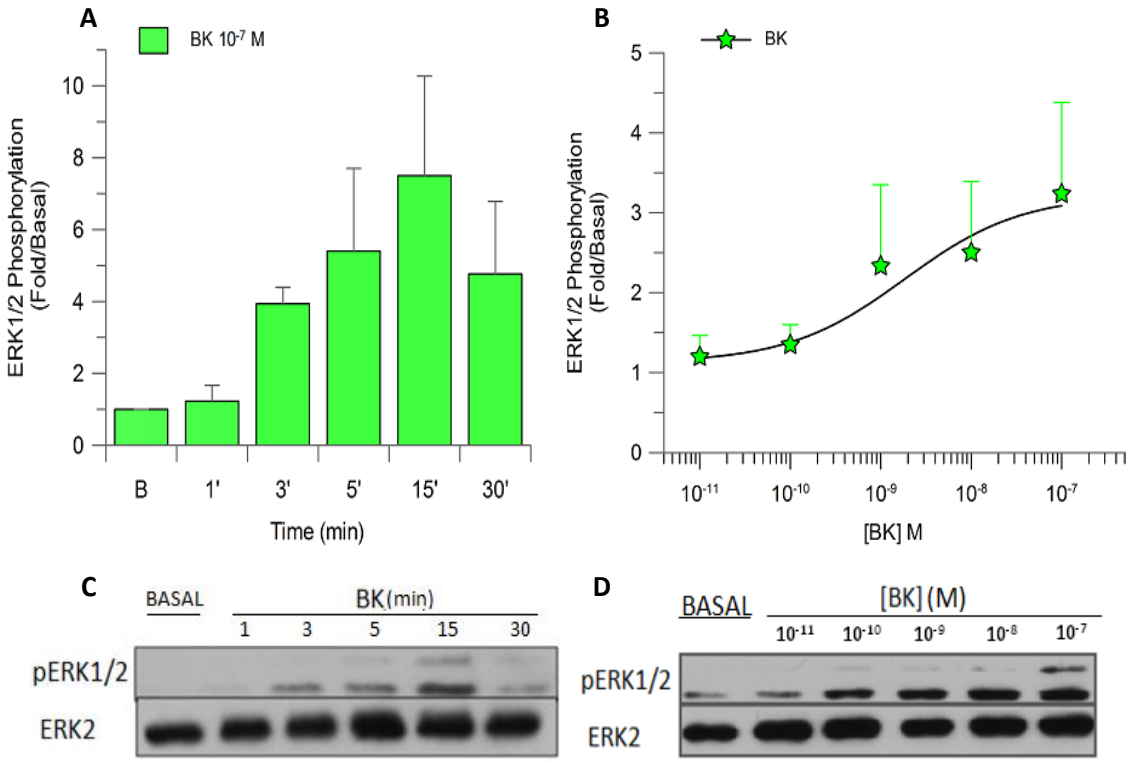

Figure 14: BK-induced activation of ERK1/2
Western blots and densitometric analysis of lysates of RASMCs treated with **(A):**10^{-7} M of BK for the indicated times, or **(B):** increasing concentrations of BK (10^{-11} to 10^{-7} M) for 10 min. Protein bands are representative of pERK1/2 and total ERK2. Results are mean ± SE of three independent experiments.

3. B2R and TP Respectively Mediate BK and TX Pharmacological Responses in RASMCs:

We next wanted to determine the type of receptors involved in the pharmacological effects of BK and TX in RASMCs. Therefore, cells were treated with either HOE140, B2R selective antagonist, or SQ29548, TP potent antagonist. Pretreatment of RASMCs with 10^{-6} M of HOE140, 30 min prior to stimulation with increasing concentrations of BK inhibited the effect of BK on ERK1/2 phosphorylation (Figure 15). This implies that B2R mediates the effects of BK in RASMCs. Similar output was gained with total inhibition of IBOP-induced effects on RASMCs, when cells were pre-incubated with 10^{-5}M of SQ29548 for 30 min before stimulation with increasing concentrations of IBOP (Figure 16). Thus, B2R and TP, respectively, mediate BK and TX pharmacological responses in RASMCs.

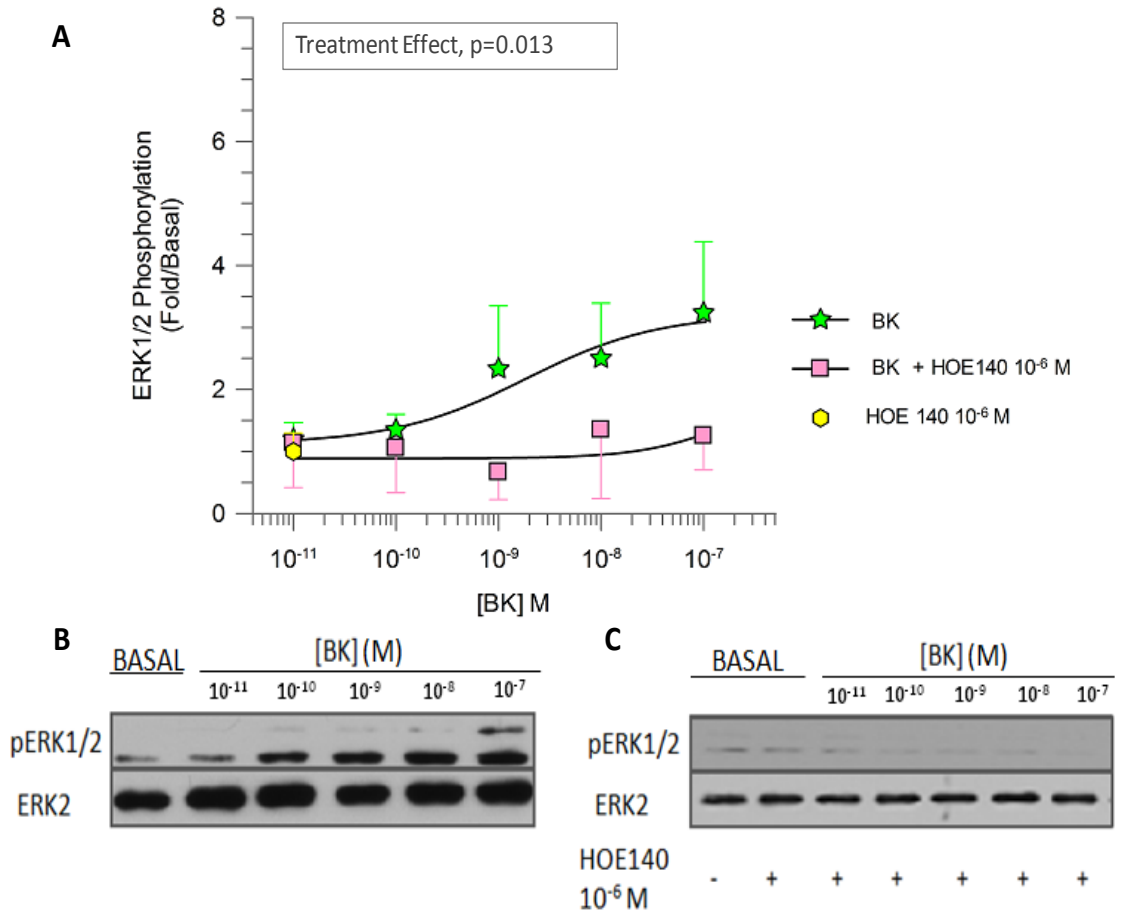

Figure 15: BK elicits its action on RASMCs through B2R
(A): Concentration-response curve of the "fold over basal" ERK1/2 phosphorylation after treatment with increasing concentrations of BK (stellar), versus that obtained when cells were pretreated with 10^{-6} M of HOE140 (B2R selective antagonist) prior to stimulation with BK (squared). Also represented is the single effect of 10^{-6} M of HOE140 on fold/basal ERK1/2 phosphorylation (mean of three independent experiments). HOE140 inhibited the activity of BK on RASMCs, pointing that BK's activation of ERK1/2 is mediated by B2R. **(B and C):** Western blots representing bands of pERK1/2 and total ERK2 for concentration-response treatment of RASMCs with BK alone (B), or after pretreatment with HOE140 (C). Results are mean ± SE of three independent experiments.

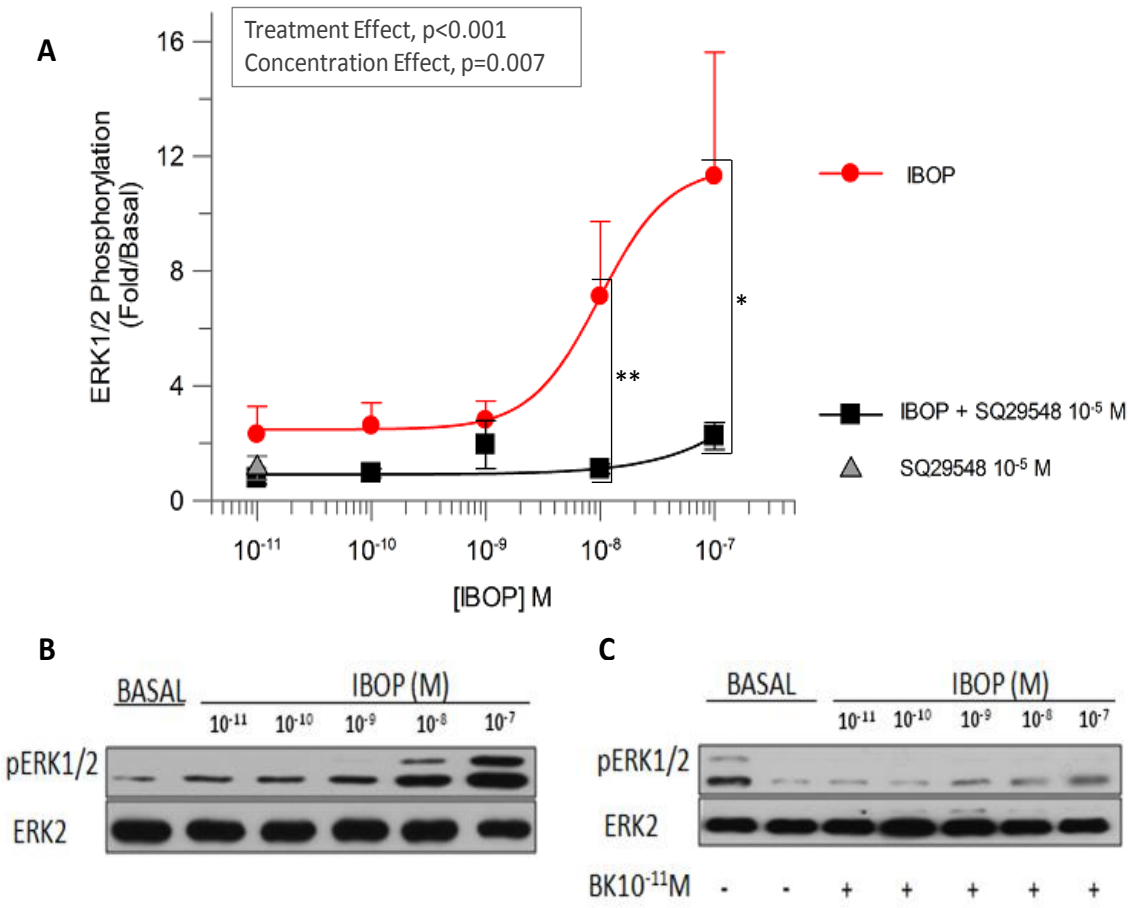

Figure 16: TP mediates IBOP-induced ERK1/2 phosphorylation in RASMCs
A: Concentration-response curve of the "fold over basal" ERK1/2 phosphorylation after treatment with increasing concentrations of IBOP (circular), versus that obtained when cells were pretreated with 10^{-5} M of SQ29548 (TP antagonist) prior to stimulation with IBOP (squared). Also represented is the single effect of 10^{-5} M of SQ29548 on fold/basal ERK1/2 phosphorylation (mean of three independent experiments). SQ29548 totally inhibited the activity of IBOP on RASMCs, pointing that TX elicits its effect on ERK1/2 pathway in RASMCs through TP. **(B and C):** Western blots representing bands of pERK1/2 and total ERK2 for RASMCs treated with BK alone (B), or after pretreatment with HOE140 (C). Results are mean ± SE of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ (IBOP Vs. IBOP + SQ29548) (Two Way ANOVA, *post hoc* Bonferroni)

CHAPTER V

STUDIES CONDUCTED ON RASMC

A- Synergistic Cooperation between B2R and TP in RASMCs:

Subsequently, we wanted to study the bi-directional crossmodulation between B2R and TP on the ERK1/2 pathway in VSMCs. Therefore, simultaneous treatment of RASMCs with a minimal concentration of one agonist, and increasing concentrations of the other were performed and analyzed.

