

Vascular effects of hydrogen sulphide - influence of O_2

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Abstract

Hydrogen sulphide (H_2S), a toxic gas, has recently been shown to be generated endogenously by biological tissue and it has emerged as a novel and important biological mediator in the vascular system. In blood vessels, H_2S has been shown to produce complex actions, including both vasorelaxation and vasoconstriction, through different mechanisms, depending on vessel origin and oxygen levels. This thesis examined the effects of H_2S on blood vessels and examined the influence of oxygen concentration on vascular responsiveness and the mechanisms involved. This study also investigated whether H_2S contributed to the hypoxic vascular response. Porcine splenic and pulmonary vessels and human chorionic blood vessels were set up in an isometric tension recording system, blood vessels were pre-contracted and responses to the H_2S donor, NaHS, were produced in the presence and absence inhibitors, under 95% O_2:5% CO_2, 95% Air: 5% CO_2 and 95% N_2:5% CO_2 gassing conditions. Western blot analysis was carried out to determine Cystathionine β synthase (CBS) and Cystathionine γ lyase (CSE) expression in porcine splenic and pulmonary arteries.

Changing oxygen concentrations altered the nature of the response observed with NaHS. Generally, in both porcine splenic arteries (PSA) and porcine pulmonary veins (PPV), gassing with physiological levels of O_2 (95% air: 5% CO_2), was associated with NaHS causing a contractile response, while, gassing with sub-physiological levels (95% N_2: 5% CO_2), NaHS caused a relaxant response in PSA. The nitric oxide (NO) synthase inhibitor L-NAME, significantly abolished contractile response in PSA, indicating an interaction with NO. In porcine splenic veins (PSV) and porcine pulmonary arteries (PPA)

under physiological gassing condition (95% N_2: 5% CO_2), NaHS-induced vasorelaxation was observed. The vasorelaxatory responses were abolished by L-NAME, showing that NaHS is activating NOS to generate NO and cause vasorelaxation. In human chorionic blood vessels, NaHS caused vasoconstriction under physiological gassing condition (95% N_2: 5% CO_2). L-NAME was without effect on the contraction, and so NO is not involved in this human blood vessel.

Both CBS and CSE are expressed in porcine splenic and pulmonary arteries. L-cysteine induced a contractile response in PSA and PPA but this was not blocked by the H_2S synthesis- enzymes inhibitors, indicating that L-cysteine-elicited contraction was not through a H_2S-dependent mechanism. Hypoxia caused a biphasic response in the PSA; a transient contraction followed by prolonged relaxation. The relaxation response was attenuated by AOAA, suggesting that H_2S participates in vascular responses to hypoxia with CBS the likely source of H_2S production. Hypoxia-induced vasoconstriction was observed in both PSA and PPA. The vasocontractile responses were abolished by L-NAME, showing that hypoxia decreases the sustained release of NO from the vascular endothelium.

In summary, these data show that O_2 influenced H_2S responses. At more physiological levels of O_2 the predominant response is vasoconstriction, often mediated by H_2S 'mopping up' endogenous NO. The predominant response in PSV and PPA was vasorelaxation, evoked by activating NOS to generate NO. Responses to hypoxia, in part, involve endogenous H_2S generation, suggesting that H_2S can act as an oxygen sensor in PSA.

Thesis related abstract publications

1. **EO Yousef**, L Leach, WR Dunn. (2016). Effects of hydrogen sulphide on human chorionic blood vessels. In Lumps and Pumps. Nottingham.

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"*In the name of God, the Gracious, the Merciful - Praise be to God, Lord of the Worlds*"

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Abbreviations

^0C	Temperature degrees in Celsius
3-MST	3-Mercaptopyruvate sulfurtransferase
4-AP	4- aminopyridine
AA	Arachidonic acid
AC	Adenylyl cyclase
Ach	Acetylcholine
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AOAA	Aminooxyacetic acid
ATP	Adenosine triphosphate
AT_1	Angiotensin II
BK	Bradykinin
BK_{ca}	Large conductance calcium-activated potassium channel
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
Ca_i	Intracellular calcium concentration
CaM	Calcium-calmodulin complex
CAT	Cysteine aminotransferase
CBS	Cystathionine β-synthase
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin-gene related peptide
CO	Carbon monoxide
CO_2	Carbon dioxide
COX	Cyclooxygenase
CSE	Cystathionine γ-lyase
$CuSO_4$	Copper sulphate
CVS	Cardiovascular system
DAG	Diacylglycerol
DAO	D-amino acid oxidase
DMSO	Dimethyl sulphoxide
EDH	Endothelial derived hyperpolarising

EDTA	Ethylenediaminetetraacetic acid disodium salt
EET_S	Epoxyeicosatrienoic acids
ET-1	Endothelin-1
g	Grams
GABA	Gamma-aminobutyric acid
GC	Guanylyl cyclase
GSSH	Glutathione persufide
GSSG	Glutathione disulfide
GTP	Guanosine triphosphate
HNO	Nitroxyl
H_2O_2	Hydrogen peroxide
HPV	Hypoxic pulmonary vasoconstriction
H_2S	Hydrogen sulfide
Ig	Immunoglobulin
IK_{Ca}	Intermediate conductance potassium channel
IP_3	Inositol-1, 4, 5 triphosphate
K^+	Potassium ion
K_{ATP}	Adenosine triphosphate sensitive potassium channel
KCa	Calcium-activated potassium channels
KCl	Potassium chloride
KIR	Inwardly rectifying potassium channels
Kv	Voltage-gated potassium channels
L-NAME	N^G-nitro-L-arginine methyl ester
LTP	Long Term Potentiation
M	Molar
Mg^{2+}	Magnesium ion
ml	Millimetre
MAPK	Mitogen-activated protein kinase
ML	Myosin light chain
MLC	Myosin-light-chain
MLCP	Myosin-light-chain phosphatase
MLCK	Myosin light chain kinase
mmHg	Millimeters of Mercury
mM	Millimolar

MPO	Myeloperoxidase
NaHS	Sodium hydroxide
Na_2S	Sodium sulfide
NADPH	Nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl-d-aspartate
NO	Nitric oxide
NO_2	Nitrogen Dioxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
NOS_1	Neuronal nitric oxide synthase
NOS_2	Inducible nitric oxide synthase
NOS_3	Endothelial nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
iNOS	Inducible nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
O_2	Oxygen
O_2^-	Superoxide radical
$ONOO^-$	Peroxynitrite
PAG	DL-propargylglycine
PDE-5	Phosphodiestrase-5
PGE_2	Dinoprostone
PGI_2	Prostacyclin
PIP_2	Phosphotidylinositol 4, 5-Bisphosphate
PKC	Protein Kinase C
PKA	Protein Kinase A
PLC	Phospholipase C
PLP	Pyridoxal-5- phosphate
PO_2	Partial pressure of oxygen
ppm	parts per million
PSS	Physiological salt solution
P-value	Probability value
ROS	Reactive oxygen species
R-SO	Sulfenyl

RyR	Ryanodine Receptor
SAM	S-Adenosylmethionine
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sGC	Soluble guanylate cyclase
SKca	Calcium sensitive potassium channels
S.E.M	Standard error of mean
SMCs	Smooth muscle cells
SNP	Sodium nitroprusside
$S_2O_3^{2-}$	Thiosulfate
SO_3^{2-}	Sulfite
SO_4^{2-}	Sulfate
SQOR	Sulfide quinine oxidoreductase
SR	Sarcoplasmic reticulum
ST	Sulfur transferase
TASK	Acid-sensitive potassium channel
TB	Transfer buffer
TBST	Tris-buffered saline and tween 20
$TRPV_1$	Transient receptor potentials vanilloid receptor 1
TSMT	Thiol-s-methyl transferase
TST	Thiosulfate sulfurtransferase
U46619	9, 11-dideoxy-9α, 11α -epoxymethanoprostaglandin F2α
UK	United Kingdom
USA	United States of America
V_1	Vasopressin receptor
v/v	Volume in milliltres per unit volume in millilitres
VSMCs	Vascular smooth muscle cells
w/v	Weight in milligrams per unit volume in millilitres
XO	Xanthine oxidase
µM	Micromolar

Chapter 1:

General introduction

1-Introduction

1.1 The Heart and vasculature

The function of the heart and blood vessels is to facilitate the exchange of gases, fluids, electrolytes and other molecules, ensuring that an adequate blood flow is distributed to organs by maintaining an appropriate cardiac output and blood pressure. The delivery of blood to the regions of the body involves passage through large and small arteries and arterioles, and the return of blood to the heart via the venous circulation (Klabunde, 2012a). As blood move away from the heart blood vessels decreases in size (arteries and arterioles), ending in the capillaries, before they increase in size as they move toward the heart (venules and veins). The function of large arteries is mass transport while small arteries and arterioles contribute to vascular resistance and the distribution of blood flow. Capillaries participate in the exchange of nutrients and gases in tissue while venules contribute to postcapillary resistance, and larger venules and veins regulate the total capacitance of the circulatory system (figure1.1). (Klabunde, 2012a)

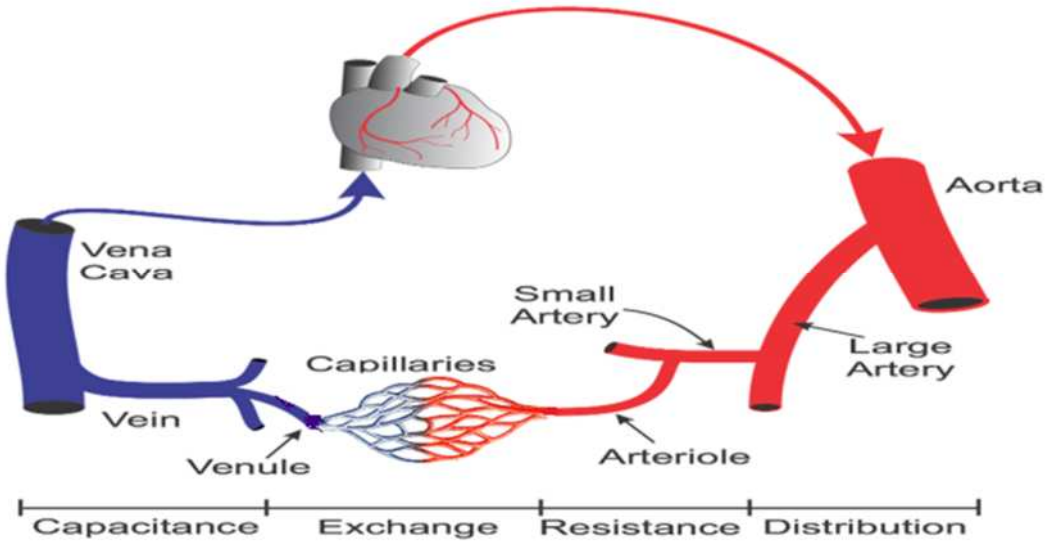


Figure 1.1 A diagram representing the circulation. Figure adapted from Klabunde, (2005a).

