VASORELAXANT AND ANTIHYPERTENSIVE EFFECTS OF 3,4,7,2',4'-PENTAHYDROXYFLAVONE IN *IN VITRO* AND *IN VIVO* MODELS

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LIST OF SYMBOLS

%	Percentage
~	Approximate
µl	Microliter
µM	Micromolar
ºC	degree Celsius
g	Gram
g/l	gram per liter
mg	Milligram
mg/dl	milligram per deciliter
mg/kg	milligram per kilogram
mg/ml	milligram per milliliter
min	minute
ml	milliliter
mM	millimolar
mm	millimeter
mmHg	millimeter of mercury
mmol/l	millimole per liter
nM	nanomolar
U/l	units per liter
µmol/l	micromole per liter

LIST OF ABBREVIATIONS

2-APB	2-Aminoethoxydiphenyl Borate
4-AP	4-Aminopyridine
A/G	Albumin/Globulin Ratio
AA	Arachidonic acid
AC	Adenylyl Cyclase
ACE	Angiotensin-Converting Enzyme
ACh	Acetylcholine
ADP	Adenosine Diphosphate
Akt	Protein kinase B
AlbP	Albumin Protein
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
ARASC	Animal Research and Service Centre
ARBs	Angiotensin II receptor blockers
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
$BaCl_2$	Barium Chloride
BilD	Direct Bilirubin
BiliT	Total Bilirubin
BK_{Ca}	Big-Conductance Calcium-Activated Potassium Channels
BP	Blood Pressure
BUSE	Blood Urea Serum Electrolyte
Ca^{2+}	Calcium Ion
Ca^{2+}-CaM	Calcium Calmodulin Complex
$CaCl_2$	Calcium Chloride
cAMP	Cyclic Adenosine Monophoaphate
CCBs	Calcium Channel Blockers
cGMP	Cyclic Guanosine Monophosphate
Cl	Chloride
Cl^-	Chloride Ion
CMC	Carboxymethyl Cellulose
CO_2	Carbon Dioxide
COX	Cyclooxygenase
CPG	Clinical Practice Guidelines
CVD	Cardiovascular diseases
DAG	Diacylglycerol

DBP	Diastolic blood pressure
DOCA	Deoxycorticosterone acetate
EC_{50}	Half Of Maximum Effective Concentration
EDHFs	Endothelium-Derived Hyperpolarising Factors
EDRFs	Endothelium-Derived Relaxing Factors
EDTA	Ethylenediaminetetraacetic Acid
EETs	Epoxyeicosatrienoic Acid
EGTA	Ethylene Glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic Acid
eNOS	Endothelial Nitric Oxide Synthase
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GGT	Gamma-Glutamyl Transferase
GPCRs	G-protein coupled receptors
GTP	Guanosine triphosphate
HF	High Fructose
HVA	High Voltage-Activated
IK_{Ca}	Intermediate-Conductance Calcium-Activated Potassium Channel
iNOS	Inducible Nitric Oxide Synthase
IP	Prostacyclin Receptor
IP_3	Inositol Triphosphate
IP_3R	Inositol Triphosphate Receptor
JNC 7	Seventh Joint National Committee Report
K	Potassium
K^+	Potassium Ion
K_{Ca}	Calcium-Activated Potassium Channels
KCl	Potassium Chloride
KH_2PO_4	Potassium Dihydrogen Phosphate
K_{ir}	Inwardly-Rectifying Potassium Channels
KPS	Krebs-Henseleit Physiology Solution
K_V	Voltage-activated Potassium Channel
LFT	Liver Functional Test
L-NAME	L-N^G-Nitro Arginine Methyl Ester
LVA	Low Voltage-Activated
M_3	Muscarinic Receptor
MAP	Mean Arterial Pressure
MB	Methylene Blue
$MgSO_4$	Magnesium Sulphate
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
Na	Sodium
Na^+	Sodium Ion

NaCl	Sodium Chloride
$NaHCO_3$	Sodium Bicarbonate
NCD	Non-communicable diseases
NHANES	National Health and Nutrition Examination Survey
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2	Oxygen
ODQ	1H-[1,2,4]Ox-adiazolol[4,3-α]quinoxaline-1-one
pD_2	Potency Of Drug
PE	Phenylephrine
PGH_2	Prostaglandin H2
PGI_2	Prostacyclin
PH	Potential Of Hydrogen
PIP_2	Phosphatidylinositol 4,5-Bisphosphate
PKA	Protein kinase A
PKC	Protein Kinase C
PKG	Protein kinases G
PLA_2	Phospholipase A_2
PLB	Phospholamban
PLC	Phospholipase C
PLC-β	Phospholipase C-β
RAS	Renin-Angiotensin System
RFT	Renal Function Test
R_{MAX}	Maximum Relaxation
ROCC	Receptor-Operated Calcium Channel
ROS	Reactive Oxygen Species
RyR	Ryanodine Receptor
S.E.M	Standard Error Mean
SBP	Systolic blood pressure
SD	Sprague-Dawley
SERCA	Sacro/Endoplasmic Reticulum Ca2+-ATPase
sGC	Soluble Guanynyl Cyclase
SHRs	Spontaneously Hypertensive Rats
SK_{Ca}	Small-Conductance Calcium-Activated Potassium Channels
SOCC	Store-Operated Calcium Channel
SR	Sarcoplasmic Reticulum
SUR	Sulfonylurea Receptor
TEA	Tetraethylammonium Chloride
TP	Total Protein
TRPV4	Transient Receptor Potential Vanilloid 4
VOCC	Voltage-Operated Calcium Channel
VSMCs	Vascular smooth muscle cells

WHO	World Health Organization

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KESAN VASORELAKSAN DAN KESAN ANTIHIPERTENSIF 3,4,7,2'4-PENTAHYDROXYFLAVONE DALAM MODEL *IN VITRO* DAN MODEL *IN VIVO*