1. BK Synergistically Potentiates IBOP-induced Activation of MAPKs:

RASMCs were co-stimulated with a minimal effective concentration of BK (10^{-11} M) and increasing concentrations of IBOP (10^{-11} – 10^{-7} M) for 10 min. The obtained results revealed a significant increase in ERK 1/2 phosphorylation when BK was added, at concentrations of IBOP equivalent to 10^{-9} M, 10^{-8} M, and 10^{-7} M: (10.98 ± 3.31), (16.89 ± 3.23), and (20.51 ± 5.12) folds, respectively as compared to basal. Besides, a significant increase ($p < 0.05$) in IBOP–induced effects on ERK was realized when cells were co-stimulated with 10^{-9}M of IBOP and 10^{-11}M of BK: (10.98 ± 3.31) folds as compared to the data corresponding to equivalent IBOP concentrations in cells individually treated with IBOP: (2.8 ± 0.66) folds (Figure 17). Besides, a significant shift in EC_{50} was observed ($p < 0.05$), which varied from ($9.84 \times 10^{-9} \pm 2.76 \times 10^{-9}$) M for IBOP concentration-response curve to ($5.55 \times 10^{-10} \pm 4.28 \times 10^{-10}$) M in case of co-stimulation with minimal concentration of BK and increasing concentrations of IBOP.

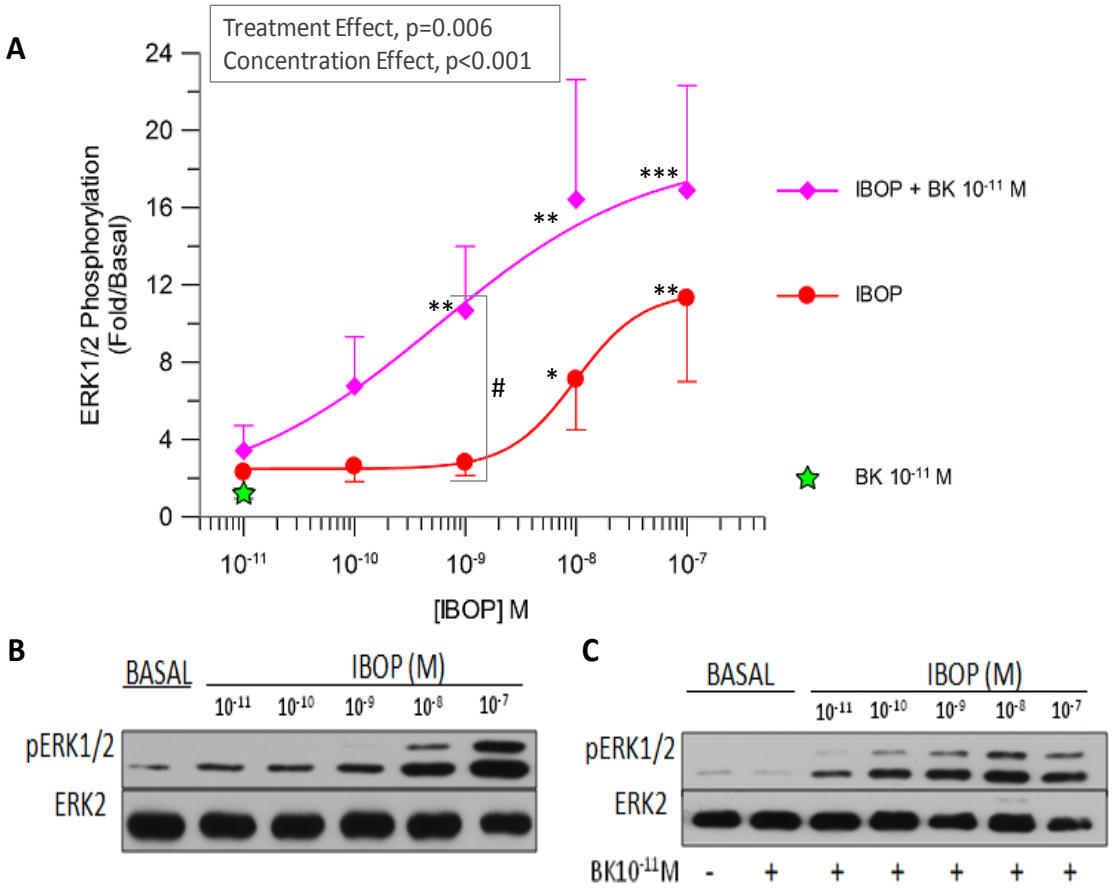

Figure 17: BK Potentiates IBOP-induced Activation of MAPKs
A: Concentration-response curves of fold/basal ERK1/2 phosphorylation in RASMCs after treatment with increasing concentrations of IBOP alone (circular), or upon co-treatment with minimal concentration of BK (diamond). Fold over basal ERK1/2 phosphorylation induced by BK 10^{-11} M (mean of three independent experiments) is also presented. **B and C:** Western blots representing bands of pERK1/2 and total ERK2 for (**B:** IBOP concentration-response treatment alone), or (**C:** with BK 10^{-11} M). Results are mean ± SE of four (C) or five (B) independent experiments.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to basal. # $p < 0.05$ (IBOP Vs. IBOP + BK 10^{-11} M) (Two Way ANOVA, *post hoc* Bonferroni)

To assess whether the realized positive modulatory effect of BK on IBOP-induced ERK1/2 phosphorylation was additive or synergistic, we performed a hypothetical simulation of the additive effect of BK10^{-11}M + IBOP at the different concentration combinations by summing the corresponding values of fold/basal ERK1/2 phosphorylation that were calculated for each combination of concentrations (in M) of IBOP-BK (10^{-11} – 10^{-11} ; 10^{-10} – 10^{-11} ; 10^{-9} – 10^{-11} ; 10^{-8} – 10^{-11} ; 10^{-7} – 10^{-11}). Our findings allow us to propose a synergistic effect of BK (10^{-11} M) when co-treated with concentrations of IBOP between 3.6 x 10^{-11} M and 10^{-7} M (Figure 18).

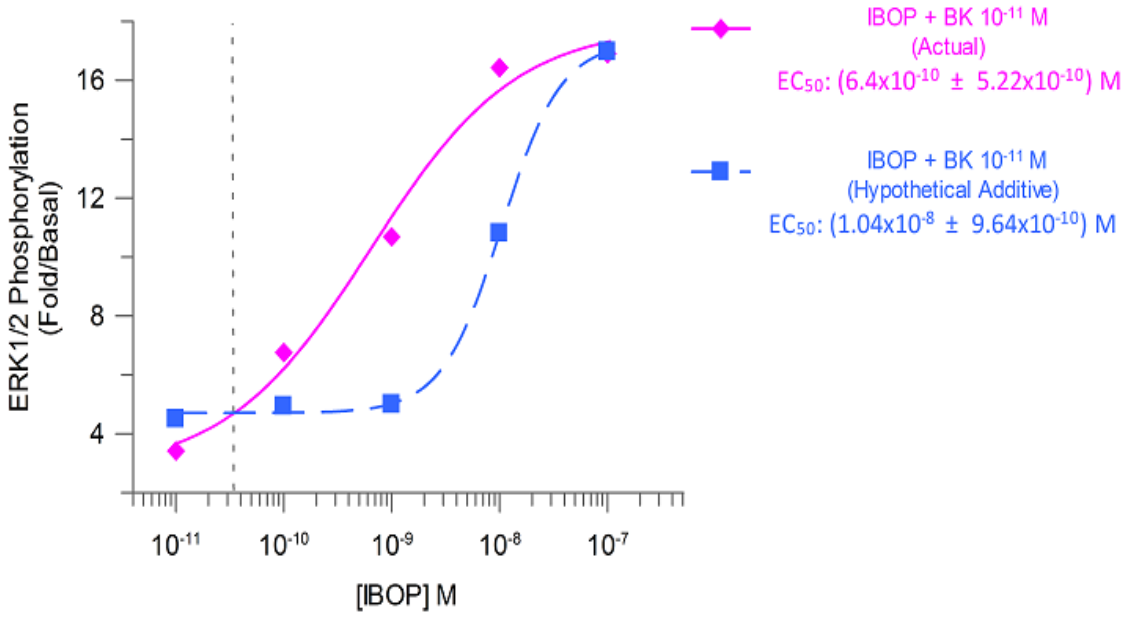

Figure 18: Hypothetical additive (squared-broken line) and actual (diamond-closed line) concentration–response curves of the effects of co-stimulation with IBOP (10^{-11} to 10^{-7} M) and BK 10^{-11} M on the phosphorylation of ERK1/2 in RASMCs treated for 10 min.

The vertical line indicates the concentration at which the combined effects of BK and IBOP were additive. Synergy could be achieved for concentrations of IBOP between 3.6 x 10^{-11} M and 10^{-7} M.

2. IBOP Enhances BK-induced Activation of MAPKs:

RASMCs were simultaneously treated with a minimal concentration of IBOP (10^{-11} M) and increasing concentrations of BK (10^{-11} - 10^{-7} M) for 10 min. Although our results reflect an increase in BK responses on RASMCs when IBOP was added, this increase in fold/basal phosphorylation of ERK1/2 was only slight at concentrations of BK below 10^{-9} M (Figure 19). However, a statistically significant increase in ERK1/2 phosphorylation was seen at concentrations of BK between 10^{-9} M and 10^{-7} M when RASMCs were co-stimulated with a minimal concentration of IBOP (10^{-11} M) as compared to the basal. For instance, ERK1/2 phosphorylation increased (3.78 ± 1), (4.84 ± 1.2), and (6.97 ± 1.8) folds for concentrations of BK equivalent to 10^{-9}, 10^{-8}, and 10^{-7} M respectively, when co-stimulation with 10^{-11} M of IBOP was applied. Comparison between the concentration-response curve representative of single treatment with BK and that corresponding to co-stimulation with a minimal concentration of IBOP and increasing concentrations of BK revealed a statistically significant difference both in the treatment effect ($p = 0.004$) and concentration effect ($p < 0.001$) applied (Figure 19).

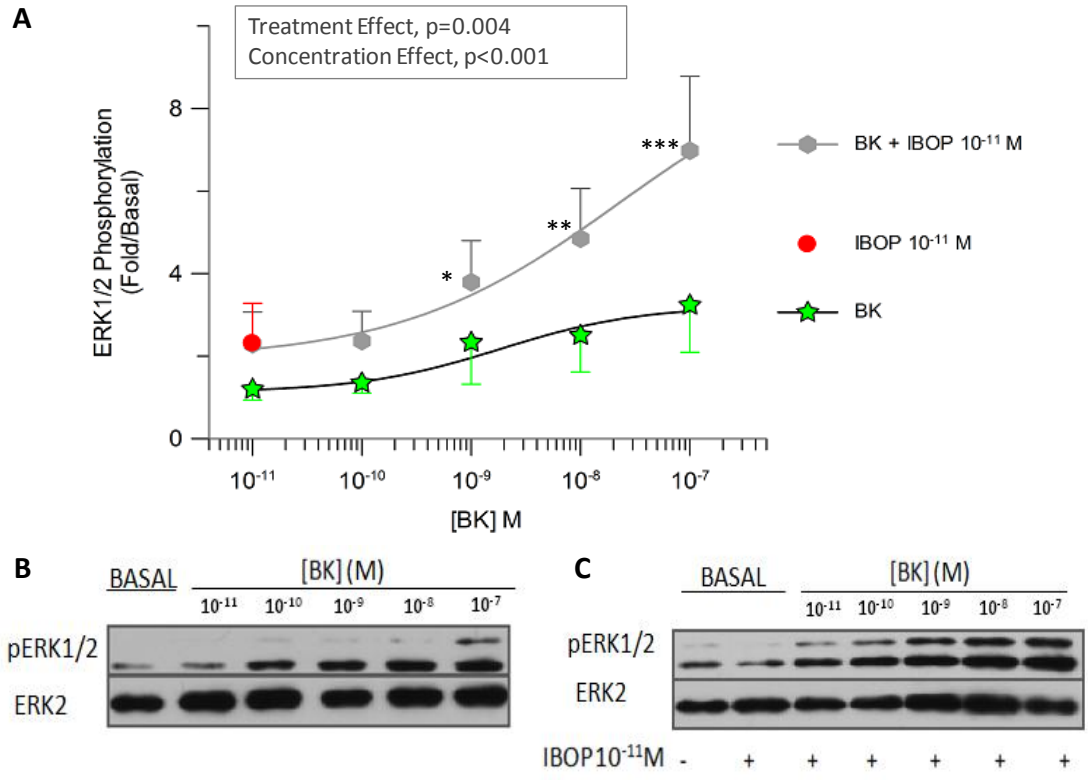

Figure 19: IBOP Enhances BK-induced ERK1/2 Phosphorylation in RASMCs
A: Concentration-response curves of fold/basal ERK1/2 phosphorylation in RASMCs after treatment with increasing concentrations of BK alone (stellar), or upon co-stimulation with a minimal concentration of IBOP (hexagon). Fold over basal ERK1/2 phosphorylation of IBOP 10^{-11} M (mean of three independent experiments) is also presented. **B and C:** Western blots representing bands of pERK1/2 and total ERK2 for (**B:** BK concentration response treatment alone), or (**C:** with IBOP 10^{-11} M). Results are mean ± SE of three (B) or four (C) independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to basal (Two Way ANOVA, *post hoc* Bonferroni)

As in the case of co-stimulation with minimal BK concentration and increasing concentrations of IBOP, we subsequently assessed whether the realized positive modulatory effect of IBOP on BK-induced ERK1/2 phosphorylation was additive or synergistic. So, we performed the same aforementioned hypothetical simulation.

