Nicotine Alters cAMP Signaling in Vascular Smooth Muscle

By

NAVID SINGHRAO
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmacology and Toxicology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

____
Madeline Nieves-Cintrón, Chair

____
Manuel F. Navedo

____
Eleonora Grandi

Committee in Charge

2023

"Fai battere il cuore" – Enzo Ferrari

Acknowledgments

First and foremost, I would like to thank my supervisor, Dr. Madeline Nieves-Cintrón for her guidance and academic supervision. I am also grateful to Dr. Manuel Navedo for his valuable input and guidance throughout my Ph.D. journey. I must thank my academic advisor, Dr. Pam Lein, for providing straightforward, honest advice and counsel. Finally, to Dr. Ele Grandi for being the final guiding light I needed to navigate achieving my Ph.D.

I need to give special thanks to my mentor and friend, Dr. Victor A. Flores-Tamez, for the ideas, arguments, and long days that helped pushed this project across the finish line. As we always said, "Nothing stops this train."

I am fortunate enough to have such strong support from so many friends. Firstly, to the Powell sisters (Kristin, Kerry, and in particular, Katie) for always playing country music and providing me sanctuary. Next, to Kristina and Nick Lawson for the deep conversations about Avatar: The Last Airbender. To the Boys, you are the original pillars of encouragement and support through the years. My thanks to Donna L. Hirschfield for her excellent advice in helping me maintain my balance. Finally, to Dr. Armando R. Hernandez, thank you for always holding the torch to show me the way forward.

To my family. Kamal, thank you for encouraging me forward with this doctoral program and being a support to me moving onward in my career. To Ruby, I would not be here without your sacrifices that have propelled me forward into life. To my Mum, you are such a beautiful and hopeful spirit, being the light in the darkest moments of my life. To my Dad, you have always provided me with such strength, guidance, and laughter, and still always found time to listen to me.

Table of Contents

Acknowledgments ... iii

List of Figures and Illustrations ... vi

List of Symbols, Abbreviations, and Nomenclature ... vii

Abstract ... ix

Chapter 1 Introduction ... *1*

1.1 Background and Significance ... 1

1.2 Aims and Outline of the Dissertation ... 2

1.3 Vascular System ... 3
1.3.1 Macro and Microvasculature ... 4

1.4 Arteries ... 6
1.4.1 Tunica Externa ... 6
1.4.2 Tunica Media ... 7
1.4.3 Tunica Intima ... 8

1.5 Regulation of vascular smooth muscle function ... 9
1.5.1 Regulation of VSM by Ion Channels ... 10
1.5.2 Voltage-gated Calcium Channels in VSM ... 10
1.5.3 Transient Receptor Potential Channels ... 12
1.5.4 IP_3 receptors ... 13
1.5.5 Large-conductance calcium-activated potassium (BK) channels ... 14
1.5.6 Voltage-gated Potassium Channels ... 15
1.5.7 GPCR Regulation of VSM ... 17
1.5.8 VSM α-adrenergic regulation ... 17
1.5.9 VSM β-adrenergic regulation ... 18

1.6 cAMP Synthesis and Regulation ... 19
1.6.1 cAMP Synthesis ... 20
1.6.2 cAMP Degradation ... 21
1.6.3 Protein Kinase A ... 22
1.6.4 Exchange Proteins Directly activated by cAMP ... 22

1.7 Role of cAMP in VSM ... 23
1.7.1 Proliferation ... 24
1.7.2 Contractile Phenotype ... 24
1.7.3 Vasodilation ... 24

1.8 Nicotine ... 25
1.8.1 Nicotine History ... 25
1.8.2 Nicotinic Acetylcholine Receptors ... 26
1.8.3 Absorption, distribution, metabolism, and excretion Profile ... 27

1.9 Effects of Nicotine on the Vasculature ... 29

1.9.1 Nicotine and its General Effects on the Vasculature 29
1.9.2 Nicotine and Vascular Pathology.. 32

1.10 Summary of Chapter One and Knowledge Gaps 33
1.10.1 Chapter Summary 33

Chapter 2 Nicotine Impairs cAMP Signaling in aortic smooth muscle cells *35*

Abstract.. 36

2.1 Introduction.. 36

2.2 Methods 38
2.2.1 Animals 38
2.2.2 Secondhand Smoke.. 38
2.2.3 Nicotine Infusion and Serum Nicotine Levels... 38
2.2.4 Wire Myography 39
2.2.5 FRET.. 40
2.2.6 Western Blot 41

2.3 Results.. 43
2.3.1 SHS exposure reduces receptor-mediated cAMP levels in VSM 43
2.3.2 Nicotine exposure impairs receptor-mediated cAMP signaling in-vivo.............. 44
2.3.3 Reduced iso-induced vasodilation in nicotine-exposed mice 46
2.3.2 PDE inhibition restores iso-induced dilation 46
2.3.3 Nicotine Effect on Expression of cAMP pathway components.......................... 49

2.4 Discussion.. 51

Chapter 3 Discussions and Conclusions 54

3.1 Summary of Major Findings and Significance 54

3.2 Contextualizing Research Results: Insights into Vascular Physiology 56
3.2.1 Consequences of Lower cAMP Levels in VSM 56
3.2.2 PDE activity and the association with vascular pathologies.............................. 58
3.2.3 PDE3 prominence and the possible explanation.. 59
3.2.4 Effects of nicotine on gender 60
3.2.5 Alterations in cAMP and PDE activity, and their links to cardiovascular complications in nicotine users 61

3.3 Limitations and future work 62

3.4 Concluding Remarks... 64

References 66

List of Figures, Illustrations and Tables

Figure 1.1 – cAMP is a critical modulator of multiple functions inside VSM. 2

Figure 1.2 – An overview general view of the micro and macrovessels. 6

Figure 1.3 – The layers of the VSM. 9

Figure 1.4 – General overview of β_2-AR stimulating cAMP levels to cause VSM relaxation. ... 19

Figure 2.1 – Decrease receptor-mediated cAMP levels in VSM form SHS exposed mice. 43

Figure 2.2 – Chronic nicotine infusion impairs cAMP signaling in freshly isolated aortic rings. 45

Figure 2.3 – Impaired iso-induced vasodilation in nicotine-exposed mice. 48

Figure 2.4 – Chronic nicotine infusion impairs AC6 expression. 50

Table 1.1 Summary of the effects that different ion channels have on the VSM. 17

List of Symbols, Abbreviations, and Nomenclature

Symbol	Definition
α-AR/s	Alpha-adrenergic receptor/s
β-AR/s	Beta-adrenergic receptor/s
β_1-AR/s	Beta-1 adrenergic receptor/s
β_2-AR/s	Beta-2 adrenergic receptor/s
AA	Arachidonic acid
AC/s	Adenylyl Cyclase/s
AR/s	Adrenergic receptor/s
BK channels	Large conductance calcium-activated potassium channels
Ca^{2+}	Calcium
CaM	Calmodulin
cAMP	3',5'-cyclic adenosine monophosphate
$CAMPER_{SM}$	Mouse expressing a cAMP biosensor specifically in smooth muscle
cGMP	Guanosine 3',5'-cyclic monophosphate
Cilo	Cilostamide
DAG	Diacylglycerol
DPBS	Dulbecco's Phosphate Buffered Saline
EC_{50}	Half-maximal effective concentration
ENDS	Electronic Nicotine Delivery Systems
eNOS	Endothelial nitric oxide synthase
EPAC	Exchange protein directly activated by cAMP
FRET	Fluorescence resonance energy transfer
FSK	Forskolin
GPCRs	G protein-coupled receptors
G_SPCRs	G_S protein-coupled receptors
IBMX	3-isobutyl-1-methylxanthine
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor/s
ISO	Isoproterenol
K+	Potassium
KREBs	KREBs-Ringer solution
K_V channels	Voltage-gated potassium channels
MgPSS	Magnesium physiological salt solution
MLCK	Myosin light chain kinase

MLCP	Myosin light chain phosphatase
N	Neuronal
nAChR	Nicotinic acetylcholine receptors
NE	Norepinephrine
Nicotine Replacement Therapies	NRTs
nNOS	Neuronal nitric oxides synthase
NO	Nitric Oxide
PDE/s	Phosphodiesterase/s
PDE3	Phosphodiesterase 3
PDE4	Phosphodiesterase 4
PE	Phenylephrine
PKA	Protein Kinase A
PLC	Phospholipase C
PM	Plasma Membrane
ROCE	Receptor-operated calcium entry pathways
Roli	Rolipram
RT	Room Temperature
RyR	Ryanodine receptors
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SHS	Secondhand smoke
SOCE	Store-operated calcium entry pathways
SR	Sarcoplasmic reticulum
TCs	Tobacco cigarettes
TRP	Transient receptor potential
TS	Tobacco smoke
VGCC/s	Voltage Gated Calcium Channel/s
VSM	Vascular smooth muscle
VSMC/s	Vascular smooth muscle cell/s

// Abstract

Cyclic adenosine monophosphate (cAMP) plays a significant role in vascular smooth muscle (VSM) biology, including proliferation, and differentiation. Moreover, cAMP signaling regulates VSM contractility. For instance, agonists acting through β-adrenergic receptors (β-AR) produce vasodilation by triggering adenylyl cyclases (ACs) activity and increasing cAMP levels VSM. [1-8]

It has been shown by our group that VSM from mice exposed to secondhand smoke (SHS) had an attenuated cAMP level in response to the β-AR agonist isoproterenol (ISO), resulting in altered vascular reactivity. Tobacco smoke (TS) contains over 7,000 chemicals, including nicotine. [9] Nicotine is the primary addictive component in tobacco cigarettes (TCs) and has been shown to alter cellular cAMP production. [10,11] Yet, how nicotine impacts cAMP signaling in the vasculature, particularly in VSM, and the implications of these changes in vascular reactivity are unclear.

Here we addressed this knowledge gap by systematically evaluating the effects of nicotine on cAMP production in native VSM from mice. Firstly, we determined the effects of nicotine on ISO-induced β-AR-mediated cAMP levels using a mouse expressing a cAMP biosensor specifically in smooth muscle ($CAMPER_{SM}$). Then we determined the vascular reactivity in vessels of mice infused with nicotine using wire myography. Finally, we determined the expression of key proteins involved in cAMP synthesis and degradation. These experiments were designed to understand how nicotine alters cAMP signaling and the physiological consequences of nicotine exposure at the artery level.

Our findings suggest that nicotine alters ISO-induced cAMP levels in VSM from intact arteries. This was correlated with impaired VSM relaxation in response to ISO. Our studies show that the altered ISO-induced cAMP synthesis and VSM relaxation in nicotine-treated mice can be

rescued by inhibiting phosphodiesterases (PDEs). We also found that nicotine-infused mice have a decrease in AC6 abundance, which could contribute to impaired ISO-induced cAMP levels in VSM. Overall, this study uncovered mechanisms mediating nicotine impairment of cAMP synthesis and VSM relaxation, which may underlie alterations in vascular function in people exposed to or using nicotine products.

Chapter 1 Introduction

1.1 Background and Significance

TS exposure is associated with altered vessel excitability, poor arterial integrity, coronary artery disease, hypertension, and stroke. [12,13] Multiple components of TS can contribute to its vascular toxicity, including particulate matter inhalation, toxicant exposure, and acute and chronic effects of nicotine, which can incite inflammation, oxidative stress, and impaired contractility. [14-16] The implementation of smoke-free policies, together with public awareness, has resulted in a reduction in smoking among individuals. [17] However, in recent years, there has been a general rise in the usage of alternative-nicotine delivery devices, such as electronic nicotine delivery systems (ENDS), resulting in the increased consumption of nicotine. [18-22] Since combustion products are minimized in these alternative nicotine delivery systems, they are deemed “safer” than conventional cigarettes. However, this notion of safety has been challenged by research showing that nicotine can contribute to vascular dysfunction. [23-25]

Nicotine is a naturally occurring alkaloid extracted from the leaves of the tobacco plant *nicotiana tabacum*. [26-30] Chronic nicotine use is associated with cardiovascular diseases such as hypertension, hypertensive heart disease, and elevated arterial constriction. [18,31-33] Many of these effects are linked to nicotine's persistent sympathetic stimulation. [25,33-38] The vascular system receives inputs from sympathetic nerves that regulate function by activating G protein-coupled receptors (GPCRs). Stimulatory G_SPCRs activate AC to synthesize the second messenger, cAMP, a critical modulator of many vascular functions, including contraction and excitability (Figure 1.1). [1,2,39-42] Thus, the deleterious cardiovascular effects of nicotine could be mediated through

alterations in vascular cAMP signaling. In this chapter, we summarize the current knowledge of the vascular effects of nicotine with an emphasis on nicotine modulation of cAMP signaling in the VSM.

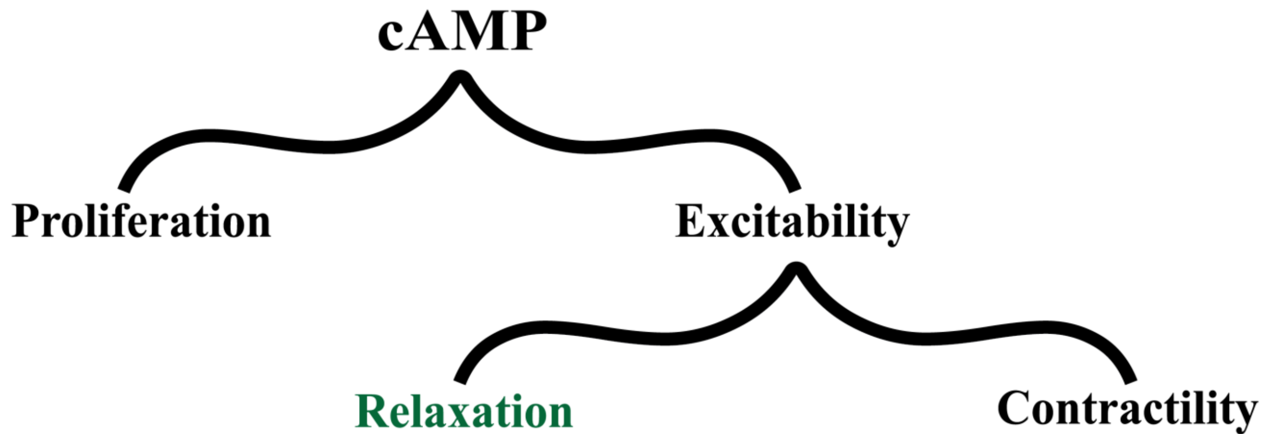

Figure 1.1 – cAMP is a critical modulator of multiple functions inside VSM.

Displayed are three of the many functions that cAMP modulates in VSM that are discussed throughout this chapter.

1.2 Aims and Outline of the Dissertation

Through this dissertation, we developed and designed a study to elucidate the effects of nicotine on cAMP signaling in VSM. This work's central hypothesis is that nicotine impairs receptor-mediated cAMP signaling in VSM, leading to altered vascular reactivity. To test our hypothesis, we pursued two specific aims:

Aim 1. Determine the effect of nicotine on receptor-stimulated cAMP levels in VSM. We hypothesized that nicotine impairs receptor-stimulated cAMP levels in VSM. To test this hypothesis, the effects of nicotine on β-AR-mediated cAMP levels were examined in VSM from a mouse expressing a fluorescence resonance energy transfer (FRET)-based cAMP biosensor

specifically in VSM (e.g., $CAMPER_{SM}$). We used a pharmacological approach to provide insight into the molecular participants involved in nicotine-induced alterations in cAMP levels.

Aim 2. Determine the effects of nicotine-induced impaired cAMP levels on vascular reactivity. We hypothesize that nicotine-induced alterations in cAMP levels impair βAR-mediated vasodilation. To test this hypothesis, we used wire myography to assess vascular reactivity in the vessels from sham (control) and nicotine-infused mice. We examined the role of PDEs, and the expression of key proteins involved in cAMP synthesis and degradation.

Chapter 1 presents a general background to VSM, emphasizing the relevance of cAMP signaling in regulating VSM function. This is then followed by a background of nicotine and its impact on the vasculature to tie in the impact that nicotine has on the VSM and cAMP specifically.

Chapter 2 delves into the experimental work performed to address the hypothesis outlined. This chapter describes the methodology and results revealing the impact of nicotine on VSM cAMP signaling, the mechanisms mediating nicotine-induced alterations in cAMP in VSM, and the functional consequences to vascular reactivity.

Finally, Chapter 3 addresses the implications of the research findings, contextualizing them into the current knowledge research about nicotine and cAMP, and future research directions illuminated by the findings.

1.3 Vascular System

The cardiovascular system is a closed system comprised of the heart, which is connected to a network of blood vessels of different sizes that transports blood and nutrients throughout the

body.[43-46] The heart is the epicenter of the vascular system, responsible for pumping blood, while the blood vessels are responsible for distributing blood throughout the body.[47]

Arteries are blood vessels that carry oxygenated blood away from the heart to the rest of the body.[46,48] Arteries branch out into smaller vessels called arterioles, which eventually lead to capillaries.[45,46,48] Capillaries are the final step for the exchange of oxygenated blood to the body's cells (it should be noted that they also exchange other vital products for the organs).[43,45,48] Capillaries eventually lead to venules, then to veins, to carry deoxygenated blood back to the heart.[48] This system plays a crucial role in maintaining the health and function of the body.

1.3.1 Macro and Microvasculature

The vascular system can be generally split into two types of blood vessels: macrovessels (larger blood vessels), and microvessels (smaller blood vessels). The macrovasculature refers to arteries with diameters that vary in size from as large as 25mm to 5mm, allowing them to hold approximately 15% of total blood volume.[48,49] Some examples include the aorta, pulmonary, and carotid artery.[50] All of these have a diameter of >5mm, with the aorta being the largest at 25mm in diameter (Figure 1.2).[48,51] Elastic arteries such as the aorta and pulmonary artery have a high content of elastic components (elastin) and less VSM compared to muscular arteries to allow more compliance.[48,49] Compliance is a property that blood vessels have, and it refers to the ability of blood vessels to expand in response to changes in pressure or volume when the heart pumps blood.[48] Muscular arteries, like the carotid, brachial or femoral arteries, contain a larger number of VSM that gives them the ability to distribute blood down the vascular tree into small resistance vessels and arterioles.

The microvasculature is composed of smaller-diameter arteries, arterioles, capillaries, and venules.[48,49] Arterioles have an average diameter of 40 µm, next venules at 20 µm, and capillaries have the

smallest diameter of 2 μm (Figure 1.2). [48,49] Microcirculation is the circulation of blood to the smallest blood vessels such as smaller diameter arteries and arterioles and these play a large role in nutrient and waste removal that occurs at the level of the microvasculature. [48] These vessels in the microvasculature have several functions. [48] Firstly, they assist with resistance and blood pressure regulation. By altering their diameter, these vessels can adjust resistance to blood flow. [48] The contractile state of VSM directly regulates arterial diameter and is responsible for its ability to regulate arterial resistance and regulate hemodynamics. [48] This is vital for maintaining stable blood pressure and ensuring that blood flow matches the metabolic demands of tissues. Secondly, in vasoconstriction and vasodilation, these smaller arteries and arterioles can alter their inner diameter to allow them to contract, a decrease in internal vessel diameter, or relax, an increase in internal vessel diameter. [48,52] Vasoconstriction reduces blood flow to specific areas, redirecting it to where it's most needed, while vasodilation increases blood flow to fulfill increased demands. [48] Another important function of smaller vessels and arterioles is tissue perfusion. [52] Regulation of blood flow to different tissues and organs by small vessels and arterioles ensures that oxygen and nutrients are delivered appropriately. [48] For example, during physical activity, arterioles lead to muscles dilating to increase blood supply and oxygen delivery, due to the body's increased demand for oxygen. Whereas during digestion, blood is redirected to the digestive organs to facilitate nutrient absorption.

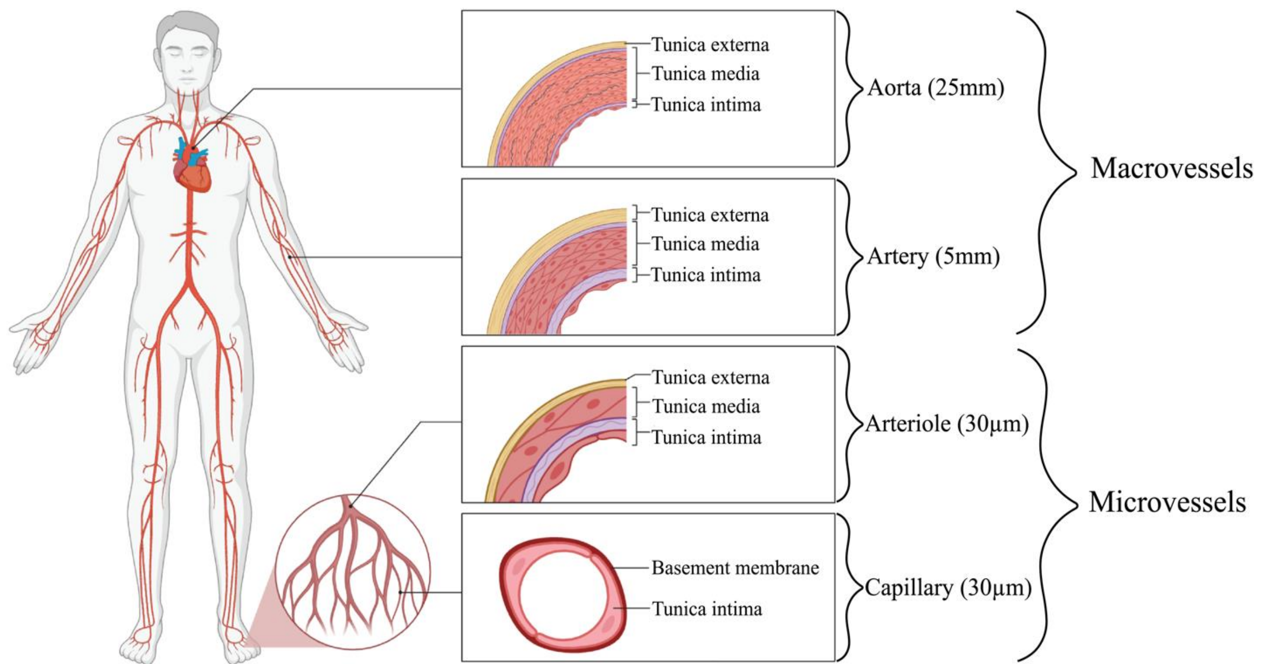

Figure 1.2 – An overview of the micro and macrovessels.

As the blood flows from the macrovessels to the microvessels, the diameter of these gets extremely small, starting at 25mm and eventually ending up at 30µm.

1.4 Arteries

Arteries have thick walls that allow them to withstand the pressure generated by the heart as it pumps blood. [48,49] Arteries are made up of an outer layer of connective tissue termed tunica externa, a medial layer comprised of smooth muscle cells or tunica media, and an inner layer of endothelial cells, also known as tunica interna (Figure 1.3). [48,53]

1.4.1 Tunica Externa

The tunica externa, also known as the tunica adventitia, is the outermost layer of arteries (Figure 1.3). [48,54] It is composed of connective tissue and collagen, which provides structural support for the vessel walls. [54,55] These larger types of vessels are categorized into elastic arteries,

containing more elastin to cope with the increased pressure from oxygenated blood, allowing for a constant, smooth, and even flow of blood from each heartbeat. [43,45,48,49] In addition, the elastic properties increase the efficiency of the stroke volume that would be lost if it were a rigid structure. [48,49,56] As a result, elastic arteries contain longitudinally oriented collagen fibers that assist in this. [57] This layer is highly important for blood vessel stability as it prevents the blood vessels from bursting under the high pressure of oxygenated blood. [58] In pathological conditions, such as atherosclerosis, the tunica externa can become inflamed and contribute to plaque buildup in the vessel walls, narrowing the vessel lumen and reducing blood flow to the tissues supplied by the affected vessel. [54,55] The tunica externa is an important component of blood vessel structure, providing support and protection to the vessel walls.