ABSTRAK

Hipertensi merupakan salah satu keadaan perubatan kronik yang dicirikan oleh tekanan darah tinggi yang berterusan dikenakan ke atas dinding saluran darah. Ia telah menjadi salah satu kebimbangan kesihatan global utama disebabkan oleh peningkatan prevalensinya di seluruh dunia. Walaupun terdapat banyak ubat antihipertensi yang tersedia di pasaran, banyak yang dilaporkan memiliki keberkesanan yang rendah dan seringkali datang dengan kesan sampingan atau kontraindikasi. Oleh itu, kajian ini direka untuk mengenal pasti ejen antihipertensi yang berpotensi mengelakkan semua isu yang berkaitan dengan ubat-ubatan yang tersedia di pasaran pada masa ini. Morin, sejenis flavonol yang dikenali oleh pelbagai sifat farmaseutikalnya, dipilih untuk menilai aktiviti vasodilatasi dan antihipertensi, masing-masing menggunakan pendekatan *in vitro* dan *in vivo*. Kesan vasodilatasi morin dan mekanisme asasnya dinilai secara *in vitro* menggunakan ujian cincin aorta. Hasilnya menunjukkan bahawa morin memberi kesan vasodilatasi melalui pelbagai mekanisme, termasuk laluan yang bergantung kepada endotelium dan laluan yang tidak bergantung kepada endotelium. Kesannya yang mendalam dalam vasodilatasi morin boleh dikaitkan dengan keupayaannya untuk meningkatkan pengeluaran faktor relaksan yang berasal dari endotelium (EDRF), mengaktifkan reseptor bergandingan G, dan mengantagoniskan aktiviti saluran kalsium. Kesan antihipertensi morin kemudiannya dinilai secara *in vivo* melalui pemberian oral sub-kronik selama 28 hari dalam tikus hipertensi spontan (SHR). Hasilnya menunjukkan bahawa morin secara signifikan menurunkan tekanan

darah dalam SHR tanpa menyebabkan tanda-tanda tidak normal dalam urea darah, elektrolit serum, fungsi buah pinggang, dan fungsi hati SHR. Kesimpulannya, morin berupaya berfungsi sebagai agen antihipertensi dan boleh digunakan sebagai alternatif untuk merawat atau mencegah hipertensi.

VASORELAXANT AND ANTIHYPERTENSIVE EFFECTS OF 3,4,7,2',4'-PENTAHYDROXYFLAVONE IN *IN VITRO* AND *IN VIVO* MODELS

ABSTRACT

Hypertension is one of the chronic medical conditions characterised by consistently high blood pressure exerted against the wall of blood vessels. It has emerged as a major global health concern due to its increasing worldwide prevalence. Although numerous antihypertensive medications are available in the market, many are reported to have low efficacy and frequently come with adverse effects or contraindications. Consequently, the current study was designed to identify a potential antihypertensive agent which could avoid all the issues associated with currently market available medications. Morin, a flavonol which was known for its diverse pharmaceutical properties, was selected to evaluate its vasodilatory and antihypertensive activities using *in vitro* and *in vivo* approaches, respectively. The vasodilatory effect of morin and its underlying mechanism were evaluated *in vitro* using aortic rings assay. The results revealed that morin exerts its vasodilatory effects through multiple mechanisms pathways, including endothelium-dependent and endothelium-independent pathways. The profound vasodilatory effects of morin can be ascribed to its ability to enhance production of endothelium-derived relaxing factors (EDRFs), activate G-protein coupled receptors, and antagonise the activity of calcium channels. The antihypertensive effects of morin were evaluated *in vivo* via 28 days sub-chronic oral administration in Spontaneously Hypertensive Rats (SHRs). The findings revealed that morin significantly lowered the blood pressure in SHRs without causing abnormal signs in blood urea serum electrolyte, renal function, and liver

function of SHRs. In conclusion, morin may serve as a promising antihypertensive agent and can be used as an alternative for treating or preventing hypertension.

CHAPTER 1

INTRODUCTION

1.1 Hypertension

Hypertension is a classic worldwide health problem and known to be “silent killer” in the world. It is a chronic disease characterised by consistently high blood pressure exerted against the arterial wall, specifically when the systolic blood pressure (SBP) surpasses 140 mmHg and diastolic blood pressure (DBP) exceeds 90 mmHg. It has been identified as a paramount risk factors for development of cardiovascular diseases (CVD) and renal diseases (Mahadir Naidu et al., 2019).

Hypertension is usually asymptomatic and has been rated as one of the factors causing premature death worldwide (Cisse et al., 2021). Due to its asymptomatic nature many individuals remain unaware of their hypertensive status until they develop severe complications. Such complications can emerge after years and include life-threatening events such as stroke, ischemic heart disease, and heart failure. The concomitant consequences encompass not only life-threatening conditions but also complications in eyes, limbs, and kidneys (Naing et al., 2016). This has brought to its recognition as a leading factor contributing to premature death on a global scale.

A comprehensive pool analysis conducted by Non-communicable Diseases (NCD) Risk Factors Collaboration has demonstrated a significant increment in global prevalence of hypertension over the past few decades. The affected population has surged from 594 million in 1975 to 1.13 billion in 2015 (Li et al., 2021). According to comprehensive data published by the World Health Organization (WHO), it has been

revealed that, in the year 2008, approximately 40% of the global adult population, aged 25 and above, suffered from hypertension. By the year 2017, hypertension has been identified as the predominant risk factor contributing to premature mortality and morbidity across diverse populations. Systemic investigation on clustering cardiovascular diseases distributed across 21 global regions has shown a concerning scenario. Over the period from 1990 to 2010, complications directly attributed to hypertension have resulted in more than 9 million mortality cases (Lim et al., 2012, Mahadir Naidu et al., 2019).

Uncontrolled and untreated hypertension amplifies the risk of cardiovascular mortality among the general hypertensive population (Gu et al., 2010). As elucidated by previous study, patients with extremely high blood pressure levels face a risk of myocardial infarction that is 2.5-fold greater than individuals with normal blood pressure levels. Moreover, those individuals with pre-hypertension stages are at a 1.5-fold higher susceptibility to the development cardiovascular diseases in contrast to individuals with normal blood pressure levels. Additionally, patients with higher blood pressure ranges face a risk of developing congestive heart failure that is 2 times greater compared to those within lower ranges (Qureshi et al., 2005, Wang et al., 2014, Yusuf et al., 2004).

Based on the data portrayed by the National Health and Nutrition Examination Survey (NHANES), there was a notable increase in the hypertension incidence among US adult populations. The rate of individuals suffering from hypertension has increased from 23.9% during the period of 1988-1994 to 29% in 2007-2008. In the Southeast Asian region, hypertension prevalence was about 27%. Meanwhile, in China, approximately 325 million adults from China aged 18 and above were

diagnosed with hypertension in year 2010. In Thailand, a quarter of Thai populations struggles with hypertension and nearly 50% of them remain unaware of their condition (Egan et al., 2010, Guessous et al., 2012, Wang et al., 2014, Zaki et al., 2021).