1.2 Circulatory system

The cardiovascular system provides oxygen and nutrients to tissues while removing waste driven by a dual pump delivering blood to the systemic and pulmonary circulation.

1.2.1 Systemic circulation

The systemic circulation is a circuit of vessels that carries oxygen - rich blood from the heart through the body via the aorta and returns deoxygenated blood from the tissues of the body back to the heart via the venous circulation. The main function of the systemic circulation is to distribute oxygen and nutrients throughout the body whilst removing waste products (Widmaier *et al.,* 2016).

Oxygenated blood from the lungs passes into the left atrium of the heart from the pulmonary veins. Blood then flows into the left ventricle and leaves the heart through the aortic valve into the aorta which subsequently branches into many small arteries which carry oxygenated blood to the different organs in the body. Arteries branch into arterioles within organs or tissues before delivering blood to the capillaries. Blood enters the capillaries where the exchange of oxygen and nutrients occurs .Waste products and carbon dioxide are moved back to the blood and deoxygenated blood flows backs via the venules and veins to return to the right atrium of the heart through two large veins called the superior vena cava and inferior vena cava in order to circulate back to the heart before delivering to the pulmonary circulation to allow the exchange of gases in the lungs (Tortora and Derrickson, 2017) (figure1.2).

1.2.2 Pulmonary circulation

The main function of the pulmonary circulation is to enable gas exchange. From the right ventricle, the oxygen-deprived blood pumps to the pulmonary circuits through the pulmonary trunk, which divides into the right and left pulmonary arteries carrying blood to the right and left lungs, respectively. This blood (low oxygen) is carried to the alveoli of lungs, where oxygen exchanges from the tiny air sacs in the lungs, via the walls of capillaries, into the blood. Carbon dioxide moves from the blood into the air sacs. Once the blood is oxygenated, it returns back to the left atrium of the heart via four pulmonary veins (two each lung), completing the vascular loop of the pulmonary circuit (Tortora and Derrickson, 2017, Widmaier *et al.*, 2016) (figure1.2).

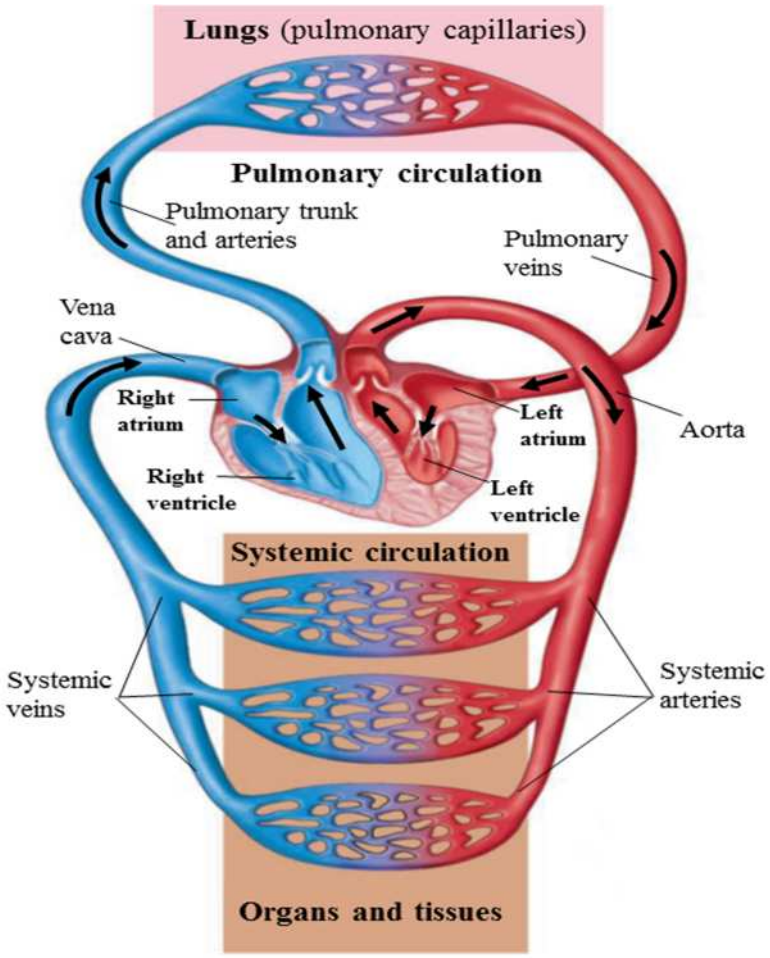


Figure 1.2 Circulatory system. Figure adapted from Widmaier *et al.*, (2016).

1.2.3 Placental circulation

One of the original initiation of this thesis was to examine blood vessels obtained from the human placenta. The placenta has a dual circulation: (1) the maternal-placental (uteroplacental) and (2) the fetal-placental (fetoplacental) circulation (contained in the system of umbilical and placental vessels) (figure 1.3). The uteroplacental circulation starts with the maternal blood flow under reduced pressure into the intervillous space via spiral arteries. In the intervillous space exchange of oxygen and nutrients takes place as the maternal blood flows around terminal villi (Carlson, 2018). The stream of maternal arterial blood pushes deoxygenated blood into the endometrial and then uterine veins back to the maternal circulation (plate of the placenta). Through the two umbilical arteries the fetal-placental circulation allows the carriage of deoxygenated and nutrient-consumed fetal blood from the fetus to the villous core fetal vessels. After the exchange of oxygen and nutrients, the umbilical vein carries fresh oxygenated and nutrient-rich blood circulating back to the fetal systemic circulation (Wang, 2010).

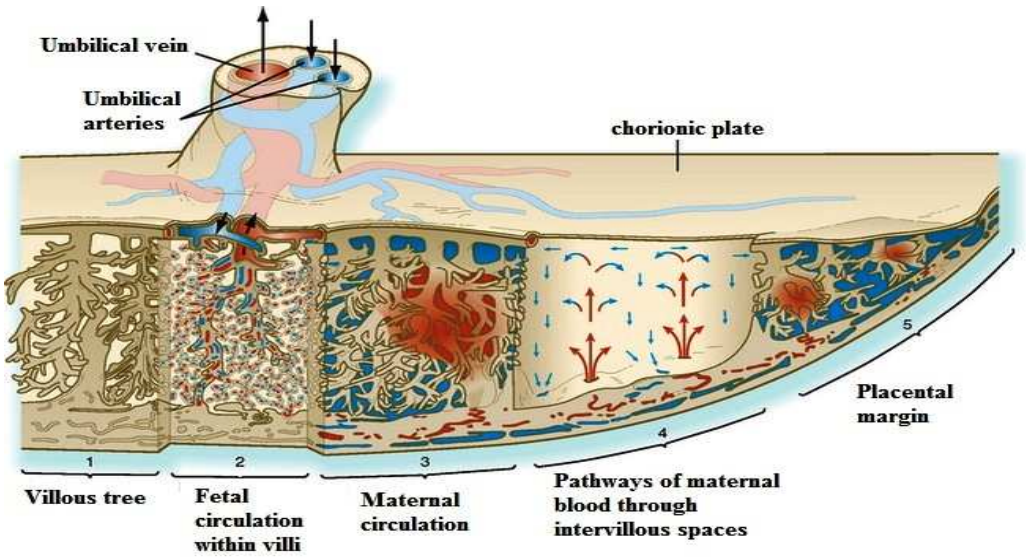


Figure 1.3 Human placental structural and circulation. Figure adapted from Fawcett (1994).

1.2.3.1 Placental structure and function

The human placenta is a complex vascular organ that acts as the mediator between the mother and fetus. It plays a pivotal role in pregnancy as it represents the vascular loop by which exchange of gas, nutrients, fetal waste removal, and secretions of hormones occurs.

The mature placenta is a disc-shaped organ with a thickness of 2-3 cm, a diameter of 15-20 cm and weighs about 500 g. The basic structural unit of the placenta is the chorionic villus. The villi are vascular projections of fetal tissue surrounded by chorion. The villus core consists of two cellular layers: (1) the outer syncytiotrophoblast which is in direct contact with maternal blood within the intervillous space, and (2) the inner cytotrophoblast (Carlson, 2018). At the junction of umbilical cord and placenta, the umbilical arteries divide into eight or more terminal chorionic plate arteries and traverse the fetal surface of the placenta in the chorionic plate and branch further before they enter into the villi and end as capillaries within the villi. The chorionic arteries are easily recognized because they always cross over the chorionic veins (Wang, 2010). The mean luminal diameter of the chorionic vessels is reported to be in the range of 250 to 350 μm (Wareing *et al.*, 2006). In the absence of nerves in the placenta, vasomotor control of the resistance arteries is performed by one, or a combination of, the following: the vascular endothelium (producing a variety of vasoactive substances such as NO), circulating factors, local paracrine effects or local physical factors (Wareing *et al.*, 2003).

1.3 Vascular structure and function

Large blood vessels are composed of three layer namely the intima (which lines the blood vessels and is composed of a single layer of simple squamous epithelium known as endothelial cells), the media (composed of vascular smooth muscle cells connected together by an extracellular matrix of elastin and collagen), and the adventitia (containing fibroelastic tissue and collagen fibers, sympathetic efferent nerves that innervate the vessel wall, and vasa vasorum) (in large vessels) (figure1.4). Vascular smooth muscle cells (VSMCs) are present in the media and align circumferentially. They have a spindle shape and a typical size of 5-10 µm in diameter and their sizes vary from 50-200 µm in length (Consighny, 1991, Klabunde, 2012a, Matsumoto and Nagayama, 2012).