Our obtained results point to an additive effect of IBOP (10^{-11} M) when co-treated with concentrations of BK greater than or equal 10^{-8} M. Although, at

concentration of BK equal to 10^{-7} M, this effect appeared to be higher than the hypothetical additive, it still cannot be considered as synergistic.

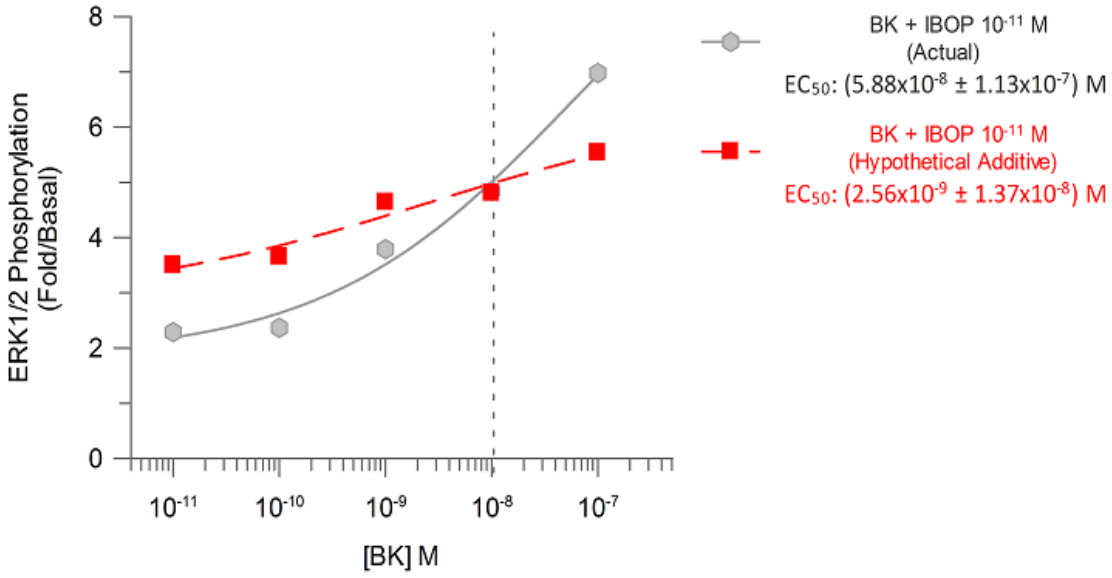

Figure 20: Hypothetical additive (red broken line) and actual (grey closed line) concentration–response curves of the effects of co-stimulation with BK (10^{-11} to 10^{-7} M) and IBOP 10^{-11}M on the phosphorylation of ERK1/2 in RASMCs treated for 10min.
The vertical line indicates the concentration at which effects were additive. Additive effect could only be achieved at concentrations of BK equal to about 10^{-8} M. Also, at [BK] above 10^{-8} M, this effect could be more likely considered as additive rather than synergistic.

3. Comparison between Curves of Reciprocal Activation of ERK1/2 induced by BK and IBOP:

While BK synergistically potentiates IBOP-induced ERK1/2 phosphorylation in RASMCs, IBOP's reciprocal action on BK-induced ERK phosphorylation results in an overall lower phosphorylation of ERK1/2. Comparison between both curves reveals a significant difference in the treatment effect ($p = 0.007$) and concentration effect ($p < 0.001$) applied. This reflects a statistically significant difference between BK

synergistic effects and IBOP enhancing effects when added to increasing concentrations of IBOP and BK respectively. For instance, for the combination of (10^{-11} M BK + 10^{-9} M IBOP), ERK1/2 phosphorylation increased by (10.68 ± 3.31) folds; however, for the combinations of (10^{-11} M IBOP + 10^{-9} M BK), ERK1/2 phosphorylation was only (3.78 ± 1) folds respectively. The realized difference in the effect of the aforementioned treatment combinations on ERK1/2 phosphorylation in RASMCSs was statistically significant with a $p < 0.05$.

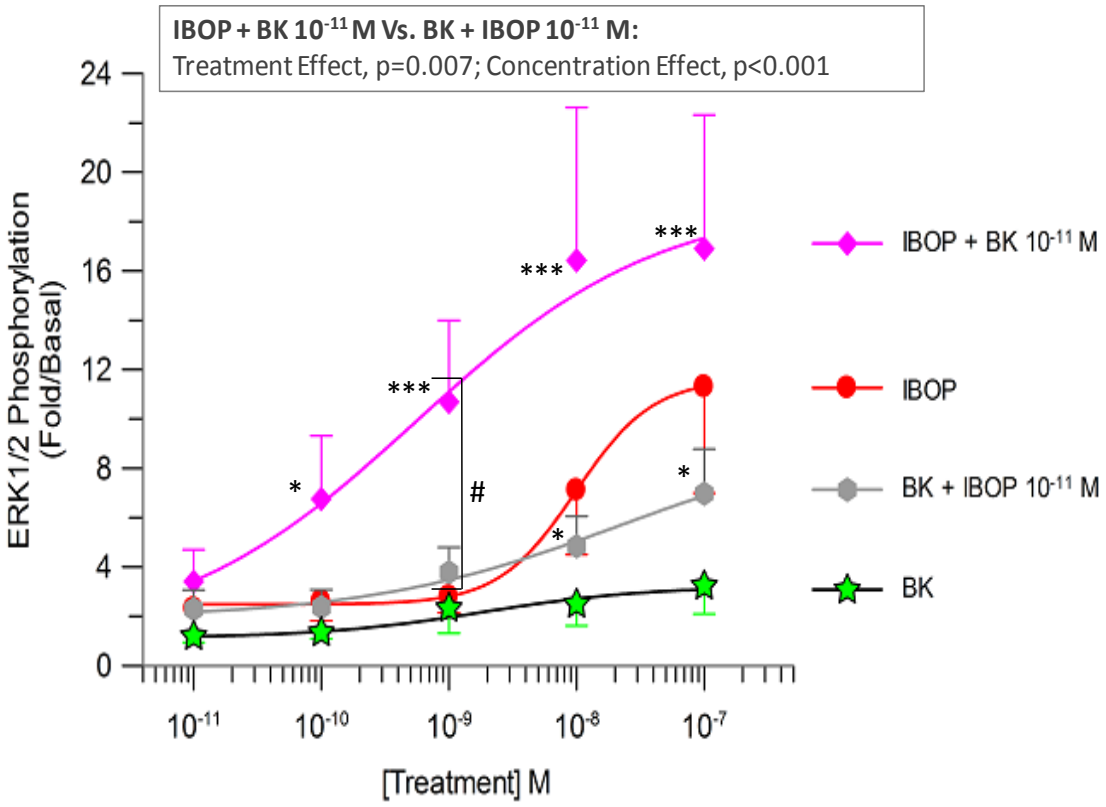

Figure 21: BK potentiation of IBOP-induced ERK1/2 phosphorylation is greater than that elicited by IBOP on BK-induced ERK1/2 phosphorylation in RASMCs. Results are mean ± SE of four independent experiments. Results were statistically analyzed using Two Way ANOVA, followed by *post hoc* Bonferroni.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to basal .
# $p < 0.05$ (IBOP + BK 10^{-11} M Vs. BK + IBOP 10^{-11} M)

B- Effects of Cross-Antagonists on IBOP- or BK- induced ERK1/2 Activation:

1. HOE 140 Attenuates TP -mediated MAPK Signaling:

Pretreatment of RASMCs with 10^{-6} M of HOE140 for 30 min prior to stimulation with increasing concentrations of IBOP (triangular) resulted in an attenuated phosphorylation of ERK1/2 as compared to cells treated with IBOP alone (circular) (Figure 22).

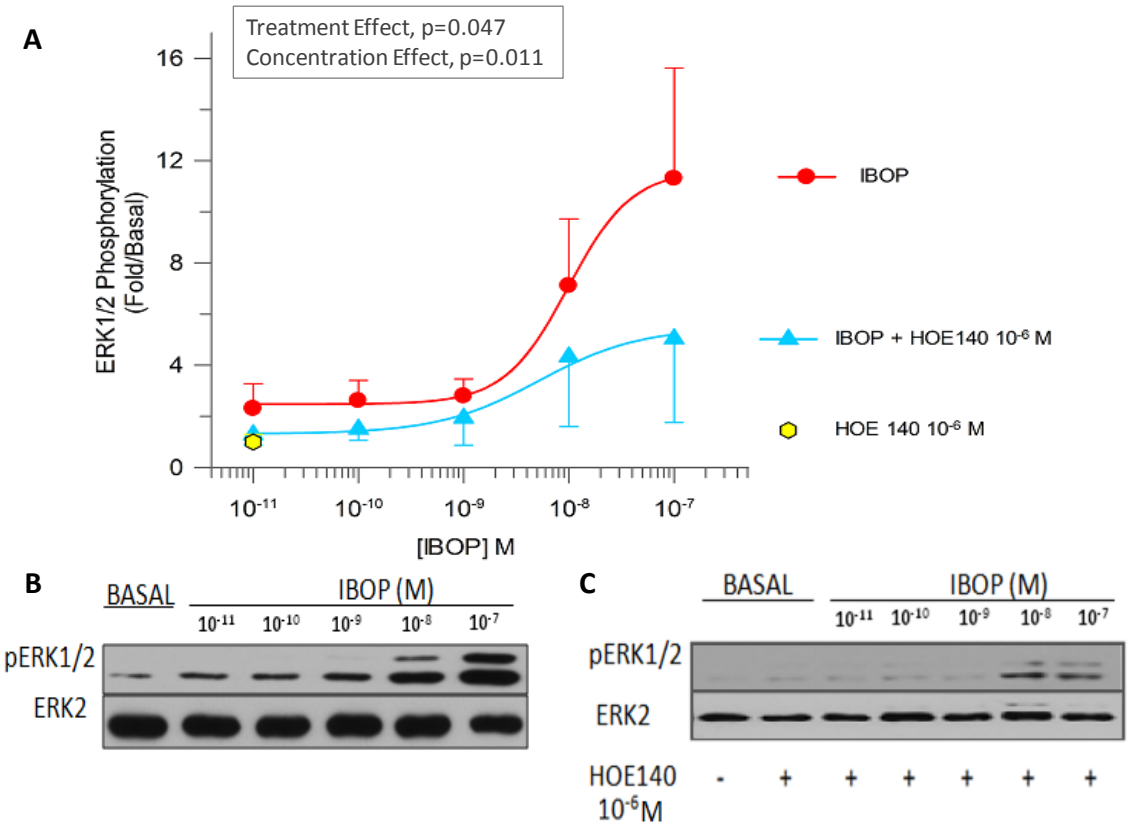

Figure 22: HOE 140 partially antagonizes IBOP activity on ERK1/2 in RASMCs
A: Comparison between concentration-response curves of fold change in ERK1/2 phosphorylation in RASMCs pretreated (triangular) or untreated (circular) with 10^{-6} M of HOE140 for 30 min prior to stimulation with increasing concentrations of IBOP for 10 min. **B and C:** Protein bands of Western blots representing pERK1/2 and total ERK2 for cells treated **(C)** or not **(B)** with HOE140 prior to stimulation with IBOP. Results are mean ± SE of three independent experiments.