According to comprehensive reports of Hypertension Clinical Practice Guidelines (CPG) of Malaysia released in 2018, there was an elevation in the incidence of hypertension among distinct age groups within Malaysia populations. In the population with age of 18 and above, the data reveals that prevalence of hypertension marked an increase by 1.7 % within 5 years, from 33.6 % in 2011 to 35.3 % in 2015. On the other hand, in older demographic encompassing individuals aged 30 and above, the prevalence of hypertension has increased by 0.9 %, growing from 43.8 % to 44.7 % over the same time frames (MOHMalaysia, 2018).

Previous epidemiological studies showed that individuals adhering to a vegetarian dietary pattern tend to exhibit lower blood pressure compared to those having non-vegetarian diet. These beneficial effects can be partly attributed to the presence of multiple phytochemicals with antioxidant properties in fruits and vegetables. Among these phytochemicals, phenolic compounds are the prominent phytochemicals found in plants (Appel et al., 1997).

Flavonoids, a prominent subclass of polyphenolic compounds which have proved to have ability in exhibiting a spectrum of pharmacological effects. They have garnered significant attention due to their beneficial effects toward cardiovascular health including treating or preventing hypertension. Characterised by their various phenolic structure, flavonoids are distributed in diverse sources, including fruits,

fruits, vegetables, grains, bark, stems, roots, flowers, and plant-based beverages (Panche et al., 2016).

Morin, chemically denoted as 3,5,7,2′,4′-pentahydroxyflavone, is classified as a member of the flavonol subclass within flavonoids family. It can be naturally found in various plant sources such as guava leaves, onions, apples, mills, and other members of the *Moraceae* family (Lotito and Frei, 2006, Rattanachaikunsopon and Phumkhachorn, 2010, Xie et al., 2006). It was renowned for its broad range of beneficial pharmacological effects including antioxidant, antimutagenic, antiallergic, anticarcinogenic, anti-inflammatory, nephroprotective, neuroprotective and cardiovascular protective (Francis et al., 1989, Rajput et al., 2021, Subash and Subramanian, 2009). It has previously been proven to carry the capability to ameliorate vascular damage caused by oxidative stress and hyperlipidaemia, hence reducing the blood pressure in the deoxycorticosterone acetate (DOCA)-induced and high fructose (HF)-induced hypertensive rats (Kang et al., 2004, Prahalathan et al., 2012). Some studies demonstrated that morin exhibits vasodilatory effect on vascular tissue by integrating in two mechanism pathways (Formica and Regelson, 1995, Herrera et al., 1996, Taguchi et al., 2020). In the context of vasodilation, various mechanism signalling pathways are involved. Morin was proved able to exert its vasodilatory activity via more than one mechanism, but its potential in other possible mechanisms has yet to be comprehensively studied.

1.2 Problem statement

The presented figures from various reports and studies done by various associations or researchers have indicated a growing trend in hypertension across different populations. These statistical data also depict the potential health challenges that could burden the nation's healthcare system. Consequently, these data emphasise the critical demand for implementing health strategies to control the rising incidence of hypertension in the global population. Therefore, it is an urgent need to seek a potent antihypertensive agent in order to reduce the hypertension incidence and enhance health quality among the global population.

Currently there are various antihypertensive medications available in the global market, including diuretics, β-blockers, calcium channel blockers (CCBs), angiotensin-converting enzyme (ACE) inhibitors, and angiotensin II receptor blockers (ARBs). However, each of them has come with their own adverse effects and contraindications. Their efficacy in treating hypertension is also limited as they target only specific mechanistic pathways and tend to develop resistance. Therefore, the discovery of a new antihypertensive agent employing multiple signalling pathways is highly desired.

Previous studies have demonstrated the potential antihypertensive effects of morin in DOCA-salt induced hypertensive rats' model and high fructose induced hypertensive rats' model. However, these studies predominantly underscored its antioxidative and insulin-modulating effects as the primary mechanism responsible for its antihypertensive effects. Nonetheless, none of these studies delved into antihypertensive effects resulting from morin-induced vasodilation. Furthermore,

morin has been substantiated as potent vasodilator by integrating with diverse mechanistic pathways. However, the understanding of the complete spectrum of mechanistic pathways contributing to morin-induced vasodilation remains far from comprehensive.

Thus, the vasodilatory and antihypertensive effects of morin, a promising nutraceutical compound, are investigated in present study. The vasodilatory and antihypertensive effect of morin were assessed via *in vitro* and *in vitro* approaches.

1.3 Objectives

I. To investigate the vasodilatory and its underlying mechanisms through *in vitro* aortic rings assay.

II. To investigate the effectiveness of antihypertensive effects of morin through *in vivo* antihypertensive study using Spontaneously Hypertensive rats.

III. To assess the impact of morin on liver function, renal function, and blood urea serum electrolyte of Spontaneously Hypertensive Rats (SHRs) after 28 days oral administration.

CHAPTER 2

LITERATURE REVIEW

2.1 Hypertension

Hypertension is recognised as one of the contributing factors that lead to cardiovascular diseases (CVD). Its prevalence has been increased over the past decades worldwide due to various factors. In many cases, patients may not be aware of their hypertension condition due to its asymptomatic nature, and it can only be detected through opportunistic screening. The symptoms only become significant when the BP reached a critical level (Chobufo et al., 2020, Schmidt and de Wit, 2020). Some patients tolerate high BP for significant periods without experiencing any adverse symptoms, even if their BP level is critically high, which might put them at risk of acute events such as stroke or organ damage.

According to the guidelines outlined in the Seventh Joint National Committee report (JNC7), clinical hypertension can be categorised into several stages. These staged including normal stages characterised by systolic blood pressure (SBP) and diastolic blood pressure (DBP) values less than 120 mmHg and 80 mmHg, respectively; prehypertensive stages, designated by SBP range within 120-139 mmHg and DBP within 80-89 mmHg; stage 1 hypertension, typified by SBP ranging from 140-159 mmHg and DBP 90-99 mmHg; and stage 2 hypertension, characterised by SBP higher than 160 mmHg and DBP more than 100 mmHg. Patients with stage 2 hypertension are at a higher risk in facing life-threatening events such as ischemic myocardial infarction and renal dysfunction (Gebreselassie and Padyab, 2015, MOHMalaysia, 2014).