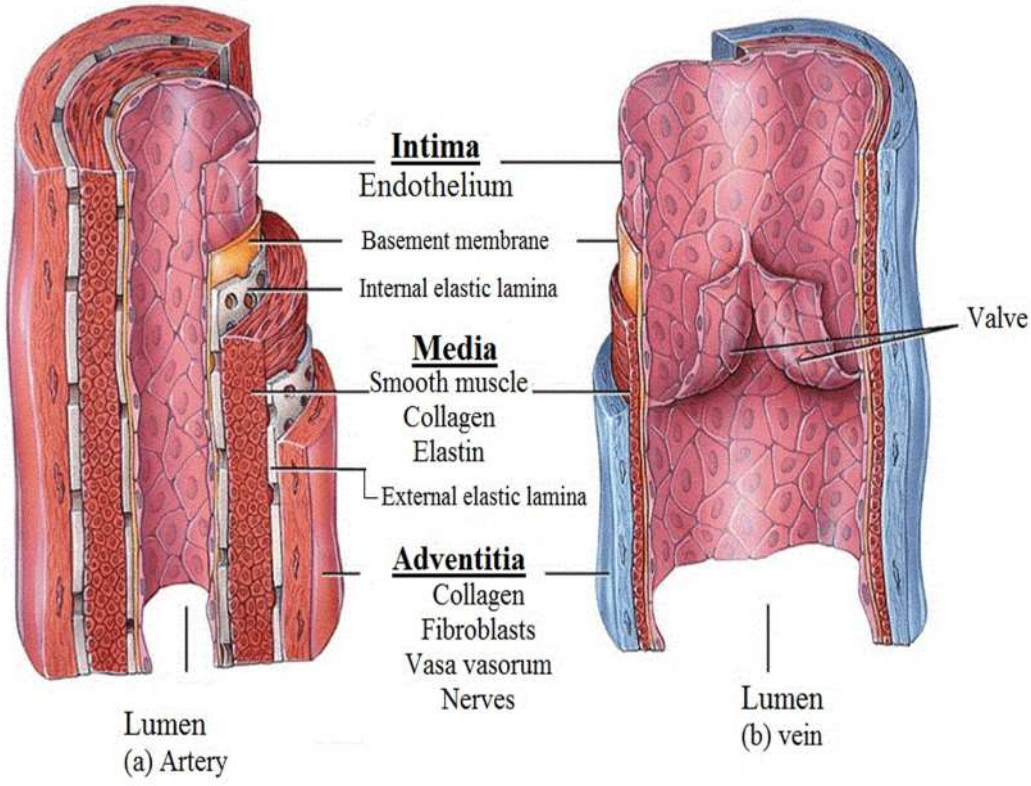


Figure 1.4 A diagram representing the blood vessel components. Figure adapted from Tortora and Derrickson (2017).

Alterations in vascular smooth muscle activity is responsible for the control of total peripheral resistance, arterial and venous tone, and the distribution of blood flow through the body. Vascular smooth muscle cells contain large numbers of thin filaments and smaller numbers of thick myosin filaments, these filaments are arranged in the long axis of the cell (Pappano and Wier, 2013).

1.4 Vascular smooth muscle contraction

Vascular smooth muscle cells contract by either, electromechanical coupling which refers to stimuli directly initiating a change in membrane potential (for instance, cell stretch) or pharmacomechanical coupling which refers to receptor – mediated stimuli or a combination of both. This can involve activation of G-protein signalling and generation of second messengers (including IP_3 and DAG) (Matsumoto and Nagayama, 2012). Vascular smooth muscles contract slower than it is in skeletal muscle and the longer period of contraction develops high forces (Pappano and Wier, 2013). The contractile activity of VSMCs is generally controlled by neuronal or humoral stimuli although this varies in different vessels. In smooth muscle cells, the interaction between myosin and actin, which leads to contraction, is controlled by the cytoplasmic calcium concentration (Pappano and Wier, 2013).

1.4.1 Calcium-dependent mechanisms vascular smooth muscle contraction.

Raised Ca^{2+} levels in response to mechanical, humoral, or neural stimuli is a key event in vascular smooth muscle contraction (Touyz *et al.*, 2018). Intracellular calcium is increased in different ways. Ca^{2+} ions can enter the through L-type voltage operated calcium channels which are activated by membrane depolarization which can occur in response to the stretching of

smooth muscle cells. Receptor operated calcium channels are activated via certain agonists acting on membrane receptors; or store–operated calcium channels (activated after depletion of sarcoplasmic reticulum's calcium stores) (Pappano and Wier, 2013). The resultant increase in free calcium leads to a specific calcium binding protein called calmodulin forming a calcium-calmodulin complex (CaM), resulting in activation of the important CaM target enzyme (myosin lights chain Kinase) and phosphorylation catalysis of myosin light chain (ML) in the presence of adenosine triphosphate (ATP). This phosphorylation reaction triggers cross bridge cycling of myosin heads along the actin filaments and causes contraction (figure 1.5) (Walsh, 2011, Pappano and Wier, 2013).

Ca^{2+} is also released into the cystol from the sarcoplasmic reticulum (SR) (cellular stores) by activation of either ryanodine receptor channels, or Inositol-1, 4, 5 triphosphate (IP_3) receptor channels. Many circulating hormones and neurotransmitters including noradrenaline, adrenaline (α_1-adrenoreceptor), angiotensin II (AT_1 receptor), Vasopressin (V1) receptor and endothelin-1 (ET-1) stimulate phospholipase C via the G_q protein, leading to the formation of IP3 from phosphatidylinositol (PIP_2). After the generation of IP3 and diacyglycerol (DAG) from PIP_2. IP_3 activates the SR to release Ca^{2+}, and DAG stimulates protein kinase C that can modulate vascular tone through protein phosphorylation (Klabunde 2012b, Pappano and Wier, 2013). PKC has contraction-promoting effects such as phosphorylation of L-type Ca^{2+} channels or cross bridge proteins, L-type Ca^{2+} channels in the membrane also activate and open in response to depolarization of the membrane by stretching of smooth muscle cells (figure 1.5) (Webb, 2003).

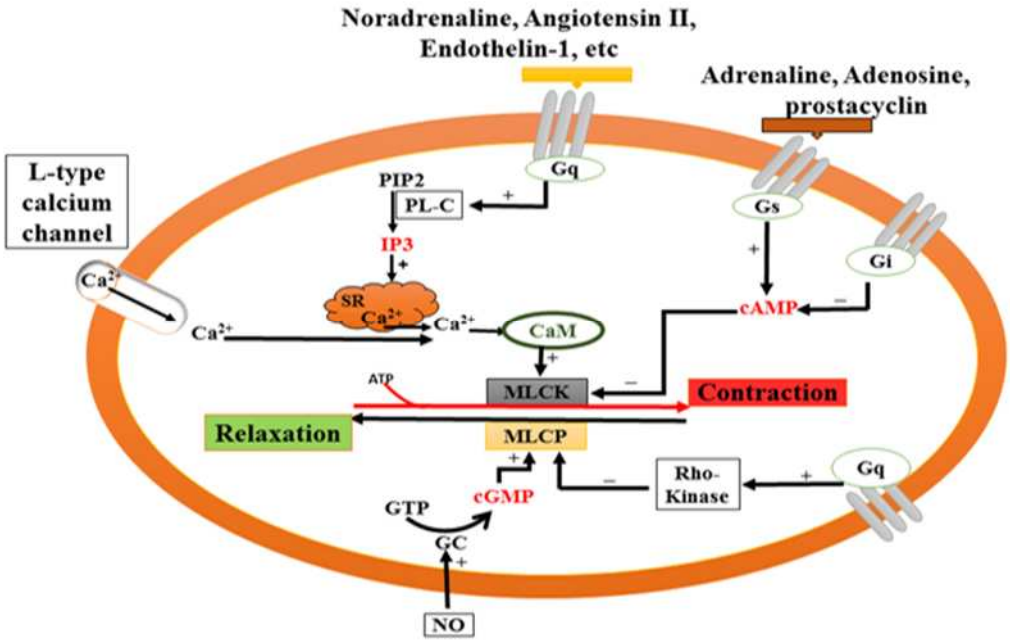


Figure 1.5 Receptor and Signal transduction pathways that regulate smooth muscle contraction and relaxation. PIP_2, phosphatidylinositol; PL-C, phospholipase C; IP3, Inositol-1,4,5 triphosphate; SR, sarcoplasmic reticulum; Ca^{2+}, Calcium; CaM, calmodulin; cAMP, cyclic adenosine monophosphate; MLCK, myosin lights chain Kinase; MLCP, myosin lights chain Phosphatase; ATP, adenosine triphosphate; cGMP, cyclic guanosine monophosphate; GTP, guanosine triphosphate; GC, guanylyl cyclase; NO, Nitric Oxide; Gs, stimulatory G-protein; Gi, inhibitory G-protein; Gq, phospholipase C-coupled G-protein. Adapted from (Klabunde, 2012b).

1.4.2 Other mechanisms of vascular smooth muscle contraction.

Other processes regulate vascular smooth muscle contraction by influencing the sensitivity of MLC to Ca^{2+}. Several signalling pathways have been implicated in mediating Ca^{2+} sensitization, including the DAG-PLC-PKC pathway and the RhoA-Rho kinase (ROCK) pathway. The mechanism of Ca^{2+} sensitization is by regulation of the phosphorylation state of MLC20, independently of calcium–calmodulin–MLCK signalling (Touyz *et al.*, 2018). All of these signalling pathways terminate in inhibition of MLCP and an increase in myosin phosphorylation, which accounts for the contractile response (Deng *et al.*, 2001). DAG acts as a second messenger inducing activation of PKC. Activated PKC induces phosphorylation of CPI-17, a smooth muscle-specific

inhibitor of MLC phosphatase (MLCP), which inhibits phosphatase activity, promoting contraction. PKC also induces phosphorylation of actin-binding proteins, which promote actin–myosin interaction and vascular smooth muscle cell contraction (Khalil, 2013, Ringvold and Khalil, 2017). The other system that sensitizes myosin to Ca^{2+} involves Rho kinase. Rho kinase influences Ca^{2+} sensitization via stimulating phosphorylation of myosin-binding subunits of MLC phosphatase, inhibiting its activity and promoting contraction (Rahman *et al.,* 2014, Behuliak *et al.,* 2017)

1.5 Vascular smooth muscle relaxation

The relaxation of vascular smooth muscle is elicited by decreasing the intracellular Ca^{2+} concentration and/or increasing MLC Phosphatase activity. This occurs either through removal of the contractile agonist or by directly inhibiting the contractile mechanisms. The decrease of cytosolic Ca^{2+} ions involves the sarcoplasmic reticulum and plasma membrane. ATP hydrolysis is responsible for Ca^{2+} ions uptake into the SR. The translocation of Ca^{2+} ions stored in the lumen of the SR depends on the phosphorylated Ca, Mg-ATPase. The Ca, Mg-ATPase enzyme is present in the plasma membrane and it provides a mechanism to decrease intracellular calcium concentration. The concentration of Ca^{2+} in the cell can also be decreased through a Na/Ca exchange channel which is located on the plasma membrane. In addition, decrease activity of receptor-operated and voltage-gated channels are important in decreasing Ca^{2+} influx and reducing contraction of the smooth muscle (Webb, 2003).