No significant change in EC_{50} was observed when pretreatment with HOE140 was applied. However, fold change in ERK1/2 phosphorylation dropped down from (11.3 ± 4.31) folds upon treatment with 10^{-7} M of IBOP alone to (5.04 ± 3.27) folds after pre-incubation with HOE140. Thus, it could be implied that HOE140 lowers the efficacy of IBOP on the ERK1/2 pathway without affecting its potency.

2. SQ29548 Weakens Signaling Through B2R on MAPK in RASMCs:

The TP antagonist, SQ29548, at concentrations of 10^{-5} M, was able to inhibit the effects of BK at concentrations of the latter less than or equal to 10^{-8} M. However, at concentrations of BK between 10^{-8} and 10^{-7} M, an increase in ERK1/2 phosphorylation was noticed, which was more pronounced than that induced by BK alone for equivalent concentrations. When cells were treated with 10^{-7} M of BK for 10 min after pretreatment with SQ29548 for 30 min, a significant increase ($p < 0.05$) in fold over basal phosphorylation of ERK1/2 was observed and accounted for (5.78 ± 1.78) folds as compared to (0.97 ± 0.21) folds observed when cells were treated with 10^{-11}M of BK after pretreatment with SQ29548 for 30 min (Figure 23).This was also higher than that observed upon treatment with 10^{-7} M of BK alone. Such finding could be pointing to a partial agonist effect exerted by SQ29548 on the ERK1/2 pathway downstream of B2R in RASMCs.

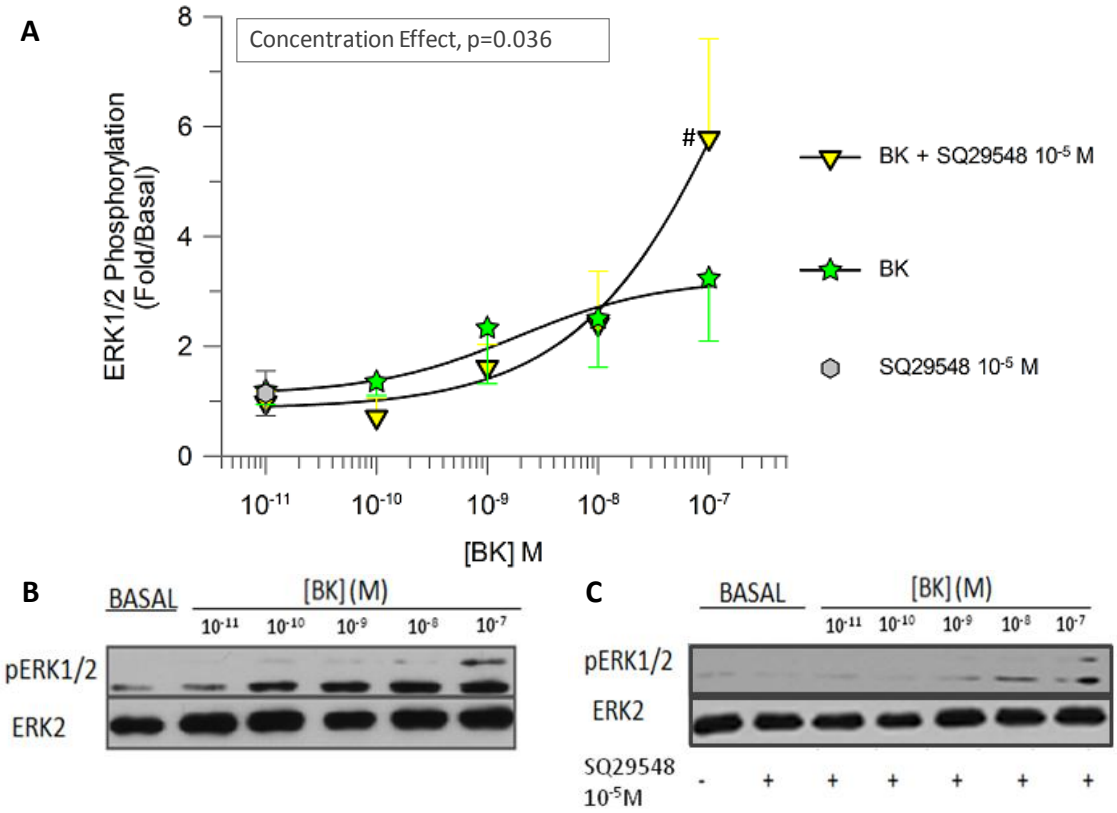

Figure 23: Effect of SQ29548 on BK-induced ERK1/2 phosphorylation in RASMCs
A: Comparison between concentration-response curves of fold change in ERK1/2 phosphorylation in RASMCs pretreated (triangular) or untreated (stellar) with 10^{-5} M of SQ29548 for 30 min prior to stimulation with increasing concentrations of BK for 10 min. **B and C**: Protein bands of Western blots representing pERK1/2 and total ERK2 for cells treated **(C)** or not **(B)** with SQ29548 prior to stimulation with BK. Results are mean ± SE of three independent experiments. # $p < 0.05$ (BK alone Vs. BK + 10^{-5} M SQ29548). (Two Way ANOVA, *post hoc* Bonferroni)

C- Effects of Triple Treatment on ERK1/2 Phosphorylation in RASMCs:

1. Effects of Antagonists on BK-induced Synergy:

a- <u>HOE140 Does not Reverse the Synergistic Effect of BK on IBOP-induced ERK1/2 Phosphorylation:</u>

To gain further insight whether HOE140 could prevent the synergy observed between BK and TX in RASMCs, cells were pretreated with HOE140 for 30 min prior to co-stimulation with a minimal concentration of BK and increasing concentrations of

IBOP for 10 min. Surprisingly, HOE140 could not reverse the synergy induced by the minimal concentration of BK when added to IBOP. On the contrary, it resulted in fold over basal phosphorylation of ERK1/2 almost equivalent to that obtained upon co-stimulation of RASMCs with a minimal concentration of BK and increasing IBOP concentrations (Figure 24). Fold changes in ERK1/2 phosphorylation were then quantified and compared to those obtained when RASMCs were stimulated with IBOP alone for 10 min. The difference in the extent of ERK1/2 phosphorylation was significant between both groups of treatments with a $p < 0.001$. In the curve of triple treatment, a significant increase in fold over basal phosphorylation of ERK1/2 was observed, and was dependent on the concentrations of IBOP applied; with a $p < 0.01$ when comparing combinations of treatments containing 10^{-9} M of IBOP to those of the basal, and ($p < 0.001$) when those treatments included 10^{-8} M and 10^{-7} M of IBOP. Finally, significance was also achieved with $p < 0.01$ when the effect of the triple treatment combination containing 10^{-9} M of IBOP (11.72 ± 4.01 folds) was compared to that of single treatment with 10^{-9} M of IBOP (2.8 ± 0.66 folds).

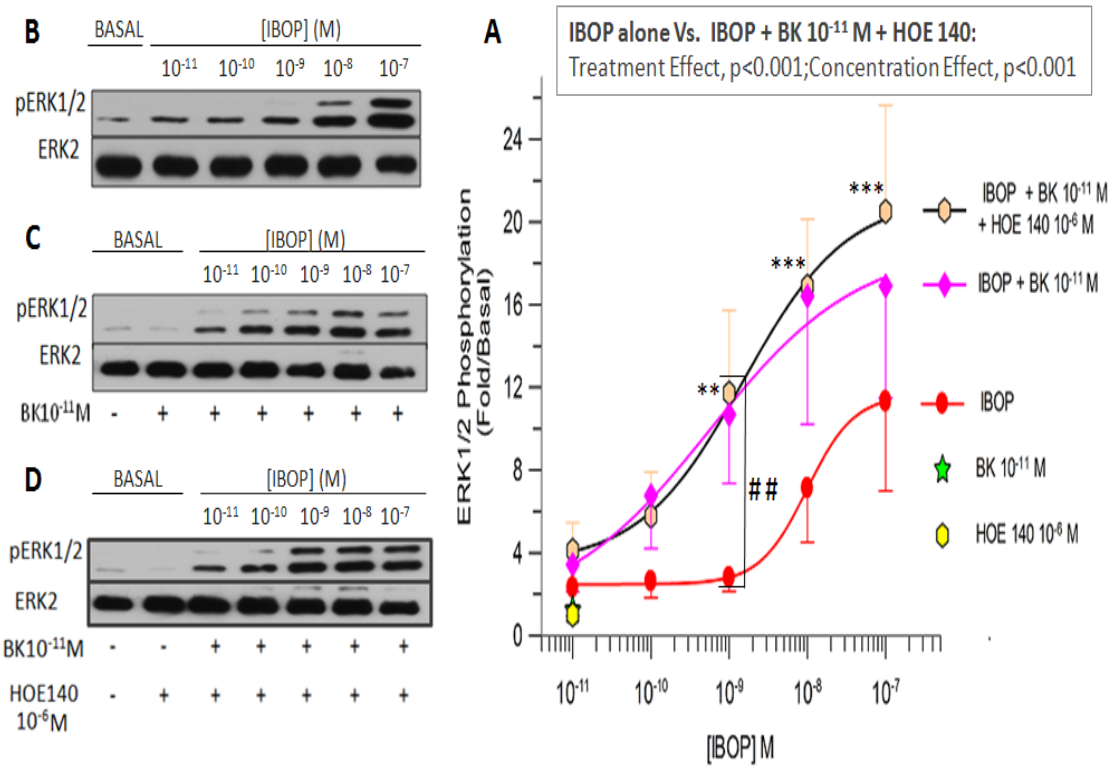

Figure 24: BK synergistic effect on IBOP-induced ERK1/2 phosphorylation was not inhibited by HOE140.

A: Concentration-response curves of fold increase in ERK1/2 phosphorylation in RASMCs pretreated (hexagon) or untreated (diamond) with 10^{-6} M of HOE140 for 30 min prior to co-stimulation with 10^{-11} M of BK and increasing concentrations of IBOP for 10 min compared to the concentration-response curve corresponding to single treatment with IBOP (circular). **B, C, and D**: Protein bands of Western blots representing pERK1/2 and total ERK2 for cells **(B):** individually treated with IBOP; or cells **(D):** treated or **(C):** untreated with HOE140 prior to co-stimulation with 10^{-11} M of BK and increasing concentrations of IBOP. Results are mean ± SE of three (D) or four (C) or five (B) independent experiments. ** $p < 0.01$ and *** $p < 0.001$ as compared to unstimulated basal. ## $p < 0.01$(IBOP alone Vs. IBOP + BK 10^{-11}M + HOE140 10^{-6} M). (Two Way ANOVA, *post hoc* Bonferroni)

b- <u>SQ29548 Inhibits the Potentiating Effect of BK on IBOP-induced ERK1/2 Phosphorylation:</u>

Unlike HOE140, SQ29548 totally inhibited any increase in ERK1/2 phosphorylation, when RASMCs were pretreated with SQ29548 for 30 min before co-stimulation with a minimal concentration of BK (10^{-11} M) and variable

concentrations of IBOP (10^{-11} – 10^{-7} M). This resulted in the significant reversal of ERK1/2 phosphorylation back to the basal level at all the treatment combinations applied (Figure 25).