2.1.1 Etiology of hypertension

Hypertension is a multifactorial condition, meaning it can be caused by multiple factors. Hypertension can be classified into two types: primary (essential) and secondary hypertension. Primary hypertensions predominate as the prevailing form, and it has no identifiable secondary causes. This type of hypertension typically develop gradually over years with several potential contributing factors such as genetics, ageing, unhealthy diet, obesity, or physical inactivity (Hall et al., 2015, Law et al., 1991, Levy et al., 2000). Conversely, secondary hypertension usually arises as consequence of other medical condition such as kidney disease, endocrine disorders, medications, and those condition causing narrowing of blood vessel (Aronow, 2017, Guyton, 1991, Kario et al., 2019).

Currently, a variety of antihypertensive medications are available in global markets. Among these, vasodilators are one of the prominent classes of antihypertensive agents employed for blood pressure regulation and reducing the incidence of hypertension. Generally, vasodilators promote dilation of blood vessels, which reduces the blood pressure and allows greater blood flow to various organs, thereby maintaining their normal physiological function. Notably, the current market offers several classes of vasodilators, comprising ACE inhibitors, angiotensin receptor blockers, nitrates, calcium channel blockers, minoxidil, hydralazine and beta-blockers (Hariri and Patel, 2023). However, these vasodilatory agents predominantly targeted on specific factors or one signalling pathway, thus limiting their efficacy in treating hypertension caused by multiple diseases or conditions. Moreover, they might bring some contraindications for certain patients. Therefore, there is an imminent need to seek new effective medication which brings no contraindications.

2.2 Overview of signaling mechanism pathways

2.2.1 Structure and functions of blood vessels

Blood vessels are the channels tasked with systemic transportation of blood throughout the entire body, facilitating the circulation and distribution of essential substances. They also serve to remove metabolic byproducts, such as carbon dioxide and waste products away from body tissues. The vascular system comprises three principal types of blood vessels: the arteries, veins, and capillaries. The arteries play a major role in nourishing the body tissues, allowing them to sustain their physiological function by distributing nutrients, oxygen, and water. The veins are tasked for carrying waste and carbon dioxide containing deoxygenated blood away from body tissues back to heart. Capillaries, being smallest and most abundant blood vessels in the body, serving as the site for the exchange of materials between blood and tissue cells (Jakala et al., 2009, Tucker et al., 2023, Yildiz et al., 2013).

The blood vessels are composed of three distinct layers: *tunica adventitia* (the outermost layer), *tunica media* (middle layer), and *tunica intima* (inner layer). The tunica media characterised by substantial presence of vascular smooth muscle (VSMCs). It primarily functions to regulating arterial vascular tone and providing essential structural stability to regulate blood flow dynamic. Tunica adventitia is entirely made up of connective tissue, contributing to the structural integrity of the blood vessels (Jakala et al., 2009, Yildiz et al., 2013). Additionally, this layer accommodate other components, including nerves, progenitors, adipocytes, and immune cells. In larger blood vessels, vasa vasorum, the capillaries that supply nutrients to the blood vessels walls, can also be found in tunica adventitia. Contrarily, tunica intima is the thinnest layer, comprising of a single layer of endothelial cells that

facilitate the blood perfusion. These endothelial cells play a pivotal role in modulating the vascular tone through vasodilation upon various stimuli. Among the three primary types of blood vessels, both arteries and veins possess all three layers, where the capillaries are characterised by a monolayer of endothelial cells (tunica intima) supported by its basement membrane (Ma et al., 2023).

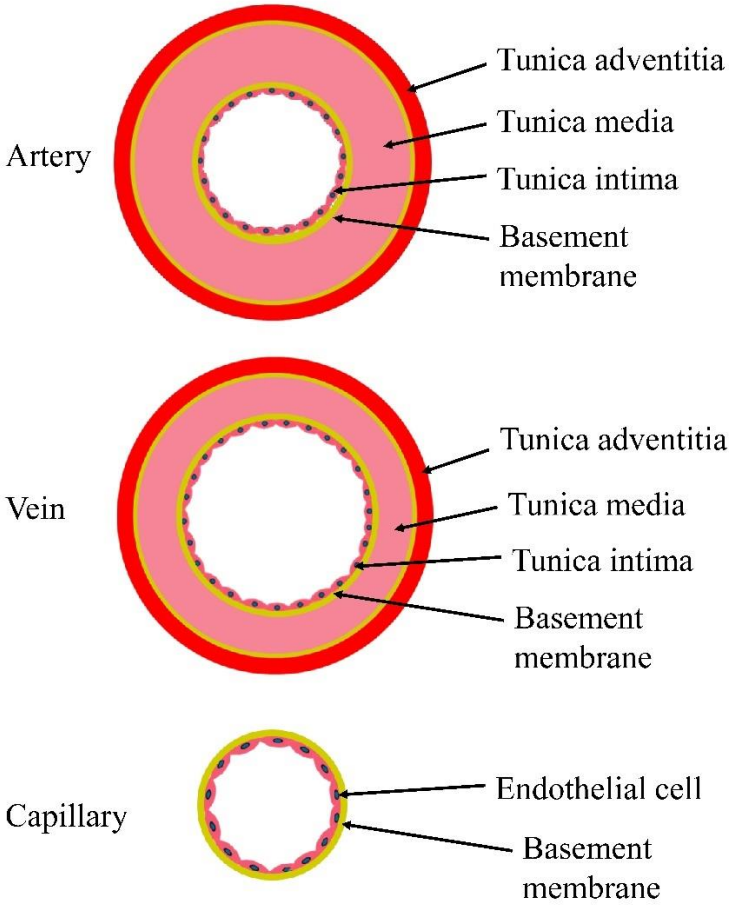

Figure 1: Structure of the three primary types of blood vessels.

2.2.2 Endothelium-derived relaxation factors and enzyme-linked receptors

Vascular endothelium plays a central role in regulation of cardiovascular homeostasis. It not only serves as a physical barrier between static vascular wall and flowing blood, but also a source for producing vasorelaxation and vasoconstricting agents, crucial for blood pressure modulation (Kang, 2014). The equilibrium between vasodilators and vasoconstrictors is perturbed in CVD, leading to hypertension. In response to mechanical shear stress and external chemicals stimuli, vascular endothelial cells engage in production of endothelium-derived relaxing factors (EDRFs) or induced membrane hyperpolarisation. These processes collectively regulate the contractile activity of neighbouring vascular smooth muscle cells (VSMCs). This physiological phenomenon is known as endothelium-dependent vasodilation.