1.5.1 Cyclic nucleotide-mediated vasorelaxation pathway

Vascular smooth muscle relaxation can be elicited by the elevation of cyclic nucleotides (cAMP and cGMP) levels in VSM cells. They are generated by the activity of adenylate and guanylate cyclases, respectively. They exert their intracellular effects by activating a specific protein kinase: cAMP-dependent protein kinase (PKA) or cGMP-dependent protein kinase (PKG), accordingly.

1.5.1.1 cAMP-dependent vasorelaxation pathway

Cyclic AMP is synthesized from intracellular ATP by adenylate cyclase, which is activated by certain agonists such as (β_2-adrenergic agonists (adrenaline, isoproterenol), adenosine, or prostaglandins (PGE_2 and PGI_2)). By binding to their receptors there is subsequent activation of $G_{\alpha s}$ -proteins which stimulate adenylyl cyclase which in turn accelerates the production of cAMP by dephosphorylating ATP. Each G-protein is a trimer composing of Gα, Gβ and Gγ subunits. Gα consists of Gi and Gs that is specifically responsible for inhibition and activation of adenylyl cyclase, respectively. Adenylate cyclase converts ATP into cAMP once activated. Cyclic AMP activates cAMP-dependent protein kinase (AMPK). This kinase provides arterial relaxation by (a) phosphorylating myosin light chain kinase at a site that decreases the affinity of kinase for the calcium-calmodulin complex, (b) promoting calcium pumps in the cell membrane and sarcoplasmic reticulum, and (c) opening potassium channels through which potassium can exit and thereby hyperpolarize the cell. Cyclic AMP is readily converted into an inactive form, 5'AMP, by phosphodiesterase enzymes and therefore the duration of cAMP activation of the kinase is limited (Sassone-Corsi, 2012).

1.5.1.2 The endothelium-dependent nitric oxide–cGMP pathway

Over two decades ago, the effect of NO on blood vessels was examined (Furchogott and Zawadzki, 1980). NO is a colourless, odourless and flammable gas. It is oxidised in air to NO_2 (Li *et al*., 2009). NO is synthesized physiologically by the group of enzymes known as nitric oxide synthases, through the conversion of the amino acid L-arginine to citrulline in the presence two co-factors; oxygen and adenine dinucleotide phosphate (NADPH) (figure 1.6).

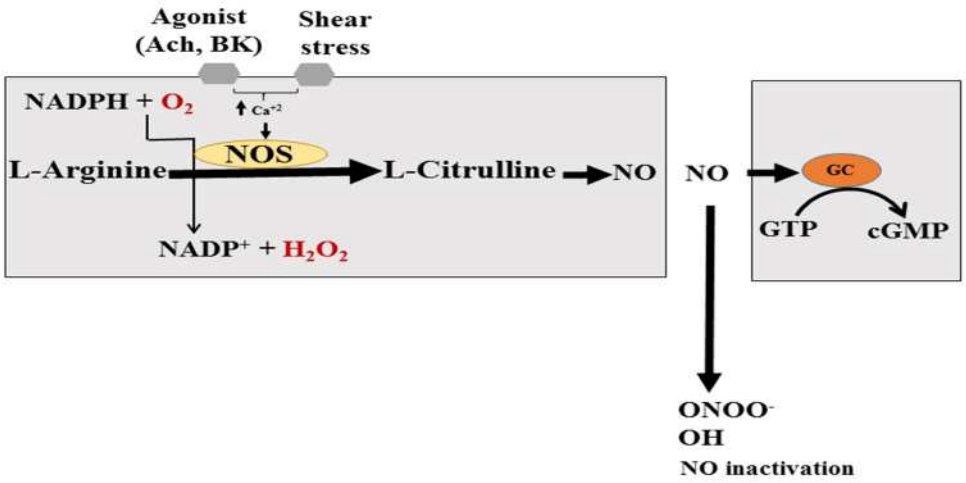


Figure 1.6 Biosynthesis of NO from (L)-arginine.

There are three isoforms of NOS named on the basis of their action as follow:

(1) Neuronal NOS (nNOS, also referred as NOS1) which is present both in the central and peripheral nervous system. In the CNS, NO acts as a neurotransmitter and has been implicated in the regulation of neuronal excitability and in memory and learning processes (Bryan *et al*., 2009, Zhao *et al*., 2015). In addition, nNOS is expressed in some non adrenergic non cholinergic peripheral autonomic nerve fibers (or "nitrergic" nerves) (Toda and Okamura, 2003). nNOS participates in neurogenic vasodilation in the rat skin

microvasculature (Merhi *et al*., 1998), and in the maintenance of penile erectile function (Burnett, 2006). The expression of nNOS is induced by acute hypoxia while the activity of nNOS is increased by chronic hypoxia (Semenza, 2005, Ward *et al*., 2005).

(2) Inducible NOS (iNOS, also referred as NOS2) is not constitutive, calcium insensitive and continuously synthesizes NO once it is expressed. Under physiological conditions, the expression of iNOS is low and the induction of iNOS occurs in response to inflammatory or pathogenic stimuli (Kroncke *et al*., 1998). For example, exposure to pro-inflammatory cytokines caused the induction of iNOS within vascular smooth muscle cells (Spink *et al*., 1995). In addition, iNOS is induced in response to infection and chronic inflammation as a host defence mechanism (Moncada and Higgs, 2006). The upregulation and increased activation of iNOS promotes the synthesis of $ONOO^-$ and may contribute to endothelial dysfunction (Viaro *et al*., 2000).

(3) Endothelial NOS (eNOS, also referred as NOS_3) is the major isoform in the vasculature. It regulates vascular function and is responsible for producing a basal amount of NO in the vascular system (Forstermann *et al*., 1994). The production of NO can change rapidly and transiently by eNOS activation to permit NO to modulate vascular tone (Dinerman *et al*., 1993). Vascular endothelial cells continuously produce NO and that production can be increased by specific agonists (such as acetylcholine and bradykinin), or by an increase in the shear force caused by blood flow or by cytokines released during inflammation (Klabunde 2012b). NO diffuse out of endothelial cells to cause smooth muscle relaxation, or inhibit platelets aggregation, via increasing cGMP formation (Klabunde 2012b).

Cyclic GMP is synthesized in VSM cells by two main types of guanylate cyclases that differ in their cellular location and in their activation by specific compounds: (i) a particulate guanylyl cyclase (pGC), present at the plasma membrane, is activated by natriuretic peptides, and (ii) a soluble guanylyl cyclase (sGC), which can be activated by either nitric oxide (NO) (which produced by endothelial NO synthase) or NO donors. Soluble guanylyl cyclase (sGC) enzyme converts GTP to cGMP, which then activates cGMP dependent protein kinase. This kinase evokes relaxation by (a) increasing the sequestration of calcium within the sarcoplasmic reticulum via phospholamban phosphorylation and activation of SR Ca^{2+}-ATPase, (b) enhancing the active extrusion of cytosolic calcium via stimulation of plasma membrane Ca^{2+}-ATPase, (c) impairing the release of calcium from the SR, via phosphorylation of the SR IP_3 receptor and/or inhibition of IP_3 synthesis and (d) reducing influx of extracellular calcium via receptor-operated calcium channels (Cornwell *et al*., 1991, Cohen *et al*., 1999, Schlossmann *et al*., 2000, Zhao *et al*., 2015).

Reduced NO formation can occur due to the deficiency of L-arginine, the precursor of NO, or a decrease in eNOS expression (Zhao *et al*., 2015). NO is inactivated in the presence of oxygen and various reactive oxygen species (ROS) such as O^-_2, H_2O_2 and OH^-. It reacts with molecular oxygen to form nitrite, or with superoxide to form peroxinitrite (Ignarro *et al*., 1981, Rubanyi *et al*., 1986). In addition, it can be scavenged by oxyheamoglobin to produce methaemoglobin and inorganic nitrite (Kelm *et al*., 1988). The biological half-life of NO differs as a consequence of the oxygen tension and the O^-_2 concentration (Ignarro *et al*., 1981, Rubanyi *et al*., 1986). H_2S decrease NO production in the endothelium

through inhibition of endothelial NOS (Kubo *et al.*, 2007, Geng *et al.*, 2007). Kubo *et al.*, (2007) demonstrated that, at low levels, NaHS caused contraction in both rat and mouse aorta. They also found that NaHS produced dilation instead of contraction when either the endothelium was removed in the mouse aorta or in the endothelium-intact aorta in the presence of L-NAME. They suggested that H_2S might block the activity of eNOS and thus H_2S inhibits NO formation in the vascular endothelium. Geng *et al.*, (2007) further showed NaHS administration in rats resulted in a reduced aortic eNOS activity and concentration of NO metabolites in plasma, suggested that H_2S could directly block eNOS.

1.5.2 Endothelium-dependent smooth muscle hyperpolarisation - mediated vasorelaxation pathway

In vascular smooth muscle, endothelium –dependent relaxation can occur by the released of an endothelium-dependent hyperpolarization factor (EDH). While the identify of EDH is not fully described a number of factors have been proposed including epoxyeicosatrienoic acids (EETs), K^+ ions, hydrogen peroxide (H_2O_2), and hydrogen sulfide (H_2S). (Campbell *et al.*, 1996, Edwards *et al.*, 1998, Matoba *et al.*, 2000, Mustafa *et al.*, 2011). Endothelial derived hyperpolarisation (EDH) is stimulated in endothelial cells by a rise in intracellular calcium levels, which can activate small conductance calcium activated K^+ channels (SKca), intermediate conductance calcium activated K^+ channels (IKca) and large conductance calcium activated K^+ channels (BKca) (Busse *et al.*, 2002, Garland *et al.*, 2011). The principle mechanisms for EDH mediated vascular smooth muscle hyperpolarisation occurs by: (1) producing

and accumulation of K^+ in the intracellular space between endothelial cells and smooth muscle cells via endothelial cell K_{ca} channels to activate inwardly rectifying potassium channels (Kir) and/or Na^+/K^+ ATPase pump in vascular smooth muscle (2) alternatively an increase in endothelial [Ca^{2+}] elicits the production of a cytochrome P450 metabolite that is essential for the subsequent EDHF-evoked responses; (3) or the hyperpolarization is spread to the vascular smooth muscle via myoendothelial gap junctions (Busse *et al.*, 2002, Edwards *et al*., 1998, McGuire *et al.*, 2001, Nelson *et al.*, 1995).