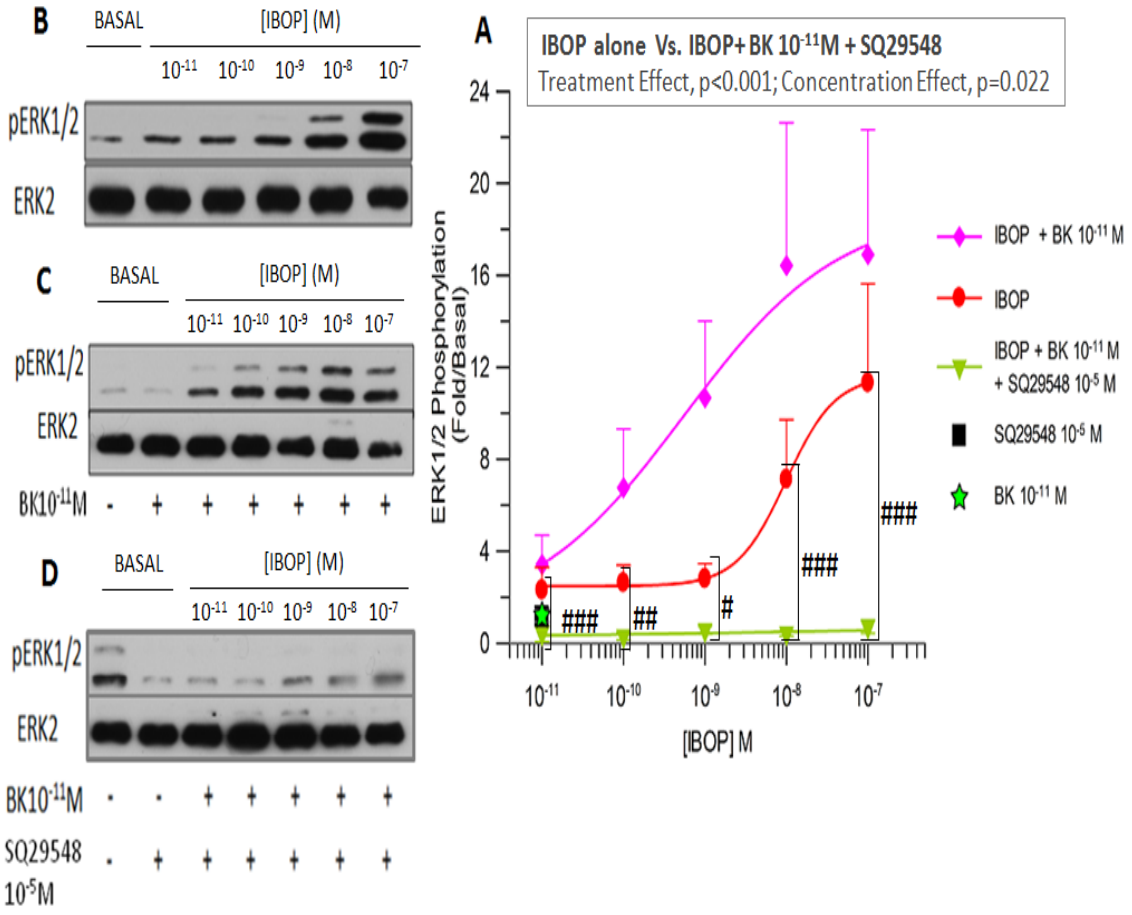

Figure 25: SQ29548 totally inhibited ERK1/2 phosphorylation after co-treatment of RASMCs with a minimal concentration of BK and increasing concentrations of IBOP.

A: Comparison between Concentration-response curves of fold change in ERK1/2 phosphorylation in RASMCs single-treated with IBOP (circular) to that corresponding to cells pretreated (triangular) or not (diamond) with 10^{-5} M of SQ29548 for 30 min prior to co-stimulation with 10^{-11} M of BK and increasing concentrations of IBOP for 10 min. **B**, **C, and D**: Protein bands of Western blots representing pERK1/2 and total ERK2 for cells **(B):** individually treated with IBOP; or cells **(D):** pre-incubated or **(C):** not with SQ29548 prior to co-stimulation with 10^{-11} M of BK and increasing concentrations of IBOP. Results are mean ± SE of three (D) or four (C) or five (B) independent experiments. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ (IBOP alone Vs. IBOP + BK10^{-11} M + SQ29548 10^{-5} M). (Two Way ANOVA, *post hoc* Bonferroni)

2. Effects of Antagonists on IBOP Near-additive Effects:

a- HOE140 Does not Reverse the Effect of IBOP on BK-induced ERK1/2 Phosphorylation:

Pretreatment of RASMCs with 10^{-6} M of HOE140 for 30 min, prior to stimulation with a minimal concentration of IBOP (10^{-11} M) and increasing concentrations of BK (10^{-11} - 10^{-7} M) resulted in fold over basal phosphorylation of ERK1/2 almost equivalent to that obtained upon co-stimulation of RASMCs with a minimal concentration of IBOP and increasing BK concentrations, in treatment combinations that included concentrations of BK below 10^{-8} M. However, at BK concentrations of 10^{-8} and 10^{-7} M, ERK1/2 phosphorylation was further enhanced and exceeded that observed upon co-treatment of RASMCs with a minimal IBOP concentration and increasing concentrations of BK. RASMCs pretreated with HOE140, followed by co-stimulation with 10^{-11} M of IBOP + 10^{-7} M of BK revealed a significant increase ($p < 0.01$) in ERK1/2 phosphorylation of (12.05 ± 2.05) folds as compared to the unstimulated basal. This was also significantly greater ($p < 0.01$) than that observed when cells were stimulated with 10^{-7} M o f BK alone: (3.23 ± 1.14) folds (Figure 26).

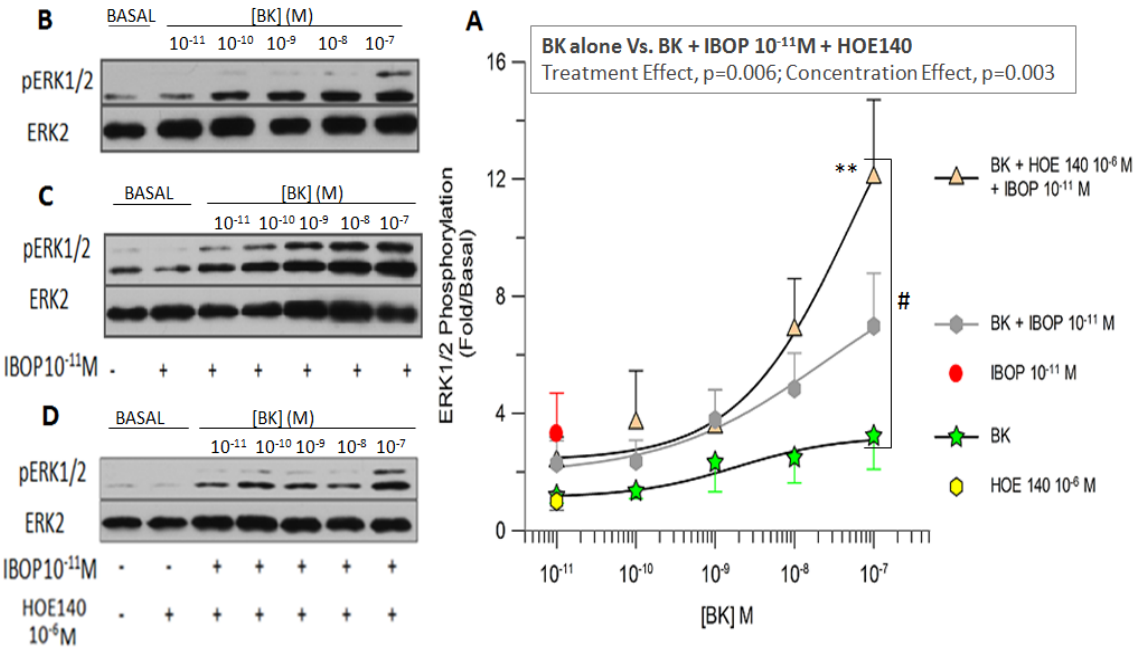

Figure 26: Pretreatment of RASMCs with HOE140 followed by co-stimulation with minimal concentration of IBOP and increasing concentrations of BK.
A: Concentration-response curves of fold change in ERK1/2 phosphorylation in RASMCs pretreated (triangular) or untreated (hexagon) with 10^{-6} M of HOE140 for 30 min prior to co-stimulation with 10^{-11} M of IBOP and increasing concentrations of BK for 10 min compared to the curve corresponding to single treatment with BK (stellar). **B, C, and D:** Protein bands of Western blots representing pERK1/2 and total ERK2 for cells **(B):** individually treated with BK, or cells **(D):** treated or **(C):** untreated with HOE140 prior to co-stimulation with 10^{-11} M of IBOP and increasing concentrations of BK. Results are mean ± SE of three (B and D) or four (C) independent experiments. ** $p < 0.01$ as compared to unstimulated basal. # $p < 0.05$ (BK alone Vs. BK + IBOP 10^{-11}M + HOR140 10^{-6} M). (Two Way ANOVA, *post hoc* Bonferroni)

b- <u>SQ29548 Inhibits The Potentiating Effect of IBOP on BK-induced ERK1/2 Phosphorylation</u>

To check whether the inhibitory effect of SQ29548 on the cooperation observed between TX and BK in RASMCs was bi-directional, cells were pre-incubated with SQ29548 for 30 min before applying simultaneous treatment of 10^{-11} M of IBOP and variable BK concentrations. Likewise, SQ29548 exerted an inhibitory effect by reverting the enhancing effect of IBOP for treatment combinations including 10^{-11}, 10^{-10}, 10^{-9} and 10^{-8} M of BK, and thus resulting in fold over basal phosphorylation of

ERK1/2 almost similar to that gained when cells were treated with equivalent concentrations of BK alone. However, at concentrations of BK equal to 10^{-7} M, SQ29548 could no longer exhibit any antagonizing effect, and the extent of ERK1/2 phosphorylation was equal to that observed when cells were treated with 10^{-11} M of IBOP + 10^{-7} M of BK (Figure 27).

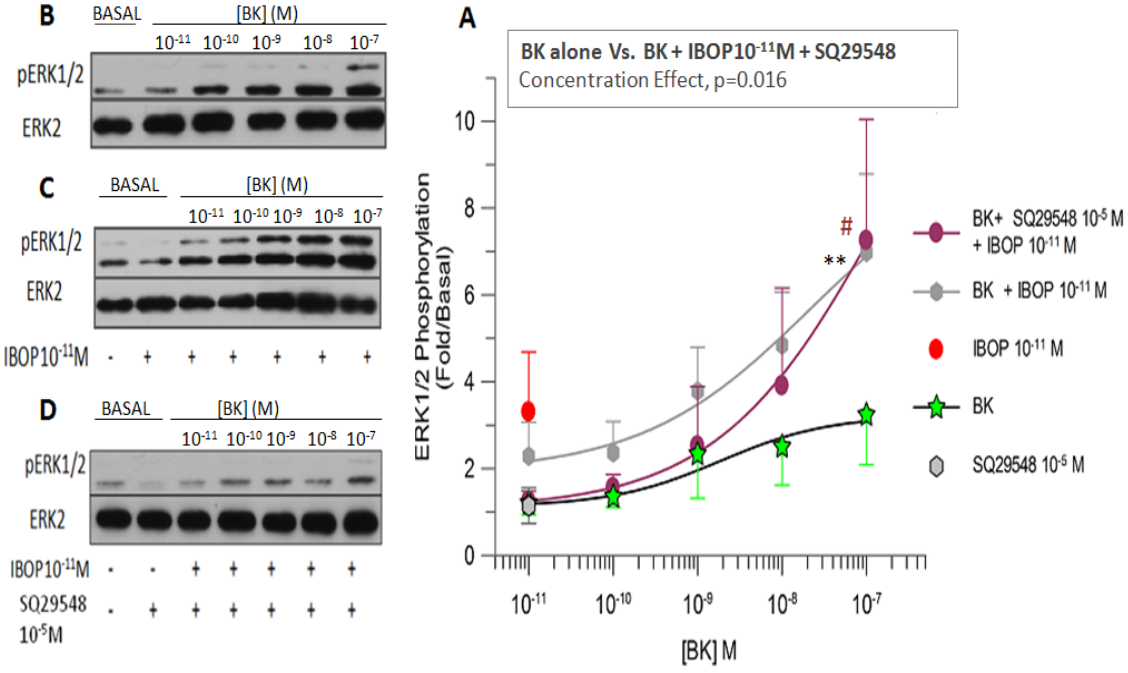

Figure 27: SQ29548 reverses enhancing effects of IBOP on ERK1/2 phosphorylation

A: Concentration-response curves of fold change in ERK1/2 phosphorylation in RASMCs pretreated (circular) or not (hexagon) with 10^{-5} M of SQ29548 for 30 min prior to co-stimulation with 10^{-11} M of IBOP and increasing concentrations of BK for 10 min compared to the curve corresponding to single treatment with BK (stellar). **B**, **C, and D**: Protein bands of Western blots representing pERK1/2 and total ERK2 for cells **(B):** individually treated with BK, or cells **(D):** treated or not **(C):** with SQ29548 prior to co-stimulation with 10^{-11} M of IBOP and increasing concentrations of BK. Results are mean ± SE of three (B and D) or four (C) independent experiments. ** $p < 0.01$ as compared to unstimulated basal. # $p < 0.05$ (BK alone Vs. BK + IBOP 10^{-11}M + SQ29548 10^{-5} M). (Two Way ANOVA, *post hoc* Bonferroni)

CHAPTER VI

STUDIES CONDUCTED ON HEK293-T CELLS

In the previous chapter, we were interested in studying the possibility of pharmacological crosstalk between B2R and TP, both of which are endogenously co-expressed in RASMCs. However, this endogenous system does not allow the study of the effect of cross-agonists and -antagonists on single receptors. Thus, our next aim was to identify the effect of cross-agonists and -antagonists on receptor trafficking properties of human B2R and human TPα when those receptors were individually overexpressed in HEK293T cells. However, there was a problem in the plasmid corresponding to HA-hTPα, so the presented results only included systems overexpressing B2R-YFP (YFP-B2R-HEK-Ts).