1.4.2 Tunica Media

The tunica media is comprised of many layers of spindle-shaped VSM that form the middle portion of the arterial wall (Figure 1.3). [53] This layer of VSM allows the artery to constrict and dilate, modulating the diameter of the artery and in turn, regulating blood flow through the center of the artery, ultimately controlling the blood flow to end-organs and even blood pressure. [45,49,53]

This important ability to contract and relax can be removed in pathological states such as atherosclerosis, vascular calcification, and hypertension, where a phenomenon named, phenotypic switching, occurs. [54,59-61] This is the VSM changing from contractile to a synthetic, noncontractile phenotype, resulting in arterial wall stiffening. [60,61] This simple change alters the ability of the whole vasculature to deliver nutrients and oxygen to the entire body causing major complications. Therefore, it cannot be underestimated how impactful the tunica media is in health and disease. In short, arteries are essential for the proper functioning of the cardiovascular system. They help to maintain blood pressure and ensure that oxygen and nutrients are delivered to the various tissues

in the body.

1.4.3 Tunica Intima

The tunica intima primarily comprises endothelial cells that line the inner surface of blood vessels, including arteries. [48,62,63] They are a single thin layer of cells that form a barrier between the blood and the surrounding tissues (Figure 1.3). [48,62,63] Endothelial cells perform critical functions within the vascular system, encompassing the regulation of blood flow, blood pressure, and preservation of the integrity of blood vessel walls. [48,62] Endothelial cells also play a significant role in the contraction and relaxation of VSM. [48,62] They can assist in smooth muscle relaxation in several ways, firstly, they contain prostacyclin's synthase I_2, and this synthesizes prostaglandin I_2, which in turn increases cAMP production in VSM to induce relaxation. It should be noted that this product is synthesized from arachidonic acid (AA) and that via the endothelium, another way increase cAMP is through the cyclooxygenase (COX) pathway, which is also synthesized from AA. [48,62] However, the endothelium has a potent vasodilator in that of nitric oxide (NO). [48,62] The chemical is synthesized by one of three nitric oxide synthases (NOS): neuronal NOS (nNOS), endothelial NOS (eNOS,), and inducible NOS (iNOS). [62,64] In brief, the NOS work in the same way in that they produce NO from L-arginine. [62] The study of the endothelium is significant due to its essential role in maintaining vascular homeostasis. [50] The endothelium serves as a physical barrier and releases various bioactive substances that play a crucial role in regulating vascular function. [50,65] Endothelial dysfunction, implicated in numerous pathologies, can lead to the development of conditions such as atherosclerosis, hypertension, reduced blood flow, and capillary rarefaction. [65] The endothelium's direct contact with blood flow and its responsiveness to shear stress make it a critical component in understanding these pathologies. [62,64,66] To induce vasoconstriction, the endothelium can synthesize endothelin, which has been associated with

several cardiovascular diseases such as atherosclerosis and hypertension. [48,62,64,66,67]

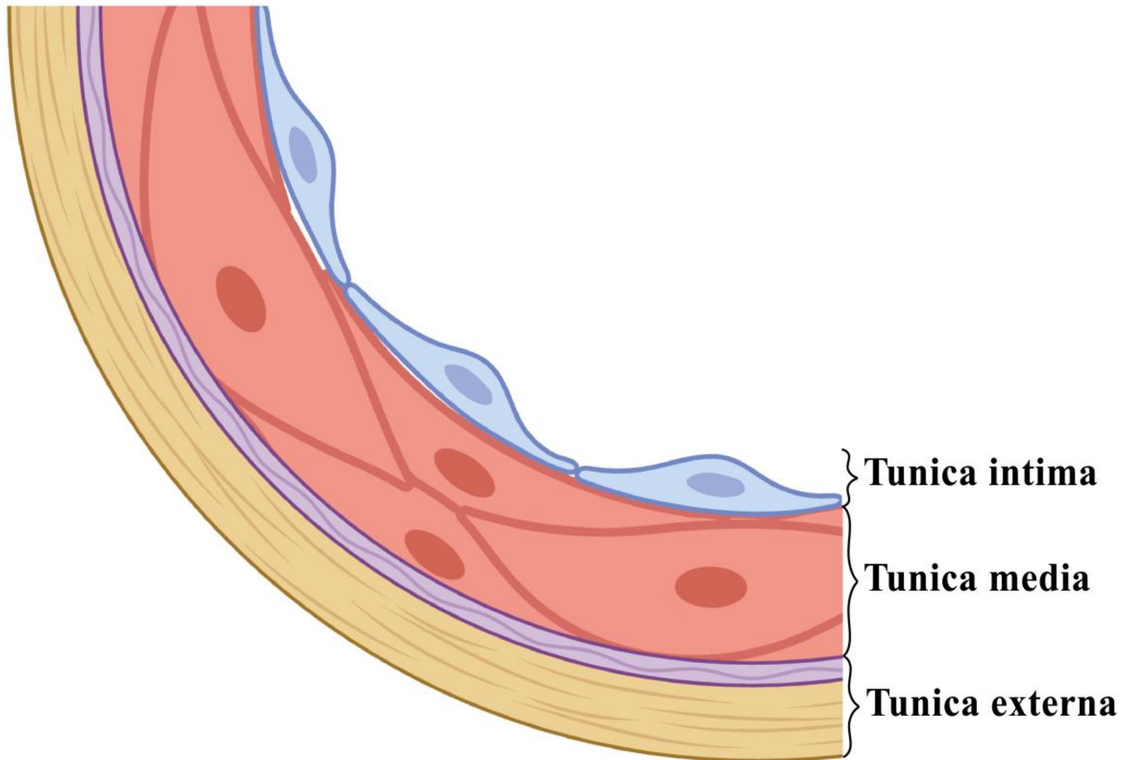

Figure 1. 3 – The layers of the VSM.

The three essential layers of the VSM, they all play an important role however the tone and blood flow regulation are ultimately controlled by the tunica media where the VSMCs are located. This image also includes a purple section in the Tunica externa that is an external elastic membrane.

1.5 Regulation of vascular smooth muscle function

Contraction of the VSM is initiated by calcium (Ca^{2+}). Once Ca^{2+} has entered the cell it binds to calmodulin (CaM), a calcium-handling protein that activates the enzyme myosin light chain kinase (MLCK). [48,68] Ca^{2+}/CaM increases the MLCK activity by 10,000-fold. [48,69,70] MLCK uses adenosine triphosphate (ATP) to phosphorylate myosin light chains located on the tips of myosin to form links with actin resulting in cross-bridging, producing tension and, therefore,

contraction. [48,68] The action of MLCK is opposed by myosin light chain phosphatase (MLCP). This phosphatase dephosphorylates the myosin light chains, decreasing the calcium sensitivity of the cytoskeleton and leading to relaxation. [68]

1.5.1 Regulation of VSM by Ion Channels

Ion channels are proteins that are found in the cell's plasma membrane (PM) and the membrane of intracellular organelles. This protein spans the membrane to allow specific ions to move in and out of the cells or, in the case of intracellular ion channels, in and out of intracellular organelles (e.g., sarcoplasmic reticulum, SR). VSM expresses several ion channels that play a critical role in regulating VSM physiology. [3,68,71] Although ion-channel physiology is not the focus of this dissertation, a general overview of major ion-channels contributing to regulating VSM function is provided for two main reasons: 1) ion-channels function is critical for regulating VSM excitability and contraction. 2) cAMP signaling (the focus of this work) can impinge on VSM function by activating effector proteins (e.g., protein kinase A, PKA) that regulate these ion channels. Here we discuss the general roles of voltage-gated calcium channels (VGCCs), transient receptor potential (TRP), voltage-gated potassium channels (K_V), large conductance calcium-activated potassium (BK) channels, and IP_3 receptors (IP_3R), and ryanodine receptors (RyRs) to provide perspective on their role on VSM function, see Table 1.1.

1.5.2 Voltage-gated Calcium Channels (VGCC)

Voltage-gated calcium channels (VGCC) are calcium-permeable ion channels located at the membrane of the cells. As their name indicates these channels are activated by changes in membrane potential (i.e., depolarization). VGCC are classified by their electrophysiology properties: L–type calcium channels (LTCC), have large conductance and long-lasting current at strong depolarizations; T-type calcium channels (TTCC), have tiny conductance and transient

current at weak depolarizations. [72,73] Whereas the N-type calcium channels have neither L nor T-type but rather an intermediate profile. Subsequent research also identifies P/Q and R types of VDCC based on their biophysical and pharmacological profile. [72,73]

T-type and L-type VGCC have been identify in VSM. While some studies suggest that T-type contributes to the regulation of VSM contraction, it is well established that Ca^{2+} influx through the L-type VGCC is the principal mediator of pressure-induced arterial constriction or myogenic tone. [3,48,74-78] Ca^{2+} influx through L-type VGCC also regulates transcription in VSM. Recent studies show that exposure to SHS causes functional up-regulation of L-type VGCC, and this was related to increased contractility and changes in the expression of BK channels. [79] Moreover, altered L-type VGCC function in vascular smooth muscle has been found in many diseases, including hypertension, diabetes, and atherosclerosis. [3,71,74] It is important to understand the function and regulation of VGCCs in VSM as alterations in these VGCCs are responsible for declines in cardiovascular health.

Intracellular signaling pathways can regulate the function of L-type VGCC, thereby modulating intracellular calcium and contraction. Indeed, cAMP has been shown to regulate the L-type VGCC through the effector protein, protein kinase A (PKA). However, this regulation is complex, with some studies indicating that PKA reduces L-type activity [3,48,74,80] and others showing that it increases the activity of the channel. [81,82] In general, in studies where PKA activity was increased globally by dialyzing the catalytic subunit of PKA into the cell or via broad activation of ACs in the cell (by use of forskolin, a potent AC activator), the activity of the L-type VGCC was blunted. [83] In contrast, in experiments using sub-micromolar concentrations of the β-AR isoproterenol (iso) the L-type activity was potentiated, whereas μM concentrations of iso led to a decrease in L-type activity. [84] Accordingly, the study by Syed et.al., using cAMP biosensors,

showed that a hyperglycemic stimulus elicited a mild increase in cAMP that was associated with a PKA-dependent potentiation of the L-type activity. [85] In this same study, the authors showed that the application of forskolin led to the expected global increase in cAMP, leading to vasodilation even in the presence of glucose. [85] This distinct regulation by cAMP/PKA suggests that low cAMP levels may enhance L-type VGCC activity and contraction, while high levels may activate vasodilatory pathways that blunt L-type activity (e.g., K^+ channels) and override L-type-mediated Ca^{2+} influx.

1.5.3 Transient Receptor Potential Channels

TRP channels are a large family of non-selective cation channels that are found in all cell types, including VSM. [71,74,86] VSMCs and TRP channels contribute to the depolarization of the cell membrane and the following increase in intracellular Ca^{2+} levels leading to VSM contraction. [3] There are 28 TRP channels identified and sub-grouped into 6 families; however, the most relevant families of TRP in VSM are canonical (TRPC), melanostatin (TRPM), polycystin (TRPP), and vanilloid (TRPV). [3] The vast array of TRP channels and their many subfamilies in VSM is extremely diverse and has a large degree of influence on both vasodilation and vasoconstriction in VSM. [3] A discussion of TRP channels is not within the scope of this dissertation. Refer to Earley and Brayden's review for more information. [86]

1.5.4 Ryanodine receptors

RyRs are intracellular Ca^{2+} release channels that play a crucial role in Ca^{2+} signaling in various cell types, including vascular smooth muscle cells. [3,48] Some of the functions that RyRs regulate in the vasculature are vascular tone and the contraction of blood vessels. [3] There are three isoforms of RyRs, RyR1 is predominantly located in the heart, RyR2 is the predominant isoform expressed in vascular smooth muscle and RyR3 is expressed mainly in skeletal muscle

and the brain. [3,74,87] RyRs are located on the SR, a specialized organelle that stores and releases Ca^{2+} ions. [3,61,74] When activated, RyRs allow the release of Ca^{2+} from the sarcoplasmic reticulum. [3,61,74] In cardiac and skeletal muscle RyRs contribute to contraction by increasing intracellular Ca^{2+}. In VSMCs, however, the activation of RyRs produces a local Ca^{2+} signal, known as Ca^{2+}spark. [3,61,74] Ca^{2+} sparks do not contribute significantly to global intracellular Ca^{2+} concentration. These events are coupled to the activation of Ca^{2+}-activated potassium channels, which hyperpolarize the membrane and contribute to the negative feedback regulation of L-type VGCC (see the section on BK channels below).

The activity of RyRs is regulated by various factors, including Ca^{2+} itself, as well as intracellular signaling, such as cAMP/PKA.[3,48] Indeed, forskolin, a broad AC activator, is a strong vasodilator that has been shown to increase Ca^{2+}spark frequency. [88] Interestingly, inhibition of RyRs significantly reduces the vasodilatory effects of forskolin. [88] Studies characterizing vasodilators that engage cAMP/PKA signaling have found similar results. Suggesting that vasodilators that engage cAMP/PKA could exert their effects in part by increasing Ca^{2+} spark frequency, leading to increased activity of BK channels, membrane potential hyperpolarization, decreased L-type VGCC activity, and vasodilation. Alterations in cAMP levels by substances, such as nicotine, could impact the regulation of RyR receptors by PKA with profound implications for the regulation of VSM contractility.

1.5.5 IP_3 receptors

Inositol trisphosphate receptors (IP_3Rs) are intracellular Ca^{2+} release channels that play a significant role in calcium signaling in various cell types, including vascular smooth muscle cells. [3,48,61,74] IP_3Rs are activated by inositol trisphosphate (IP_3), which is produced upon the activation of $G_{q/11}$-coupled GPCRs located on the PM. [3,48,61,74] In VSM vasoconstrictors such as

norepinephrine (NE), for example, that engage $G_{q/11}$PCRs signaling and phospholipase C (PLC) triggering the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into IP_3 and diacylglycerol (DAG). [3,48,61,74] Activation of IP_3Rs by IP3 results in the release of SR-stored Ca^{2+} into the cytoplasm, known as a calcium wave, which in turn activates calcium-sensitive contractile proteins, including actin and myosin, promoting smooth muscle contraction. [3,48,61,74] In contrast, studies in airway SMC and other cell types suggest that cAMP/PKA signaling inhibits IP_3R function. [89,90] Moreover, cAMP/PKA inhibits PLC, thus decreasing the production of IP_3. [91] cAMP/PKA signaling could contribute to vasodilation by decreasing the IP3R-mediated calcium release. Lower cAMP levels, as we observed with nicotine treatment (see Chapter 2), could compromise the regulation of IP_3R by PKA, and impact vascular function by favoring vasoconstriction.

1.5.6 Large-conductance calcium-activated potassium (BK) channels

VSM expresses BK channels which contribute to membrane potential regulation. [3,71] As their name indicates these channels are activated by calcium. In VSM Ca^{2+} release events from the SR through RyRs, also known as Ca^{2+} sparks, activate BK channels. Ca^{2+} sparks activate multiple BK channels simultaneously, leading to hyperpolarization of the cell membrane potential, which provides negative feedback regulation of VGCC, resulting in the relaxation of VSM. [61,74]

BK channels are regulated by cAMP/PKA signaling, with there being clear links to how the two interact indirectly. It is thought that with the use of vasodilators that increase cAMP, there is an increase in Ca^{2+} sparks and SERCA activity to speed up Ca^{2+} reuptake into the SR, which is mediated by PKA-dependent phosphorylation. [3] There also appears to be a spatial-temporal relationship between β_2-AR receptors, VGCC, and the BK channel, through the $G\alpha_s$ subunit in the

aortic VSM. [3] It is widely accepted that there is a close relationship between cAMP and the BK channel, as many studies have shown that increased cAMP levels in the vasculature increase the vasodilatory response in part through BK channels. [3,6,92-94]

1.5.7 Voltage-gated Potassium Channels

Kv channels are expressed widely in VSM and play an important role in the membrane potential and myogenic tone regulation. [3,95,96 71,96] They are comprised of 12 families, Kv 1-12, and are made up of tetramer α subunits that form an ion pore with auxiliary β subunits. [3,71] VSM, express multiple subtypes, namely, Kv 1, 2, 7, and 9. Kv channels are opened by membrane potential depolarization and are responsible for the efflux of potassium from the cytosol, which hyperpolarizes the cell membrane contributing to the resting membrane potential and myogenic tone regulation. [95,97]

The cAMP/PKA pathway has been implicated in the modulation of Kv. [3,98] Essentially, the mechanism of action through the β-adrenergic receptors results in cAMP-dependent PKA-phosphorylation of these particular Kv channels resulting in increased activity through this channel, causing vasodilation.[98] However, overstimulation of this channel via this cAMP-dependent pathway may result in the downregulation of Kv channels. [3,98] Again, the prominence of cAMP, as a second messenger in its altering of vascular function through the modulation of ion channels, underlies the critical role of this second messenger for VSM function and highlights the relevance of understanding its regulation in health and disease and in response to an exogenous stimulus such as nicotine.

Summary of the effects that different ion channels have on the VSM.

Name of Ion Channel	Effects	Regulation by cAMP signaling
VGCC	Contraction, depolarization increases the open probability of VGCC, which allow the influx of extracellular Ca^{2+} ions into the cytosol, leading to VSM contraction. [3,48,61,74]	cAMP regulates the VGCC through PKA, decreasing Ca^{2+} influx and causing vasodilation. [3,48,61,74]
TRP	Contraction, contribute to depolarization of PM then increasing in intracellular Ca^{2+} levels. [3,74]	Effects vary depending on subfamily, cAMP effects PKA modulates some TRP channels leading to contraction, whilst others lead to dilation. [3,74]
BK	Vasodilation, activated BK channels in the plasmalemma increase hyperpolarization of the cell, promoting relaxation of VSM. [3,48,61,74]	Increases in cAMP, increase Ca^{2+}sparks and SERCA, mediated by PKA-dependent phosphorylation, pumping to speed up Ca^{2+} reuptake into the SR. [3,48,61,74]
Kv	Vasodilation, depolarization of the PM activates Kv	βAR agonism of cAMP-dependent PKA-

	channels causing subsequent efflux of potassium from the cytosol. [3,48,61,74]	phosphorylation results in increased current through this channel. [3,48,61,74]

Table 1.1 Summary of the effects that different ion channels have on the VSM.

Above is a general overview of the ion channels and the effects they have on the VSM. In addition, is an overview of the links to how each of these ion channels interact with cAMP.

1.5.8 Adrenergic Regulation of VSM

Adrenergic receptors are part of the large family of seven transmembrane domain receptors that are coupled to GPCRs. [99] There are three subunits located near the intracellular aspect of the GPCR: α, β, and γ. Ligand binding triggers a change in the confirmation of the Gα subunit, allowing for GDP (inactive state) to dissociate and GTP to bind (active state). [48,99] Gα subunits are organized into four families according to their α subunit: $G\alpha_i$ (inhibitory), $G\alpha_s$ (stimulatory), $G\alpha_{12/13}$, and $G\alpha_q$. [100] ARs are categorized into four types α1, α2, β1, and β2. [101,102] Each of these receptors is associated with a particular Gα subunit and their activity regulates VSM function.

1.5.9 VSM α-adrenergic regulation

α-adrenergic receptors (α-ARs) are a type of GPCR expressed in the vasculature, including VSMCs, where they are involved in regulating contraction. [48,102] There are two main subtypes of α-ARs: α_1 and α_2. While they share some similarities in their downstream effects, there are fundamental differences in the transduction pathways engaged by each receptor subtype. [48,102] The α_1 receptor engages $G\alpha_q$ protein, and ligand binding to the receptor activates phospholipase C (PLC). PLC, in turn, hydrolyzes phosphatidylinositol 4,5-bisphosphate producing the second messenger's inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). [48,102,103] These second messengers induced contraction via two mechanisms: IP_3 binds to inositol IP_3Rs on the SR,

increasing Ca^{2+} release from intracellular stores. Meanwhile, DAG activates PKC, and PKC phosphorylates CIP-17, a phosphorylation-dependent inhibitor of myosin phosphatase. [48,68,102] Inhibiting MLCP tips the balance towards higher MLC phosphorylation and vasoconstriction. [48,68,102]

The activity of α_2-AR receptors leads to vasocontraction, albeit through different mechanisms than those engaged by α_1-AR receptor activation. [48,104,105] α_2-ARs are coupled to inhibitory $G\alpha_i$ protein and, when activated by NE, inhibit AC from producing cAMP leading to vasoconstriction. [48,105] Dysregulation of α-receptors in the vasculature will result in multiple cardiovascular diseases, such as hypertension and atherosclerosis. [105,106] For example, it is known that the coronary arteries that have a damaged endothelium have excessive vasoconstriction when α_1-AR are activated, leading to myocardial ischemia. [107] Indeed, myocardial ischemia is linked to high blood pressure, and one treatment is α-adrenergic blockers, such as doxazosin, whereby the mechanism of action is to block the binding of NE to the α_1-AR. [108] These two receptor subtypes are an important part of the intricate regulation of the vasomotion in the vasculature, having a large influence on the vasoconstriction and resistance of the vasculature.

1.5.10 VSM β-adrenergic regulation

Another type of GPCR that is involved in the regulation of VSM function is the β-AR. As mentioned, there are two subtypes of β-AR expressed in VSM: β_1-AR and β_2-AR. β_1-AR and β_2-AR are coupled to G_S activation of the receptor leading to the increased activity of AC, subsequently increasing intracellular levels of cAMP. [48,68,102,109-111] The increased cAMP then activates PKA, which in turn, phosphorylate downstream targets leading to vasodilation by

regulating the Ca^{2+} sensitivity of the contractile machinery and decreasing intracellular Ca^{2+}, see Figure 1.4. [48,68,109]

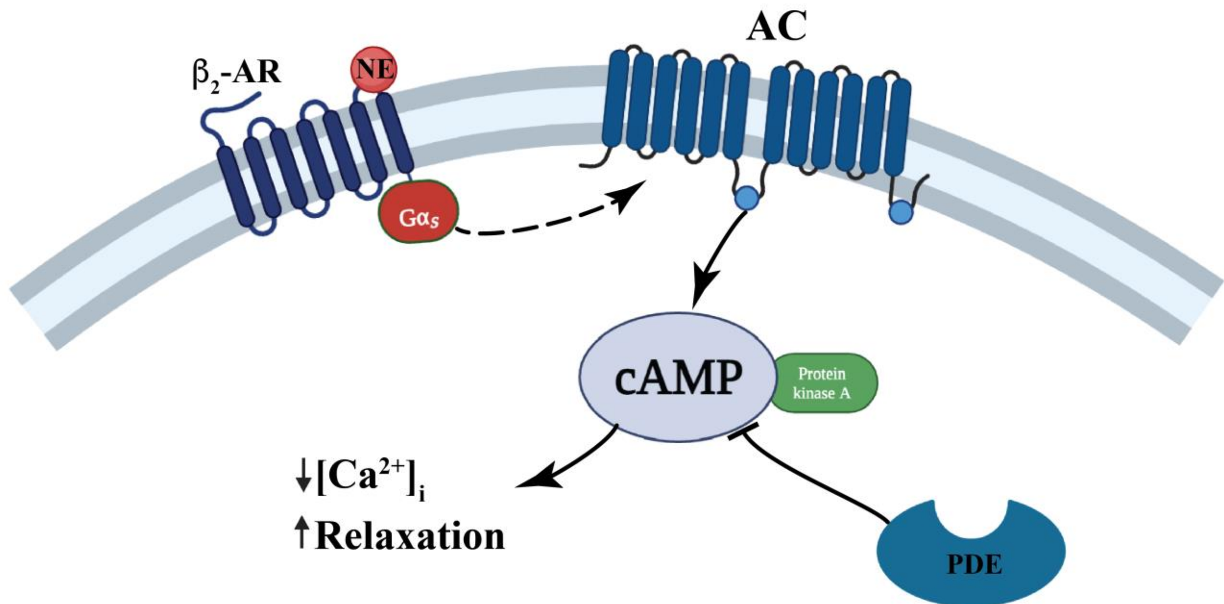

Figure 1.4 – General overview of β_2-AR stimulating cAMP levels to cause VSM relaxation.

After the binding of the endogenous ligand NE (NE) to the β2-AR this allows the disassociation of Gαs to bind to the AC. After activation of the AC cAMP is increased which in turn leads to the eventual decrease of Ca. PDEs tightly regulate cAMP levels by hydrolyzing them to make them inactivate.