In addition to NO being identified as an endogenously formed gasotransmitter, carbon monoxide (CO) and hydrogen sulphide (H_2S) have also been shown to be endogenously synthesized and to cause important biological actions.

1.6 Carbon Monoxide (CO)

Carbon monoxide is a colourless, odourless, and chemically stable gas (Li *et al*., 2009). CO is synthesized principally during the process of heme degradation catalysed by the heme oxygenase enzyme. CO is able to activate soluble guanylyl cyclase; and hence cGMP which act as an intracellular signal to stimulate smooth muscle relaxation, inhibition of platelet aggregation and it may also be involved in synaptic transmission (Morse *et al.*, 2002). CO exerts anti-inflammatory and anti-apoptosis effects by blocking the synthesis of pro-inflammatory cytokines through mitogen-activated protein kinase (MAPK) signalling (Ryter *et al*., 2002).

1.7 Hydrogen Sulphide (H_2S)

The physiological importance of H_2S was first recognized by the finding of endogenous H_2S formation in mammalian cells and it has since been shown to have many physiological functions mediated through various molecular targets. (Wang, 2012).

1.7.1 Physical and chemical properties of hydrogen sulphide

H_2S is a colourless, water soluble, flammable and pungent toxic gas, it has a molecular weight of 34.08 Daltons and a vapour density of 1.19 i.e. (heavier than air). H_2S is the sulphur analogue of water and can be oxidized in a series of reactions to form sulphur dioxide. The half-life of H_2S in air varies from 12 to 37 hrs. H_2S is a weak acid in aqueous solution and dissociates into H^+ and hydrosulphide anion (HS^-) (Wang, 2012). H_2S can cross plasma membranes as its solubility in lipophilic solvents is greater than it is in water. Therefore, the gas can easily diffuse through cells and reach intracellular compartments (Mancardi *et al.,* 2009). Sodium hydrosulfide (NaHS) is commonly applied as an H_2S donor since it dissociates to Na^+ and HS^-; the latter then partially binds H^+ to form undissociated H_2S (lowicka and Beltowski, 2007).

1.7.2 Toxicology of Hydrogen Sulphide

Until recently H_2S gas was considered as a wide spectrum toxic agent with most organ systems susceptible to H_2S effects, based on concentration, time and rate of exposure (Reiffenstein *et al*., 1992). Acute exposure to 50–100 ppm H_2S causes eye irritation, neurological disturbances, skin symptoms, behaviour changes, cardiovascular abnormalities, and respiratory disorders. A

concentration of H_2S more than 500 ppm leads to unconsciousness and respiratory arrest (Wang, 2012).

1.7.3 Biosynthesis of hydrogen sulphide and regulation of its synthesizing enzymes.

H_2S is synthesized in tissue through enzymatic and non-enzymatic pathways. H_2S is produced endogenously from enzymatic desulfydration of cysteine that is catalysed by cystathionine δ lyase (CSE) and cystathionine β synthase (CBS) in the presence of pyridoxal 5-phospate (vitamin B6) as a co-factor (Li *et al*., 2011, Wang, 2012). Recently, the role of 3-mercaptopyrovate sulfurtransferase (3-MST) along with cysteine aminotransferase (CAT) in the production of H_2S has been examined in some tissues. The 3-mercaptopyruvate is derived from l-cysteine by the CAT enzyme in the presence of α-ketoglutarate, and then 3-MST enzyme produced H_2S and pyruvate from 3-mercaptopyruvate (figure 1.7) (Kajimura *et al.*, 2010).

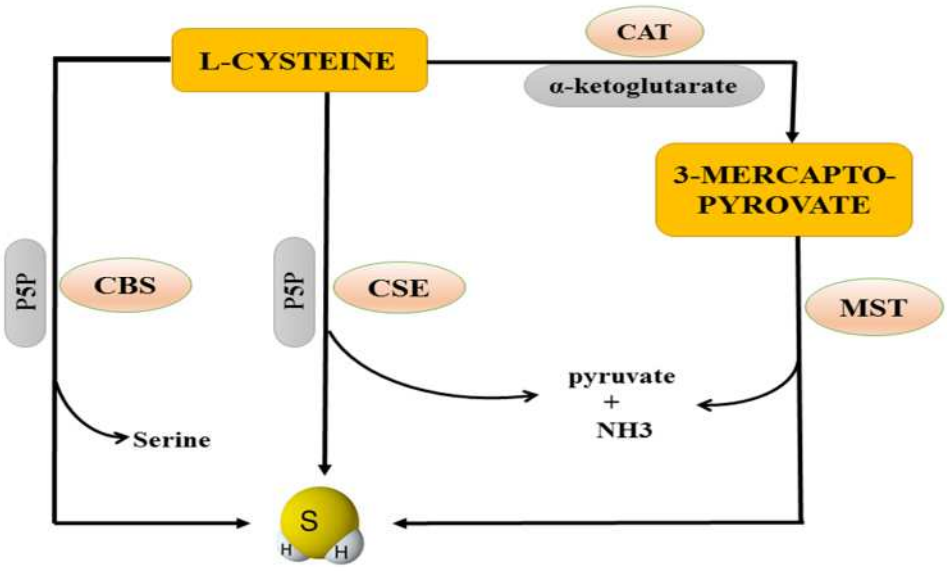


Figure 1.7 Biosynthesis of H_2S in mammalian cells.

Expression of CSE and CBS are widespread in many cells and a variety of tissues. Originally, CBS was thought to be highly expressed in nervous system tissue, while, CSE was thought to be most prominent in the vasculature and peripheral tissue (Li and Moore, 2007, Ishii *et al.*, 2004). However, it is now known CBS can be found in vasculature. For example, Baragatti *et al*., (2013) has shown that CBS is located within the vascular endothelium and smooth muscle cells.

1.7.4 Enzymatic production and inhibition of H_2S

1.7.4.1 Cystathionine β-synthase (CBS)

CBS (EC 4.2.1.22) was isolated under the name "serine sulfhydrase" by Braunstein *et al*., in 1969 (Braunstein *et al*., 1969). It is cytosolic pyridoxal-5-phosphate (PLP)-dependent enzyme (Li *et al*., 2011). In the presence of L-cysteine, CBS catalyses the generation of H_2S by a replacement reaction. The reaction catalysed by CBS is the condensation of homocysteine and serine to generate cystathionine and H_2S (Chen *et al*., 2004). CBS is a homotetramer consisting of 63-kDa subunits. It is a modular protein that is comprised of three structural domains. The NH_2 terminal binds both PLP and heme. CBS can be regulated by redox mechanisms due to the presence of heme in the N-terminal domain. The central core contains catalytic domain and the COOH terminus of CBS contains a regulatory domain which provides an autoinhibitory role for CBS activity and. The activity of CBS is regulated by binding of the allosteric activator, S-Adenosylmethionine (SAM), to this domain (Ereno-Orbeaa *et al*., 2014). CBS can be inhibited by Aminooxyacetic acid (AOAA), it is also called carboxymethoxylamine, which has been used as a nonspecific inhibitor for CBS.

1.7.4.2 Cystathionine γ-lyase (CSE)

CSE (EC 4.4.1.1) is also a cytosolic pyridoxal-5-phosphate (PLP)-dependent enzyme. (Li *et al*., 2011). It is also known as cystathionase, cysteine lyase, cysteine desulfhydrase. CSE generates H_2S, pyruvate and ammonium ions after hydrolysing L-cysteine. Alternatively, as a result of activation of CSE, cysteine can be converted to thiocysteine, and consequently to H_2S and cysteine (Qu *et al.*, 2008). CSE is also a homotetrameric enzyme and can be regulated by redox mechanisms. The activity of CSE is influenced by Ca^{2+} ion concentration in cells. At low intracellular Ca^{2+} ion concentrations, CSE activity is increased with subsequent H_2S generation, possibly evoked through an interaction with calmodulin. At high level of Ca^{2+} ions in the cells the activity of CSE is decreased. CSE is expressed in a wide range of cells and tissues. It is particularly highly expressed in the mammalian cardiovascular and respiratory systems (Hosoki *et al.,* 1997, Zhao *et al.,* 2001). CSE can be inhibited by DL-propargylglycine (PPG or PAG; C5H7NO2). This is an irreversible CSE inhibitor.

1.7.4.3 3-mercaptopyruvate sulfurtransferase (3MST)

MST (EC 2.8.1.2) is the most recently discovered H_2S generating enzyme, unlike CBS and CSE, it is PLP independent. It has been localized to both the cytosol and mitochondria, but most of the H_2S production by 3-MST occurs in the mitochondria, since, the levels of cysteine are much higher in the mitochondria than the cytoplasm (Nagahara *et al.*, 2007; Shibuya *et al.*, 2009). 3-MST catalyses the conversion of 3-mercaptopyrovate to H_2S. In this pathway, 3-mercaptopyrovate is generated by the action of cysteine aminotransferase (CAT) on L-cysteine via the incorporation of α–ketoglutarate into the reaction,

before 3-MST generates pyruvate and an intermediate persulphide compound that is reduced to produce H_2S in presence of reductants like thioredoxin, dihydrolipolic acid, and dithiothreitol (Nagahara *et al.,* 2007, Shibuya *et al.,* 2009, Mikami *et al.,* 2011, Rashid *et al.,* 2013, Yadav *et al.,* 2013). In addition to L-cysteine, D-cysteine can also generate H_2S via the 3MST pathway, through peroxisomal D-amino acid oxidase (DAO) formation of 3-mercaptopyrovate (Shibuya *et al.,* 2013). 3MST is expressed in many tissues and a variety cells such as neuroglial cells in the brain, cardiomyocytes in the heart, pericentral hepatocytes in the liver, and the proximal tubular epithelium of the kidney (Nagahara *et al.,* 1998). It is also expressed in aortic endothelium and smooth muscle cells (Shibuya *et al.,* 2009). H_2S generated by 3MST can be inhibited by aspartate which is a potent CAT inhibitor (Akagi, 1982).