A- BK Activates B2R and Results in its Rapid Internalization

Several studies have provided evidence on the rapid desensitization and internalization of B2R upon stimulation with BK (reviewed in [**17**]). This part of work was conducted on YFP-B2R-HEK-Ts to monitor receptor trafficking upon different treatment combinations. So, we checked the action of BK on B2R endocytosis as a positive control. Whereas cells kept at the basal unstimulated state (NS) displayed membranous distribution of B2R-YFP, obvious internalization of B2R was obtained in cells stimulated with 10^{-7} M of BK for 30 min (Figure 28). Thus, our data came consistent with previously published data regarding BK-induced B2R internalization

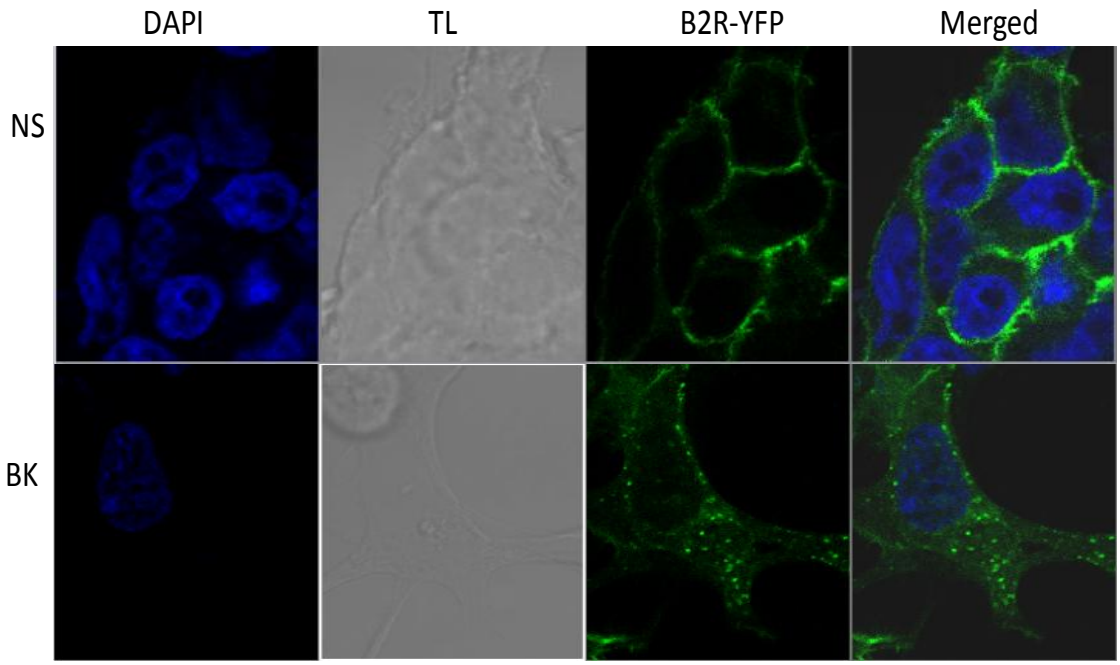

Figure 28: Internalization of B2R following treatment of HEK293-T cells overexpressing B2R-YFP with BK for 30 min.
Representative fluorescence microscopy images of transfected HEK-T cells show the cell sufrace localization of B2R-YFP on the plasma membrane at the basal unstimulated (NS) state. However, intracellular retention of B2R-YFP is noticed after treatment with 10^{-7} M of BK . The left panel shows fluorescence images of nucleii stained with DAPI. The middle panels show transmisssson light (TL) images, and fluorescence microscopy images of B2R-YFP. The right panel (merged) presents overlay images.

B- IBOP Exerts No Effect on B2R Internalization

We next wanted to check whether IBOP has any effect on the binding of BK to its receptor, which might interfere with its additive effect in VSMCs. So, we first treated YFP-B2R-HEK-Ts with 10^{-7} M of IBOP for 30 min to check its individual action of B2R trafficking. Fixed cells were subsequently visualized using confocal microscopy, whereby acquired images were compared to those corresponding to NS and BK-treated as negative and positive controls, respectively.

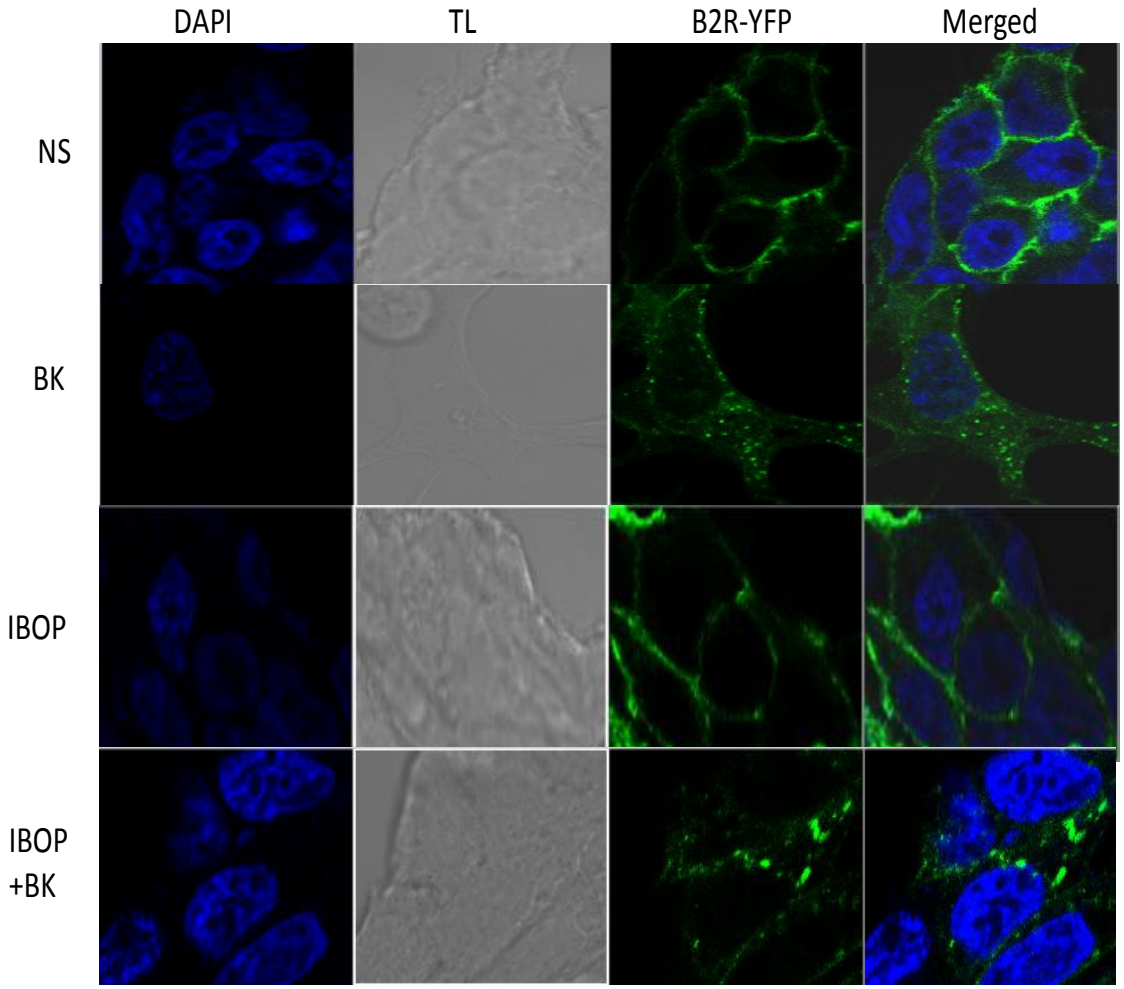

Figure 29: Internalization of B2R following simultaneous treatment of HEK293-T cells overexpressing B2R-YFP with IBOP and BK for 30min.
Top row: Representative fluorescence microscopy images of transfected HEK-T cells show the cell sufrace localization of B2R-YFP on the plasma membrane at the basal unstimulated (NS) state. **Second row:** Intracellular retention of B2R-YFP is noticed after treatment with 10^{-7} M of BK. **Third row:** Membranous distribution of B2R-YFP is maintained after treatment with 10^{-7} M of IBOP. **Bottom row**: Intracellular localization of B2R-YFP after co-stimulation with 10^{-7} M of BK and 10^{-7} M of IBOP for 30 min.The left panel shows fluorescence images of nucleii stained with DAPI. The middle panels show transmissson light (TL) images, and fluorescence microscopy images of B2R-YFP. The right panel (merged) presents overlay images

Tracking B2R-YFP localization in these cells led us to propose the lack of any effect of IBOP on B2R internalization. This was revealed through the strict membranous distribution of B2R-YFP in a pattern that highly resembles that of the negative control (Figure 29-third row).

C- IBOP-BK Co-stimulation Does not Revert BK Effects on B2R Trafficking

We next aimed to inspect the effect of IBOP on BK-induced B2R endocytosis when cells were simultaneously treated with 10^{-7} M of each of IBOP and BK for 30 min. Obvious internalization of B2R could be noticed, which means that IBOP could not affect BK-induced B2R internalization (Figure 29-bottom row).

D- HOE140 Partially Antagonizes BK-induced B2R Endocytosis

Treatment of YFP-B2R-HEK-Ts with 10^{-6} M of HOE140 for 30 min resulted in internalization of B2R. On the other hand, pretreatment with 10^{-6} M of HOE140 for 30 min, prior to stimulation with BK for other 30 min, displayed both membranous and intracellular localization of B2R-YFP. However, the extent of B2R internalization was less than that seen in cells treated with BK alone (Figure 30).

Previously, several studies provided evidence of a weak partial agonist effect of HOE140 on B2R [**17**]. In our case, the differential localization of B2R-YFP upon pretreatment with HOE140 might account for such role of HOE140 on B2R.