1.6 cAMP Synthesis and Regulation

The ubiquitous second messenger, cAMP can be triggered by the activation of numerous GsPCRs expressed in arterial myocytes to regulate many cellular events, including contraction, proliferation, and relaxation. [1,2,39] Indeed, many vasodilators, such as low-dose epinephrine, histamine, and prostacyclin, act on arterial myocytes by activating GsPCR receptors. [92,112] When GsPCRs are activated, they stimulate the activity of membrane-bound ACs, which converts ATP into cAMP. [109] This increases intracellular cAMP concentration, stimulating PKA activity. Once activated, it phosphorylates several downstream targets that decrease the Ca^{2+} sensitivity of the

contractile apparatus and reduces intracellular Ca^{2+} concentration by modulating the activity of several proteins at the PM and SR, which contributes to vasodilation. [6,92-94] Thus, cAMP plays a critical role in regulating arterial myocyte excitability and vascular reactivity.

1.6.1 cAMP Synthesis

There are 10 AC isoforms, 9 of which are membrane-bound. [2,113] These ACs are further split into 3 subclasses: class I (AC1,3 and 8) class II (AC2, 4, and 7), and finally class III (AC5, and 6). [2,113,114] However the most abundant in VSMCs are AC 3, 5, and 6 and this is where the focus of this discussion will be. [2] Whilst all ACs are activated by G_S, only the latter two are inactivated by G_i, whilst AC3 is inactivated by Ca^{2+}. [2,115,116]

The mechanism of action by which ACs are activated is well understood. [2,48] After an agonist binds the G_SPCR there is a confirmational shift with the α and βγ, allowing the α subunit to exchange GDP for GTP, the latter being the active state. [2,48] Then the α-GTP travels to the ACs activating it to convert ATP to cAMP, causing an increase in the basal concentration of cAMP. This second messenger can then promote many downstream signals to induce proliferation, relaxation, and contraction (discussed later in this chapter). [2,48]

Although, when there is an increase in cAMP throughout the body there are, "nanodomains" of cAMP. [2,117-119] These are discrete local pools of cAMP that assist with the signaling cascade within the cell. [2,117-119] These nanodomains allow cAMP to generate a multitude of unique signals within a confined area to activate local PKA to specific parts of the cell. [119] For example, cAMP is involved with fight or flight responses and specifically, within cardiomyocytes, the precise control of cAMP to induce certain signals over others. [120] Where it has been shown in cardiomyocytes that when these nanodomains are altered it is a factor within cardiovascular disease. [121] This is a highly important concept that needs to be kept in mind when discussing levels

of increasing cAMP, as firstly, it shows the reason behind the numerous signals within the cell that are a result of cAMP being activated and secondly, it implies that the altered levels of cAMP can disrupt some signals more than others.

1.6.2 cAMP Degradation

As with everything in the body, balance is key, with the creation of cAMP through ACs, resulting in specific effectors being activated in the cell, there must be a way to break cAMP down. This is done by PDEs that hydrolyze cAMP and are a key component of cAMP degradation and regulation. [122,123] There are 11 families of PDEs encoded by 21 genes and over 80 types of isoforms, that work to hydrolyze Guanosine 3',5'-cyclic monophosphate (cGMP) and cAMP to either 5'AMP or GMP. [124,125] However, of the 11 families only PDE 4,7,8, hydrolyze cAMP to AMP, exclusively, whilst PDE 1-3, 10-11 have dual specificity for both cAMP and cGMP. [122,126] Intriguingly, the most abundant PDEs in VSM are PDE3 and PDE4. Commonly used therapies involve the increase of cAMP by inhibiting these PDEs. [126,127] For example, inhibition of PDE3 and PDE4 has been shown to decrease chronic obstructive pulmonary disorder (COPD) symptoms, a disease strongly linked to tobacco smoking and impairment in airway smooth muscle cells. [128-130] Gao et.al., showed the important role of PDE4 whilst studying abdominal aortic aneurysms. In their study, they explored that PDE4 was elevated in humans who had abdominal aortic aneurysms and in angiotensin (ANGII) mice. [131] They found that PDE4 knockout mice showed a decreased likelihood of abdominal aortic aneurysms. [131] They also showed that the PDE4 inhibitor rolipram (roli) could curb abdominal aortic aneurysms in mice. [131] Their results suggested that PDE4 is elevated in abdominal aortic aneurysms and that PDE4 deficiency suppressed smooth muscle cell apoptosis through the cAMP-PKA pathway. [131] The research described in Chapter 2 shows that nicotine reduces cAMP levels in VSM through a mechanism that involves, in part, PDE

modulation. The deleterious vascular effects of nicotine and containing products, including a higher risk of cardiovascular-related mortality, may be modulated through alterations in vascular cAMP signaling due to changes in PDE signaling.

1.6.3 cAMP Effectors

cAMP activates multiple proteins within the cell to affect cellular function. Two prominent and well-studied cAMP effectors are PKA and EPAC. [2] [1,2,119] Whilst they have their similarities, they are ultimately very different in how they transmit the signals that cAMP elicits.

1.6.4 Protein Kinase A

Discovered in the 1970s PKA is classified as a holoenzyme, in its active state, and is by far the most researched and explored effector protein of cAMP. [2,132,133] PKA has two isoforms, PKA I, found within the cytosol working by diffusion, where after binding cAMP it becomes catalytically active. [133,134] Whilst, PKA II is anchored in the PM by AKAPs, this is to allow highly specific domain targets, such as $Ca_{v1.2}$. [2,133,135] The way that PKA II can anchor close to these targets is a fundamental reason behind cAMP microdomains. [2,119] Mechanistically, both isoforms work the same, whereby two cAMP molecules bind to PKA causing a confirmational shift, this then allows the kinase to become active. [2] One study found that inhibition of PKA during vascular development causes "hypersprouting" of vessels, which has the possibility of leading to tumors and ischemia. [136,137] cAMP activates PKA; thus, impaired cAMP signaling could lead to vascular dysfunction by compromising PKA regulatory effects.

1.6.5 Exchange Proteins Directly activated by cAMP

Exchange proteins directly activated by cAMP (EPAC) function as a guanine nucleotide exchange factor (GEF) for Rap1 and Rap2. In the inactive state, Rap1 and Rap2, are bound to guanosine diphosphate (GDP), unable to exert the functions dictated by EPAC. Activation of

EPAC occurs when cAMP binds to EPACs regulatory domain. Then EPAC catalyzes the exchange of GDP for guanosine triphosphate (GTP) on Rap1 and Rap2, leading to their activation and allowing them to exert the functions of EPAC. [138,139] EPACs are separate from PKA and are cAMP-regulated GEFs. [2,134,139] With regard to the VSM, EPAC promotes vasodilation in several ways. [140] One such way EPAC accomplishes this is through the regulation of SR Ca^{2+} release through the RyR. [140] The RyR is close in proximity to the BK channel and a local release of Ca^{2+} in this region causes the BK channel to activate and hyperpolarize the PM, thereby closing VGCC and stopping the entry of new Ca^{2+} into the cytosol. [140] By limiting Ca^{2+} influx through the VGCC this causes vasodilation. [134,139,140] In pathological conditions, EPAC has been associated with a phenomenon in VSMCs known as “phenotypic switching”, whereby VSMCs change from contractile to non-contractile. [2,60,140] This process has been linked to hypertension and atherosclerosis. [54,60] EPAC emerges as a noteworthy participant within VSM, particularly in its interaction with cAMP, to facilitate the essential function of vasodilation, adding to the vast array of proteins and signals that are regulated by this influential second messenger. Where lower cAMP levels, because of nicotine exposure, can increase the chances of cardiovascular diseases by altering the essential function of vasodilation, which EPAC directly affects.

1.7 Role of cAMP in VSM

As discussed above cAMP regulates many processes, such as proliferation and excitability within the VSM. [2,109,134] Where these downstream processes begin with the agonism of GPCRs activating ACs to produce cAMP. [1,2] While cAMP plays a key role in numerous physiological and pathological processes in VSM such as proliferation, of interest to this work is how cAMP can control VSM excitability and vascular reactivity.

1.7.1 Proliferation

cAMP assists with differentiated VSMCs to maintain a low proliferation rate. [2] One such example of this is how cAMP can decrease VSMC proliferation after injury to VSM, but in healthy VSM it will hinder the production of VSMCs. [141] The importance of maintaining a low proliferation rate rests in, “phenotypic switching” (see 1.4.2). [2,60,140,141] The vasculature relies on its ability to respond quickly to changes in blood flow by contracting and relaxing. [48,141] However when the proliferation rate of VSMC increases vasculature impairment of the contraction and relaxation mechanisms in the VSM, results in diseases such as atherosclerosis and hypertension. [60,141] Namely, cAMP plays a large role in VSMC proliferation, which is also intertwined with its influence on vascular excitability.

1.7.2 Contractile Phenotype

cAMP significantly impacts the VSM's maintaining a contractile phenotype, which allows the blood vessels to increase or decrease in diameter, vasodilation, or vasorelaxation. [2,60] This process links with the concept described above whereby VSMC takes on a synthetic phenotype from a contractile one. [2,60,141] However the effect of cAMP described above involves increasing proliferation rates of VSM that in turn quells the vasomotion properties of the vasculature. [2,60,141] Here the difference is that the ability of the vasculature to contract or relax is simply gone when cAMP signaling is impaired, but the same pathologies of atherosclerosis and hypertension are the result. [54,60,141] Again showing the interconnectedness of how cAMP can alter not just the proliferative state of the VSM but also its contractile phenotype.

1.7.3 Vasodilation

One of the most prominent effects of cAMP is its ability to induce relaxation in the vasculature. [1,2,48,110] As discussed earlier cAMP is synthesized by ACs and then cAMP binds to

effector proteins that increase downstream signaling. In the case of increasing vasodilation, cAMP binds PKA where it phosphorylates downstream targets that decrease the Ca^{2+} sensitivity of the contractile apparatus and reduce intracellular Ca^{2+} concentration by modulating the activity of several proteins at the PM and sarcoplasmic reticulum. [6,92-94] Indeed much of this vasodilation is due to the agonism of β_2-AR directly increasing cAMP levels. [48,142,143] Where diseases such as hypertension are associated with β_2-AR dysfunctions. [142,144] This dysfunction is directly linked to cAMP-dependent signaling as this molecule is a powerful and ubiquitous second messenger. This emphasizes the importance of this second messenger and how much control it has over such a fundamental function of the human body.

1.8 Nicotine

1.8.1 Nicotine History

The history of nicotine and tobacco is invariably linked, as it is the primary addictive component in tobacco and was initially purified from tobacco leaves. [26-28] The use of tobacco was widespread throughout the Americas, long before European colonizers landed. [145] Approximately 2500 years ago Mayans used tobacco in religious settings by burning and inhaling the smoke. The Aztecs mixed tobacco with the charred remains of poisonous animals for consumption. [145] After Europeans colonized the Americas, tobacco use spread with the Spanish, Portuguese, and then English throughout the 1500s. [146] It was during this time that nicotine in its crude form was known but took until 1828 to have a purified form of nicotine. [147] During the 19th century commercial cigarettes became widespread with the invention of the cigarette rolling machine. [146] In the 20th century it was becoming clear that tobacco use did have adverse health effects, particularly on the cardiovascular system, with elevated heart rate and increased vasoconstriction. [148] Yet, it took until

1988 for the surgeon general to publish a report naming nicotine as the primary addictive component in tobacco reports. [149] The history of nicotine use is complex but consistently shows the highly addictive nature of this chemical.

1.8.2 Nicotinic Acetylcholine Receptors

Nicotine binds to nicotinic acetylcholine receptors (nAChR) selectively and competitively. [150,151] There are two nAChRs neuronal (N) and muscle-type (M) types. Neuronal nAChRs are categorized into N1 and N2, with the former on skeletal muscle at the neuromuscular junction, whereas N2 is located in the peripheral and central nervous systems. [152] N2 receptors are also located on cell bodies of postganglionic neurons in the parasympathetic and sympathetic nervous systems. [152] M-type nAChRs are located on the neuromuscular junctions. [153] nAChRs are pentameric with 5 protein subunits designated, α, β, δ, γ, ε, as they are located at postsynaptic nerve terminals throughout the body, and their endogenous ligand of binding is acetylcholine (ACh). [154] In the context of nicotine, it is thought that nicotine addiction is mediated by α4 β2, adrenal gland, and cardiovascular responses have α3 β4 present, and α7 homomeric receptors, are present in the brain and endothelial and inflammatory cells. [18] The binding of the endogenous ligand, Ach, to nAChRs, causes membrane depolarization through the increased flow of cations of sodium and Ca^{2+} ions into the cell and potassium out of the cell. [155,156] Nicotine acts directly on the nAChRs, like ACh, but is not regulated by the body like ACh. Nicotine exerts its effects on the cardiovascular system, such as increased heart rate and blood pressure through the activation of nAChRs, located on peripheral postganglionic sympathetic nerve endings and on the adrenal medulla. [156]

1.8.3 Absorption, distribution, metabolism, and excretion Profile

Nicotine is a naturally occurring alkaloid with a unique absorption, distribution, metabolism, and excretion profile. [29] The absorption of nicotine is highly dependent on the nature in which is it consumed, and this can be split into two large categories: combustible and non-combustible products. [157-160] Combustible tobacco products, such as traditional cigarettes, cigars, and hookahs, must be ignited to be consumed. Whilst non-combustible tobacco products do not have to be ignited at all and therefore cover far more products, this includes inhaled products such as ENDS (e.g., Juul) and oral products (such as nicotine patches and chewing tobacco). [160] For combustible and non-combustible tobacco products, that are inhaled, the primary absorption method is the lungs, due to the airways and alveoli having a large surface area, in addition to the higher pH 7.4 increasing the unionized nicotine content. [29] Oral absorption, through chewing tobacco, for example, is facilitated by the lower pH in mucus, increasing the unionized nicotine content. [29] The rate of absorption for oral absorption is much slower than inhaled absorption, with peak absorption for the latter at 10 minutes then rapidly declining, and oral absorption peaking in 30 minutes. [29]

Much research has been conducted on the pharmacological distribution of nicotine through tobacco smoking but there is sufficient information on nicotine distribution through nicotine replacement therapies (NRTs). Nicotine's distribution has the lowest abundance in adipose tissue but the most in the liver (where it is primarily metabolized) for tobacco smokers. [28,29] For cigarettes, the distribution of nicotine reaches its peak level in the bloodstream in approximately 5 minutes, meaning the peak nicotine level, approximately 15ng mL^{-1}, drops to a third of this within one hour. [29] However, for alternative cigarette options, such as NRTs, this effect is much longer: for example chewing tobacco reaches a peak nicotine level of 15ng mL^{-1} after 30 minutes and stays at this level

for a further 2 hours. [29] This shows that the distribution of nicotine within these NRTs is slower but stays in the vasculature for longer. [29,161] Whilst nicotine distribution through the vasculature via tobacco cigarettes is well understood, there is vast evidence in the field to show that NRTs keep nicotine in the bloodstream longer.

The liver primarily metabolizes nicotine into six known metabolites, the main metabolite being cotinine. Humans metabolize about 80% of nicotine to cotinine through the cytochrome P450 CYP2A6 liver enzyme. [29,161,162] Nicotine in the bloodstream is 69% unionized and 31% ionized due to the blood pH of 7.4, with the unionized form able to cross the blood-brain barrier. [29,163] Regarding the vasculature, nicotine is known to increase vasoconstriction, whilst its metabolite cotinine is associated with vascular inflammation by increasing oxidative stress. [18,164] It should be noted that multiple factors, such as age, sex, diet, and others can alter the rate of metabolism of nicotine. One such example is in adults over 65 which shows the decrease in nicotine metabolism partly due to a decrease in liver blood flow. [29]

The excretion of nicotine is done primarily through urine. [29] Nicotine's half-live in humans is approximately 2 hours compared to cotinine which is 16 hours, due to this large difference ways to measure nicotine in the human body are by serum nicotine/cotinine levels. [29,165 165] A quarter of nicotine ingested is excreted unchanged and the reabsorption of it is highly dependent on pH. [29,166] However, some studies show that this number is reflective of nicotine metabolites, particularly in smokers. [167] Nevertheless, the primary way the body rids itself of nicotine is through renal clearance, whether this value is reflective of total nicotine metabolites or total nicotine excreted.

1.9 Effects of Nicotine on the Vasculature

1.9.1 Nicotine and its General Effects on the Vasculature

Nicotine impacts VSM function through endothelium-dependent and independent mechanisms. Nicotine impairs endothelial functions by increasing reactive oxygen species (ROS). [150] Generally, the mechanism of action by how nicotine increases ROS is by disrupting endothelial nitric oxide synthase (eNOS), which produces the potent vasodilator nitric oxide (NO). [168] An essential component of NO synthesis is oxygen radicals and the dysfunction of eNOS by nicotine causes an increase in ROS and impairment of NO-dependent vasodilation. [150,168] This is not a phenomenon that is not just linked to conventional tobacco cigarette smoking, rather with the rise of vaping from 2010 onwards, there have been increasing links of vaping causing impairment in NO-dependent relaxation. [150,169] It is generally accepted that endothelial dysfunction is present in both forms of nicotine use. [150,169] Whilst it does appear that NRTs are not as impactful to endothelial dysfunction they do exert an effect. [170,171] Nicotine circulating the body is carried by the blood and the first area of contact is the endothelium, therefore the impact on it is highly relevant but it is one component of a vast system.

The effect that nicotine has on ion channels within the vasculature is broad and, in some cases, irreversible. Many studies show the effect of cigarettes on vascular ion function, one such example is the work of Le et al. where mice were exposed to secondhand smoke (SHS) for 12 weeks. [79] It is shown by this work that SHS impairs mesenteric arterial myocyte myogenic tone, and that ion channel function is altered. [79] There was a clear observation of a decrease in the BK channel current amplitude whereby the SHS-exposed mice had a depolarized membrane potential compared to the sham, which was also correlated with increased LTCC function. [79] The literature is clear on this subject that there is a vast array of ion channel alterations that occurs with nicotine

consumption. [156,172] One such study by Tang et al, explored how nicotine alters on potassium (K+) channel currents in the VSMCs. [173] This study used arterial smooth muscle cells from a rat tail to study the K+ channels. [173] Their discovery was the first to show that nicotine diversely affects the whole-cell K+ channel currents in VSMCs. [173] Thus nicotine can affect ion channels function by altering the expression or activity.

Indeed, with the VSM, nicotine has been shown to exert a more vasoconstrictive effect and this appears to be modulated increasingly by adrenergic stimulation. [150] Whilst some of this effect is linked to the NO-dependent vasorelaxation being impaired by vascular endothelial dysfunction resulting in increasing vasoconstriction, the literature also clearly shows links to adrenergic stimulation. [150] As described nicotine increases catecholamine being released from the post-synaptic nerves and this in turn activates α-adrenoceptors in VSM causing vasocontraction. [172,174] Olfert et al conducted a study to show the cardiovascular effects of using ENDS, exposing mice to either ENDS vapor, TS, or filtered air for 8 months. [175] One experiment they conducted was to extract the mice's aortas and then measure their arterial reactivity using wire myography. [175] Beyond their general conclusions of finding that chronic ENDS exposure accelerates arterial stiffness and impairs aortic endothelial function, they specifically found that there were alterations to the adrenergic response. [175] Showing that both TC and ENDS showed an increased responsiveness to α-AR (Alpha-adrenergic receptor) agonist, phenylephrine (PE). [175] From this study they concluded that α-AR vasoconstrictor response was greater in TC and ENDS exposed to mice. [150,175]

It has also been shown that epinephrine concentration is higher in the arterial plasma of smokers, users of nicotine nasal sprays, and intravenous users. [176] These increases in epinephrine caused by nicotine stimulation of the sympathetic nervous systems increase the likelihood of

alterations in the vasculature tone of arteries. However, the downstream mechanism by how β_2-AR is altered is not known but one potential mechanism is through alterations in cAMP levels.

Due to nicotine's addictive potential, much research has been dedicated to the study of nicotine's effects on the brain and its association with addiction. There have been studies examining the role of cAMP in dopamine release and its association with nicotine addiction. [177] Hamada et al studied the regulatory role of nAChRs in dopaminergic signaling within the striatum and how this is affected by nicotine. They discovered that nicotine stimulates dopamine release through specific nAChR subtypes, which then affect the phosphorylation of Dopamine- and cAMP-regulated phosphoprotein (DARPP-32), a protein involved in signaling pathways. Another study, by Sun et al exposed rats to varying doses of nicotine that would lead to different states of activation and desensitization in nAChRs. [178] They then examined the activities of cAMP-dependent PKA and PKC in the rat brains corresponding to these nAChR states. Where they found that desensitized nAChRs in the rat brain have the ability to suppress PKA and PKC activities, which may potentially contribute to the development of nicotine dependence. [178] However, the role of nicotine and how cAMP signaling is affected, in the vasculature, is less explored. PDE4 expression has been shown to be increased in the airway smooth muscle of murine models of cigarette smoke exposure. [127] Higher levels of PDE4 expression was associated with increased airway reactivity and a blunted vasodilatory response to β-AR agonist in the animals exposed to cigarette smoke compared to sham control. [127] This study concludes that lower cAMP levels are due to increased PDE expression with SHS exposure, but the work presented in Chapter 2 does not share this same result. [127] What is missing in the literature is the effect of nicotine, without the additives that are present in tobacco or ENDS, on the cAMP signaling in the vasculature. This is of clear importance, as cAMP controls many pathways but importantly controls excitability in the

vasculature. There is a great need to understand the impact of nicotine on cAMP signaling in arterial myocytes and how this may impact receptor-mediated vasodilation.

1.9.2 Nicotine and Vascular Pathology

Nicotine consumption has been implicated in many vascular pathologies, including atherosclerosis, coronary spasms, hypertension, and aortic aneurysms, to name a few. [18,179] CVD is the leading cause of premature deaths globally, responsible for approximately 18 million deaths a year. [18,179,180] With nicotine users, primarily tobacco smokers, and growing evidence of vaping use increasing the risk factor for cardiovascular disease comorbidities. [164,179,181] Presented here are a few common vascular diseases, atherosclerosis, and hypertension that are a direct result of nicotine consumption.

Atherosclerosis and nicotine consumption are closely linked, with a large body of work to show how the latter increases the chances of getting the former. Atherosclerosis is a chronic inflammatory disease characterized by the buildup of fatty deposits, cholesterol, and calcium in the walls of arteries. [54,182] It is a major underlying cause of cardiovascular diseases such as coronary artery disease, heart attacks, and vascular complications. It is thought that nicotine accelerates atherosclerosis by promoting macrophage pyroptosis and increasing the ROS that damages NO-dependent relaxation. [183] Although the signals of nicotine and its links to atherosclerosis may not be linear, it is noteworthy that the contributions of nicotine to this disease are clear.

Nicotine increases the risk of developing hypertension [18,28,179] defined as consistently-high blood pressure in arteries that is higher than 140/90 mmHg (diastolic/systolic). [184,185] Nicotine exacerbates vasoconstriction, which will progress chronic hypertension to more malignant hypertension that will increase morbidity sooner. [18] One study showed that male dual users of tobacco cigarettes and electronic nicotine delivery systems (ENDS) had an increased risk of

hypertension compared to users that preferred one device over the other. [184] Hypertension is then considered to be the biggest risk factor in other diseases such as coronary artery disease, atrial fibrillation, and stroke. [184,186] It is important to note that the effects of nicotine on blood pressure can be acute or long-term, and this also depends on other factors such as race, age, diet, etc. It should be of concern though that nicotine and hypertension are linked, and this is the first step to other cardiovascular diseases.

1.10 Summary of Chapter One and Knowledge Gaps

1.10.1 Chapter Summary

Through the course of this first chapter, we reviewed the vasculature system, arteries, cAMP regulation, nicotine, and nicotine's effect on the vasculature. This dissertation work addresses the knowledge gap of how nicotine alters cAMP signaling in the vasculature. It is known that nicotine is linked to cardiovascular disease, whereby a clear link exists between nicotine and the impairment of NO-dependent relaxation through the endothelium. In addition, most studies focus on the connection between nicotine increasing vasoconstriction and how this links to the increased likelihood of hypertension. However, few studies have explored the effect of beta-adrenergic relaxation on the vasculature and tested if there are any alterations to this mechanism. Additionally, there have been no studies on how cAMP levels are altered by nicotine regarding vascular relaxation.