In most cases, endothelium-dependent vasodilation occur through the diffusion of EDRFs from endothelium and interact with the enzymes or receptors present at adjacent VSMCs. Nevertheless, vasodilation might also happen upon activation of potassium channels located in endothelium (Freed and Gutterman, 2017). The activated potassium channels generates hyperpolarizing current, subsequently leading to vasodilation. The nitric oxide (NO) and prostacyclin (PGI_2) are the two extensively characterised EDRFs that play central role in endothelium-dependent vasodilation. They bind to and activate enzyme linked receptors, initiating a complex signalling cascade involving multiple ligand-receptor interactions.

Nitric oxide (NO) serves as an indispensable signalling molecule participating in numerous physiological and pathological processes. It is primarily produced by three distinct isoforms of nitric oxide synthase (NOS), namely endothelial NOS

(eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS), with eNOS being the predominant isoform expressed in vascular endothelium (Forstermann and Sessa, 2012). The eNOS is phosphorylated upon increase in hemodynamic shear stress, protein kinase A (PKA) and protein kinase B (Akt). Once activated, eNOS catalyses conversion of L-arginine to NO. The NO diffuses into neighbouring VSMCs, where it engages with and activates soluble guanylyl cyclase (sGC). This interaction triggers the transformation of guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP) from. The elevation in cGMP levels subsequently phosphorylate specific cGMP-dependent protein kinases (PKG), resulting in reduction of intracellular Ca^{2+} level in VSMCs, ultimately leading to vasodilation (Freed and Gutterman, 2017). Additionally, NO has the capability to stimulate calcium-activated potassium channels (K_{Ca}) and voltage-activated potassium channel (K_V), inducing hyperpolarisation in VSMCs, resulting in vasodilation .

Other than NO, prostacyclin (PGI_2) is another EDRFs inherently produced by endothelium. It belongs to the family of endogenous prostanoids and is derived from arachidonic acid (AA) through a cascade of enzymatic conversions (Hislop et al., 2022, Ruan et al., 2010). In endothelial cells, the AA is rarely encountered as free molecule in cell due to its highly reactive nature and sensitivity to oxidation (Mitchell and Kirkby, 2019). Instead, it is primarily incorporated with phospholipid bilayer of the cell membrane. The enzymes phospholipase A_2 (PLA_2) or diacylglycerol (DAG) lipase act to hydrolyse the phospholipid and free the AA into cytosol for subsequent prostanoid synthesis process. The enzyme cyclooxygenase (COX) facilitates the formation of prostaglandin H_2 (PGH_2) from mobile AA. The prostacyclin synthase then catalyses the conversion of PGH_2 into PGI_2. A fraction of PGH_2 metabolises by thromboxane synthase, yielding produce thromboxane A_2, which counteracts the

effects of PGI_2. The PGI_2 binds to and activates the prostacyclin receptor (IP), a $G_s\alpha$ protein-coupled receptor localised on the membrane of VSMCs. The activated $G_s\alpha$ protein triggers adenylyl cyclase (AC), catalysing formation of 3',5'-cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). The elevation in cAMP level subsequently initiates the activation of PKA, which, in turn, hinders the formation of calcium-calmodulin complexes (Ca^{2+}-CaM) in VSMCs and results in vasodilation (Berumen et al., 2012, Nichols and Nichols, 2008).

Endothelium-dependent vasodilation is a multifaceted physiological activity that cannot solely be explained by the action of NO and PGI_2 (Scotland et al., 2005). There are other chemical entities that have been suggested to be released from endothelium and contributing to vasodilation, such as potassium ions (K^+), lipophilic compounds, proteins, radicals, and certain gaseous molecules. These mediators were termed as endothelium-derived hyperpolarising factors (EDHFs) (Schmidt and de Wit, 2020). The EDHFs play their role in vascular regulation by generating electrical signals that induce hyperpolarisation current on endothelial cell membrane. The hyperpolarisation current is then propagated to adjacent VSMCs, leading to their relaxation and subsequent vasodilation. In vasculature, EDHFs can be produced through diverse biochemical reactions. For instance, a fraction of the AA enzymatically breaks down into epoxyeicosatrienoic acids (EETs), which activate intermediate calcium-activated potassium channels (IK_{Ca}) and calcium-activated potassium channels (SK_{Ca}) located at the endothelial cell membrane. The activation of these channels induces the translocation of potassium ions (K^+) from cytosol into extracellular space, thereby instigating the generation of a hyperpolarising current across the cell membrane (Loh et al., 2018). The hyperpolarisation signals subsequently transferred to adjacent VSMCs through myoendothelial gap junction,

triggering VSMCs hyperpolarisation and relaxation. In the context of action potential, the efflux of K^+ leads to elevation of K^+ concentration in myoendothelial space. The changes in K^+ concentration in myoendothelial space activate inwardly rectifying potassium channels (K_{ir}), initiating K^+ influx into cytosol of VSMCs. The increase in cytosolic K^+ in turn activates big-conductance calcium-activated potassium channels (BK_{Ca}), triggering K^+ efflux to extracellular space. This process led to hyperpolarisation and subsequently induces vasodilation (Pires et al., 2013).

In addition to potassium channels, involvement of calcium channels has been observed in vasodilation induced by EDHFs. EETs have the capability to activate transient receptor potential vanilloid 4 (TRPV4) situated on endothelium and trigger calcium-induced calcium release from sarcoplasmic reticulum (SR), contributing Ca^{2+} sparks (Liu et al., 2021). These sequential events activate IK_{Ca} and SK_{Ca}, resulting in hyperpolarisation and vasodilation. Furthermore, the increase in cytosolic Ca^{2+} enhances formation of Ca^{2+}-CaM in endothelium, which in turn activates eNOS and triggers the NO/sGC/cGMP signalling pathway (van Nieuw Amerongen et al., 1998). The EDRFs involved in vasodilation have been summarised in Table 1.

Table 1: The endothelium-derived relaxing factors involved in vasodilation.