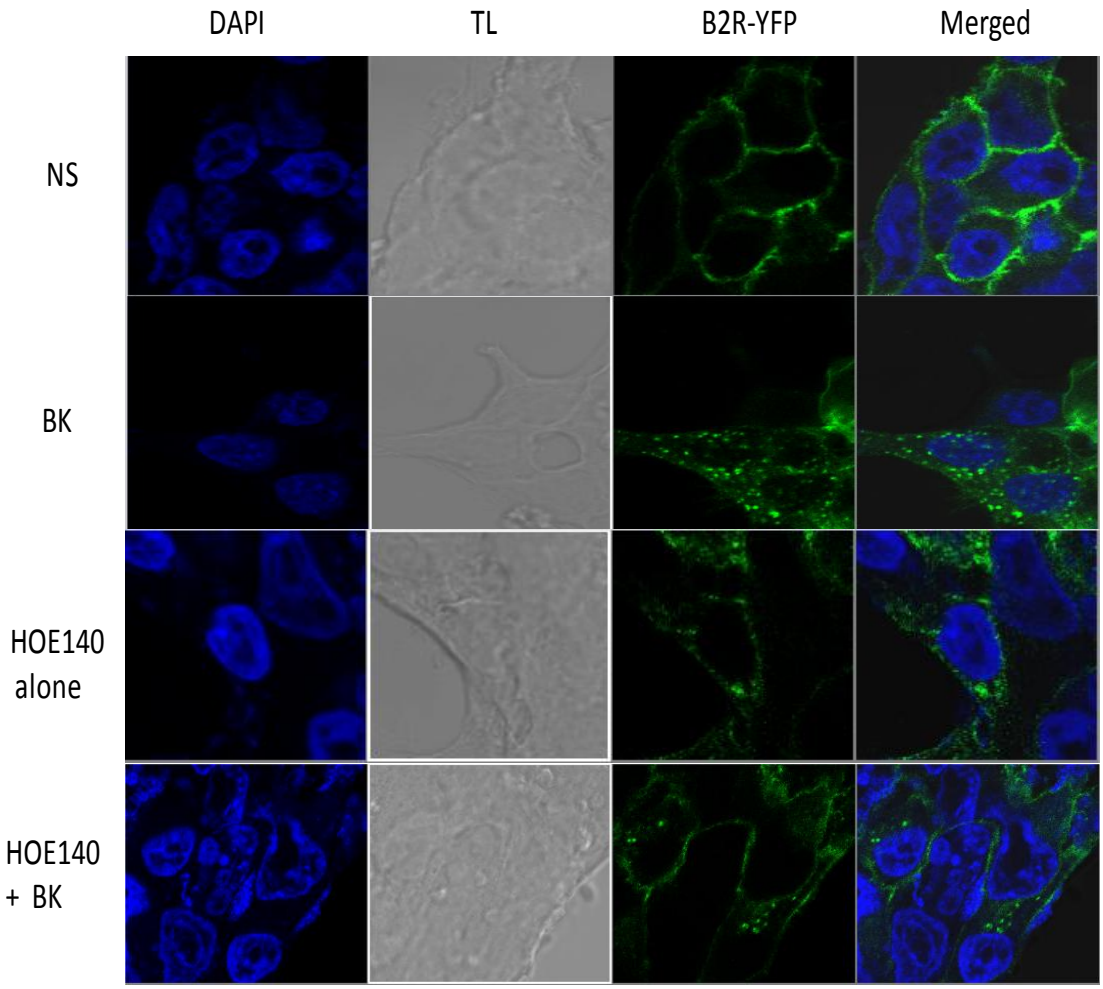

Figure 30: HOE140 Partially Antagonizes BK-induced B2R endocytosis.
Top row: Representative fluorescence microscopy images of transfected HEK-T cells show the cell sufrace localization of B2R-YFP on the plasma membrane at the basal unstimulated (NS) state. **Second row:** Intracellular retention of B2R-YFP is noticed after treatment with 10^{-7} M of BK alone. **Third row:** Intracellular retention of B2R-YFP is noticed after treatment with 10^{-6} M of HOE140 alone. **Bottom row**: Partial internalization of B2R-YFP after pretreatment with 10^{-6} M of HOE140 prior to stimulation with 10^{-7} M of BK for 30 min. The left panel shows fluorescence images of nucleii stained with DAPI. The middle panels show transmissson light (TL) images, and fluorescence microscopy images of B2R-YFP. The right panel (merged) presents overlay images

E- SQ29548 Has no Inhibitory Effect on BK-induced B2R Trafficking

We finally wanted to speculate any possible effect of SQ29548 on the endocytotic properties of B2R. Thus, YFP-B2R-HEK-Ts were pretreated with 10^{-5} M of SQ29548 for 30 min, then stimulated or not with 10^{-7} M of BK for other 30 min. Single treatment with SQ29548 did not cause any internalization of B2R-YFP. However, images corresponding to cells pretreated with SQ29548 followed by treatment with BK revealed obvious internalization of B2R-YFP in a pattern that highly resembled the individual effect of BK treatment on B2R (Figure 31). Thus, SQ29548 is neither antagonizing the effect of BK on it receptor, nor interfering with its binding to B2R. This infers the specificity of BK-B2R binding properties.

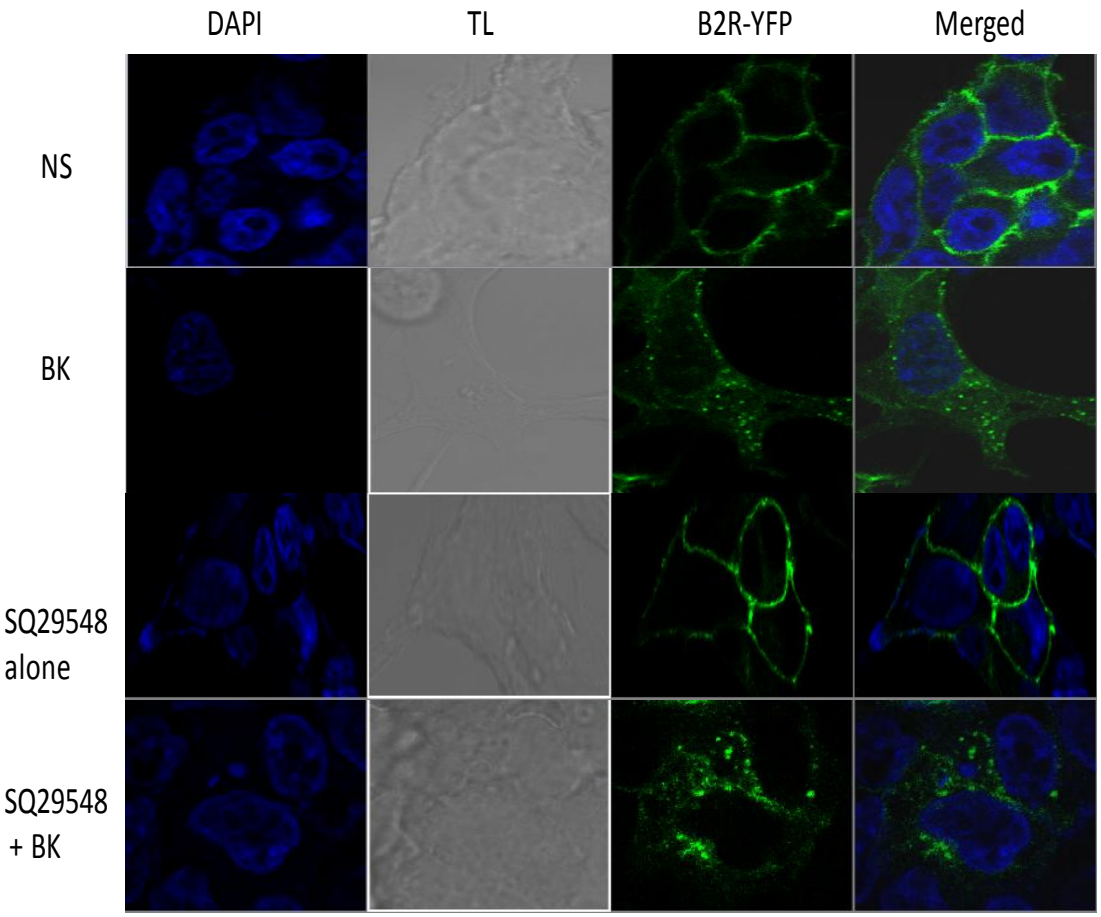

Figure 31: SQ29548 neither alters BK-induced B2R endocytosis nor affects BK binding to it receptor.
Top row: Representative fluorescence microscopy images of transfected HEK-T cells show the cell sufrace localization of B2R-YFP on the plasma membrane at the basal unstimulated (NS) state. **Second row:** Intracellular retention of B2R-YFP is noticed after treatment with 10^{-7} M of BK. **Third row:** Membranous distribution of B2R-YFP is maintained after individual treatment with 10^{-5} M of SQ29548. **Bottom row**: Internalization of B2R-YFP after pretreatment with 10^{-5} M of SQ29548 prior to stimulation with 10^{-7} M of BK for 30 min.The left panel shows fluorescence images of nucleii stained with DAPI. The middle panels show transmissson light (TL) images, and fluorescence microscopy images of B2R-YFP. The right panel (merged) presents overlay images.

CHAPTER VII

DISCUSSION

Endothelial injury is the preliminary step for the formation and aggravation of vascular diseases and atherogenesis. The subsequent development and enlargement of the atherosclerotic lesion is highly dependent on the proliferative and constrictive state of VSMCs [**5, 4, 6**]. Thus, two prominent vascular players, BK and TX, have been extensively studied for their individual promotion of proliferation in cultured VSMCs. Agonist-induced activation of B2R or TP in these cells led to the successive phosphorylation and activation of ERK1/2, which resulted in enhanced VSMC proliferation. However, the possible cross-modulation of the ERK1/2 pathway between both receptors in VSMCs is yet undefined.

It has been previously shown that functional modulation between two GPCRs could occur through multiple routes: 1- receptor heterodimerization; 2- receptor modulation of scaffold and kinase proteins that could affect the function of the other receptor; 3- receptor alteration in the activity or expression of important downstream signaling molecules; 4- each receptor could regulate the expression of the other receptor; 5- each receptor could lead to paracrine release of agonists or antagonists of the other receptor [**109**]. It has also been previously demonstrated that upon binding to B2R, BK activates intracellular signaling cascades such as the arachidonic acid/prostaglandin pathway, and thus leads to increased production of TXA2 [**110, 111, 112**]. However, the receptor-receptor interactions between B2R and TP have never been addressed.

Knowing that each of TP and B2R could form heterodimers and thus affect the functional and trafficking properties of other GPCRs, we suspected a possible similar crosstalk to occur between both receptors in VSMCs. Hence, the aim of this study was to inspect some of the possible pharmacological and physiological mechanisms that could be involved in the crosstalk between B2R and TP in systems endogenously expressing both receptors, such as VSMCs, as well as in overexpressing systems.

Initially, our work was conducted on RASMCs to determine the crosstalk between the intrinsically expressed receptors (B2R and TP) on the ERK1/2 pathway. Our findings provide pharmacological evidence of cooperative ERK1/2 activation when both receptors were simultaneously activated by their corresponding agonists. However, this cooperation was synergistic when a minimal effective concentration of BK was simultaneously added to concentrations of IBOP above 3.6×10^{-11} M. On the other hand, by shifting our focus to the TP-dependent modulation of B2R pharmacological responses, only additive effects could be realized, and were restricted to concentrations of BK equivalent or greater than 10^{-8} M. Besides, a statistically significant difference in the fold over basal phosphorylation of ERK1/2 was detected between data of TP-modulated B2R responses and those of B2R-modulated TP responses, when comparing equivalent concentrations of 10^{-9} M of each of BK and IBOP respectively.

Now that this cooperation was obtained, we sought to test the role of cross-antagonists in the modulation of BK- or IBOP-induced individual effects. Both antagonists, SQ29548 and HOE140, proved effective in attenuating the subsequent activation of ERK when RASMCs were pretreated with the antagonists prior to single stimulation with either BK or IBOP. However, while pretreatment with HOE140 allowed some TP-mediated remedial activation of ERK when cells were stimulated with

10^{-8} and 10^{-7} M of IBOP, SQ29548 could inhibit any BK-induced ERK1/2 phosphorylation at concentrations of the latter less than or equal to 10^{-8}M . However, pretreatment with SQ29548 prior to stimulation with 10^{-7} M of BK revealed a partial agonist effect of SQ29548, which resulted in enhanced ERK1/2 activation.

We next examined the effect of antagonists on the cooperative activation of ERK1/2 that was observed between B2R and TP in RASMCs. Pretreatment with SQ29548 totally inhibited the modulatory effects of BK on IBOP-induced ERK1/2 activity. On the other hand, SQ29548 was able to antagonize IBOP modulatory effects on BK-induced ERK activation only for concentrations of BK less than or equal to 10^{-8} M, whereby above this concentration SQ29548 lost its antagonizing property and the cooperative fold increase in ERK1/2 phosphorylation was maintained. These findings point to the possibility that a physical interaction could be taking place between TP and B2R in VSMCs, and could account for their reciprocal regulation by cross-antagonists.