1.7.5 Catabolism of Hydrogen Sulphide

H_2S catabolism occurs by four pathways namely oxidation, methylation, expiration and scavenging (figure 1.8).

1.7.5.1 Oxidation

H_2S is oxidized, mainly in mitochondria, to thiosulphate ($S_2O_3^{2-}$) and then to sulfite (SO_3^{2-}) and sulfate (SO_4^{2-}) (Caliendo *et al.,* 2010). Thiosulphate generation is probably a non-enzymatic processes, but thiosulphate conversion to sulphite and/or sulfate is catalysed by thiosulfate: cyanide sulfurtransferase (TST, Rhodanese). The sulphite that is generated in this reaction is oxidized quickly by sulphite oxidase to sulfate, which is the major end-product of H_2S metabolism under physiological normoxic conditions. Sulfate is excreted in the urine either free or as conjugated sulfate (Curtis *et al.,* 1972, Beauchamp *et al.,*

1984). In mitochondria, H_2S can also be oxidized to persulfide by sulfide quinine oxidoreductase (SQOR) and then by rhodanese persulfide producing thiosulfate (Jackson *et al.,* 2012).

1.7.5.2 Methylation

The second pathway of H_2S catabolism is methylation. It mainly occurs in the cytosol, and H_2S is methylated into methanethiol and dimethylsulfide by thio-S-methyltransferase (TSMT). Methanethiol can be further methylated to nontoxic compound, dimethylsulfide. TSMT activity has been reported in the colonic and cecal mucosa, liver, lung, and kidney (Weiseger *et al.,* 1980, Furne *et al.,* 2001). The methylation of sulphide is slower than the oxidation, thus under physiological conditions, this route of H_2S metabolism is less important.

1.7.5.3 Expiration

Endogenous H_2S may be metabolised by exhalation as a readily diffusible gas through the lungs. In healthy subjects, the free concentration of H_2S in blood is very low due to metabolism, thus little of H_2S escapes through the lungs (Furne *et al.,* 2008). However, in pathological conditions such as chronic obstruction pulmonary disease and septic shock, the mammalian lungs provide an important excretory route for H_2S when it is generated in larger than normal amounts (Furne *et al.,* 2008, Yun *et al.,* 2013).

1.7.5.4 Scavenging

H_2S can be scavenged by methemoglobin to produce sulfo-methemoglobin. This limits the half-life of free H_2S in the blood. The H_2S binds

to metallo or disulfide-containing molecules such as oxidized glutathione (GSSG) to form glutathione persufide (GSSH) (Smith and abbanat, 1966, Beauchamp *et al.,* 1984). Hemoglobin may act as a common sink for H_2S and compete with oxygen for binding, thus contributing to its toxicity on exposure to high concentrations (Liu *et al.*, 2012).

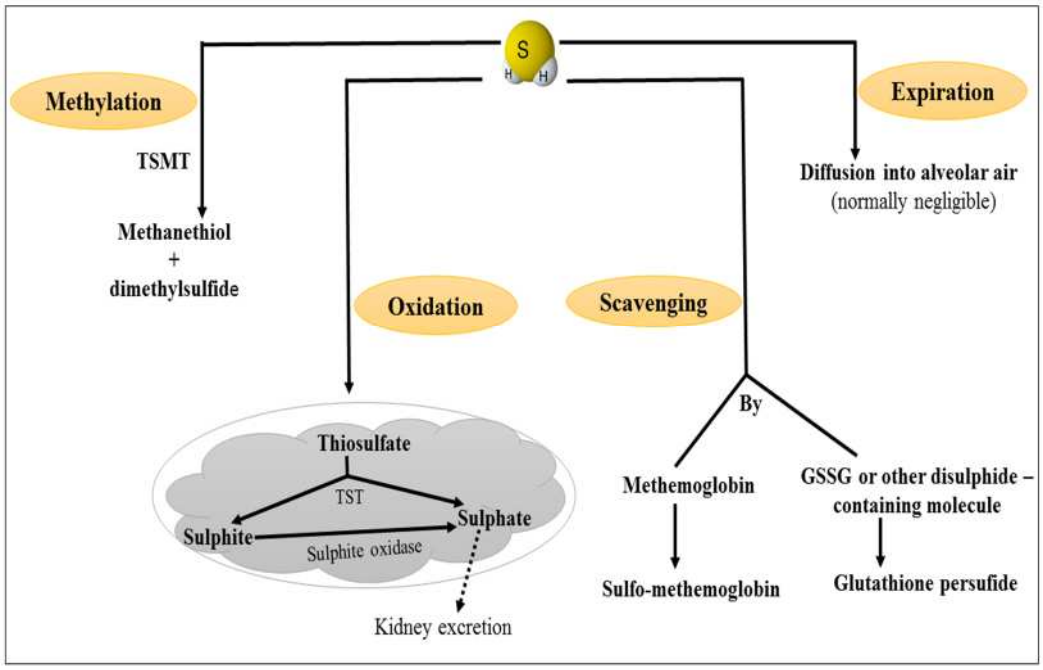


Figure 1.8 Catabolism of Hydrogen Sulphide.

1.7.6 Physiological functions of hydrogen sulphide

1.7.6.1 Hydrogen sulphide in the central nervous system

H_2S has been reported to have effects in central nervous system, and to be involved in processes including neurotransmission and neuroprotection (Wang, 2012). H_2S has the potential to modulate several neurotransmitters including glutamate and GABA (Wang, 2012). Glutamate is a major excitatory amino acid neurotransmitter. It has a role in learning, memory and perception of pain, by activating NMDA receptors. Abnormalities in metabolism of glutamate

can cause excitatory neuron cell injury (Wang, 2012). Several studies indicate that H_2S may cause physiological or pathological outcomes through regulating NMDA receptors. For example, Abe and Kimura (1996) and Kimura (2002) suggested that physiological levels of H_2S increase NMDA receptor-mediated currents and accelerated the induction of hippocampal Long Term Potentiation (LTP) in rats. By contrast, H_2S at high concentrations can harm the brain and cause reduced learning and memory (Partlo *et al.*, 2001). Prolonged stimulation of NMDA receptors leads to calcium overload in cells and causes cell death (Cheung *et al*., 2007). Garcia-Bereguiain *et al*., (2008) showed that H_2S stimulates glutamate release and may cause cell death, as shown since H_2S induced cell neuron death was reversed using a NMDA receptor antagonist (MK-801).

1.7.6.2 Hydrogen sulphide in the inflammation.

H_2S has been shown to exert both pro-inflammatory (Nishimura *et al*., 2009, Tamizhselvi *et al.*, 2008, Zhang and Bhatia, 2008) and anti-inflammatory actions (Esechie *et al*., 2008, Li *et al.*, 2008, Zanardo *et al.*, 2006), that may depend upon concentration. Li *et al*., (2005) reported that NaHS caused inflammation of liver and lung tissue by increasing the activity of myeloperoxidase (MPO). In animal models of inflammation such as septic and endotoxic shock, and pancreatitis, the plasma H_2S concentration and tissue H_2S producing activity and CSE expression are increased (Li *et al*., 2005, Mok *et al*., 2004). Furthermore, in rats, H_2S formation is increased in septic shock induced by cecal puncture (Hui *et al*., 2003). Unuma *et al.*, (2019) showed that, NaHS caused bronchitis and increased respiratory mucosal secretions. Di Villa Bianca

et al., (2010) showed that injection of NaHS and L-cysteine into mouse paw caused oedema. The mechanism for the pro-inflammatory action of H_2S is that it activates sensory nerve endings, releasing endogenous tachykinins, for instance, substance P and calcitonin-gene related peptide (CGRP) (Wang, 2012). By contrast there is considerable evidence supporting an anti-inflammatory action of H_2S. For example, Whiteman *et al.,* (2010) showed that H_2S has an anti-inflammatory effect in a mouse model of endotoxic shock. In addition, oedema was enhanced by inhibiting endogenous production of H_2S (Zanardo *et al.,* 2006). Furthermore, H_2S prevented the inflammation-associated lowering of gastric mucosal blood flow (Li *et al.,* 2006) and promoted ulcer healing in the rat (Wallace *et al.,* 2007). Fiorucci *et al.,* (2007) demonstrated that mesalamine (H_2S releasing agent) diminished colitis-associated expression of various pro-inflammatory cytokines and leukocyte infiltration. These rather conflicting data demonstrate that endogenous H_2S may mediate inflammation or have anti-inflammatory properties depending on a number of factors, for example, the concentrations, administration routes for H_2S and the experimental model or conditions employed (Wang, 2012).

1.7.6.3 Hydrogen sulphide in the digestive system

The regulation of many GI functions such as control of motility, secretions and inflammation are influenced by H_2S. H_2S reduces the motility of jejunum and colon in humans, mice, and rats (Wang, 2012). Hosoki *et al*., (1997) and Teague *et al.,* (2002) showed that, in rat and guinea pigs, pre-contracted ileum was relaxed by NaHS. The spontaneous contraction of the rabbit isolated ileum was also prevented by H_2S (Teague *et al.,* 2002). Schicho *et al.,* (2006)

found that both NaHS and L-cysteine led to concentration-dependent rises in the release of chloride in human and guinea- pig submucosal and mucosal preparations. These effects were reduced by capsaicin suggesting that TRPV1 receptors play an important role in H_2S-mediated ion secretions. In addition, Fiorucci *et al*., (2005a) showed that H_2S attenuates gastric mucosal injury induced by acetylsalicylic acid, perhaps by regulation of the gastric microcirculation.

1.7.6.4 Hydrogen sulphide effects in the vasculature

1.7.6.4.1 Vasorelaxation effects of H_2S

Hosoki *et al*., (1997) were the first to show that H_2S can relax portal vein and thoracic aorta. Subsequently, H_2S has been reported to cause vasorelaxation in many blood vessels including the rat aorta (Zhao *et al*., 2001), rat hepatic artery (Siebert *et al*., 2008), rat mesenteric resistance arteries (Cheng *et al*., 2004, Tang *et al*., 2005), rat coronary artery (Cheang *et al*., 2010) and piglet cerebral arterioles (Liang *et al*., 2011). In addition, H_2S has been shown to relax human blood vessels including internal mammary artery (Webb *et al*., 2008) and placental chorionic artery (Cindrova-Davies *et al.*, 2013).