EDRFs	**Precursor**	**Enzyme**	**Mechanism of actions**
Nitric oxide	L-arginine	Endothelial nitric oxide synthase	Activate sGC and sCMP, eventually reduce Ca^{2+} in VSMCs
Prostacyclin	Arachidonic acid	Cyclooxygenase, Prostacyclin synthase	Activate AC/cAMP pathway which eventually reduce cytosolic Ca^{2+} in VSMCs
Epoxyeicosatrienoic acids	Arachidonic acid	CYP epoxidase	Induce hyperpolarising current through activation of IK_{Ca} and SK_{Ca}

2.2.3 G-protein coupled receptors

G-protein coupled receptors (GCPRs), alternatively referred to as 7-transmembrane domain receptors, become functionally activated upon binding with extracellular agonists, enabling the transmission of signals from the extracellular environment into the cell. These receptors are associated with a group of guanine nucleotide binding proteins (G-proteins) composed of three subunits, termed alpha (Gα), beta (Gβ) and gamma (Gγ). The G-proteins are categorised into four subtypes based on the constituent Gα subunit, into $G_q\alpha$, $G_i\alpha$, $G_{12/13}\alpha$ and $G_s\alpha$. The $G_s\alpha$ and $G_i\alpha$ are responsible for regulating the activity adenylyl 1 cyclase, whereas G_q activates phospholipase C-β (PLC-β). Additionally, $G_{12/13}$ functions to initiate the activation of small GTPase families (Nieves-Cintron et al., 2018).

The GPCRs expressed in VSMCs, and endothelium play a pivotal role in vascular tone regulation (Brinks and Eckhart, 2010). Activation of G-protein is initiated when GPCRs bind with their respective agonist. This binding induces conformational changes in GPCRs, allowing them to transiently function as guanine nucleotide exchange factor (GEF) (Wettschureck and Offermanns, 2005). The GPCRs activate the G-proteins by exchanging the bound guanosine diphosphate (GDP) into guanosine triphosphate (GTP). Upon activation, the heterotrimeric G-proteins dissociate into Gα-GTP monomer and Gβγ trimers. Both Gα-GTP monomer and Gβγ trimers subsequently engage in their respective signalling mechanisms. The Gα-GTP monomer activate the different signalling pathways depending on the type of Gα subunit, while Gβγ dimers tend to activate specific types of signalling molecules, encompassing ion channels, lipid kinases, phospholipases as well as initiating their

own signalling cascades (Bar-Shavit et al., 2016, Dorsam and Gutkind, 2007, Neves et al., 2002, Yuen et al., 2010).

2.2.3(a) $G_q\alpha$ protein coupled receptors

In vascular tissue, $G_q\alpha$ proteins are linked to two distinct signalling pathways, phospholipase C (PLC) pathway and Rho-kinase pathway. In PLC pathway, the activated $G_q\alpha$ protein triggers the activation of PLC-β, which hydrolyses of phosphatidylinositol-4,5,-bisphosphate (PIP_2) into secondary messengers, namely inositol triphosphate (IP_3) and DAG. These secondary messengers amplify G_q-mediated signals through intracellular calcium mobilisation, involving Ca^{2+} release from SR store and the activation DAG-dependent protein kinase C (PKC). The IP_3 binds to the IP_3 receptor (IP_3R) located on the sarcoplasmic reticulum, triggering intracellular release of Ca^{2+} from SR into cytosol. Simultaneously, DAG activates PKC, thereby leading to an increase in cytosolic Ca^{2+} (Mizuno and Itoh, 2009). The M_3-muscarinic receptor (M_3) is one of the $G_q\alpha$ protein-coupled receptors predominantly found in vascular endothelial cells. Hence, its activation leads to an increase of cytosolic Ca^{2+} in vascular endothelium and causes vasodilation (Ishii and Kurachi, 2006).

2.2.3(b) $G_s\alpha$ protein coupled receptors

The activated $G_s\alpha$ protein initiates the AC/cAMP/PKA pathway and results in vasodilation. In VSMCs, there exist at least two prominent $G_s\alpha$ protein-coupled receptors present in VSMCs, namely β_2-adrenergic receptor (β_2) and PGI_2 receptor (IP) (Jakala et al., 2009, Klabunde, 2011). Activation of these receptors decreases cytosolic Ca^{2+} in VSMCs, consequently leading to vasoconstriction.

2.2.3(c) $G_i\alpha$ protein coupled receptors

In vasculature, α_2-adrenergic receptor (α_2) is classified as a member of under $G_i\alpha$ protein-coupled receptors and is found in the VSMCs. $G_i\alpha$ protein-coupled receptors are functionally opposed to the $G_s\alpha$ protein-coupled receptors, primarily engaging in inhibition of the cAMP-dependent AC activity. This inhibitory event thereby prevent the conversion of ATP into cAMP, leading to an elevation in cytosolic Ca^{2+}, consequently, vasoconstriction (Klabunde, 2011, Qin et al., 2008).

2.2.4 Channel-linked receptors

In the context of vascular tone regulation, channel-linked receptors serve as crucial modulators by receiving and integrating numerous inputs originating from various factors. They are also known as ligand-gated ion channels or ionotropic receptors (Loh et al., 2018). They are a group of transmembrane ion channels facilitating the movement of ions (Na^+, K^+, Ca^{2+} and Cl^-) across the cellular membrane upon specific ligands stimulation. In the vascular system, these ion channels play vital roles in regulating the contractile activity of VSMCs by modulating depolarisation and hyperpolarisation mechanisms. Among these ion channels, potassium (K^+) channels and calcium (Ca^{2+}) channels emerge as the two primary contributors to vascular tone regulation (Jackson, 2000).

2.2.4(a) Potassium channels

Potassium channels are the most widely distributed class of ion channels and virtually present in all living organisms (Littleton and Ganetzky, 2000). In VSMCs, they predominantly function to regulate membrane potential and modulate vascular tone (Nelson and Quayle, 1995, Nieves-Cintron et al., 2018). The activated K^+

channels permit the efflux of K^+ ions from the cell into extracellular milieu and create hyperpolarizing current on the membrane. Four distinct types of K^+ channels, namely the calcium-activated K^+ channel (K_{Ca}), ATP-sensitive K^+ channel (K_{ATP}), inwardly rectifying K^+ channel (K_{ir}), and voltage-gated K^+ channel (K_V), have been extensively studied in relation to their role in vascular tone regulation. Furthermore, K_{Ca} can further subdivided into three subtypes: large conductance K_{Ca} channels (BK_{Ca}), intermediate-conductance K_{Ca} (IK_{Ca}) and small-conductance K_{Ca} (SK_{Ca}) (Jackson, 2000).