Chapter 2 explores nicotine's specific impact on receptor-mediated cAMP synthesis and vascular reactivity. We hypothesized that nicotine impairs receptor-mediated cAMP signaling in arterial myocytes. Here it is shown that in nicotine-infused mice with activation of the β-AR pathway resulted in reduced cAMP synthesis and impaired vascular dilation. Show that the

alterations in vascular cAMP signaling have the potential to modulate the deleterious vascular effects of nicotine, such as increased incidence of cardiovascular disease and higher risk of cardiovascular-related mortality. [187-189]

Chapter 2 Nicotine Impairs cAMP Signaling in Aortic Smooth Muscle Cells

The content of this chapter is in preparation for publication:

N Singhrao, VA Flores, GR Reddy, AE Burns, Kent Pinkerton, Chao-Yin Chen, MF Navedo, M Nieves-Cintrón., "Nicotine impairs cAMP signaling in aortic smooth muscle cells", Microcirculation

Abstract

Cigarette smoke has detrimental cardiovascular effects. Mice exposed to cigarette smoke showed an attenuated synthesis of cAMP in response to the β-AR agonist ISO. Multiple components of cigarette smoke can contribute to its cardiovascular toxicity, including acute and chronic nicotine effects. The study's goal is to determine the specific impact of nicotine on receptor-mediated cAMP synthesis and vascular reactivity. We hypothesize that nicotine impairs receptor-mediated cAMP signaling in arterial myocytes. Consistent with our hypothesis, the smoke effects on ISO-induced cAMP synthesis were recapitulated in arterial myocytes from mice infused with nicotine. This nicotine-induced reduction in receptor-mediated cAMP synthesis was correlated with reduced ISO-induced vasodilation. Our results suggest that nicotine impairs receptor-mediated cAMP signaling leading to altered vascular reactivity.

2.1 Introduction

TS exposure is associated with poor arterial integrity, coronary artery disease, stroke, altered arterial contractility, and hypertension. [12,13,79] The implementation of smoke-free policies and public awareness have reduced smoking rates in the last decades. [17] However, alternative-nicotine delivery systems that minimize or eliminate tobacco combustion products (e.g., nicotine patches, gum, ENDS, etc.) have gained popularity as "safer" alternatives to conventional cigarettes. This notion of safety has been challenged by studies showing that nicotine can contribute to arterial dysfunction, including arterial stiffness and enhanced arterial constriction. [23,24,31,187-189] Although, all the vascular alterations have well-established endothelial components, the roles and potential mechanisms underlying nicotine-mediated modifications in arterial smooth muscle function are

less clear. The increase in the prevalence of nicotine usage globally warrants a better understanding of the specific impact and mechanisms of nicotine effects on vasculature responses. [9,27,190,191]

Nicotine is a strong sympathomimetic with significant effects on the cardiovascular system[18] and has been shown to alter cellular cAMP production. [10,11,129] Arterial myocytes receive many inputs from sympathetic nerves that regulate vascular reactivity by activating G_SPCRs. [192] The ubiquitous second messenger cAMP is synthesized by ACs in response to the activation of various G_SPCRs and plays a critical role in vascular function. [6,7,193,194] Importantly, arterial myocyte cAMP signaling has been traditionally linked with relaxation. [1-8] However, how nicotine impacts cAMP signaling in the vasculature, particularly in arterial myocytes, and the functional implications of these changes are unclear.

This study tested the hypothesis that nicotine impairs beta-adrenergic-mediated cAMP signaling in arterial myocytes. The effects of nicotine on cAMP signaling and vascular function were systematically tested in acutely isolated aortic rings from mice expressing the cAMP sensor $^{T}Epac^{VV}$ ($CAMPER_{SM}$) in VSM infused with nicotine. Our data revealed a blunting effect of nicotine on beta-adrenergic-mediated cAMP levels. This change in cAMP signaling correlated with impaired beta-adrenergic-mediated vasodilation. PDE inhibition rescued beta-adrenergic-mediated vasodilation. Moreover, aortic tissue from nicotine-exposed mice showed decreased protein expression of adenylyl cyclase 6 (AC6). These results highlight a mechanism whereby nicotine impacts vascular reactivity by affecting cAMP synthesis and degradation. These changes in cAMP signaling may contribute to the sequelae of cardiovascular risks observed in users of nicotine-containing products.

2.2 Methods

2.2.1 Animals

All animal-related procedures were performed in strict compliance with protocols approved by the Institutional Animal Care and Use Committee at the University of California, Davis. Eight-week-old C57BL/6J mice were acquired from The Jackson Laboratory. We crossed the $CAMPER_{SM}$ reporter mouse (which ubiquitously expresses the latest-generation cAMP sensor, $^{T}Epac^{VV}$, preceded by *loxP-Stop-loxP*)[195] with the SM22-Cre mouse for smooth muscle-specific expression.

2.2.2 Secondhand Smoke

Adult C57BL/6J male mice were randomized into filter air (FA; control group) or SHS exposed group. Mice were allowed to acclimatize to the exposure facility before starting the exposures. Humidified 3R4F research cigarettes (Tobacco Health Research Institute; Lexington, KY) were used to produce smoke. An automatically metered puffer generated smoke under Federal Trade Commission conditions (35 mL puff, 2 s duration, and 1 puff/minute). The target smoke concentration was set at 3 mg/m^3.

2.2.3 Nicotine Infusion and Serum Nicotine Levels

Mice were surgically implanted with osmotic minipumps (model 1007D, Alzet Durect Corporation, Cupertino, CA). The nicotine concentration was 6 mg/kg/day. Mice were infused with nicotine for 7 days. Blood was collected via cardiac puncture. Blood was spined down in serum separator tubes, and the serum samples were stored in cryogenic vials at -20°C until nicotine/cotinine measurements were conducted. Samples were analyzed for nicotine using a Varian GC-MS/MS system, with a 450-gas chromatograph interfacing a 320 triple quadrupole

mass spectrometer with electron ionization and positive/negative chemical ionization source at the Clinical Pharmacology laboratory at the University of California San Francisco.

2.2.4 Wire Myography

The entire length of the thoracic aorta from the heart to the diaphragm was carefully removed and placed in a Petri dish with 4°C Krebs-Ringer solution (KREBs) buffer consisting of (in mM: 119 NaCl, 4.7 KCl, 0.24 $NaHCO_3$, 1.18 KH_2PO_4, 1.19 $MgSO_4$, 5.5 glucose, and 1.6 $CaCl_2$). The connective tissue was carefully removed, and the aorta was cut into 3-4 mm segments. Aortic segments were mounted in a wire myograph (DMT Multi Wire Myograph System - 620M) with a vertical wire system, using a 40 µm diameter wire, then connected to an isometric strain gauge transducer. Transducer signals were fed into an 8-channel PowerLab A/D converter and recorded on a PC using LabChart software.

The organ bath containing 5 mL of KREBs solution was maintained at 37°C and bubbled with 5% CO_2: 95% O_2. An extra 500 mL of KREBs solution was bubbled and kept at 37°C for washing the organ bath. [196,197] The tension of the aortic segments was then set to a normalizing tension of 10 mN (~1g) over 10 min. period (approximately 1mN/min). [197,198] The stabilization period was followed by a 5-minute contraction using 60 mM KCl KREBs, then washing twice for 5 minutes with 37°C KREBs solution, allowing tension to return to 10 mN. [197] This step was repeated but with 10 min contraction of 60 mM KCl, KREBs to fully contract the artery, followed by washing with 37°C KREBs twice. [197] Aortic rings were precontracted with PE (1 µM; Sigma 1001166140) for 10 mins. Arteries with a PE-evoked wall tension of < 1mN were excluded from the analysis. After contraction by 1µM PE, a dose-response curve was conducted using (+)-ISO (+)-bitartrate salt (ISO; Sigma 14638-70-1), ranging from 1nM to 10µM by cumulatively adding increasing ISO concentrations into the wire myographs organ bath. The aorta segment was allowed

to equilibrate for 5 mins in the organ chamber after adding each ISO concentration. The phosphodiesterase 3 (PDE3) or 4 (PDE4) inhibitors cilo (100 nM, Caymen Chemical 14455) and roli (100 nM, Abcam ab120029), respectively, were added simultaneously with PE. Vessel tension was allowed to stabilize for 10 mins. after addition. An ISO dose-response curve was conducted as before. At the end of each experiment, 10µM forskolin (FSK) (Millipore Sigma 66575-29-9), dissolved in Ca^{2+}-free PSS (in mM: 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 7 glucose, 24 $NaHCO_3$, and 5 EGTA) was added to each organ chamber to get maximal relaxation of the aorta segments.

2.2.5 FRET

CAMPER$_{SM}$ mice were implanted with osmotic pumps containing either saline or nicotine (6mg/kg/day). After 7 days with the pumps, CAMPER$_{SM}$ mice were sacrificed, and the aorta was carefully extracted and cleaned of connective tissue and fat in Magnesium physiological salt solution (MgPSS) at 4°C. The segments were then canulated in a stage containing 3 mL Dulbecco's Phosphate Buffered Saline (DPBS, Cat-No 14190144) at 4°C. Live-FRET images were acquired using a Leica DMI3000B inverted fluorescence microscope (Leica Biosystems, Buffalo Grove, IL) equipped with a Hamamatsu Orca-Flash 4.0 digital camera (Bridgewater, NJ), controlled by Metafluor software (Molecular Devices, Sunnyvale, CA). Images were collected using a 10x air objective lens (Olympus UPlanFL N 10x). The donor fluorophore was exited at 430-455nm, and emission fluorescence was detected with two filters (475DF40 for cyan and 535DF25 for yellow). Images were acquired every 20 s, and the background was subtracted. Arterial rings were allowed to equilibrate for 5 minutes, and a FRET fluorescence baseline was established by calculating the donor/acceptor FRET ratio and then normalizing it to the ratio without the stimulator. After establishing the baseline, the aortic rings were challenged with

100nM ISO. At the end of the experiments, 100μM 3-isobutyl-1-methylxanthine (IBMX; CAS number: 28822-58-4, ToCris, China) and 10μM FSK (CAS-No: 6675-29-9, Sigma-Aldrich, USA) were added simultaneously to obtain maximal cAMP stimulation. Averages of normalized curves and maximal response to stimulation were graphed based on FRET ratio changes. Experiments were performed at RT (22-25°C).

2.2.6 **Western Blot**

Thoracic aortas were collected from adult mice and kept at 4°C in a nominal Ca^{2+}-free solution (containing mM: 5 KCl, 140 NaCl, 2 $MgCl_2$, 10 Glucose, 10 HEPES). Aortas were cleaned from connective tissue and homogenized in RIPA lysis buffer solution consisting of (mM): 50 Tris-HCl, 0.04% sodium dodecyl sulfate (SDS), 150 NaCl, 1 EDTA, 1% Triton X-100 with protease inhibitors (complete mini EDTA-free, Roche, 11836170001) and phosphatase inhibitors (phosphatase inhibitor cocktail set V, Calbiochem, Germany, 524629) supplemented with 2 μM microcystin LR (EMD Millipore, Germany, 475815). Four to five independent experiments pooling tissue from two mice per condition were conducted. Arterial lysates were centrifugated at 12,000 rpm for 20 min at 4° C. The supernatant was collected, and the total protein was quantified with a BCA protein assay kit (ThermoFisher, 23235) and assay plate reader (CLARIOstar Plus BMG LABTECH). The protein samples were heated to 65°C for 20 minutes in the SDS sample buffer (Bio-Rad; 1610737).

A precast stain-free gradient SDS-polyacrylamide (4-20%) gel (Bio-Rad; 4568094) was used to separate proteins (1hr, 200V, room temperature). Proteins were transferred (20 h at 50 V and 4°C) to polyvinylidene difluoride membranes (Merk Millipore; IPFL00005). Transfer buffer consisted of (mM): 25 Tris base,192 mM Glycine, 10% methanol, and 0.05% SDS. Membranes were incubated in a blocking buffer comprised of 5% albumin bovine fraction V (Research

Products International; A30075) in tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h at room temperature (25°C) before overnight incubation in primary antibodies at 4°C. Rabbit anti-AC5 (1:200, Thermo Fisher, PA5-38843) and rabbit-antic AC6 (1:200, Thermo Fisher; PA5-107053) were used to detect the adenylyl cyclase isoforms AC5 and AC6, respectively. β_1 and β_2-ARs were detected with rabbit anti-β1 antibody (1:200, Thermo Fischer, PA1-049) and rabbit anti-β2 (1:200, Biorbyt, orb22348) respectively. PDE isoforms were detected with specific rabbit anti-PDE3 (1:50, Thermo Fischer Scientific, PA1-31151) and PDE4 (1:200, Thermo Fischer, PA5-29481). Following AB incubation, membranes were washed 3 times/ 5 mins with a blocking buffer. Membranes were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody (1:2000; Invitrogen, 31460) with a blocking buffer. We used a stripping solution of 6M GnHCl, 0.2% Nonidet P-40 (NP-40), 0.1M β-mercaptoethanol, 20mM Tris-HCl, pH7.5 to dissociate antibodies from the western blots without removing significant amounts of the transferred proteins. [199] When noted, the detection of PDE4 was carried out after the stripping solution and re-probing it with the anti-PDE4 antibody in a 5% BSA in TBST solution. [200] The same was done for AC5 and AC6 protein antibodies. The same was done for AC5 and anti-AC6 protein antibodies. Chemiluminescent Substrate SuperSignal West Pico (catalog # 34579), Femto (catalog # 34096), or Atto (catalog # A38554) (Thermo Fisher Scientific), and a Chemidoc MP system (Bio-Rad) was used to detect target proteins. Immunoreactive bands were analyzed by using Image Lab version 6.1 (Bio-Rad). The densitometric signal of the target proteins was normalized to the total protein signal obtained from the stain-free bolts and then expressed as a relative density ratio of target protein/ total protein. [201]

2.3 Results

2.3.1 SHS exposure reduces receptor-mediated cAMP levels in VSM

Aortic VSM from mice exposed to environmentally relevant SHS concentration showed a decrease in cAMP synthesis in response to the β-adrenergic agonist ISO (Fig. 2.1A-B), but this was reversed by using the PDE inhibitor IBMX (Fig. 2.1C-D). These results are consistent with research showing lower cAMP levels in the airways epithelial and smooth muscle cells from animal models of cigarette smoke exposure. [9,127] Multiple components of TS can contribute to its vascular effects, including nicotine. [14-16] To determine the specific impact of nicotine on arterial myocyte cAMP signaling, in the next series of experiments, we tested the effects of in-vivo infusion of nicotine on cAMP signaling in VSM.

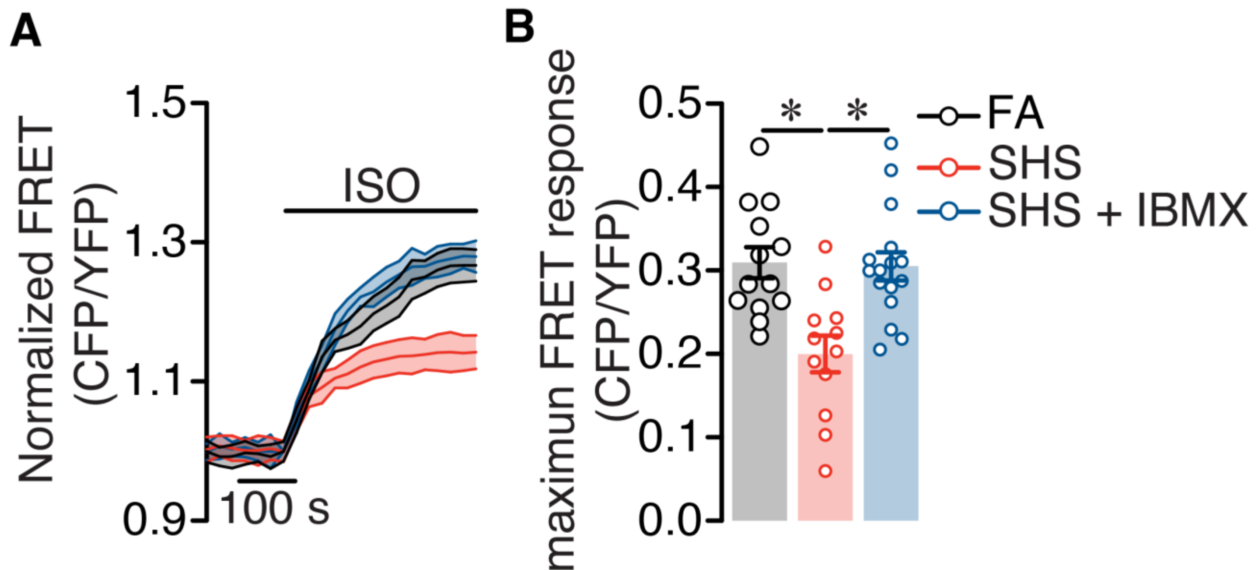

Figure 2.1 – Decrease receptor-mediated cAMP levels in VSM form SHS exposed mice.

Time-course of iso-induced cAMP changes (A) and bar plot of maximum FRTE response (B) in aortic smooth muscle cells from filter air (FA) and second-hand smoke-exposed mice (SHS) in the absence and presence of the PDE inhibitor IBMX.

2.3.2 Nicotine exposure impairs receptor-mediated cAMP signaling in-vivo

We next determined how in-vivo nicotine infusion impacts cAMP signaling in freshly isolated aortas. We used genetically engineered mice expressing a cAMP biosensor, specifically in VSM ($CAMPER_{SM}$) (Fig. 2.2A-B). The $^{T}Epac^{VV}$-based sensor (Fig. 2.2A) in the $CAMPER_{SM}$ mice has a large dynamic range and robust tissue expression, thus allowing us to assess cAMP in freshly isolated aortic rings. The $CAMPER_{SM}$ mice were infused with nicotine (6 mg/Kg/day) for 7 days via osmotic minipump. Serum samples were analyzed for nicotine; mice infused with nicotine had average plasma levels of 8.0 ± 2.0 ng/mL; nicotine was not detected in blood samples from sham mice (Fig. 2.2C). Life-FRET imaging was performed in aortic rings from acutely isolated aortas from sham and nicotine-infused $CAMPER_{SM}$ mice. cAMP was stimulated by adding 100 nM ISO to the bath. Stimulating with iso (100 nM) produced a decrease in the FRET signal, indicating an increase in cAMP levels (Fig 2.2D-F). ISO-induced cAMP responses were significantly lower in aortic rings from nicotine-infused mice (4.819±1.083%) compared to sham mice (15.14±0.9925%) (Fig. 2.2F), suggesting that nicotine impairs cAMP signaling in VSM.

Cigarette smoke has been shown to increase the expression of PDE4 and PDE3 in airway smooth muscle, which has been implicated in airway overactivity in individuals exposed to cigarette smoke Field. [129] We use a pharmacological approach to probe the involvement of these PDEs in the nicotine-induced reduction of cAMP in VSM. Iso-induced cAMP responses were measured in freshly isolated aortas from sham and nicotine-infused mice in the presence and absence of the specific PDE4 and PDE3 inhibitors roli (10nM) and cilo (100nM), respectively (Fig. 2.2E *ii* and *iii*). Simultaneous application of iso + roli or iso + cilo produced an increase in cAMP in aortic rings from sham mice that were 34.62±3.925% and 35.62±5.563%, higher, respectively, than iso alone (Fig. 2.2D-F). Both roli (Fig. 2.2E *ii* & F) and cilo (Figure 2.2E *iii* & F) significantly

increase iso-induced cAMP in aortic rings from nicotine-infused mice. Together these results suggest that nicotine impairs cAMP signaling through a mechanism involving at least in part PDE3 and PDE4.

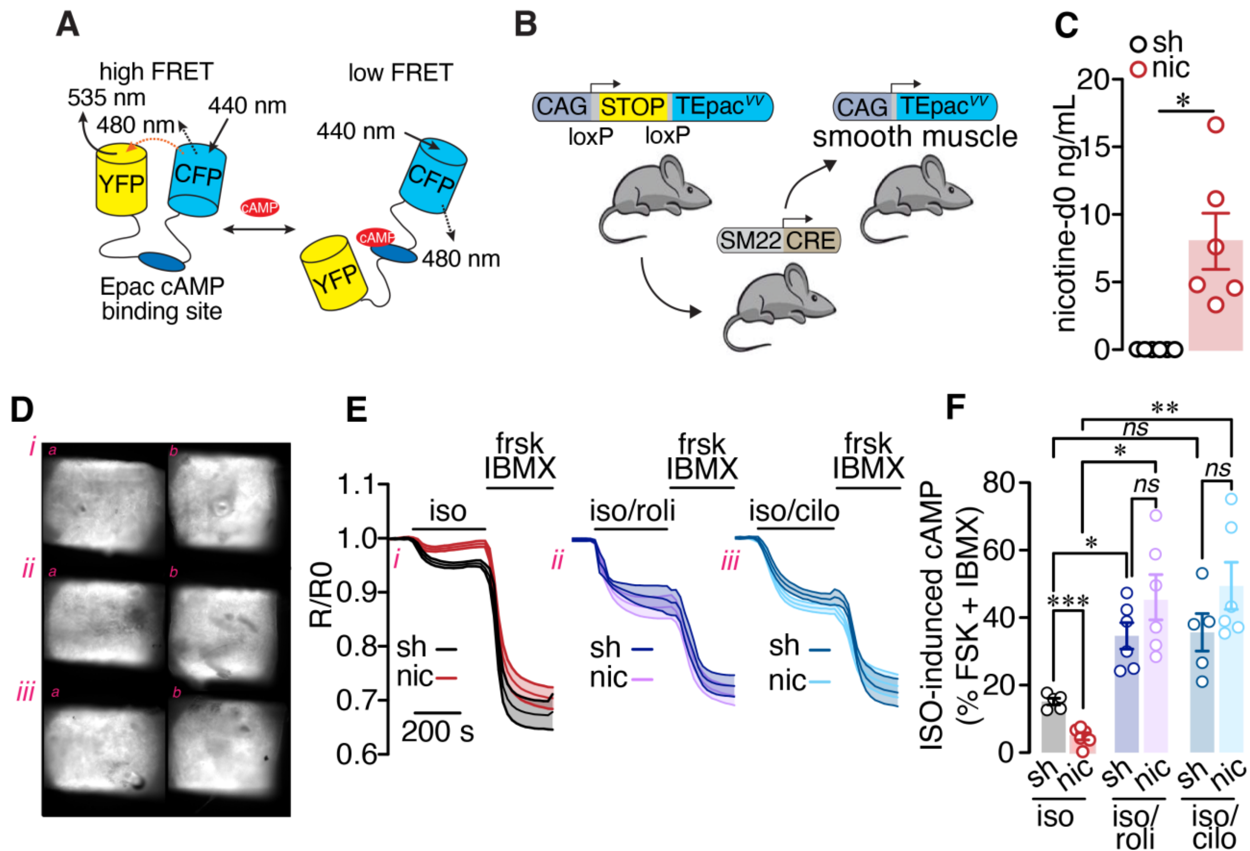

Figure 2.2 – Chronic nicotine infusion impairs cAMP signaling in freshly isolated aortic rings.

A) Schematic representation of the ^{T}Epac^{VV}-based sensor and B) breeding strategy to generate smooth muscle-specific CAMPER$_{SM}$ mice. C) Bar-plot of nicotine levels in sham and nicotine-infused mice. D) Representative-pseudo color images of acutely isolated aortic rings. E) Representative traces of iso-induced changes in cAMP detected in acutely isolated aortic rings from sham (sh) and nicotine-infused (nic) mice in the absence (*i*) and presence of PDE inhibitors rolipram (roli; *ii*) and cilostamide (cilo, *iii*). F) Bar-plot of iso-induced cAMP normalized to the maximum response obtained after simultaneous application of the broad adenylyl cyclase activator and PDE inhibitor FSK and IBMX, respectively.

2.3.3 Reduced iso-induced vasodilation in nicotine-exposed mice

We use wire myography to test the functional consequences of reduced cAMP signaling after nicotine exposure. We started by measuring the vasodilatory responses elicited by iso on aortic rings from sham and nicotine-infused mice that had been pre-constricted with PE (1μM). After stable tension to PE was established, increasing concentrations of iso were cumulatively added to the bath to generate a concentration-response curve. The iso-response curve for aortic rings from nicotine-infused mice shows a rightward shift compared to the sham control (Fig. 2.3A&F). The average half-maximal effective concentration (EC_{50}) for iso-induced dilation was 0.53 ± 0.18 μM for aortic rings from sham mice and 1.4 ± 0.19 μM for nicotine-infused mice (Fig. 2.3E, Table 1). These results suggest an impairment of β-adrenergic-mediated dilation in animals exposed to nicotine.