1.7.6.4.1.1 Mechanisms of H_2S-induced vasorelaxation

1.7.6.4.1.1.1 Opening of K_{ATP} and/or voltage-dependent K^+ channels

In rat aorta, Zhao *et al*., (2001) showed that H_2S induced a vasorelaxation through the opening of K_{ATP} channels in vascular smooth muscle, since glibenclamide, a K_{ATP} channel antagonist, significantly attenuated the vasodilatory effect of H_2S. Similar observations were made by Tang *et al*.,

(2005) where H_2S evoked a vasorelaxation via directly activating K_{ATP} channels and caused membrane hyperpolarization in the vascular smooth muscle of rat small mesenteric arteries. Webb *et al*., (2008) also showed that high concentrations of H_2S produced relaxation of human mammary artery and this relaxation was partially inhibited by glibenclamide. Furthermore, H_2S caused vasorelaxation both in vivo and in vitro in canine coronary arteries, by stimulating K_{ATP} channels (Casalini *et al.,* 2014).

Despite this, a number of studies have reported that the vasorelaxation evoked by H_2S is only partially blocked by glibenclamide or unaffected (Cheng *et al*., 2004, Kiss *et al*., 2008, Webb *et al*., 2008, Cheang *et al*., 2010). Other potassium channels have been suggested to be involved. For example, Martelli *et al*., (2013) showed that H_2S evoked vasorelaxation by activating K_v7 voltage-gated potassium channels on vascular smooth muscle cells isolated from rat aortic rings. Hedegaard *et al*., (2014) produced similar results, in porcine coronary arteries. In addition, Cheang *et al*., (2010) showed that 4-aminopyridine (4-AP, a specific K_v inhibitor) decreased NaHS-induced vasorelaxation in the rat coronary artery. Jackson-Weaver *et al.,* (2013) showed that H_2S stimulated BKca channels located in the endothelium in small mesenteric arteries, to produce hyperpolarization and relaxation. Additionally, Wen *et al*., (2018) showed that Kca channels involved in the cerebrovascular relaxation to H_2S.

1.7.6.4.1.1.2 Activate of cyclic guanosine monophosphate (cGMP)

The vasorelaxation effect of H_2S has also been proposed to occur through decreasing cGMP degeneration by blocking phosphodiesterase-5 (PDE-5) activity in vascular smooth muscle of the rat aorta (Bucci *et al*., 2012). The

cGMP levels increased when incubation of cultured rat aortic SMC with exogenous NaHS in a concentration-dependent action (Bucci *et al*., 2012). In addition, the sGC/cGMP cascade plays an important role in the relaxation action in trout branchial arteries (Dombkowski *et al*., 2004).

1.7.6.4.1.1.3 Arachidonic acid pathway (Prostaglandin Production)

Arachidonic acid (AA) is the precursor of prostaglandins. Prostaglandins are produced when AA is released from the plasma membrane by the action of the phospholipase A_2 enzyme. The AA is metabolised by two COX isoforms, COX-1 and COX-2, to prostaglandin H_2, which is precursor to the production of prostacyclin, and other prostaglandins (Norregaard *et al.,* 2015). H_2S induced vasorelaxation in mesenteric artery beds was blocked by 4-(4-octadecylphenyl)-4-oxobutenoic acid, a phospholipase A_2 inhibitor (Di Villa Bianca *et al.*, 2011). In addition, in rat aorta, indomethacin, a COX inhibitor, enhanced the contractile effects of H_2S in rat aorta, suggesting that AA metabolites mediate H_2S-induced vasorelaxation (Koenitzer *et al.*, 2007).

1.7.6.4.1.1.4 Intracellular acidification

The regulation of vascular tone may be affected by the intracellular pH. In most vascular beds, intracellular acidification has a vasorelaxant effect, whereas alkalinization causes vasoconstriction. Lee *et al.*, (2007) showed that H_2S stimulate $Cl^-/HCO3^-$ exchanger activity leading to intracellular acidosis, and an associated vasodilation. Kiss *et al.*, (2008) found that, in rat aorta, an inhibitor of $Cl^-/HCO3^-$ channels (1mM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate, DIDS) abolished NaHS-induced relaxation, suggesting that H_2S caused vascular relaxation through activation of

the $Cl^-/HCO3^-$ exchanger, causing intracellular acidosis with subsequent activation of K_{ATP} channels.

1.7.6.4.2 Vasoconstriction effects of H_2S

H_2S has been shown to contract blood vessels, and different mechanisms have been proposed to underlie this effect. H_2S-induced vasoconstriction is brought about by mopping up NO, decrease in cAMP level, and/or downregulation of endothelial NOS.

1.7.6.4.2.1 Mechanisms of H_2S-induced vasoconstriction

1.7.6.4.2.1.1 Mopping up NO

Ali *et al.*, (2006) observed that at low levels NaHS caused a contraction in rat aorta. Low levels of NaHS also reversed the NO-generated vasodilation response promoted by acetylcholine. The pro-contractile actions of H_2S were prevented by denuding the endothelium, or blockage of eNOS, leading them to suggest that H_2S acted to delete the basal vasorelaxant influence of NO to cause a contraction. They also found that low doses of NaHS increased mean arterial blood pressure in anesthetized rats, and this action was abolished in animals pre-treated with L-NAME. It was suggested that H_2S reacts with endogenous NO leading to loss of NO-derived vasorelaxant action and an increase in blood pressure (Ali *et al.*, 2006). They also showed that mixing the NO donor, SNP, with NaHS decreased the vasorelaxation effect compared with SNP alone, indicating H_2S inactivates the NO (Ali *et al.*, 2006). Similarly, Kubo *et al.*, (2007) demonstrated that, at low levels, NaHS caused contraction in both rat and

mouse aorta. They also found that NaHS produced dilation instead of contraction if either the endothelium was removed or in the presence of L-NAME. In support of this, Whiteman *et al.*, (2006) found that H_2S could interact with NO to produce a nitrosothiol compound. Ali *et al*., (2006) showed that $CuSO_4$ (200 nM) (which is known to convert nitrosothiols to nitrite and nitrate; Stubauer et al., 1999) blocked the contractile response of NaHS. In contrast, Filipovic *et al*., (2013) and Yong *et al*., (2010) demonstrated that the reaction between H_2S and NO did not produce nitrosothiol but generate nitroxyl (HNO) compound, which could possibly have caused the contraction.

1.7.6.4.2.1.2 Decrease in cAMP level

Other mechanisms that may cause contraction in blood vessels include modulation of cAMP levels. The reduction in the level of cAMP leads to activation of MLC kinase, causing vasoconstriction. In rat aorta, Lim *et al*., (2008) demonstrated that low concentrations of H_2S caused contraction. They showed that NaHS reversed the vasodilation action produced by isoprenaline and salbutamol (β_2-adrenoreceptors) and forskolin (adenylyl cyclase activator) suggesting that the contractile activity of H_2S occurred by blocking cAMP accumulation (Lim *et al*., 2008). Similarly, Li *et al.,* (2015) demonstrated that NaHS attenuated the vasorelaxant responses of isoprenaline and forskolin and suggested H_2S induced vasoconstriction of cerebral artery occurred, at least in part, by inhibiting the cAMP/adenylyl cyclase pathway (Li *et al*., 2015).

1.7.6.4.2.1.3 Downregulation of endothelial NOS

As mentioned earlier, H_2S decrease NO production in the endothelium through inhibition of endothelial NOS. Kubo *et al.,* (2007) found that NaHS produced dilation instead of contraction when either the endothelium was removed in the mouse aorta or in the endothelium-intact aorta in the presence of L-NAME. They suggested that H_2S might block the activity of eNOS and thus H_2S inhibits NO formation in the vascular endothelium.

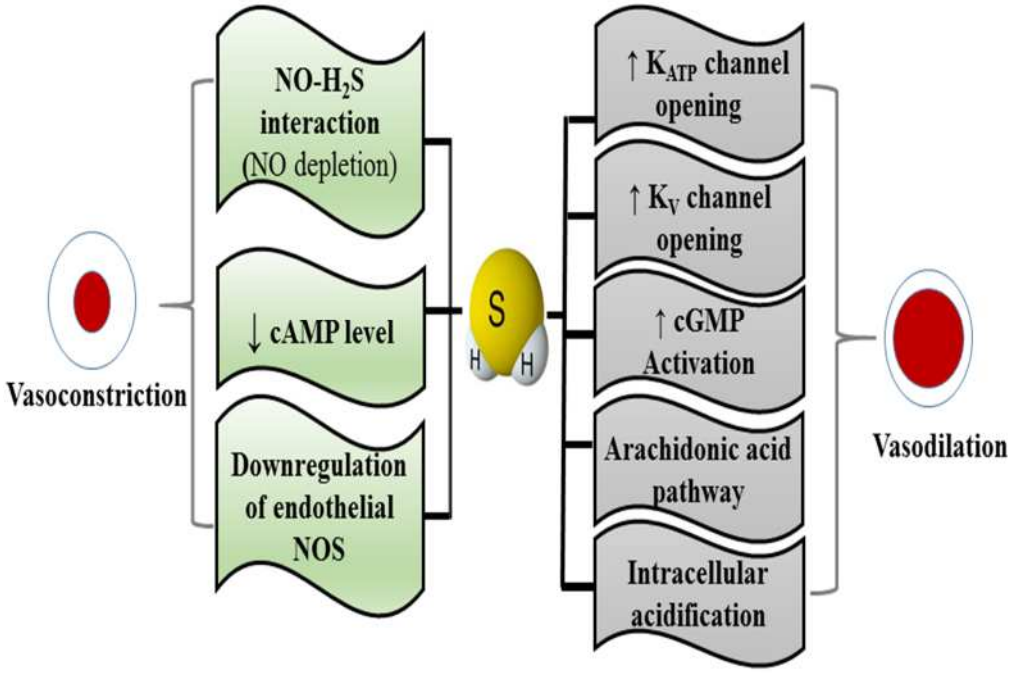


Figure 1.9 Mechanisms of H_2S-induced vasorelaxation and vasoconstriction

1.7.6.5 Influence of experimental conditions on H_2S responses

H_2S responses have been shown to be variable. Some of this variability may reflect the experimental conditions employed to study the vascular responsiveness, for example, the level of O_2 tissues are exposed to.