Among the diverse population of K^+ channel types in VSMCs, BK_{Ca} emerged as the most prominent K^+ channel and is ubiquitously expressed in VSMCs membrane (Archer and Rusch, 2001). They are functionally activated in response to an elevation of intracellular Ca^{2+} level. They facilitate efflux of K^+ ions from the cytosol and generate hyperpolarizing current that subsequently results in closure of Ca^{2+} channels. The reduction in cytosolic Ca^{2+} hinders the activity of MLCK, leading to vasodilation (Félétou and Vanhoutte, 2006, Schumacher et al., 2001). In addition to direct activation by Ca^{2+}, BK_{Ca} can be indirectly activated by PKA and PKG (Archer and Rusch, 2001, Robertson and Nelson, 1994, Scornik et al., 1993). In contrast to BK_{Ca}, IK_{Ca} and SK_{Ca} are more abundantly expressed in vascular endothelium and operate independently with voltage changes (Gueguinou et al., 2014, Sheng et al., 2009). However, they are highly sensitive to changes in cytosolic Ca^{2+} and calmodulin levels. Therefore, they are located close to SR store and cell membrane calcium channels (Adelman et al., 2012, Stocker, 2004) . It has been proposed that IK_{Ca} and SK_{Ca} play a role in generating EDHF signals and influence adjacent VSMCs (Archer and Rusch, 2001).

Other than K_{Ca}, K_V channels are another class of K^+ channels that are highly prevalent in VSMCs. These channels are activated in response to membrane depolarisation and are functionally correlated with voltage operated Ca^{2+} channel (VOCC), crucially contributing to maintenance of VSMCs membrane potential. In general, K_V channels participate in the negative feedback mechanism and restore the membrane potential from depolarised state to steady state (Chadha et al., 2014, Jackson, 2017, Nelson and Quayle, 1995). K_V channels comprise of two primary subtypes: alpha subunits and beta subunits. These channels could potentially be involved in the mechanistic action of both vasodilators and vasoconstrictors. Vasoconstrictors such as phenylephrine and angiotensin II, demonstrate the capability to inhibit K_V. Conversely, vasodilators possess the potential to activate K_V channels through the cAMP/PKA pathway (Jackson, 2017). The activated K_V channels induce hyperpolarisation and relaxation in VSMCs by reducing Ca^{2+} influx through calcium channels (Nelson and Quayle, 1995). During repolarisation phase, the activated K_V channels hasten the efflux of K^+ from the cytosol into the extracellular milieu, thereby facilitating the restoration of negative membrane potential, with minimal activation of Ca^{2+} channels. Furthermore, activated PKA indirectly increases the magnitude of K_V currents and reduces cytosolic Ca^{2+} by hastening the activation of PKC (Aiello et al., 1995, Jakala et al., 2009, Nelson and Quayle, 1995, Robertson and Nelson, 1994, Yildiz et al., 2013).

Inwardly rectifying K^+ channels (K_{ir}) are found abundantly in smooth muscles of small resistance blood vessels. They are classified as the members of K_V channels because they possess a pore domain that is homologous to K_V. Activation of K_{ir} channels occurs upon binding with PIP_2. Different from other K^+ channels, K_{ir} allows inward move of K^+ into the cell, rather than an outward flow. This action aids in re-

establishment of the membrane potential from hyperpolarised state to the resting state and stabilised the resting membrane potential (Edwards and Weston, 1995, Jackson, 2000). In vascular endothelium, activated K_{ir} may induce hyperpolarising current and resulting in vasodilation (Edwards et al., 1998).

K_{ATP} channels are heteromultimeric complex consisting of four pores forming $K_{ir}6$ subunits and four sulfonylurea receptor (SUR) subunits (Akrouh et al., 2009). K_{ATP} channels are classified within K_{ir} family. They distinguish themselves from other K_{ir} channels by their heightened sensitivity to intracellular ATP levels, thus designation as ATP-sensitive. K_{ATP} channels are functionally activated by increasing intracellular ADP and inhibited while intracellular ATP increases (Ashcroft and Ashcroft, 1990, Boyd et al., 1990, Ko et al., 2008, Standen et al., 1989). After activation, these channels mediate the efflux of K^+ from the cytosol and creates hyperpolarizing current, consequently resulting in vasodilation (Dogan et al., 2019, Jackson, 2017, Teramoto, 2006).

2.2.4(b) Calcium channels

The changes in cytosolic Ca^{2+} concentration is the principal mechanism in regulating the contractile state of VSMCs. After activation by vasoconstrictors, the Ca^{2+} channels promote a transmembrane movement of Ca^{2+} from extracellular space and/or intracellular Ca^{2+} store (sarcoplasmic reticulum) into the cytosol, collectively elevating cytosolic Ca^{2+} level. The cytosolic Ca^{2+} binds to calmodulin in the VSMCs, forming the Ca^{2+}-CaM complex, which serve as an activator for myosin light chain kinase (MLCK). The activated MLCK, in turn, phosphorylates MCL at serine residue-19, allowing for the formation of cross-bridge with actin filaments. This process contributes to muscular contraction through myosin-actin cycling, which is known to

be a fundamental element of the sliding filament theory (Hill-Eubanks et al., 2011, Kuo and Ehrlich, 2015, Ottolini and Sonkusare, 2021). Conversely, the myosin light chain phosphatase (MLCP) take a counterbalance role by dephosphorylating phosphorylated MLC, leading to termination the muscular contraction and triggering vasodilation.

In vasculature, VOCC serves as one of the prominent receptors involved in vascular tone regulation. There are two main types of Ca^{2+} channels found in VSMCs, namely high voltage-activated (HVA) L-type and low voltage-activated (LVA) T-type channels. In the context of arterial contraction, L-type Ca^{2+} channels play a pivotal role in by mediating Ca^{2+} influx from extracellular space. In contrast, the contribution of T-type Ca^{2+} channel to arterial contraction is considered negligible (Cribbs, 2006). The normal physiological Ca^{2+} level in the extracellular space is upheld at ~3-4 mM, a concentration that is thousand-fold greater than the cytosolic Ca^{2+} level, which is maintained within the range of 50-100 nM (Boyd et al., 1990). When the membrane potential of VSMCs achieves depolarised state, the activated VOCC facilitate Ca^{2+} influx from extracellular space into cytosol, leading to vasoconstriction (Furberg et al., 1995).