2.3.2 PDE inhibition restores iso-induced dilation

Since PDE4 and PDE3 inhibition rescues iso-induced cAMP, we tested whether inhibiting these PDEs will restore iso-induced vasodilation. We started by characterizing the effect of PDE4 and PDE3 inhibitors roli and cilo, respectively, on PE-induced tension. PE elicited an average tension of similar magnitude in aortic rings from sham and nicotine-infused mice (Fig. 2.3B). Roli (10 nM) or cilo (100 nM) were added simultaneously with PE (1 μM) to induce tension and determine the effect of PDE inhibitors on PE-induced tension. In sham aortic rings, the tension caused by PE+roli and PE+cilo (1.65 ± 0.219 and 2.56 ± 0.219 mN) was significantly lower than the tension caused by PE alone (4.15 ± 0.517 mN; $P < 0.05$). In aortic rings from nicotine-infused mice, PE, and PE+roli-induced tension was 4.01 ± 0.379 and 2.76 ± 0.293 mN, respectively ($P >$ 0.05). On the other hand, PE+cilo induced a tension of 1.55 ± 0.120 mN, which was significantly

lower than PE alone ($P < 0.05$). These results indicate that inhibiting PDE4 and PDE3 impact basal PE-induced tension. Thus, the effects of PDE4 and PDE3 in modulating iso-induced dilation after chronic nicotine infusion were determined in aortic rings from sham and nicotine-infused mice after inducing tension by the simultaneous addition of PE+roli or PE+cilo.

Once a stable tension with PE+roli or PE+cilo was achieved, increasing iso concentrations were added to the bath as before to generate dose-response curves. As illustrated in Fig. 2.3 C&D, for both sham and nicotine, there was a leftward shift in the iso-response in arteries pre-contracted with PE in the presence of roli (Fig. 2.3C) or cilo (Fig 2.3D) compared to PE alone (dotted lines). The iso-response curve for aortic rings from nicotine-infused mice precontracted with PE/roli was left-shifted compared to PE/roli sham (Fig 2.3C). The difference in EC_{50} for iso-induced dilation was not statistically significant, however for PE+roli pre-constricted aortic rings from nicotine and sham animals (0.71 ± 0.18 μM, 0.22 ± 0.31; respectively, $P = 0.17$), respectively (Fig. 2.3C&F; Table 1). Cilo, however, brings the iso-response curves to the same level as sham arteries precontracted with PE+cilo (Fig. 2.3D&F, Table 1). These results suggest that PDE4 and PDE3 may contribute to blunted iso-induced dilation in nicotine-infused mice. The data point to a

stronger contribution of PDE3 to impaired iso-induced dilation in nicotine-infused mice.

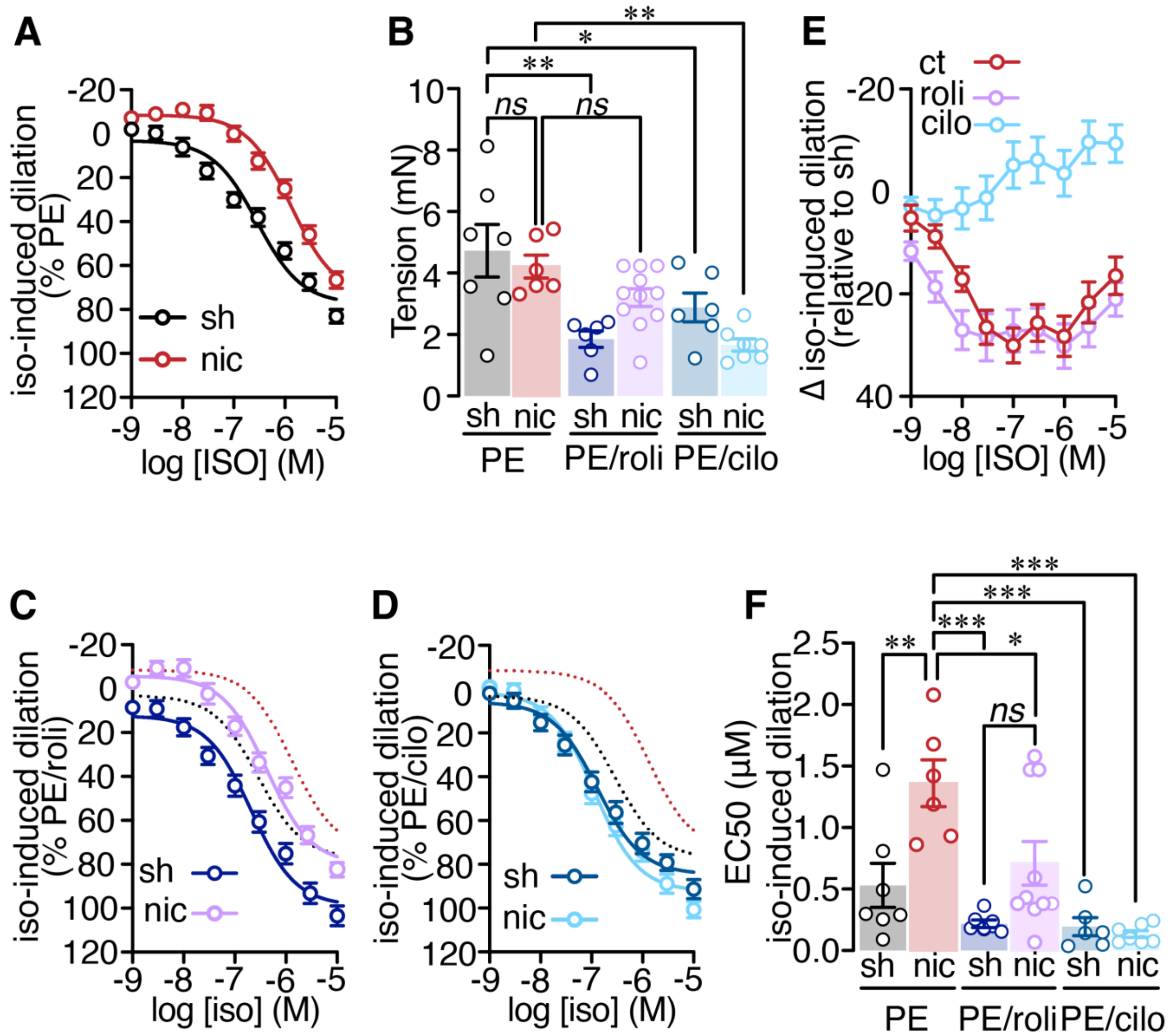

Figure 2.3 – Impaired iso-induced vasodilation in nicotine-exposed mice.

A) Dose-response curve for iso-induced dilation of aortic rings precontracted with phenylephrine (PE, 1μM). B) Bar-plot of PE-induced tension in aortic rings from sham and nicotine-infused mice in the presence and absence of roli and cilo. Dose-response curve for iso-induced dilation of aortic rings precontracted with PE 1μ in the presence of roli (10nM) (C) or cilo (D). The dotted lines represent the iso-induced dilation of aortic rings precontracted with phenylephrine alone (shown in panel A). E) Graphical representation of the change in tension as a function of iso-concentration between sham and nicotine in the absence (ct) and presence of roli and cilo. F) Bar-plot of EC_{50} for iso-induced dilation.

2.3.3 Nicotine Effect on Expression of cAMP pathway components

Next, we examined the expression of relevant up and downstream targets in the regulation of cAMP signaling upon β-AR stimulation, starting with the β-AR. The expression of β_1 and β_2-AR did not change significantly in aortic lysates from sham and nicotine Fig. 2.4A. Next; we examined the expression of adenylyl cyclase isoforms AC5 and AC6 and PDE3 and PDE4 to test whether changes in the expression of enzymes responsible for cAMP synthesis, degradation, or both were involved in lower cAMP levels in the aorta of nicotine-treated mice. Immunoblots analysis showed similar expression levels for AC5 in sham and nicotine-infused mice. In the case of AC6, however, western blot analysis shows a decrease in expression levels of nicotine relative to the sham control. Meanwhile, the expression levels of PDE4 and PDE3 were not significantly different between aortic tissues from nicotine or sham mice. These results suggest that decreased cAMP levels after chronic nicotine exposure are at least partly due to decreased expression of AC6.

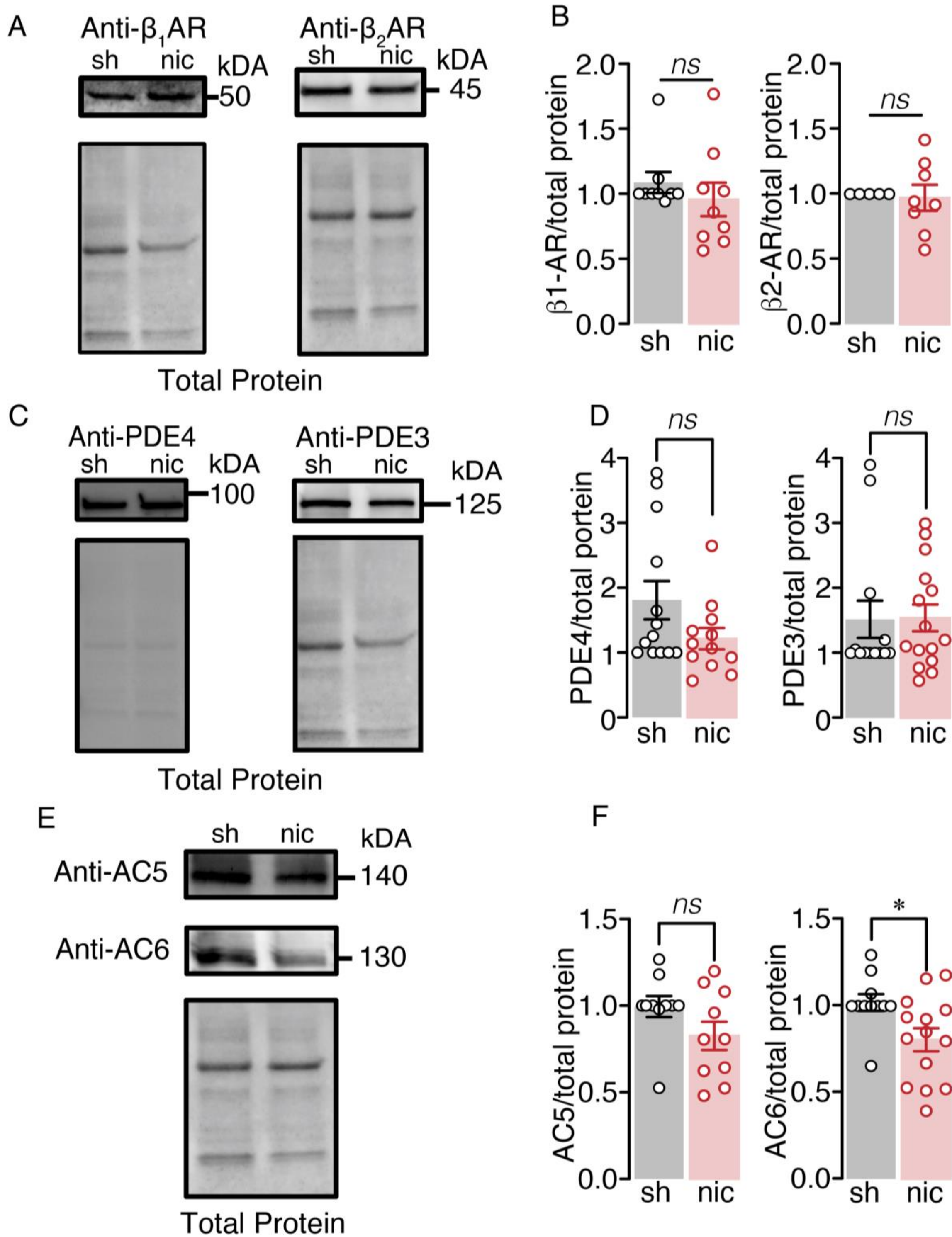

Figure 2.4 – Chronic nicotine infusion impairs AC6 expression.

Immunoblot analysis of expression levels of β1 and β2-AR (A), PDE 3 and PDE4 (B), and AC5 and AC6 (C).

2.4 Discussion

TS exposure is associated with altered vessel excitability, poor arterial integrity, coronary artery disease, hypertension, and stroke. [12,13] Multiple components of TS can contribute to its vascular toxicity, including particulate matter inhalation, toxicant exposure, and acute and chronic effects of nicotine. [14-16] Research suggests that nicotine can contribute to arterial dysfunction, including arterial stiffness, vascular calcifications, and enhanced arterial constriction. [23,24] Although the vascular alterations have well-established endothelial components, the roles and potential mechanisms underlying nicotine-mediated modifications in arterial smooth muscle function are less clear. In this study, we evaluated the effects of chronic nicotine exposure on βAR-mediated cAMP signaling in smooth muscle from acutely isolated aortic rings. We showed that arterial myocytes from mice exposed to cigarette smoke had decreased cAMP in responses to the β-adrenergic agonist ISO. Moreover, acutely isolated aortic rings from mice expressing the cAMP sensor $^{T}Epac^{VV}$ ($CAMPER_{SM}$) in smooth muscle infused with nicotine showed reduced iso-induced cAMP levels. These results are consistent with studies showing impaired cAMP in airway cells in an animal model of cigarette smoke exposure. [129]

AC isoforms synthesize the ubiquitous second messenger cAMP in response to G_SPCRs activation. cAMP regulates many processes in arterial myocytes, including growth, differentiation, and excitability. [109] For example, cAMP signaling has been shown to maintain low rates of proliferation while promoting the contractile phenotype of differentiated arterial myocytes. [109,202] In differentiated arterial myocytes, cAMP signaling strongly correlates with relaxation. [112] Accordingly, we found that reduced cAMP levels resulted in impaired iso-induced vasodilation of precontracted aortic rings. Our data shows that PE-induced tension was not different between sham and nicotine-infused mice. This result contrasts with research suggesting nicotine may promote

contraction via increased responsiveness to alpha-adrenergic agonists (e.g., NE, PE). This discrepancy could be due to differences in exposure methods (inhaled vs. subcutaneous nicotine administration in our study. It could be due to sex differences since the reference study used female mice while ours used male mice. Future studies should address potential sexually dimorphic reactivity to α-AR agonists.

cAMP signaling is regulated by PDEs, which degrades cAMP. [123,203] Nicotine has been shown to increase PDE activity in airway cells and cavernosal VSM. [129] Our experiments showed that acute inhibition of PDE4 and PDE3 isoforms increases cAMP in sham aortic rings and rescues receptor-mediated cAMP signaling aortic rings from mice exposed to nicotine. [194,204] At the functional level, acute inhibition of PDE4 improved iso-mediated vasodilation. Although a rightward shift in the ISO-dose response curve was still observable, the EC_{50} for iso in the presence of roli was not statistically different between sham and nicotine conditions. Meanwhile, acute PDE3 inhibition rescue the iso-induced vasodilation to sham levels. These results suggest that PDE4 and PDE3 regulate cAMP basally and contribute to nicotine-induced changes in receptor-mediated cAMP signaling in arterial myocytes. Moreover, the data indicate a stronger contribution of PDE3 to nicotine-induced changes in cAMP signaling in VSM.

cAMP signaling was restored when PDEs were inhibited, indicating that nicotine may act on the cAMP signaling pathway beyond direct action on the GsPCR. Accordingly, protein expression levels of β_1 and β_2-AR were no different between sham and nicotine-exposed mice. Although the nicotine-induced decrease in ISO-induced cAMP and dilation was ameliorated by PDE inhibitors roli and cilo, we didn't find a significant difference in PDE4 and PDE3 protein levels between sham and nicotine-infused mice. We, however, found a decreased expression of ACs isoform AC6. Thus, lower cAMP levels may be due to reduced expression of AC6. But

enough AC enzyme activity remains that cAMP can be restored by inhibiting PDE. These, however, do not entirely explain the more substantial effect of PDE3-inhibitor cilo in rescuing ISO-induced vasodilation. One possible explanation is that nicotine leads to a redistribution of PDE3, bringing its hydrolyzing activity closer to beta-AR and AC enzymes. Future studies should determine nicotine effects on the subcellular distribution of PDE isoforms. [205]

cAMP orchestrates various functions in VSM; it regulates contractility and arterial wall stability. In mice lacking expression of stimulatory protein alpha (Gs_{α}), lower cAMP levels were associated with increased aneurysm formation. [206] Moreover, PDE3 and PDE4 inhibitors have been shown to ameliorate aortic aneurism in animal models. [131,207] Thus, deleterious vascular effects of nicotine, including increased incidence of cardiovascular disease and higher risk of cardiovascular-related mortality, may be modulated through alterations in vascular cAMP signaling. [7–9]

Chapter 3 Discussions and Conclusions

3.1 Summary of Major Findings and Significance

This dissertation aimed to understand the effects of nicotine exposure on cAMP and how this alters VSM physiological function. Currently, it is understood that nicotine can contribute to arterial dysfunction, including arterial stiffness, vascular calcifications, and enhanced arterial constriction, and it impairs endothelium-dependent vasodilation. [23,24,172] However, the roles and potential mechanisms underlying nicotine-mediated modifications in the function of arterial myocytes. [169] cAMP plays a critical role in the regulation of VSM excitability and reactivity. Therefore, there is high significance in understanding how nicotine impacts cAMP signaling in VSMCs and how this may impact receptor-mediated vasodilation.

In Chapter 2, explored the effects of nicotine on cAMP in the VSM and tested the hypothesis that nicotine impairs receptor-mediated cAMP signaling in arterial myocytes. We made several novel observations. Firstly, SHS decreases cAMP levels in mice cells, and these altered cAMP levels can be restored to control levels using IBMX, a non-selective PDE inhibitor. Next, we found that cAMP levels in mice exposed to nicotine, through osmotic pumps, significantly decreased compared to sham mice. Where normal cAMP levels were restored using either PDE3 or PDE4 inhibition by cilo and roli respectively. Using this information, the physiological effects of decreased cAMP levels were investigated. It was found that nicotine-exposed mice have a substantial decrease in vasodilation responses with increased ISO (nonselective β-AR agonist). From these experiments, it was discovered that inhibition of PDE3, by cilo, not PDE4 inhibition, by roli, played a prominent effect in returning nicotine-exposed mice to sham levels of relaxation. The final set of experiments showed that AC6 protein expression levels are decreased compared

to sham levels, showing an alteration in the production of cAMP. Overall, there are three conclusions that can be drawn from these data: 1) Nicotine decreases receptor-mediated cAMP signaling in arterial myocytes, 2) nicotine decreases cAMP-mediated vasorelaxation, and 3) nicotine-exposed mice have alterations in cAMP vasorelaxation can be restored using PDE3, not PDE4 inhibition.

This work occupies a unique place within the research on nicotine and vasculature, to our knowledge we are the first to show that nicotine alters receptor-mediated cAMP levels in the VSM. Nicotine usage has primarily been linked with vasoconstriction and the research presented here displays that the picture is far more complex. [18] As discussed in Chapter 1, nicotine exacerbates vasoconstriction leading to chronic hypertension. [18] Rather than implicating vasoconstriction further though, the work here shows that there is an alteration in cAMP levels leading to a blunted vasodilation response. Furthermore, we add to the knowledge of PDE involvement in nicotine-impaired cAMP responses by showing that inhibition of PDE3 and PDE4 recovers cAMP levels in these nicotine-exposed mice. [127,208] Specifically, the study demonstrates a novel and crucial function of PDE3 inhibition in enhancing cAMP production, leading to a significant recovery of nicotine-exposed mice, and restoring them to vasorelaxation levels. This is of particular importance when it comes to how PDE3 may be distributed within the VSMCs. Where it has been shown that PDE3 is associated more with the SR and this would indicate that perhaps with nicotine exposure there is a redistribution of PDE3 within the VSMCs. [209]

In the landscape of a society that still uses nicotine regularly as a stimulant, there are large consequences of this work. [9,27,190,191] With the increased rise of vaping among young adults, there is a need to understand nicotine's effects, especially since vaping products can deliver such high doses of nicotine. [210] The work here falls into this research landscape in a unique way, in that

explored are the effects of just nicotine and how this alters the VSM, whereas most other works showcase nicotine products, in particular vaping and tobacco products, as a function of all their components. Although studying nicotine alone has some disadvantages, there is a lack of real-world context, as most nicotine users inhale through TC or ENDS. There are potentially significant synergistic effects that could be present from the many components of nicotine interacting with other aerosolized compounds. However, studying nicotine alone has allowed us to isolate the effects of nicotine on the vasculature. It has also allowed us to reduce the confounding factors that are present when other compounds are emitted through TC or ENDS. Overall, despite the disadvantages, this study has shown the powerful effects of nicotine without additional components seen in TC or ENDS, challenging challenges the notion that nicotine is safe and providing a relevant consideration for the user of nicotine products such as patches or gum.

3.2 Contextualizing Research Results: Insights into Vascular Physiology

This new knowledge of how nicotine alters cAMP causing decreased vasodilation response needs to be placed in the context of vascular physiology. Through this next section, the work presented here will be placed in the context of the consequences of altered cAMP levels, vascular complications, and potential differences in nicotine on cAMP between the sexes, to provide insights into vascular physiology.

3.2.1 Consequences of Lower cAMP Levels in VSM

There is a significant body of work within vascular physiology discussing the potential pathologies that occur from lower cAMP levels. It has been found that as humans age, the levels of cAMP drop due to the vasculature losing its responsiveness to β-AR receptor stimulation. [211,212] This decrease in cAMP has been shown to be associated with decreased vasodilation responses as

humans age and indeed this has been linked to hypertension in elderly patients. [211,213] Schutzer et al developed a VSMC model from rat aortas to reproduce the molecular changes observed in the vasculature that occur with age. [211] When this group separated out the rats into phenotypic segregation ("young", "mature", "old" and "old-old") there was a significant reduction in ISO-mediated cAMP accumulation. [211] One consequence of this is that cardiovascular disease is common in the elderly, in part due to this. [211,212] With regard to nicotine and the data achieved in this dissertation, decreased cAMP levels may result in similar outcomes of cardiovascular disease, resulting in the increased likelihood of hypertension and other cardiovascular diseases among nicotine users.

There are links between how lower cAMP maybe assist vascular injury recovery and these mechanisms may be inhibited by nicotine use. Fantidis et al investigated if cAMP can accelerate reendothelization in swine carotid and coronary arteries after injury. [214] The animals were subjected to a balloon injury to cause intimal hyperplasia in the carotid artery and then given FSK, to increase the cAMP levels. Once the animals had been sacrificed, the carotid artery was extracted to determine reendothelization. [214] Where this group found that cAMP increases reendothelization and stops fibromuscular proliferation (this promotes thickening of the smooth muscle). [214,215] Interestingly, a study conducted in rats exposed to 6-8 cigarettes a day (considered high dose), found that cigarette smoke increases the development of intimal hyperplasia. [216] Our study shows that nicotine decreases cAMP production; thus, nicotine could contribute to increased intimal hyperplasia in smokers and users of nicotine-containing products. This would result in the inability of reendothelization in smokers, increasing the occurrences of vascular injury.

3.2.2 PDE activity and the association with vascular pathologies

It is important to understand the association of PDE activity with different vascular pathologies, as five PDEs are highly specific to hydrolyze cAMP. [122,126] There are many downstream signals where cAMP is at the inception, and PDEs help regulate these signals by regulating cAMP. It has been shown by a wide variety of literature that PDE dysregulation can cause many vascular complications such as atherosclerosis, hypertension, and subarachnoid hemorrhage. [217] The work presented in Chapter 2 implicates PDE dysregulation causing altered vasorelaxation of nicotine-exposed mice, and there are studies showing that PDE dysregulation is linked to vascular complications such as hypertension. [218] In a study by Fan et al, there was an association between increased levels of PDE4D and hypertension. [218] One of their discoveries was PDE4D promotes VSMC vasocontraction through the cAMP-PKA-MLC signaling pathway and that PDE4D knockout mice showed that VSMCs reduced Ang II-induced hypertension. [218] There is much evidence indicating how smoking can increase the likelihood of hypertension and exacerbate it in current smokers who already have it. [219-221] Whether this is solely due to nicotine is still not fully understood, with some studies showing that whilst nicotine does increase blood pressure, it is acute but long-term use could also increase the occurrence of diseases like hypertension. [18,222] Our study using nicotine-exposed mice appears to align with the association that PDE dysregulation does have the potential to increase cardiovascular diseases, such as hypertension. This was done using PDE inhibitors, cilo, and roli, to recover the responses on the nicotine-exposed mice. Within vascular pathology, there is a link between PDE dysregulation and cardiovascular diseases, and the work presented in Chapter 2 appears to show that PDE regulation is disrupted by nicotine use.