There are two possibilities by which SQ29548 could regulate the single or combined effects of BK in VSMCs. One possibility could be that SQ29548 might unspecifically bind to B2R and thus directly interfere with its ability to bind BK. Or, the other possibility could be that upon binding of SQ29548 to TP, it causes a conformational change in the ligand binding site of B2R, and thus indirectly prevents BK from inducing its effect. However, for the second possibility to occur, a physical interaction should be bridging both receptors in VSMCs.

To rule out the possibility of non-specific binding of SQ29548 to B2R, we worked on YFP-B2R-HEK-Ts, which lack endogenously functional TP receptors. Pre-incubation of YFP-B2R-HEK-Ts with SQ29548 prior to stimulation with BK could not interfere with the binding of B2R to its ligand. Accordingly, agonist-induced receptor

endocytosis was maintained and resembled that obtained after single treatment of YFP-B2R-HEK-Ts with BK. Thus, the inhibitory effect of SQ29548 on BK-induced single or cooperative regulation of ERK1/2 is more likely to occur as a result of physical interaction between B2R and TP in RASMCs. This theory could also be reinforced by our previous knowledge of the localization of each of B2R and TP on the cell membrane of VSMCs which makes such interaction highly feasible.

On the contrary, HOE140 could neither normalize the positive modulatory effect of BK on IBOP-induced ERK1/2 phosphorylation, nor could it affect the ability of IBOP to enhance the pharmacological effects of BK in VSMCs. Although previous studies have unveiled a partial agonist effect of HOE140 on B2R, this mechanism could not by itself explain the persistent cooperation that continued to occur even after pretreatment with HOE140, especially that HOE140 was able to inhibit ERK1/2 phosphorylation when single BK treatment was applied. Besides, no significant activation of ERK1/2 could be realized upon single treatment of RASMCs with 10^{-6} M of HOE140 for 30 min. Therefore, our explanation is that the physical interaction between TP and B2R could lead to a new active conformation of B2R induced by the simultaneous stimulation of RASMCs with BK and IBOP. Instead of antagonizing this conformation, HOE140 leads to its stabilization, therefore favoring persistent activation of the ERK1/2 pathway. Thus, in this case HOE140 adopts the principle of biased agonists that are known to stabilize a certain functional conformation induced by the natural unbiased ligand in favor of upregulating a certain signaling pathway [**113**].

Finally, the ability of cross-agonists and -antagonists to regulate single stimulation of B2R or TP in VSMCs, where both receptors are endogenously expressed, led us to speculate the possible involvement of a physical interaction between both

receptors and its effect on receptor trafficking properties. Interestingly, we wanted to study receptor trafficking and sequestration in HEK293T cells overexpressing either HA-hTPα or B2R-YFP or both. Unfortunately, the plasmid we used to overexpress HA-hTPα was destructed and did not yield the required receptor. So, in the meantime, we continued our work on YFP-B2R-HEK-Ts while waiting to receive a new functional HA-hTPα plasmid.

At the basal unstimulated level, B2R is known to have some basal agonist-independent sequestration [**35**]. In our work on YFP-B2R-HEK-Ts, this was very minimal. However, upon stimulation with BK for 30 min, evident endocytosis of B2R-YFP could be observed, which came consistent with evidence provided from previous studies. Subsequently, we wanted to monitor the effect of HOE140 and IBOP on B2R internalization. While the individual treatment of YFP-B2R-HEK-Ts with HOE140 for 30 min led to B2R internalization, pretreatment of YFP-B2R-HEK-Ts with HOE140 for 30 min prior to stimulation with BK for other 30min resulted in both membranous and intracellular localization of B2R-YFP. Thus, HOE140 could partially inhibit BK-induced B2R internalization, revealing its role as a partial agonist. Meanwhile, IBOP neither affected the binding properties nor did it cause the internalization of B2R. This was deduced from the membranous localization of B2R resembling that of the unstimulated basal when single treatment of YFP-B2R-HEK-Ts with IBOP was applied. Besides, simultaneous stimulation of YFP-B2R-HEK-Ts with equal concentrations of BK and IBOP did not inhibit the binding of BK to its receptor, and resulted in obvious internalization of B2R similar to that seen when cells were individually treated with BK. Thus, IBOP did not compete with BK on its binding to B2R. These results are in favor of the theory that the cooperation between TP and B2R in VSMCs is mediated by

physical interaction of receptors and excludes the possibility of unspecific binding of IBOP to B2R.

CHAPTER VIII

CONCLUSION AND RECOMMENDATIONS

Compiled together, our findings are suggestive of pharmacological and physiological cooperation between TP and B2R in VSMCs. Pharmacologically, BK-dependent modulation of the TP-induced activation of ERK1/2 resulted in a mono-directional synergy (Figure 32), while only an additive cooperation was obtained in the TP-modulation of B2R effects (Figure 33). This complex cooperation that seems to exist between BK and TX might be leading to deleterious ramifications on the progression and escalation of vascular diseases. Hence, additional work should be conducted to study the consequences of this cooperative activation of ERK1/2 through targeting the effective signaling cascades downstream of TP and B2R in VSMCs. This should be accompanied by tracking the subcellular compartmentalization of pERK1/2 after stimulating RASMCs with the different treatment combinations to determine the possible pharmacological effects that might be implicated. Furthermore, measurement of thymidine incorporation would be very helpful in the revelation of the possible involvement of such cooperation between TP and B2R in vascular proliferation and mitogenesis.

Additionally, the shift in the mode of action of SQ29548 and HOE140 from antagonists to partial agonist and biased agonist, respectively, should be further investigated to determine its pharmacological consequences on the vasculature.

Both pharmacologically and physiologically, a growing evidence of a possible heterodimerization between TP and B2R could be inferred. Hence, studying the likelihood of such heterodimerization and its functional implications on the modulation

of individual receptor signaling and internalization would help provide insights about the cooperative role of TX and BK in the vasculature. This would later provide an important platform for more effective therapies tackling vascular injury and atherosclerosis.

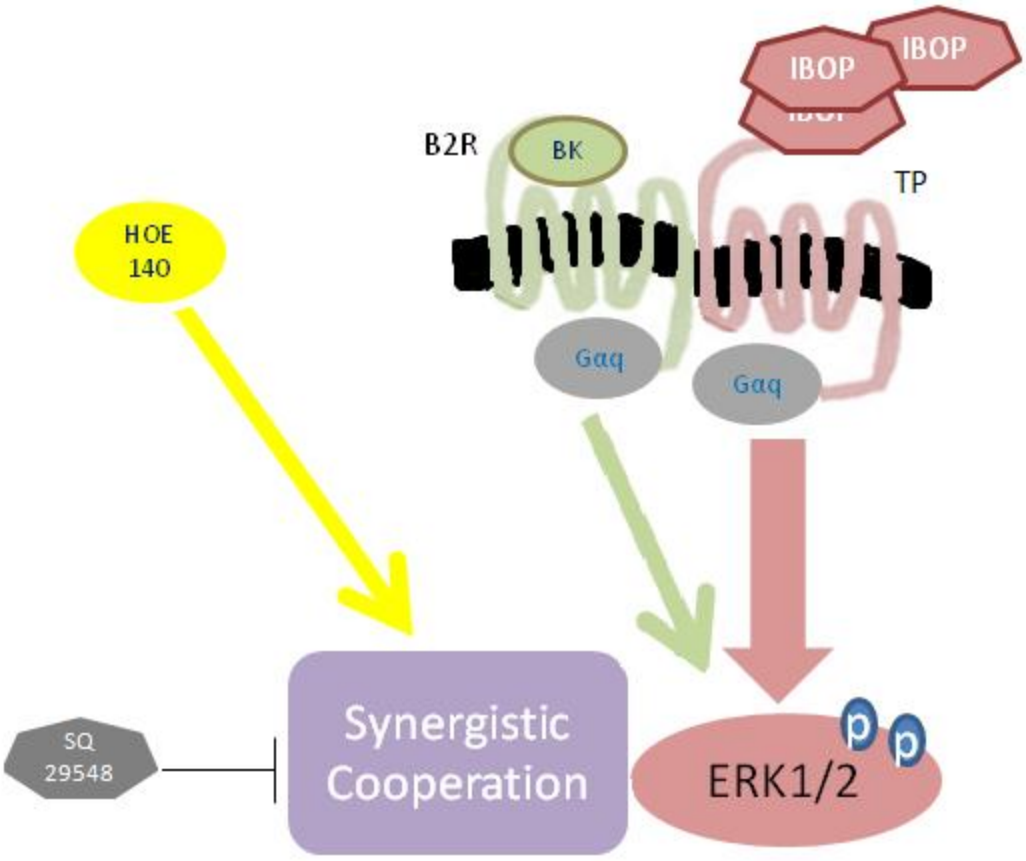

Figure 32: Schematic representing BK-synergistic modulation of TP pharmacological effects in RASMCs
Co- stimulation of RASMCs with a minimal concentration of BK and increasing effective concentrations of IBOP results in a synergistic cooperation between BK and TX on the ERK1/2 pathway that leads to enhanced ERK1/2 phosphorylation. This is further maintained after pretreatment with the B2R selective antagonist, HOE140. However, pre-incubation with the TP antagonist, SQ29548, totally inhibits ERK1/2 phosphorylation, resulting in a status resembling that of the unstimulated basal.

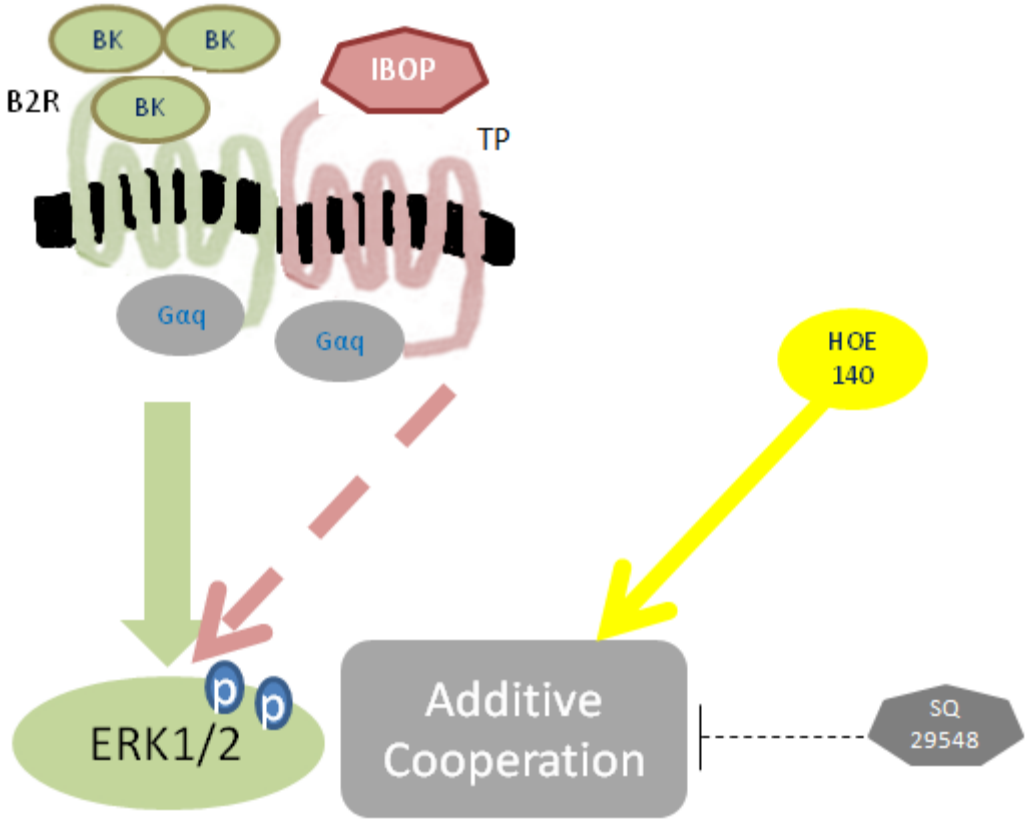

Figure 33: Schematic representing TP-additive modulation of B2R pharmacological effects in RASMCs

Co- stimulation of RASMCs with a minimal concentration of IBOP and increasing effective concentrations of BK results in an additive cooperation between BK and TX on the ERK1/2 pathway that leads to enhanced ERK1/2 phosphorylation. This is further enhanced after pretreatment with the B2R selective antagonist, HOE140. However, pre-incubation with the TP antagonist, SQ29548, reverts ERK1/2 phosphorylation to a status resembling that of single treatment with BK.

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