Other than calcium influx, intracellular release of Ca^{2+} release from the internal store is also an important mechanism for modulating cellular contraction (Kuo and Ehrlich, 2015, Ma et al., 2023, Touyz et al., 2018). Both IP_3R and ryanodine receptor (RyR) are receptor-operated calcium channels (ROCC) involved in this mechanistic action. IP_3R serves as the principal receptor involved in intracellular Ca^{2+} release. As aforementioned, it is situated on the surface of SR and becomes activated in response to the stimulation of GPCRs. The activated IP_3R promote Ca^{2+} translocations from SR

stores to cytosol, causing an increase in cytosolic Ca^{2+} and subsequent vasoconstriction. Nevertheless, RyR is less prevalent in vascular tissue and plays a subsidiary role in triggering Ca^{2+} release from the SR store. When the cytosolic Ca^{2+} increases, the RyR is activated, prompting further Ca^{2+} release from the SR store. This phenomenon is recognised as calcium-induced calcium release (CICR). The Ca^{2+} release through RyR subsequently causes a transient Ca^{2+} spark in the muscle cells (Pires et al., 2013, Quyyumi and Ozkor, 2006). The calcium channel blocker 2-aminoehtoxydiphenyl borate (2-APB), is usually used in the study involving IP_3R, where caffeine is commonly used as the antagonist for RyR.

Both VOCC and ROCC function to increase cytosolic Ca^{2+} level. On the contrary, store-operated calcium channels (SOCC) primarily function to replenish Ca^{2+} in the SR store. They are termed “store-operated” because of their distinctive responsiveness to depletion of Ca^{2+} in SR store. These channels become activated as a consequence of the depletion of Ca^{2+} in SR, which results from the release of Ca^{2+} through IP_3R (Parekh and Putney, 2005). The sacro/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), a P-type ATPase, specialises in transporting cytosolic Ca^{2+} back to the SR store and maintaining the cytosolic Ca^{2+} at a low level (50-100 nM) (Berchtold et al., 2000). The activity of SERCA is regulated by phospholamban (PLB) and calsequestrin. It remains in an inactive state when bound to PLB. Conversely, the calsequestrin residue in the lumen of SR binds to the Ca^{2+}, aiding in storage and maintenance of the concentration of Ca^{2+} at high level in the calcium store. Meanwhile, it reduces the concentration of free mobile Ca^{2+} in SR store, thereby optimizing efficient uptake of Ca^{2+} into SR through SERCA (Swietach et al., 2008). Figure demonstrates the overview of signalling pathways during vascular tone regulations.

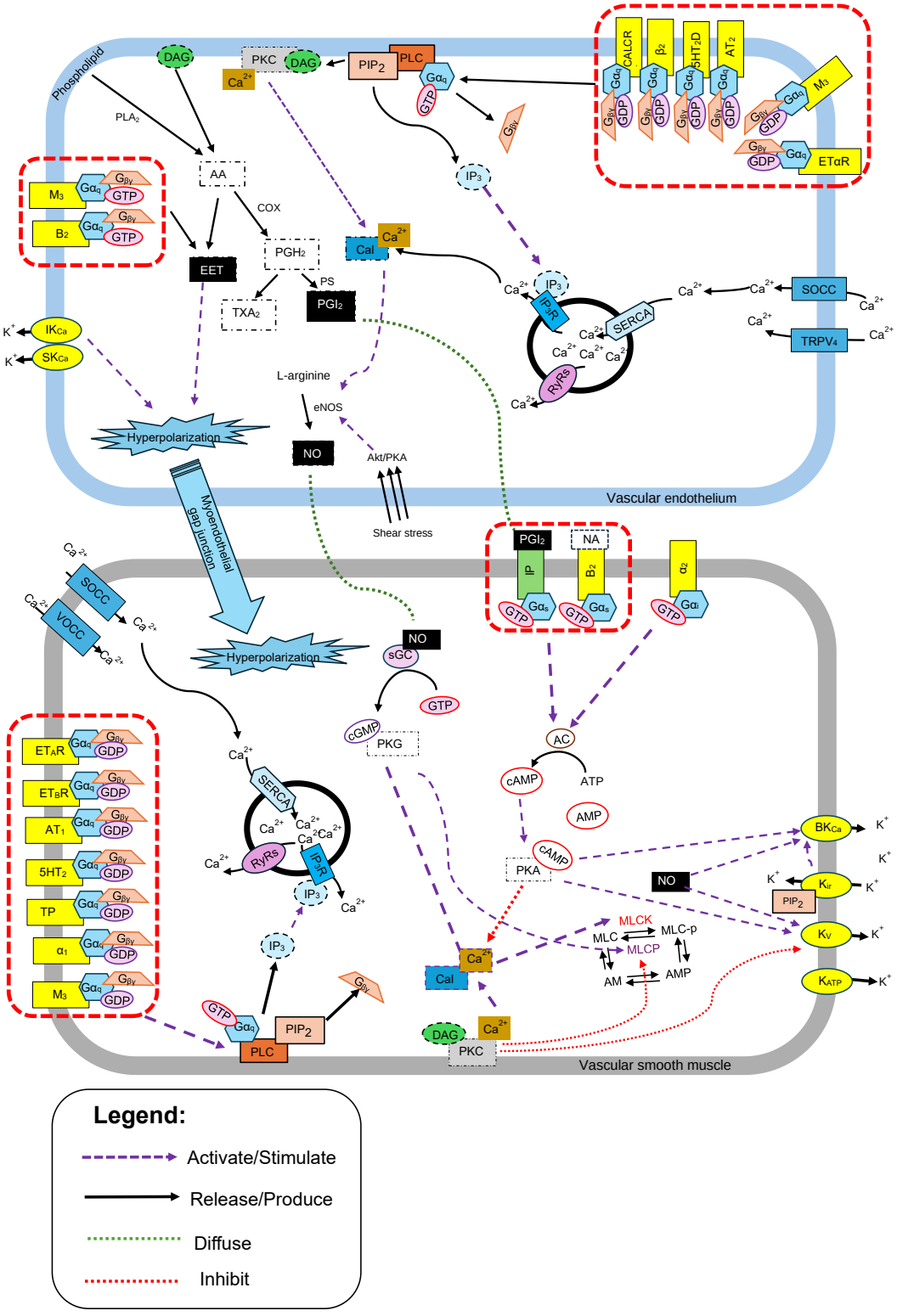

Figure 2: Overview of signalling mechanism pathway involved in vascular tone regulation.