3.2.3 PDE3 prominence and the possible explanation

As was shown in Chapter 2, PDE3 inhibition has a prominent effect on restoring vasodilation in nicotine-exposed mice and this is due to restoration of cAMP levels in nicotine exposed-mice. The prominence of PDE3 in this regard needs to be explored further but one possible explanation is that PDE3 is redistributed within the cell because of nicotine exposure. Interestingly, PDEs are not distributed throughout the cell homogenously. [209,223,224] For example, in cardiomyocytes, PDE2 is anchored to the plasma membrane, whilst PDE4 is associated with the plasma membrane and nuclear envelope. [205,209] One review suggests that the specificity of this highly organized distribution of PDEs is perhaps a reason for many pathologies, such as cancer and neurodegeneration. [209]

With respect to PDE3, there is also a highly specific distribution of this enzyme within the VSM. [209,223,224] It has been shown that PDE3 is in the VSM and intracellularly it is associated with the plasma membrane and SR. [209,223] The distribution and localization of PDE3 within the cell have been suggested by one review to be responsible pathology of diseases such as cardiomyopathy. [223] Indeed when it comes to nicotine causing intracellular dysregulation in the VSM, there appears to be a wide variety of knowledge on the subject. [150] A study by Nogueira et al suggests that mice exposed to TS impair hindlimb muscle vascular structure, with one potential reason they documented as a large impairment in the SR Ca^{2+}. [225] This alteration in SR function caused by nicotine could potentially be explained by the redistribution of PDE3 to the SR decreasing the effect of cAMP to cause less vasodilation. This would be further confirmed by the data achieved in Chapter 2 with the restoration of vasodilation function using PDE3 inhibitor, cilostamide (cilo).

3.2.4 Effects of nicotine on gender

There is a wealth of evidence showing that nicotine has different effects on both males and females regarding sexual reproduction, neuroscience, and so on. [226,227] It should be established that these differences are independent of plasma nicotine/cotinine concentrations. A review by Benowitz et al explored the potential gender differences in nicotine pharmacology and discussed any differences in nicotine/cotinine found in some studies due to weight and differing renal clearances. [226] Where men weigh more than women, but women have lower clearance than men, implying that women have a decreased nicotine plasma concentration. [226] Another factor that was not accounted for but implicated in a study by Chen et al was height. [228] Where nicotine/cotinine levels were measured in daily smokers in Pennsylvania and found, in addition to weight, height was a major contributor to differing nicotine/cotinine levels between genders. [228] Evans et al investigated the dose effects of transdermal nicotine patches on male and female smokers and found that plasma nicotine levels over a 6-hour period did not differ from gender. [229] Essentially, it has been established that there are no differences in nicotine plasma concentration.

Yet, with these similar nicotine plasma levels with regard to VSM, there is evidence to display that nicotine has differing VSM pathological effects on gender. [226] There are increasing studies revealing that nicotine consumption displays dimorphic gender effects within the same disease, such as atherosclerosis, appearing to affect females more. One such example is Centner et al, where male and female $ApoE^{-/-}$ mice were exposed to either cigarette smoke or drinking water containing nicotine for 4 months, nicotine levels being comparable to a moderate smoker (10-19 cigarettes per day). [230,231] Their findings suggested that males exposed to nicotine or smoke had more plaque in the aortic arc only, compared to females who had increased plaque in the arch and descending aorta. [231] Their findings suggested that females are at a higher risk of developing

atherosclerosis compared to males.[231] Adding to this, it has been shown that even offspring of female mice exposed to nicotine mice have differences in the degree of cardiovascular responses depending on their gender.[232] Fox et al, had C57Bl/6J female mice drink a nicotine solution 2 weeks before breeding until weaning.[232] They concluded female offspring exposed to nicotine had higher systolic, diastolic, and mean blood pressures at rest or during stress.[232] Whilst males had decreased heart rates and vascular responses to PE.[232] From the literature it appears that female nicotine users have a higher likelihood of getting cardiovascular diseases, meaning there is a sexual dimorphism of vascular disease in nicotine users.

3.2.5 Alterations in cAMP and PDE activity, and their links to cardiovascular complications in nicotine users

The larger implications of how nicotine usage can alter cAMP and PDE activity levels are of particular interest as they can increase cardiovascular complications. Firstly, Chapter 2 revealed the results that nicotine-exposed mice have lower levels of cAMP, but this is framed in the context of how this can increase the risk of cardiovascular disease. Indeed, Chang et al showed that reduced β−AR receptors and cAMP production induced hypertension in mice.[233] Albeit, this study explored the link between lead and cardiovascular disease however, their conclusions concerning cAMP production being linked to increased blood pressure are still valid for this work.[233] Rather fittingly, this displays the power cAMP dysregulation has on increasing cardiovascular disease outcomes.

The second discovery from Chapter 2 revealed the result that cAMP levels can be rescued in nicotine-exposed mice by PDE inhibitors. There are reviews that discuss the importance of PDEs regarding how dysregulation can lead to cardiovascular diseases, but one item of note is how cardiovascular diseases increases with age.[126,234] Where Elvebak et al investigated PGI_2-mediated

vasodilation and how PDE3 is affected by aging in VSM.[235] This study showed that PDE3 function is relatively preserved in healthy aging adults, with dysfunction of this leading to early aging and increasing the events of cardiovascular disease. When taken in context with the work presented in Chapter 2, there is a clear dysfunction in PDE regulation in nicotine-exposed mice. This links to increased aging and the incidence of cardiovascular disease, implying that users of nicotine are prone to increased aging, in addition to cardiovascular diseases.

3.3 Limitations and future work

Whilst our work shows that nicotine alters cAMP in arterial myocytes, there are some limitations present in this study, as there are with any. Yet, these new limitations should not be viewed as pitfalls but rather as future research areas to explore within the VSM and nicotine research. A few major areas need to be addressed within this dissertation.

Firstly, it should be acknowledged that whilst most nicotine products are inhaled this study uses osmotic pumps to have nicotine directly into the bloodstream of the mouse. Studies using inhaled nicotine do have more relevance with tobacco smoke or vaping, two common ways to consume nicotine. However, with mice studies in particular, the mice will also ingest the nicotine by grooming themselves, as well as inhaling it. This double route of ingestion increases the dosage given to the mice. Osmotic pumps have the advantage of having a precisely controlled dose given directly to the mice subcutaneously. Nonetheless, it should be acknowledged that this is a translational limitation of this work.

The work presented in Chapter 2 was conducted in male mice, and further studies need to be conducted into the effects of nicotine on female mice. Comparing sex differences across all research is often overlooked, but with recent movements in the scientific community, more

emphasis is being placed on having balanced studies with both male and female mice. [236,237] There are fundamental biological differences between males and females that need to be considered, such as females mensurating, being able to carry children, having higher fat mass lower, experiencing more strokes than men, and so on. [238,239] Due to these reasons and many more, it should be emphasized that males and females have some fundamental biological differences that will affect the outcomes of many experiments. One such example of this that is relevant to this work is that females showed less sensitivity to nicotine administered subcutaneously. [240] Lefever et al. trained male and female mice to discriminate subcutaneous nicotine from saline but they also evaluated the effects of aerosolized tobacco products. [240] Finding that nicotine, subcutaneously, had distinct effects between sexes with females showing less sensitivity and greater variability to the drug. [240] However, it is essential to acknowledge that there are fundamental differences between the sexes. Further research is needed to address these differences and decrease the potential discrepancies in the treatment of nicotine use between males and females.

The direct mechanism by which nicotine exposure alters receptor-mediated cAMP levels still must be established. Whilst we can hypothesize that increased catecholamine release results in an overstimulation of the VSMCs, causing a decrease in AC6, resulting in decreased cAMP levels, we need to test this hypothesis. The lack of knowledge in this area is partially due to the focus on research on the heart rather than the VSM. The work presented here is the beginning of this interesting connection and can be a pathway to understanding various nicotine induced VSM diseases such as hypertension.

With the increased nicotine use through vaping, for example, it would be wise to explore if the effects seen with just nicotine are exacerbated with vaping devices. Some studies claim large variations in nicotine delivery from vaping devices and this highly impacts the effect of nicotine

from person to person. [241] Our study used 6mg/mL which is the lower concentration of nicotine self-dose through ENDS and considered the average dose by TC users. [242,243] Whilst there are more studies showing the effects that vaping has on endothelial cells within the vasculature there have been no studies on how this may impact cAMP through receptor-mediated vasodilation. [180,181] This would be a highly relevant and impactful future area of exploration.

Despite the limitations, our study provides important novel information that highlights and informs future research directions. Firstly, the new discovery showing PDE3 inhibition by cilo, restores receptor-mediated vasodilation function in nicotine-exposed through increased cAMP levels back to sham levels. This provides one area of therapeutic potential in preventing and managing cardiovascular diseases such as hypertension and atherosclerosis. Although not explored in this dissertation, we show that another opportunity for research is through increasing cAMP production through ACs. Where it is shown that nicotine decreases protein expression levels of AC6 and drugs that target ACs may be another way to target increasing receptor-mediated vasodilation.

3.4 Concluding Remarks

Nicotine consumption has been present in human society for centuries, and its use, potency, and dosage have increased over time. From American Natives using tobacco leaves for ceremonies to the 1960s tobacco cigarette consumption and, more recently, to the use of ENDS, the prevalence of nicotine cannot be denied. Yet, this work tries to address a single component of this highly prominent drug within an extremely important system of the body, the vasculature. Chapter 1 provides an overview of VSM function and the complexity of its regulation by ion channels and intracellular signaling, emphasizing β-AR signaling which is the focus of this

dissertation. The relevance of cAMP is for on contractile function in the VSM was highlighted and the links between nicotine and the effect on the VSM were established to highlight the knowledge gap of how nicotine alters cAMP signaling in the vasculature.

Through Chapter 2 it was shown that receptor-mediated cAMP levels are decreased in nicotine-exposed mice, and this is consistent with β-AR mediated vasodilation being impaired in nicotine-exposed mice. Mechanistically, it was shown that is due to two reasons, AC_6 protein expression levels being decreased and PDE activity in nicotine-exposed mice being increased. Finally, it was shown that these responses can be recovered using cilo and roli that rescues cAMP responses in arteries from nicotine-exposed mice. The central finding of this dissertation underscores that nicotine exposure, coupled with alterations in cAMP signaling and vascular reactivity, may collectively contribute to the adverse cardiovascular effects associated with use of nicotine products. Whereby this work adds one more grain of knowledge to the many studies currently published and those that are yet to come, exploring not just the detrimental consequences and therapeutics of nicotine consumption to receptor-mediated vasculature excitability.

References

1 Roberts, O. L. & Dart, C. cAMP signalling in the vasculature: the role of Epac (exchange protein directly activated by cAMP). *Biochem Soc Trans* **42**, 89-97 (2014). https://doi.org:10.1042/BST20130253

2 Glorian, M. & Limon, I. in *Current Trends in Atherogenesis Current Trends in Atherogenesis* (ed Rita Rezzani) Ch. 6, 121-145 (IntechOpen, 2013).

3 Tykocki, N. R., Boerman, E. M. & Jackson, W. F. Smooth Muscle Ion Channels and Regulation of Vascular Tone in Resistance Arteries and Arterioles. *Compr Physiol* **7**, 485-581 (2017). https://doi.org:10.1002/cphy.c160011

4 Wellman, G. C., Bonev, A. D., Nelson, M. T. & Brayden, J. E. Gender differences in coronary artery diameter involve estrogen, nitric oxide, and Ca^{2+}-dependent K^{+} channels. *Circ Res* **79**, 1024-1030. (1996).

5 Wellman, G. C., Santana, L. F., Bonev, A. D. & Nelson, M. T. Role of phospholamban in the modulation of arterial Ca^{2+} sparks and Ca^{2+}-activated K^{+} channels by cAMP. *Am J Physiol Cell Physiol* **281**, C1029-1037. (2001).

6 Aiello, E. A., Walsh, M. P. & Cole, W. C. Phosphorylation by protein kinase A enhances delayed rectifier K+ current in rabbit vascular smooth muscle cells. *Am J Physiol* **268**, H926-934 (1995). https://doi.org:10.1152/ajpheart.1995.268.2.H926

7 Moore, C. L., Nelson, P. L., Parelkar, N. K., Rusch, N. J. & Rhee, S. W. Protein kinase A-phosphorylated KV1 channels in PSD95 signaling complex contribute to the resting membrane potential and diameter of cerebral arteries. *Circ Res* **114**, 1258-1267 (2014). https://doi.org:10.1161/CIRCRESAHA.114.303167

8 Ostrom, R. S. *et al.* Localization of adenylyl cyclase isoforms and G protein-coupled receptors in vascular smooth muscle cells: expression in caveolin-rich and noncaveolin domains. *Mol Pharmacol* **62**, 983-992 (2002). https://doi.org:10.1124/mol.62.5.983

9 Margolis, K. A., Bernat, J. K., Keely O'Brien, E. & Delahanty, J. C. Online Information About Harmful Tobacco Constituents: A Content Analysis. *Nicotine & Tobacco Research* **19**, 1209-1215 (2016). https://doi.org:10.1093/ntr/ntw220

10 Hu, Y. *et al.* Analyzing the genes related to nicotine addiction or schizophrenia via a pathway and network based approach. *Scientific Reports* **8**, 2894 (2018). https://doi.org:10.1038/s41598-018-21297-x

11 Oshikawa, J. *et al.* Nicotinic acetylcholine receptor alpha 7 regulates cAMP signal within lipid rafts. *Am J Physiol Cell Physiol* **285**, C567-574 (2003). https://doi.org:10.1152/ajpcell.00422.2002

12 Raghuveer, G. *et al.* Cardiovascular Consequences of Childhood Secondhand Tobacco Smoke Exposure: Prevailing Evidence, Burden, and Racial and Socioeconomic Disparities: A Scientific Statement From the American Heart Association. *Circulation* **134**, e336-e359 (2016). https://doi.org:10.1161/CIR.0000000000000443

13 Sanip, Z. *et al.* The effects of Secondhand Smoke (SHS) exposure on microvascular endothelial function among healthy women. *Tob Induc Dis* **13**, 32 (2015). https://doi.org:10.1186/s12971-015-0052-9

14 Varela-Carver, A., Parker, H., Kleinert, C. & Rimoldi, O. Adverse effects of cigarette smoke and induction of oxidative stress in cardiomyocytes and vascular endothelium. *Curr Pharm Des* **16**, 2551-2558 (2010). https://doi.org:10.2174/138161210792062830

15 Middlekauff, H. R., Park, J. & Moheimani, R. S. Adverse effects of cigarette and noncigarette smoke exposure on the autonomic nervous system: mechanisms and implications for cardiovascular risk. *J Am Coll Cardiol* **64**, 1740-1750 (2014). https://doi.org:10.1016/j.jacc.2014.06.1201

16 Zhang, W. Z., Venardos, K., Chin-Dusting, J. & Kaye, D. M. Adverse effects of cigarette smoke on NO bioavailability: role of arginine metabolism and oxidative stress. *Hypertension* **48**, 278-285 (2006). https://doi.org:10.1161/01.HYP.0000231509.27406.42

17 Dove, M. S., Dockery, D. W. & Connolly, G. N. Smoke-free air laws and secondhand smoke exposure among nonsmoking youth. *Pediatrics* **126**, 80-87 (2010). https://doi.org:10.1542/peds.2009-3462

18 Benowitz, N. L. & Burbank, A. D. Cardiovascular toxicity of nicotine: Implications for electronic cigarette use. *Trends Cardiovasc Med* **26**, 515-523 (2016). https://doi.org:10.1016/j.tcm.2016.03.001

19 Sapru, S. *et al.* E-cigarettes use in the United States: reasons for use, perceptions, and effects on health. *BMC Public Health* **20**, 1518 (2020). https://doi.org:10.1186/s12889-020-09572-x

20 Durmowicz, E. L. The impact of electronic cigarettes on the paediatric population. *Tob Control* **23 Suppl 2**, ii41-46 (2014). https://doi.org:10.1136/tobaccocontrol-2013-051468

21 Moore, G. F., Littlecott, H. J., Moore, L., Ahmed, N. & Holliday, J. E-cigarette use and intentions to smoke among 10-11-year-old never-smokers in Wales. *Tob Control* **25**, 147-152 (2016). https://doi.org:10.1136/tobaccocontrol-2014-052011

22 Drummond, M. B. & Upson, D. Electronic cigarettes. Potential harms and benefits. *Ann Am Thorac Soc* **11**, 236-242 (2014). https://doi.org:10.1513/AnnalsATS.201311-391FR

23 Chaumont, M. *et al.* Differential Effects of E-Cigarette on Microvascular Endothelial Function, Arterial Stiffness and Oxidative Stress: A Randomized Crossover Trial. *Sci Rep* **8**, 10378 (2018). https://doi.org:10.1038/s41598-018-28723-0

24 Franzen, K. F. *et al.* E-cigarettes and cigarettes worsen peripheral and central hemodynamics as well as arterial stiffness: A randomized, double-blinded pilot study. *Vasc Med* **23**, 419-425 (2018). https://doi.org:10.1177/1358863X18779694

25 Moheimani, R. S. *et al.* Sympathomimetic Effects of Acute E-Cigarette Use: Role of Nicotine and Non-Nicotine Constituents. *J Am Heart Assoc* **6** (2017). https://doi.org:10.1161/JAHA.117.006579

26 Rodgman, A. & Perfetti, T. The Chemical Components Identified in Tobacco and Tobacco Smoke Prior to 1954: A Chronology of Classical Chemistry. *Beitrage zur Tabakforschung International/ Contributions to Tobacco Research* **23**, 277-333 (2009). https://doi.org:10.2478/cttr-2013-0866

27 Le Foll, B. *et al.* Tobacco and nicotine use. *Nat Rev Dis Primers* **8**, 19 (2022). https://doi.org:10.1038/s41572-022-00346-w

28 Benowitz, N. L. Pharmacology of nicotine: addiction, smoking-induced disease, and therapeutics. *Annu Rev Pharmacol Toxicol* **49**, 57-71 (2009). https://doi.org:10.1146/annurev.pharmtox.48.113006.094742

29 Benowitz, N. L., Hukkanen, J. & Jacob, P., 3rd. Nicotine chemistry, metabolism, kinetics and biomarkers. *Handb Exp Pharmacol*, 29-60 (2009). https://doi.org:10.1007/978-3-540-69248-5_2

30 Kenton, A., Parokonny, A. S., Gleba, Y. Y. & Bennett, M. D. Characterization of the Nicotiana tabacum L. genome by molecular cytogenetics. *Mol Gen Genet* **240**, 159-169 (1993). https://doi.org:10.1007/bf00277053

31 Wagenhauser, M. U. *et al.* Chronic Nicotine Exposure Induces Murine Aortic Remodeling and Stiffness Segmentation-Implications for Abdominal Aortic Aneurysm Susceptibility. *Front Physiol* **9**, 1459 (2018). https://doi.org:10.3389/fphys.2018.01459

32 Babic, M., Schuchardt, M., Tolle, M. & van der Giet, M. In times of tobacco-free nicotine consumption: The influence of nicotine on vascular calcification. *Eur J Clin Invest* **49**, e13077 (2019). https://doi.org:10.1111/eci.13077

33 Shao, X. M., Lopez-Valdes, H. E., Liang, J. & Feldman, J. L. Inhaled nicotine equivalent to cigarette smoking disrupts systemic and uterine hemodynamics and induces cardiac arrhythmia in pregnant rats. *Sci Rep* **7**, 16974 (2017). https://doi.org:10.1038/s41598-017-17301-5

34 Jensen, K. *et al.* General mechanisms of nicotine-induced fibrogenesis. *FASEB J* **26**, 4778-4787 (2012). https://doi.org:10.1096/fj.12-206458

35 van Berlo, J. H., Maillet, M. & Molkentin, J. D. Signaling effectors underlying pathologic growth and remodeling of the heart. *J Clin Invest* **123**, 37-45 (2013). https://doi.org:10.1172/JCI62839

36 Morris, P. B. *et al.* Cardiovascular Effects of Exposure to Cigarette Smoke and Electronic Cigarettes: Clinical Perspectives From the Prevention of Cardiovascular Disease Section Leadership Council and Early Career Councils of the American College of Cardiology. *J Am Coll Cardiol* **66**, 1378-1391 (2015). https://doi.org:10.1016/j.jacc.2015.07.037

37 Carter, B. D., Freedman, N. D. & Jacobs, E. J. Smoking and mortality--beyond established causes. *N Engl J Med* **372**, 2170 (2015). https://doi.org:10.1056/NEJMc1503675

38 Shao, X. M. & Fang, Z. T. Severe Acute Toxicity of Inhaled Nicotine and e-Cigarettes: Seizures and Cardiac Arrhythmia. *Chest* **157**, 506-508 (2020). https://doi.org:10.1016/j.chest.2019.10.008

39 Manoury, B., Idres, S., Leblais, V. & Fischmeister, R. Ion channels as effectors of cyclic nucleotide pathways: Functional relevance for arterial tone regulation. *Pharmacol Ther*, 107499 (2020). https://doi.org:10.1016/j.pharmthera.2020.107499

40 Reddy, G. R. *et al.* Illuminating cell signaling with genetically encoded FRET biosensors in adult mouse cardiomyocytes. *J Gen Physiol* **150**, 1567-1582 (2018). https://doi.org:10.1085/jgp.201812119

41 Bers, D. M., Xiang, Y. K. & Zaccolo, M. Whole-Cell cAMP and PKA Activity are Epiphenomena, Nanodomain Signaling Matters. *Physiology (Bethesda)* **34**, 240-249 (2019). https://doi.org:10.1152/physiol.00002.2019

42 Sassone-Corsi, P. The cyclic AMP pathway. *Cold Spring Harb Perspect Biol* **4** (2012). https://doi.org:10.1101/cshperspect.a011148

43 Chaudhry, R., Miao, J. H. & Rehman, A. in *StatPearls* (StatPearls Publishing Copyright © 2023, StatPearls Publishing LLC., 2023).

44 Pugsley, M. K. & Tabrizchi, R. The vascular system. An overview of structure and function. *J Pharmacol Toxicol Methods* **44**, 333-340 (2000). https://doi.org:10.1016/s1056-8719(00)00125-8
45 Matienzo, D. & Bordoni, B. in *StatPearls* (StatPearls Publishing
Copyright © 2023, StatPearls Publishing LLC., 2023).
46 Aird, W. C. Discovery of the cardiovascular system: from Galen to William Harvey. *J Thromb Haemost* **9 Suppl 1**, 118-129 (2011). https://doi.org:10.1111/j.1538-7836.2011.04312.x
47 King, J. & Lowery, D. R. in *StatPearls* (StatPearls Publishing
Copyright © 2023, StatPearls Publishing LLC., 2023).
48 Pappano, A. J. & Wier, W. G. *Cardiovascular physiology*. Eleventh edition / Achilles J. Pappano, Withrow Gil Wier. edn, (Elsevier, 2018).
49 Tucker, W. D., Arora, Y. & Mahajan, K. in *StatPearls* (StatPearls Publishing
Copyright © 2022, StatPearls Publishing LLC., 2022).
50 Sandoo, A., van Zanten, J. J., Metsios, G. S., Carroll, D. & Kitas, G. D. The endothelium and its role in regulating vascular tone. *Open Cardiovasc Med J* **4**, 302-312 (2010). https://doi.org:10.2174/1874192401004010302
51 Erbel, R. & Eggebrecht, H. Aortic dimensions and the risk of dissection. *Heart* **92**, 137-142 (2006). https://doi.org:10.1136/hrt.2004.055111
52 Rahman, M. & Siddik, A. B. in *StatPearls* (StatPearls Publishing
Copyright © 2023, StatPearls Publishing LLC., 2023).
53 Yap, C., Mieremet, A., de Vries, C. J. M., Micha, D. & de Waard, V. Six Shades of Vascular Smooth Muscle Cells Illuminated by KLF4 (Krüppel-Like Factor 4). *Arterioscler Thromb Vasc Biol* **41**, 2693-2707 (2021). https://doi.org:10.1161/atvbaha.121.316600
54 Libby, P. *et al.* Atherosclerosis. *Nat Rev Dis Primers* **5**, 56 (2019). https://doi.org:10.1038/s41572-019-0106-z
55 Tonar, Z. *et al.* Vasa vasorum in the tunica media and tunica adventitia of the porcine aorta. *Annals of Anatomy - Anatomischer Anzeiger* **205**, 22-36 (2016). https://doi.org:https://doi.org/10.1016/j.aanat.2016.01.008
56 Shadwick, R. E. Mechanical design in arteries. *J Exp Biol* **202**, 3305-3313 (1999). https://doi.org:10.1242/jeb.202.23.3305
57 Witter, K., Tonar, Z. & Schöpper, H. How many Layers has the Adventitia? – Structure of the Arterial Tunica Externa Revisited. *Anatomia, Histologia, Embryologia* **46**, 110-120 (2017). https://doi.org:https://doi.org/10.1111/ahe.12239
58 Trammel, J. E. & Sapra, A. in *StatPearls* (StatPearls Publishing
Copyright © 2023, StatPearls Publishing LLC., 2023).
59 Leloup, A. J. *et al.* Elastic and Muscular Arteries Differ in Structure, Basal NO Production and Voltage-Gated Ca(2+)-Channels. *Front Physiol* **6**, 375 (2015). https://doi.org:10.3389/fphys.2015.00375
60 Chakraborty, R. *et al.* Targeting smooth muscle cell phenotypic switching in vascular disease. *JVS Vasc Sci* **2**, 79-94 (2021). https://doi.org:10.1016/j.jvssci.2021.04.001
61 Brozovich, F. V. *et al.* Mechanisms of Vascular Smooth Muscle Contraction and the Basis for Pharmacologic Treatment of Smooth Muscle Disorders. *Pharmacol Rev* **68**, 476-532 (2016). https://doi.org:10.1124/pr.115.010652

62 Félétou, M. in *The Endothelium: Part 1: Multiple Functions of the Endothelial Cells—Focus on Endothelium-Derived Vasoactive Mediators* (Morgan & Claypool Life Sciences
Copyright © 2011 by Morgan & Claypool Life Sciences Publishers., 2011).
63 Oosterhoff, L. A. *et al.* Characterization of Endothelial and Smooth Muscle Cells From Different Canine Vessels. *Front Physiol* **10**, 101 (2019). https://doi.org:10.3389/fphys.2019.00101
64 Förstermann, U. & Sessa, W. C. Nitric oxide synthases: regulation and function. *Eur Heart J* **33**, 829-837, 837a-837d (2012). https://doi.org:10.1093/eurheartj/ehr304
65 Favero, G., Paganelli, C., Buffoli, B., Rodella, L. F. & Rezzani, R. Endothelium and its alterations in cardiovascular diseases: life style intervention. *Biomed Res Int* **2014**, 801896 (2014). https://doi.org:10.1155/2014/801896
66 Chiu, J. J. & Chien, S. Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives. *Physiol Rev* **91**, 327-387 (2011). https://doi.org:10.1152/physrev.00047.2009
67 Gohar, E. Y. & Pollock, D. M. Sex-Specific Contributions of Endothelin to Hypertension. *Curr Hypertens Rep* **20**, 58 (2018). https://doi.org:10.1007/s11906-018-0856-0
68 Webb, R. C. Smooth muscle contraction and relaxation. *Adv Physiol Educ* **27**, 201-206 (2003). https://doi.org:10.1152/advan.00025.2003
69 Raina, H., Zacharia, J., Li, M. & Wier, W. G. Activation by Ca2+/calmodulin of an exogenous myosin light chain kinase in mouse arteries. *J Physiol* **587**, 2599-2612 (2009). https://doi.org:10.1113/jphysiol.2008.165258
70 Singla, S. I., Hudmon, A., Goldberg, J. M., Smith, J. L. & Schulman, H. Molecular characterization of calmodulin trapping by calcium/calmodulin-dependent protein kinase II. *J Biol Chem* **276**, 29353-29360 (2001). https://doi.org:10.1074/jbc.M101744200
71 Pereira da Silva, E. A., Martín-Aragón Baudel, M., Navedo, M. F. & Nieves-Cintrón, M. Ion channel molecular complexes in vascular smooth muscle. *Frontiers in Physiology* **13** (2022). https://doi.org:10.3389/fphys.2022.999369
72 Dolphin, A. C. A short history of voltage-gated calcium channels. *Br J Pharmacol* **147 Suppl 1**, S56-62 (2006). https://doi.org:10.1038/sj.bjp.0706442
73 Zamponi, G. W., Striessnig, J., Koschak, A. & Dolphin, A. C. The Physiology, Pathology, and Pharmacology of Voltage-Gated Calcium Channels and Their Future Therapeutic Potential. *Pharmacol Rev* **67**, 821-870 (2015). https://doi.org:10.1124/pr.114.009654
74 Ghosh, D. *et al.* Calcium Channels in Vascular Smooth Muscle. *Adv Pharmacol* **78**, 49-87 (2017). https://doi.org:10.1016/bs.apha.2016.08.002
75 Nieves-Cintrón, M., Flores-Tamez, V. A., Le, T., Baudel, M. M.-A. & Navedo, M. F. Cellular and molecular effects of hyperglycemia on ion channels in vascular smooth muscle. *Cellular and Molecular Life Sciences* **78**, 31-61 (2021). https://doi.org:10.1007/s00018-020-03582-z
76 Harraz, O. F. *et al.* Ca(V)3.2 channels and the induction of negative feedback in cerebral arteries. *Circ Res* **115**, 650-661 (2014). https://doi.org:10.1161/circresaha.114.304056
77 Harraz, O. F. *et al.* CaV1.2/CaV3.x channels mediate divergent vasomotor responses in human cerebral arteries. *J Gen Physiol* **145**, 405-418 (2015). https://doi.org:10.1085/jgp.201511361

78 El-Rahman, R. R. A. *et al.* Identification of L- and T-type Ca2+ channels in rat cerebral arteries: role in myogenic tone development. *American Journal of Physiology-Heart and Circulatory Physiology* **304**, H58-H71 (2013). https://doi.org:10.1152/ajpheart.00476.2012

79 Le, T. *et al.* Secondhand Smoke Exposure Impairs Ion Channel Function and Contractility of Mesenteric Arteries. *Function (Oxf)* **2**, zqab041 (2021). https://doi.org:10.1093/function/zqab041

80 Fuller, M. D., Fu, Y., Scheuer, T. & Catterall, W. A. Differential regulation of CaV1.2 channels by cAMP-dependent protein kinase bound to A-kinase anchoring proteins 15 and 79/150. *Journal of General Physiology* **143**, 315-324 (2014). https://doi.org:10.1085/jgp.201311075

81 Ruiz-Velasco, V., Zhong, J., Hume, J. R. & Keef, K. D. Modulation of Ca²⁺ Channels by Cyclic Nucleotide Cross Activation of Opposing Protein Kinases in Rabbit Portal Vein. *Circulation Research* **82**, 557-565 (1998). https://doi.org:doi:10.1161/01.RES.82.5.557

82 Keef, K. D., Hume, J. R. & Zhong, J. Regulation of cardiac and smooth muscle Ca2+channels (CaV1.2a,b) by protein kinases. *American Journal of Physiology-Cell Physiology* **281**, C1743-C1756 (2001). https://doi.org:10.1152/ajpcell.2001.281.6.C1743

83 Xiong, Z., Sperelakis, N. & Fenoglio-Preiser, C. Regulation of L-type calcium channels by cyclic nucleotides and phosphorylation in smooth muscle cells from rabbit portal vein. *J Vasc Res* **31**, 271-279 (1994). https://doi.org:10.1159/000159053

84 Ishikawa, T., Hume, J. R. & Keef, K. D. Regulation of Ca2+ channels by cAMP and cGMP in vascular smooth muscle cells. *Circulation Research* **73**, 1128-1137 (1993). https://doi.org:doi:10.1161/01.RES.73.6.1128

85 Syed, A. U. *et al.* Adenylyl cyclase 5-generated cAMP controls cerebral vascular reactivity during diabetic hyperglycemia. *J Clin Invest* **129**, 3140-3152 (2019). https://doi.org:10.1172/jci124705

86 Earley, S. & Brayden, J. E. Transient receptor potential channels in the vasculature. *Physiol Rev* **95**, 645-690 (2015). https://doi.org:10.1152/physrev.00026.2014

87 Nilipour, Y. *et al.* Ryanodine receptor type 3 (RYR3) as a novel gene associated with a myopathy with nemaline bodies. *Eur J Neurol* **25**, 841-847 (2018). https://doi.org:10.1111/ene.13607

88 Porter, V. A. *et al.* Frequency modulation of Ca2+sparks is involved in regulation of arterial diameter by cyclic nucleotides. *American Journal of Physiology-Cell Physiology* **274**, C1346-C1355 (1998). https://doi.org:10.1152/ajpcell.1998.274.5.C1346

89 Tertyshnikova, S. & Fein, A. Inhibition of inositol 1,4,5-trisphosphate-induced Ca²⁺ release by cAMP-dependent protein kinase in a living cell. *Proceedings of the National Academy of Sciences* **95**, 1613-1617 (1998). https://doi.org:doi:10.1073/pnas.95.4.1613

90 Bai, Y. & Sanderson, M. J. Airway smooth muscle relaxation results from a reduction in the frequency of Ca2+ oscillations induced by a cAMP-mediated inhibition of the IP3 receptor. *Respiratory Research* **7**, 34 (2006). https://doi.org:10.1186/1465-9921-7-34

91 Nalli, A. D. *et al.* Regulation of Gβγi-Dependent PLC-β3 Activity in Smooth Muscle: Inhibitory Phosphorylation of PLC-β3 by PKA and PKG and Stimulatory Phosphorylation of Gαi-GTPase-Activating Protein RGS2 by PKG. *Cell Biochemistry and Biophysics* **70**, 867-880 (2014). https://doi.org:10.1007/s12013-014-9992-6

92 Jackson, W. F. Ion channels and vascular tone. *Hypertension* **35**, 173-178 (2000). https://doi.org:10.1161/01.hyp.35.1.173
93 Nelson, M. T., Patlak, J. B., Worley, J. F. & Standen, N. B. Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *Am J Physiol* **259**, C3-18 (1990). https://doi.org:10.1152/ajpcell.1990.259.1.C3
94 Mundina-Weilenmann, C. *et al.* Endoplasmic reticulum contribution to the relaxant effect of cGMP- and cAMP-elevating agents in feline aorta. *Am J Physiol Heart Circ Physiol* **278**, H1856-1865 (2000). https://doi.org:10.1152/ajpheart.2000.278.6.H1856
95 Wilson, D. P. in *Mechanisms of Vascular Disease: A Reference Book for Vascular Specialists* (eds R. Fitridge & M. Thompson) (University of Adelaide Press
© The Contributors 2011., 2011).
96 Nieves-Cintrón, M., Syed, A. U., Nystoriak, M. A. & Navedo, M. F. Regulation of voltage-gated potassium channels in vascular smooth muscle during hypertension and metabolic disorders. *Microcirculation* **25** (2018). https://doi.org:10.1111/micc.12423
97 Zhai, J., Lin, Q. S., Hu, Z., Wong, R. & Soong, T. W. in *Ion Channels in Health and Disease* (ed Geoffrey S. Pitt) 265-292 (Academic Press, 2016).
98 van der Horst, J., Greenwood, I. A. & Jepps, T. A. Cyclic AMP-Dependent Regulation of Kv7 Voltage-Gated Potassium Channels. *Frontiers in Physiology* **11** (2020). https://doi.org:10.3389/fphys.2020.00727
99 Rosenbaum, D. M., Rasmussen, S. G. & Kobilka, B. K. The structure and function of G-protein-coupled receptors. *Nature* **459**, 356-363 (2009). https://doi.org:10.1038/nature08144
100 Kamato, D. *et al.* Structure, Function, Pharmacology, and Therapeutic Potential of the G Protein, Gα/q,11. *Front Cardiovasc Med* **2**, 14 (2015). https://doi.org:10.3389/fcvm.2015.00014
101 Paravati, S., Rosani, A. & Warrington, S. J. in *StatPearls* (StatPearls Publishing
Copyright © 2023, StatPearls Publishing LLC., 2023).
102 Farzam, K., Kidron, A. & Lakhkar, A. D. in *StatPearls* (StatPearls Publishing
Copyright © 2023, StatPearls Publishing LLC., 2023).
103 Piascik, M. T. & Perez, D. M. Alpha1-adrenergic receptors: new insights and directions. *J Pharmacol Exp Ther* **298**, 403-410 (2001).
104 Wang, S. Y. Vasoconstrictive versus Vasodilatory Effects of Alpha-2 Adrenergic Agonists on the Spinal Microcirculation. *Anesthesiology* **92**, 1488-1488 (2000). https://doi.org:10.1097/00000542-200005000-00043
105 Muszkat, M., Kurnik, D., Sofowora, G. G., Wood, A. J. & Stein, C. M. Independent regulation of α1 and α2 adrenergic receptor-mediated vasoconstriction in vivo. *J Hypertens* **29**, 251-256 (2011). https://doi.org:10.1097/HJH.0b013e3283407ffd
106 Kanagy, N. L. Alpha(2)-adrenergic receptor signalling in hypertension. *Clin Sci (Lond)* **109**, 431-437 (2005). https://doi.org:10.1042/cs20050101
107 Jensen, B. C., O'Connell, T. D. & Simpson, P. C. Alpha-1-adrenergic receptors in heart failure: the adaptive arm of the cardiac response to chronic catecholamine stimulation. *J Cardiovasc Pharmacol* **63**, 291-301 (2014). https://doi.org:10.1097/fjc.0000000000000032
108 Sica, D. A. Alpha1-Adrenergic Blockers: Current Usage Considerations. *The Journal of Clinical Hypertension* **7**, 757-762 (2005). https://doi.org:https://doi.org/10.1111/j.1524-6175.2005.05300.x

109 Indolfi, C. *et al.* Membrane-bound protein kinase A inhibits smooth muscle cell proliferation in vitro and in vivo by amplifying cAMP-protein kinase A signals. *Circ Res* **88**, 319-324 (2001). https://doi.org:10.1161/01.res.88.3.319

110 Lohse, M. J., Engelhardt, S. & Eschenhagen, T. What Is the Role of β-Adrenergic Signaling in Heart Failure? *Circulation Research* **93**, 896-906 (2003). https://doi.org:doi:10.1161/01.RES.0000102042.83024.CA

111 Chruscinski, A. *et al.* Differential distribution of beta-adrenergic receptor subtypes in blood vessels of knockout mice lacking beta(1)- or beta(2)-adrenergic receptors. *Mol Pharmacol* **60**, 955-962 (2001). https://doi.org:10.1124/mol.60.5.955

112 Nelson, C. P. *et al.* Principal role of adenylyl cyclase 6 in K(+) channel regulation and vasodilator signalling in vascular smooth muscle cells. *Cardiovasc Res* **91**, 694-702 (2011). https://doi.org:10.1093/cvr/cvr137

113 Pavan, B., Biondi, C. & Dalpiaz, A. Adenylyl cyclases as innovative therapeutic goals. *Drug Discovery Today* **14**, 982-991 (2009). https://doi.org:https://doi.org/10.1016/j.drudis.2009.07.007

114 Webb, J. G., Yates, P. W., Yang, Q., Mukhin, Y. V. & Lanier, S. M. Adenylyl cyclase isoforms and signal integration in models of vascular smooth muscle cells. *American Journal of Physiology-Heart and Circulatory Physiology* **281**, H1545-H1552 (2001). https://doi.org:10.1152/ajpheart.2001.281.4.H1545

115 Scholich, K. *et al.* Facilitation of signal onset and termination by adenylyl cyclase. *Science* **283**, 1328-1331 (1999). https://doi.org:10.1126/science.283.5406.1328

116 Qiu, L., LeBel, R. P., Storm, D. R. & Chen, X. Type 3 adenylyl cyclase: a key enzyme mediating the cAMP signaling in neuronal cilia. *Int J Physiol Pathophysiol Pharmacol* **8**, 95-108 (2016).

117 Warrier, S. *et al.* cAMP microdomains and L-type Ca2+ channel regulation in guinea-pig ventricular myocytes. *J Physiol* **580**, 765-776 (2007). https://doi.org:10.1113/jphysiol.2006.124891

118 Zaccolo, M. & Pozzan, T. Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science* **295**, 1711-1715 (2002). https://doi.org:10.1126/science.1069982

119 Surdo, N. C. *et al.* FRET biosensor uncovers cAMP nano-domains at β-adrenergic targets that dictate precise tuning of cardiac contractility. *Nature Communications* **8**, 15031 (2017). https://doi.org:10.1038/ncomms15031

120 Chao, Y. C., Surdo, N. C., Pantano, S. & Zaccolo, M. Imaging cAMP nanodomains in the heart. *Biochem Soc Trans* **47**, 1383-1392 (2019). https://doi.org:10.1042/bst20190245

121 Folkmanaite, M. & Zaccolo, M. Regulation of cardiac function by cAMP nanodomains. *Biosci Rep* **43** (2023). https://doi.org:10.1042/bsr20220953

122 Knight, W. & Yan, C. Therapeutic potential of PDE modulation in treating heart disease. *Future Med Chem* **5**, 1607-1620 (2013). https://doi.org:10.4155/fmc.13.127

123 Dodge, K. L. *et al.* mAKAP assembles a protein kinase A/PDE4 phosphodiesterase cAMP signaling module. *Embo J* **20**, 1921-1930 (2001).

124 Bolger, G. B. The PDE-Opathies: Diverse Phenotypes Produced by a Functionally Related Multigene Family. *Trends Genet* **37**, 669-681 (2021). https://doi.org:10.1016/j.tig.2021.03.002

125 Keravis, T. & Lugnier, C. Cyclic nucleotide phosphodiesterase (PDE) isozymes as targets of the intracellular signalling network: benefits of PDE inhibitors in various diseases and

perspectives for future therapeutic developments. *Br J Pharmacol* **165**, 1288-1305 (2012). https://doi.org:10.1111/j.1476-5381.2011.01729.x

126 Lorigo, M., Oliveira, N. & Cairrao, E. PDE-Mediated Cyclic Nucleotide Compartmentation in Vascular Smooth Muscle Cells: From Basic to a Clinical Perspective. *J Cardiovasc Dev Dis* **9** (2021). https://doi.org:10.3390/jcdd9010004

127 Zuo, H. *et al.* Cigarette smoke up-regulates PDE3 and PDE4 to decrease cAMP in airway cells. *Br J Pharmacol* **175**, 2988-3006 (2018). https://doi.org:10.1111/bph.14347

128 Seidler, U. & Sjöblom, M. in *Physiology of the Gastrointestinal Tract (Fifth Edition)* (eds Leonard R. Johnson *et al.*) 1311-1339 (Academic Press, 2012).

129 Zuo, H. *et al.* Cigarette smoke up-regulates PDE3 and PDE4 to decrease cAMP in airway cells. *Br J Pharmacol* **175**, 2988-3006 (2018). https://doi.org:10.1111/bph.14347

130 Devine, J. F. Chronic obstructive pulmonary disease: an overview. *Am Health Drug Benefits* **1**, 34-42 (2008).

131 Gao, R. *et al.* Phosphodiesterase 4D contributes to angiotensin II-induced abdominal aortic aneurysm through smooth muscle cell apoptosis. *Exp Mol Med* **54**, 1201-1213 (2022). https://doi.org:10.1038/s12276-022-00815-y

132 Tao, M., Salas, M. L. & Lipmann, F. Mechanism of Activation by Adenosine 3′:5′-Cyclic Monophosphate of a Protein Phosphokinase from Rabbit Reticulocytes[*]. *Proceedings of the National Academy of Sciences* **67**, 408-414 (1970). https://doi.org:doi:10.1073/pnas.67.1.408

133 Di Benedetto, G. *et al.* Protein kinase A type I and type II define distinct intracellular signaling compartments. *Circ Res* **103**, 836-844 (2008). https://doi.org:10.1161/circresaha.108.174813

134 Cheng, X., Ji, Z., Tsalkova, T. & Mei, F. Epac and PKA: a tale of two intracellular cAMP receptors. *Acta Biochim Biophys Sin (Shanghai)* **40**, 651-662 (2008). https://doi.org:10.1111/j.1745-7270.2008.00438.x

135 Brandon, E. P., Idzerda, R. L. & McKnight, G. S. PKA isoforms, neural pathways, and behaviour: making the connection. *Current Opinion in Neurobiology* **7**, 397-403 (1997). https://doi.org:https://doi.org/10.1016/S0959-4388(97)80069-4

136 Zhao, X. *et al.* Endothelial PKA activity regulates angiogenesis by limiting autophagy through phosphorylation of ATG16L1. *Elife* **8** (2019). https://doi.org:10.7554/eLife.46380

137 Yoo, S. Y. & Kwon, S. M. Angiogenesis and its therapeutic opportunities. *Mediators Inflamm* **2013**, 127170 (2013). https://doi.org:10.1155/2013/127170

138 Tsalkova, T., Blumenthal, D. K., Mei, F. C., White, M. A. & Cheng, X. Mechanism of Epac activation: structural and functional analyses of Epac2 hinge mutants with constitutive and reduced activities. *J Biol Chem* **284**, 23644-23651 (2009). https://doi.org:10.1074/jbc.M109.024950

139 de Rooij, J. *et al.* Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* **396**, 474-477 (1998). https://doi.org:10.1038/24884

140 Wehbe, N. *et al.* EPAC in Vascular Smooth Muscle Cells. *Int J Mol Sci* **21** (2020). https://doi.org:10.3390/ijms21145160

141 Smith, S. A., Newby, A. C. & Bond, M. Ending Restenosis: Inhibition of Vascular Smooth Muscle Cell Proliferation by cAMP. *Cells* **8**, 1447 (2019).

142 Ali, D. C. *et al.* β-Adrenergic receptor, an essential target in cardiovascular diseases. *Heart Failure Reviews* **25**, 343-354 (2020). https://doi.org:10.1007/s10741-019-09825-x

143 Warrier, S. *et al.* Beta-adrenergic- and muscarinic receptor-induced changes in cAMP activity in adult cardiac myocytes detected with FRET-based biosensor. *Am J Physiol Cell Physiol* **289**, C455-461 (2005). https://doi.org:10.1152/ajpcell.00058.2005

144 Gratze, G. *et al.* β-2 Adrenergic Receptor Variants Affect Resting Blood Pressure and Agonist-Induced Vasodilation in Young Adult Caucasians. *Hypertension* **33**, 1425-1430 (1999). https://doi.org:doi:10.1161/01.HYP.33.6.1425

145 Hammond, S. K. Global patterns of nicotine and tobacco consumption. *Handb Exp Pharmacol*, 3-28 (2009). https://doi.org:10.1007/978-3-540-69248-5_1

146 Dani, J. A. & Balfour, D. J. Historical and current perspective on tobacco use and nicotine addiction. *Trends Neurosci* **34**, 383-392 (2011). https://doi.org:10.1016/j.tins.2011.05.001

147 Charlton, A. Medicinal uses of tobacco in history. *J R Soc Med* **97**, 292-296 (2004). https://doi.org:10.1177/014107680409700614

148 MUSK, A. W. & DE KLERK, N. H. History of tobacco and health. *Respirology* **8**, 286-290 (2003). https://doi.org:https://doi.org/10.1046/j.1440-1843.2003.00483.x

149 Centers for Disease, C. *et al.* in *How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General* (Centers for Disease Control and Prevention (US), 2010).

150 Whitehead, A. K., Erwin, A. P. & Yue, X. Nicotine and vascular dysfunction. *Acta Physiologica* **231**, e13631 (2021). https://doi.org:https://doi.org/10.1111/apha.13631

151 Whitehead, Anna K. *et al.* Alpha7 nicotinic acetylcholine receptor mediates chronic nicotine inhalation-induced cardiopulmonary dysfunction. *Clinical Science* **136**, 973-987 (2022). https://doi.org:10.1042/cs20220083

152 Carlson, A. B. & Kraus, G. P. in *StatPearls* (StatPearls Publishing
Copyright © 2022, StatPearls Publishing LLC., 2022).

153 Cetin, H., Beeson, D., Vincent, A. & Webster, R. The Structure, Function, and Physiology of the Fetal and Adult Acetylcholine Receptor in Muscle. *Frontiers in Molecular Neuroscience* **13** (2020). https://doi.org:10.3389/fnmol.2020.581097

154 Unwin, N. Nicotinic acetylcholine receptor and the structural basis of neuromuscular transmission: insights from Torpedo postsynaptic membranes. *Q Rev Biophys* **46**, 283-322 (2013). https://doi.org:10.1017/s0033583513000061

155 Hotston, M. R. *et al.* Sildenafil inhibits the up-regulation of phosphodiesterase type 5 elicited with nicotine and tumour necrosis factor-alpha in cavernosal vascular smooth muscle cells: mediation by superoxide. *BJU Int* **99**, 612-618 (2007). https://doi.org:10.1111/j.1464-410X.2006.06618.x

156 Haass, M. & Kübler, W. Nicotine and sympathetic neurotransmission. *Cardiovasc Drugs Ther* **10**, 657-665 (1997). https://doi.org:10.1007/bf00053022

157 Sun, R., Mendez, D. & Warner, K. E. Trends in Nicotine Product Use Among US Adolescents, 1999-2020. *JAMA Network Open* **4**, e2118788-e2118788 (2021). https://doi.org:10.1001/jamanetworkopen.2021.18788

158 Choi, S., Lee, K. & Park, S. M. Combined Associations of Changes in Noncombustible Nicotine or Tobacco Product and Combustible Cigarette Use Habits With Subsequent Short-Term Cardiovascular Disease Risk Among South Korean Men: A Nationwide Cohort Study. *Circulation* **144**, 1528-1538 (2021). https://doi.org:10.1161/circulationaha.121.054967

159 Miller, C. R., Smith, D. M. & Goniewicz, M. L. Changes in Nicotine Product Use among Dual Users of Tobacco and Electronic Cigarettes: Findings from the Population Assessment of Tobacco and Health (PATH) Study, 2013–2015. *Substance Use & Misuse* **55**, 909-913 (2020). https://doi.org:10.1080/10826084.2019.1710211
160 Soneji, S., Sargent, J. & Tanski, S. Multiple tobacco product use among US adolescents and young adults. *Tobacco Control* **25**, 174 (2016). https://doi.org:10.1136/tobaccocontrol-2014-051638
161 Herman, A. I. & Sofuoglu, M. in *Interventions for Addiction* (ed Peter M. Miller) 337-343 (Academic Press, 2013).
162 Benowitz, N. L., St Helen, G., Dempsey, D. A., Jacob, P., 3rd & Tyndale, R. F. Disposition kinetics and metabolism of nicotine and cotinine in African American smokers: impact of CYP2A6 genetic variation and enzymatic activity. *Pharmacogenet Genomics* **26**, 340-350 (2016). https://doi.org:10.1097/fpc.0000000000000222
163 Gholap, V. V., Kosmider, L., Golshahi, L. & Halquist, M. S. Nicotine forms: why and how do they matter in nicotine delivery from electronic cigarettes? *Expert Opin Drug Deliv* **17**, 1727-1736 (2020). https://doi.org:10.1080/17425247.2020.1814736
164 Kumboyono, K. *et al.* Detection of Vascular Inflammation and Oxidative Stress by Cotinine in Smokers: Measured Through Interleukin-6 and Superoxide Dismutase. *Int J Gen Med* **15**, 7319-7328 (2022). https://doi.org:10.2147/ijgm.S367125
165 Tan, X., Vrana, K. & Ding, Z.-M. Cotinine: Pharmacologically Active Metabolite of Nicotine and Neural Mechanisms for Its Actions. *Frontiers in Behavioral Neuroscience* **15** (2021). https://doi.org:10.3389/fnbeh.2021.758252
166 Holloway, A. C. in *Encyclopedia of Toxicology (Third Edition)* (ed Philip Wexler) 514-516 (Academic Press, 2014).
167 Byrd, G. D., Chang, K. M., Greene, J. M. & deBethizy, J. D. Evidence for urinary excretion of glucuronide conjugates of nicotine, cotinine, and trans-3'-hydroxycotinine in smokers. *Drug Metab Dispos* **20**, 192-197 (1992).
168 Tonnessen, B., Severson, S., Hurt, R. & Miller, V. Modulation of nitric-oxide synthase by nicotine. *The Journal of pharmacology and experimental therapeutics* **295**, 601-606 (2000).
169 Neunteufl, T. *et al.* Contribution of nicotine to acute endothelial dysfunction in long-term smokers. *Journal of the American College of Cardiology* **39**, 251-256 (2002). https://doi.org:10.1016/S0735-1097(01)01732-6
170 Xue, C. *et al.* Effects of Smoking Cessation with Nicotine Replacement Therapy on Vascular Endothelial Function, Arterial Stiffness, and Inflammation Response in Healthy Smokers. *Angiology* **70**, 719-725 (2019). https://doi.org:10.1177/0003319719853458
171 Benowitz, N. L. & Gourlay, S. G. Cardiovascular toxicity of nicotine: implications for nicotine replacement therapy. *J Am Coll Cardiol* **29**, 1422-1431 (1997). https://doi.org:10.1016/s0735-1097(97)00079-x
172 Hanna, S. T. Nicotine Effect on Cardiovascular System and Ion Channels. *Journal of Cardiovascular Pharmacology* **47**, 348-358 (2006). https://doi.org:10.1097/01.fjc.0000205984.13395.9e
173 Tang, G., Hanna, S. T. & Wang, R. Effects of nicotine on K+ channel currents in vascular smooth muscle cells from rat tail arteries. *Eur J Pharmacol* **364**, 247-254 (1999). https://doi.org:10.1016/s0014-2999(98)00833-4

174 Hill, P. & Wynder, E. L. Smoking and cardiovascular disease: Effect of nicotine on the serum epinephrine and corticoids. *American Heart Journal* **87**, 491-496 (1974). https://doi.org:https://doi.org/10.1016/0002-8703(74)90174-4
175 Olfert, I. M. *et al.* Chronic exposure to electronic cigarettes results in impaired cardiovascular function in mice. *J Appl Physiol (1985)* **124**, 573-582 (2018). https://doi.org:10.1152/japplphysiol.00713.2017
176 Gourlay, S. G. & Benowitz, N. L. Arteriovenous differences in plasma concentration of nicotine and catecholamines and related cardiovascular effects after smoking, nicotine nasal spray, and intravenous nicotine. *Clinical Pharmacology & Therapeutics* **62**, 453-463 (1997). https://doi.org:https://doi.org/10.1016/S0009-9236(97)90124-7
177 Hamada, M. *et al.* Nicotine regulates DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of 32 kDa) phosphorylation at multiple sites in neostriatal neurons. *J Pharmacol Exp Ther* **315**, 872-878 (2005). https://doi.org:10.1124/jpet.105.090852
178 Sun, X., Liu, Y., Hu, G. & Wang, H. Activities of cAMP-dependent protein kinase and protein kinase C are modulated by desensitized nicotinic receptors in the rat brain. *Neurosci Lett* **367**, 19-22 (2004). https://doi.org:10.1016/j.neulet.2004.05.072
179 Benowitz, N. L. & Liakoni, E. Tobacco use disorder and cardiovascular health. *Addiction* **117**, 1128-1138 (2022). https://doi.org:10.1111/add.15703
180 Qasim, H., Karim, Z. A., Rivera, J. O., Khasawneh, F. T. & Alshbool, F. Z. Impact of Electronic Cigarettes on the Cardiovascular System. *J Am Heart Assoc* **6**, e006353 (2017). https://doi.org:10.1161/JAHA.117.006353
181 Espinoza-Derout, J. *et al.* Electronic Cigarette Use and the Risk of Cardiovascular Diseases. *Front Cardiovasc Med* **9**, 879726 (2022). https://doi.org:10.3389/fcvm.2022.879726
182 Centner, A. M., Bhide, P. G. & Salazar, G. Nicotine in Senescence and Atherosclerosis. *Cells* **9**, 1035 (2020). https://doi.org:10.3390/cells9041035
183 Mao, C. *et al.* Nicotine exacerbates atherosclerosis through a macrophage-mediated endothelial injury pathway. *Aging (Albany NY)* **13**, 7627-7643 (2021). https://doi.org:10.18632/aging.202660
184 Kim, S. Y. *et al.* High prevalence of hypertension among smokers of conventional and e-cigarette: Using the nationally representative community dwelling survey. *Frontiers in Public Health* **10** (2022). https://doi.org:10.3389/fpubh.2022.919585
185 Oparil, S. *et al.* Hypertension. *Nature Reviews Disease Primers* **4**, 18014 (2018). https://doi.org:10.1038/nrdp.2018.14
186 Kjeldsen, S. E. Hypertension and cardiovascular risk: General aspects. *Pharmacological Research* **129**, 95-99 (2018). https://doi.org:https://doi.org/10.1016/j.phrs.2017.11.003
187 Carnevale, R. *et al.* Acute Impact of Tobacco vs Electronic Cigarette Smoking on Oxidative Stress and Vascular Function. *Chest* **150**, 606-612 (2016). https://doi.org:10.1016/j.chest.2016.04.012
188 Ikonomidis, I. *et al.* Electronic Cigarette Smoking Increases Arterial Stiffness and Oxidative Stress to a Lesser Extent Than a Single Conventional Cigarette: An Acute and Chronic Study. *Circulation* **137**, 303-306 (2018). https://doi.org:10.1161/CIRCULATIONAHA.117.029153
189 Vlachopoulos, C. *et al.* Electronic Cigarette Smoking Increases Aortic Stiffness and Blood Pressure in Young Smokers. *J Am Coll Cardiol* **67**, 2802-2803 (2016). https://doi.org:10.1016/j.jacc.2016.03.569

190 He, H. *et al.* Health Effects of Tobacco at the Global, Regional, and National Levels: Results From the 2019 Global Burden of Disease Study. *Nicotine Tob Res* **24**, 864-870 (2022). https://doi.org:10.1093/ntr/ntab265
191 Bilano, V. *et al.* Global trends and projections for tobacco use, 1990-2025: an analysis of smoking indicators from the WHO Comprehensive Information Systems for Tobacco Control. *Lancet* **385**, 966-976 (2015). https://doi.org:10.1016/s0140-6736(15)60264-1
192 Sheng, Y. & Zhu, L. The crosstalk between autonomic nervous system and blood vessels. *Int J Physiol Pathophysiol Pharmacol* **10**, 17-28 (2018).
193 Cooper, D. M. F., Mons, N. & Karpen, J. W. Adenylyl cyclases and the interaction between calcium and cAMP signalling. *Nature* **374**, 421-424 (1995). https://doi.org:10.1038/374421a0
194 Polson, J. B. & Strada, S. J. Cyclic nucleotide phosphodiesterases and vascular smooth muscle. *Annu Rev Pharmacol Toxicol* **36**, 403-427 (1996). https://doi.org:10.1146/annurev.pa.36.040196.002155
195 Muntean, B. S. *et al.* Interrogating the Spatiotemporal Landscape of Neuromodulatory GPCR Signaling by Real-Time Imaging of cAMP in Intact Neurons and Circuits. *Cell Rep* **22**, 255-268 (2018). https://doi.org:10.1016/j.celrep.2017.12.022
196 Silswal, N., Parelkar, N., Andresen, J. & Wacker, M. J. Restoration of Endothelial Function in Pparα (-/-) Mice by Tempol. *PPAR Res* **2015**, 728494 (2015). https://doi.org:10.1155/2015/728494
197 Wenceslau, C. F. *et al.* Guidelines for the measurement of vascular function and structure in isolated arteries and veins. *Am J Physiol Heart Circ Physiol* **321**, H77-h111 (2021). https://doi.org:10.1152/ajpheart.01021.2020
198 Jin, L., Lipinski, A. & Conklin, D. J. A Simple Method for Normalization of Aortic Contractility. *J Vasc Res* **55**, 177-186 (2018). https://doi.org:10.1159/000490245
199 Yeung, Y. G. & Stanley, E. R. A solution for stripping antibodies from polyvinylidene fluoride immunoblots for multiple reprobing. *Anal Biochem* **389**, 89-91 (2009). https://doi.org:10.1016/j.ab.2009.03.017
200 Bass, J. J. *et al.* An overview of technical considerations for Western blotting applications to physiological research. *Scand J Med Sci Sports* **27**, 4-25 (2017). https://doi.org:10.1111/sms.12702
201 Taylor, S. C. & Posch, A. The design of a quantitative western blot experiment. *Biomed Res Int* **2014**, 361590 (2014). https://doi.org:10.1155/2014/361590
202 Zhu, Q. *et al.* Intermedin reduces neointima formation by regulating vascular smooth muscle cell phenotype via cAMP/PKA pathway. *Atherosclerosis* **266**, 212-222 (2017). https://doi.org:10.1016/j.atherosclerosis.2017.10.011
203 Fischmeister, R. & Hartzell, H. C. Cyclic AMP phosphodiesterases and Ca2+ current regulation in cardiac cells. *Life Sci* **48**, 2365-2376 (1991).
204 Netherton, S. J. & Maurice, D. H. Vascular endothelial cell cyclic nucleotide phosphodiesterases and regulated cell migration: implications in angiogenesis. *Mol Pharmacol* **67**, 263-272 (2005). https://doi.org:10.1124/mol.104.004853
205 Martinez, J. M. *et al.* Arrestin-dependent nuclear export of phosphodiesterase 4D promotes GPCR-induced nuclear cAMP signaling required for learning and memory. *Sci Signal* **16**, eade3380 (2023). https://doi.org:10.1126/scisignal.ade3380

206 Qin, X. *et al.* Smooth muscle-specific Gsalpha deletion exaggerates angiotensin II-induced abdominal aortic aneurysm formation in mice in vivo. *J Mol Cell Cardiol* **132**, 49-59 (2019). https://doi.org:10.1016/j.yjmcc.2019.05.002
207 Liu, Y. *et al.* Inhibition of adenosine uptake and augmentation of ischemia-induced increase of interstitial adenosine by cilostazol, an agent to treat intermittent claudication. *J Cardiovasc Pharmacol* **36**, 351-360 (2000). https://doi.org:10.1097/00005344-200009000-00011
208 Lugnier, C., Al-Kuraishy, H. M. & Rousseau, E. PDE4 inhibition as a therapeutic strategy for improvement of pulmonary dysfunctions in Covid-19 and cigarette smoking. *Biochem Pharmacol* **185**, 114431 (2021). https://doi.org:10.1016/j.bcp.2021.114431
209 Lugnier, C. Cyclic nucleotide phosphodiesterase (PDE) superfamily: A new target for the development of specific therapeutic agents. *Pharmacology & Therapeutics* **109**, 366-398 (2006). https://doi.org:https://doi.org/10.1016/j.pharmthera.2005.07.003
210 Cullen, K. A. *et al.* e-Cigarette Use Among Youth in the United States, 2019. *Jama* **322**, 2095-2103 (2019). https://doi.org:10.1001/jama.2019.18387
211 Schutzer, W. E., Beard, D. R., Reed, J. F. & Mader, S. L. Characterization of Clonal Vascular Smooth Muscle Cell Lines Derived from Young and Old Fischer 344 Rats. *In Vitro Cellular & Developmental Biology - Animal* **47**, 445-450 (2011). https://doi.org:10.1007/s11626-011-9430-7
212 Schutzer, W. E., Reed, J. F. & Mader, S. L. Decline in caveolin-1 expression and scaffolding of G protein receptor kinase-2 with age in Fischer 344 aortic vascular smooth muscle. *Am J Physiol Heart Circ Physiol* **288**, H2457-2464 (2005). https://doi.org:10.1152/ajpheart.01090.2004
213 Schutzer, W. E., Reed, J. F., Bliziotes, M. & Mader, S. L. Upregulation of G protein-linked receptor kinases with advancing age in rat aorta. *Am J Physiol Regul Integr Comp Physiol* **280**, R897-903 (2001). https://doi.org:10.1152/ajpregu.2001.280.3.R897
214 Fantidis, P. *et al.* [Effect of cAMP on the function of endothelial cells and fibromuscular proliferation after the injury of the carotid and coronary arteries in a porcine model]. *Rev Esp Cardiol* **54**, 981-989 (2001). https://doi.org:10.1016/s0300-8932(01)76434-2
215 Ronkainen, A. & Hernesniemi, J. in *Stroke (Fourth Edition)* (eds J. P. Mohr *et al.*) 1211-1216 (Churchill Livingstone, 2004).
216 Petrik, P. V., Gelabert, H. A., Moore, W. S., Quinones-Baldrich, W. & Law, M. M. Cigarette Smoking Accelerates Carotid Artery Intimal Hyperplasia in a Dose-Dependent Manner. *Stroke* **26**, 1409-1414 (1995). https://doi.org:doi:10.1161/01.STR.26.8.1409
217 Matsumoto, T., Kobayashi, T. & Kamata, K. Phosphodiesterases in the vascular system. *J Smooth Muscle Res* **39**, 67-86 (2003). https://doi.org:10.1540/jsmr.39.67
218 Fan, T. *et al.* Phosphodiesterase 4D promotes angiotensin II-induced hypertension in mice via smooth muscle cell contraction. *Communications Biology* **5**, 81 (2022). https://doi.org:10.1038/s42003-022-03029-0
219 Benowitz, N. L. Safety of nicotine in smokers with hypertension*. *American Journal of Hypertension* **14**, 731-732 (2001). https://doi.org:10.1016/s0895-7061(01)02171-9
220 Kaplan, R. C. *et al.* Current Smoking Raises Risk of Incident Hypertension: Hispanic Community Health Study-Study of Latinos. *Am J Hypertens* **34**, 190-197 (2021). https://doi.org:10.1093/ajh/hpaa152

221 Virdis, A., Giannarelli, C., Neves, M. F., Taddei, S. & Ghiadoni, L. Cigarette smoking and hypertension. *Curr Pharm Des* **16**, 2518-2525 (2010). https://doi.org:10.2174/138161210792062920
222 Piano, M. R. *et al.* Impact of smokeless tobacco products on cardiovascular disease: implications for policy, prevention, and treatment: a policy statement from the American Heart Association. *Circulation* **122**, 1520-1544 (2010). https://doi.org:10.1161/CIR.0b013e3181f432c3
223 Movsesian, M. A. PDE3 cyclic nucleotide phosphodiesterases and the compartmentation of cyclic nucleotide-mediated signalling in cardiac myocytes. *Basic Res Cardiol* **97 Suppl 1**, I83-90 (2002). https://doi.org:10.1007/s003950200035
224 Choi, Y. H. *et al.* Identification of a novel isoform of the cyclic-nucleotide phosphodiesterase PDE3A expressed in vascular smooth-muscle myocytes. *Biochem J* **353**, 41-50 (2001).
225 Nogueira, L. *et al.* Cigarette smoke directly impairs skeletal muscle function through capillary regression and altered myofibre calcium kinetics in mice. *J Physiol* **596**, 2901-2916 (2018). https://doi.org:10.1113/jp275888
226 Benowitz, N. L. & Hatsukami, D. Gender differences in the pharmacology of nicotine addiction. *Addict Biol* **3**, 383-404 (1998). https://doi.org:10.1080/13556219871930
227 Pogun, S. & Yararbas, G. in *Nicotine Psychopharmacology* (eds Jack E. Henningfield, Edythe D. London, & Sakire Pogun) 261-291 (Springer Berlin Heidelberg, 2009).
228 Chen, A. *et al.* Sex/Gender Differences in Cotinine Levels Among Daily Smokers in the Pennsylvania Adult Smoking Study. *J Womens Health (Larchmt)* **26**, 1222-1230 (2017). https://doi.org:10.1089/jwh.2016.6317
229 Evans, S. E., Blank, M., Sams, C., Weaver, M. F. & Eissenberg, T. Transdermal nicotine-induced tobacco abstinence symptom suppression: nicotine dose and smokers' gender. *Exp Clin Psychopharmacol* **14**, 121-135 (2006). https://doi.org:10.1037/1064-1297.14.2.121
230 Chang, J. T., Anic, G. M., Rostron, B. L., Tanwar, M. & Chang, C. M. Cigarette Smoking Reduction and Health Risks: A Systematic Review and Meta-analysis. *Nicotine Tob Res* **23**, 635-642 (2021). https://doi.org:10.1093/ntr/ntaa156
231 Centner, A. *et al.* Sex Differences in Atherosclerosis in ApoE-/- Mice exposed to Nicotine and Cigarette Smoke. *The FASEB Journal* **35** (2021). https://doi.org:https://doi.org/10.1096/fasebj.2021.35.S1.04018
232 Fox, K. A. *et al.* Sex-specific effects of nicotine exposure on developmental programming of blood pressure and vascular reactivity in the C57Bl/6J mouse. *American Journal of Obstetrics and Gynecology* **207**, 208.e201-208.e209 (2012). https://doi.org:https://doi.org/10.1016/j.ajog.2012.06.021
233 Chang, H.-R. *et al.* Reduced vascular β-adrenergic receptors and catecholamine response in rats with lead induced hypertension. *Archives of Toxicology* **71**, 778-781 (1997). https://doi.org:10.1007/s002040050460
234 Costantino, S., Paneni, F. & Cosentino, F. Ageing, metabolism and cardiovascular disease. *J Physiol* **594**, 2061-2073 (2016). https://doi.org:10.1113/jp270538
235 Elvebak, R. L., Eisenach, J. H., Joyner, M. J. & Nicholson, W. T. The Function of Vascular Smooth Muscle Phosphodiesterase III is Preserved in Healthy Human Aging. *Clinical and Translational Science* **3**, 239-242 (2010). https://doi.org:https://doi.org/10.1111/j.1752-8062.2010.00232.x

236 Murrar, S., Johnson, P. A., Lee, Y. G. & Carnes, M. Research Conducted in Women Was Deemed More Impactful but Less Publishable than the Same Research Conducted in Men. *J Womens Health (Larchmt)* **30**, 1259-1267 (2021). https://doi.org:10.1089/jwh.2020.8666

237 Liu, K. A. & Mager, N. A. Women's involvement in clinical trials: historical perspective and future implications. *Pharm Pract (Granada)* **14**, 708 (2016). https://doi.org:10.18549/PharmPract.2016.01.708

238 Bredella, M. A. Sex Differences in Body Composition. *Adv Exp Med Biol* **1043**, 9-27 (2017). https://doi.org:10.1007/978-3-319-70178-3_2

239 Regitz-Zagrosek, V. Sex and gender differences in health. Science & Society Series on Sex and Science. *EMBO Rep* **13**, 596-603 (2012). https://doi.org:10.1038/embor.2012.87

240 Lefever, T. W., Thomas, B. F., Kovach, A. L., Snyder, R. W. & Wiley, J. L. Route of administration effects on nicotine discrimination in female and male mice. *Drug Alcohol Depend* **204**, 107504 (2019). https://doi.org:10.1016/j.drugalcdep.2019.06.007

241 Raymond, B. H., Collette-Merrill, K., Harrison, R. G., Jarvis, S. & Rasmussen, R. J. The Nicotine Content of a Sample of E-cigarette Liquid Manufactured in the United States. *J Addict Med* **12**, 127-131 (2018). https://doi.org:10.1097/adm.0000000000000376

242 Dawkins, L. E., Kimber, C. F., Doig, M., Feyerabend, C. & Corcoran, O. Self-titration by experienced e-cigarette users: blood nicotine delivery and subjective effects. *Psychopharmacology (Berl)* **233**, 2933-2941 (2016). https://doi.org:10.1007/s00213-016-4338-2

243 National Academies of Sciences, E. *et al.* in *Public Health Consequences of E-Cigarettes* (eds D. L. Eaton, L. Y. Kwan, & K. Stratton) (National Academies Press (US)
Copyright 2018 by the National Academy of Sciences. All rights reserved., 2018).