1.7.6.5.1 O_2 levels and the vascular responses to H_2S.

The responses to H_2S in blood vessels are complex with either relaxation or contraction reported and the responses to H_2S may also be sensitive to the levels of O_2. Koenitzer *et al.,* (2007) observed that the effect of H_2S was dependent upon the prevailing O_2 tension. They showed that, in rat aorta, H_2S promoted vasoconstriction at high O_2 levels and vasorelaxation was produced at lower concentrations of O_2. This alteration in response was proposed to be due to oxidation of H_2S at high O_2 tension, causing a decrease in H_2S availability or, to the formation of H_2S-derived oxidation compounds that had contractile activity (Koenitzer *et al.,* 2007). Kiss *et al.,* (2008) also found an improved vasodilator response to H_2S under conditions of low O_2.

1.7.6.5.2 H_2S as vascular oxygen sensor

Oxygen tension is one of the key factors required for cellular signalling and physiological functions. Hypoxia is an important regulator of vascular tone promoting vasoconstriction in the pulmonary circulation to reduce perfusion of under-ventilated hypoxic regions, and vasodilation in the systemic vasculature to increase blood flow and oxygen delivery to hypoxic tissues (Wier and Archer 1995, Skovgaard *et al*., 2011). It is possible that all tissues and cells are able to sense changes in oxygen tension (Brij and Peacock, 1998). The oxygen sensor

can be described as an endogenously generated molecule that is instantly interact with oxygen to induce structural and functional changes that consequently produce a series of cellular and body reactions (Wang, 2012). Recently, it has been suggested that H_2S may act as an oxygen sensor/transducer of the hypoxic response in the vasculature. Hypoxia elicits mouse cerebral vasodilation via increasing H_2S level (Morikawa *et al*., 2012). Olson *et al*., (2006) showed that hypoxia induced vasodilation in rat aorta occur through generating H_2S, since PPG, a H_2S synthesis inhibitor, blocked the response. Similarly, Donovan *et al*., (2017) reported that AOAA (300µM) reduced hypoxic vasorelaxation in porcine coronary arteries, suggesting that a role of H_2S in the hypoxic response in this vessel also. Takano *et al*., (2014) also reported that hypoxia increase the expression of CBS and generation of H_2S resulting rat cerebral vasodilation, suggesting a longer lasting response involving H_2S. The proposed mechanism of this O_2 sensing system is that decline in tissue O_2 concentrations reduces the rate of H_2S oxidation, thus increasing the level of biologically active of H_2S (Olson *et al.,* 2008). O_2 influences the production and metabolism of H_2S, and the bioavailability of H_2S whether endogenous or exogenous (Li and Lancaster, 2013). At low intracellular O_2 levels, H_2S metabolism was shown to be decreased, leading to raised H_2S levels while at high oxygen tension, H_2S oxidation was increased leading to reduced levels of H_2S in the cells (Olson *et al.,* 2006, Whitfield *et al.,* 2008). In addition, the activities of CBS and CSE may be influenced by the oxygen level to modify H_2S formation (Maclean *et al.,* 2004; Stipanuk, 2004). Doeller *et al.,* (2005) showed that in various rat tissues the production of H_2S was evident under low O_2 tension while at high oxygen levels H_2S generation was decreased.

The Aims of the Current Study

Recently, H_2S has emerged as a novel and important biological mediator in the cardiovascular system. In blood vessels, it produces complex responses: vasoconstriction in some vessels, vasodilation in others, through several mechanisms. Different responses and mechanisms have been reported, the reasons for this are not immediately apparent but it is possible that experimental conditions could contribute to the variability in responses to H_2S. One of these conditions is oxygen concentrations used to gas vessels.

The aims of present research were:

- To investigate the O_2 levels impact on H_2S vascular responsiveness in systemic arteries and vein, pulmonary arteries and vein and chorionic vessels. Studies were conducted using porcine splenic and pulmonary vessels along with human chorionic arteries and veins. Vessels were gassed with 95% O_2:5% CO_2, 95% Air: 5% CO_2 or 95% N_2:5% CO_2.
- To examine the mechanisms of NaHS responses under different O_2 levels.
- To investigate whether endogenous H_2S was involved in contributing to hypoxia-induced systemic vasodilation and pulmonary vasoconstriction in PSA and PPA respectively.

Chapter 2:

Materials, General Methods and Protocols

2 Materials, General Methods and Protocols

2.1 Contractility studies

2.1.1 Tissue collection and sample preparation

2.1.1.1 Porcine tissue collection and sample preparation

Porcine spleens and lungs were obtained from Wood's and Son Abattoir (Clipston, Nottingham, UK). Porcine spleens and lungs were isolated at the abattoir and stored in ice-cold Krebs-Henseleit buffer (see table 2.1) at 4˚C during transport to the laboratory. On arrival, the porcine splenic arteries and veins and pulmonary arteries and veins were dissected from the spleens and lungs respectively and kept overnight at 4˚C in PSS pre-gassed with a 95% O_2: 5% CO_2 gas mixture. The next day, after samples reached room temperature, the isolated segments were placed in a dissection dish (sponged petri dish) full of PSS solution where the remaining adipose and connective tissue was removed whilst ensuring vessels were not stretched or damaged. The cleaned vessels were then cut into equal segments approximately 5mm in length. Vessels were kept in PSS solution and left to normalize to room temperature before being setup in the isometric tension recording system.

2.1.1.2 Placental collection, Ethical Approval and Sample preparation

Term placentas were obtained from Elective caesarean sections in women with uncomplicated pregnancies at Queen's Medical Centre, Nottingham, UK. Written informed consent was obtained from all subjects and ethical approval was obtained from The Queen's Medical Centre and University Hospital NHS

Trust Ethics committee (OG010101). The human placenta, cord left clamped, was brought to the laboratory within 30min of delivery. On arrival, the chorionic arteries and veins were carefully dissected from the placenta and kept in PSS pre-gassed with a 95% O_2: 5% CO_2 gas mixture. The cleaned vessels were then cut into equal segments approximately 5mm in length (Figure 2.1).

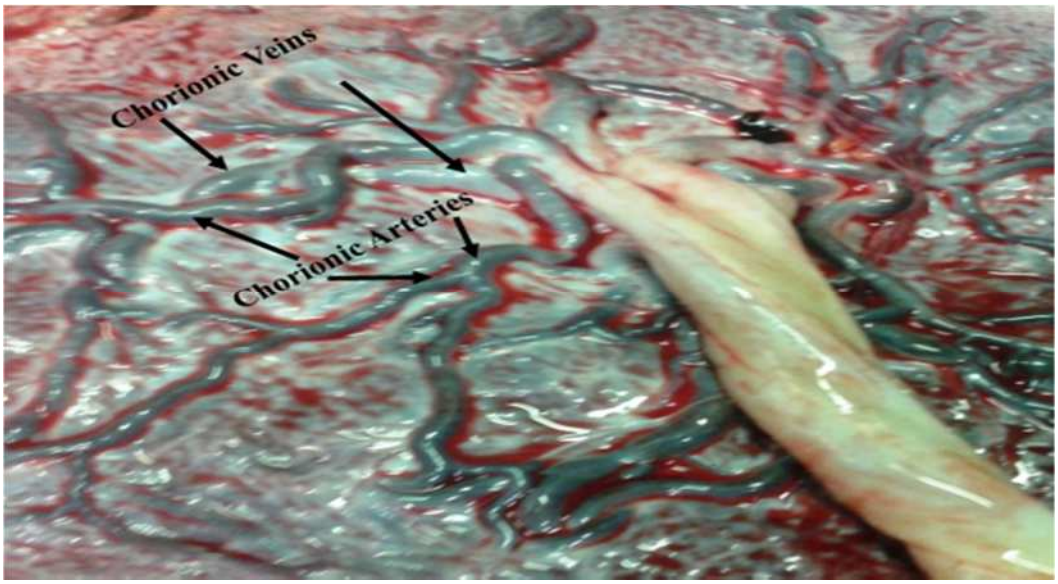


Figure 2.1 the fetal side of the placenta showing the attached umbilical cord and chorionic vessels (arteries and veins) branching from the umbilical vessels (Note: arteries usually cross over veins)

2.1.2 Set up and the general protocol for recording isometric tension

The segments of the vessels were taken out of PPS and attached to two steel wire hooks, ensuring that the hooks were not overlapping. The upper hook was attached to an isometric force transducer (AD instruments, UK) using a non-stretchable silk thread, while, the lower hook was attached directly to a glass rod. Arteries and veins were then immersed in an organ bath. The organ bath contained 20ml PSS and it was maintained at a constant temperature of 37 ˚C using a circulating water heater. The PSS was continuously gassed with the appropriate gas mixture for each experiment.

The force transducers were connected to a Bridge Amplifier (AD Instruments, UK) to measure changes in voltage registered by the transducer due to alterations in the force applied to the transducer. The Bridge Amplifier was in turn attached to a PowerLab unit (AD Instruments, UK), which connected the transducer to a computer where data was recorded using Lab Chart software (AD Instruments, UK). Transducers were calibrated daily with a 10g weight to guarantee consistency of measurements (Figure 2.2).

The cleaned vessels were set up in organ baths containing PSS at 37˚C and gassed with the required gas mixture for each experiment. The PSS was pre-gassed for approximately 30 minutes to ensure gaseous equilibrium of the PSS. Following that, an initial resting tension was applied to vessels (10 gram for PSA and PPV, 6 gram for PSV and PPA (White, 2012, Alassaf, 2018) and 2 gram for chorionic vessels (Razak *et al.,* 2018) which were allowed to relax for approximately 30-45 minutes to equilibrate and achieve a stable baseline tone (approximately 4g for PSA and PPV, 2g for PSV and PPA and around 1.0-1.5g for chorionic vessels). Thereafter, vessels were exposed to KCl (60mM) for 15 minutes and allowed to contract to confirm their viability.

After the response plateaued, the KCL was washed out with fresh PSS two times at 5 minutes intervals. Following two exposures of the vessels to KCl (60mM), any test substances applied in the experiment were added for at least 30 minutes incubation time. After this time vessels were pre-contracted to a level of 30-70% of the maximum KCl response. In most experiments, this was achieved using either, the thromboxane A2 receptor agonist, U46619 or the endothelin receptor agonist, endothelin-1. In all experiments another paired segment (from the same tissue) was set up and used as a control.

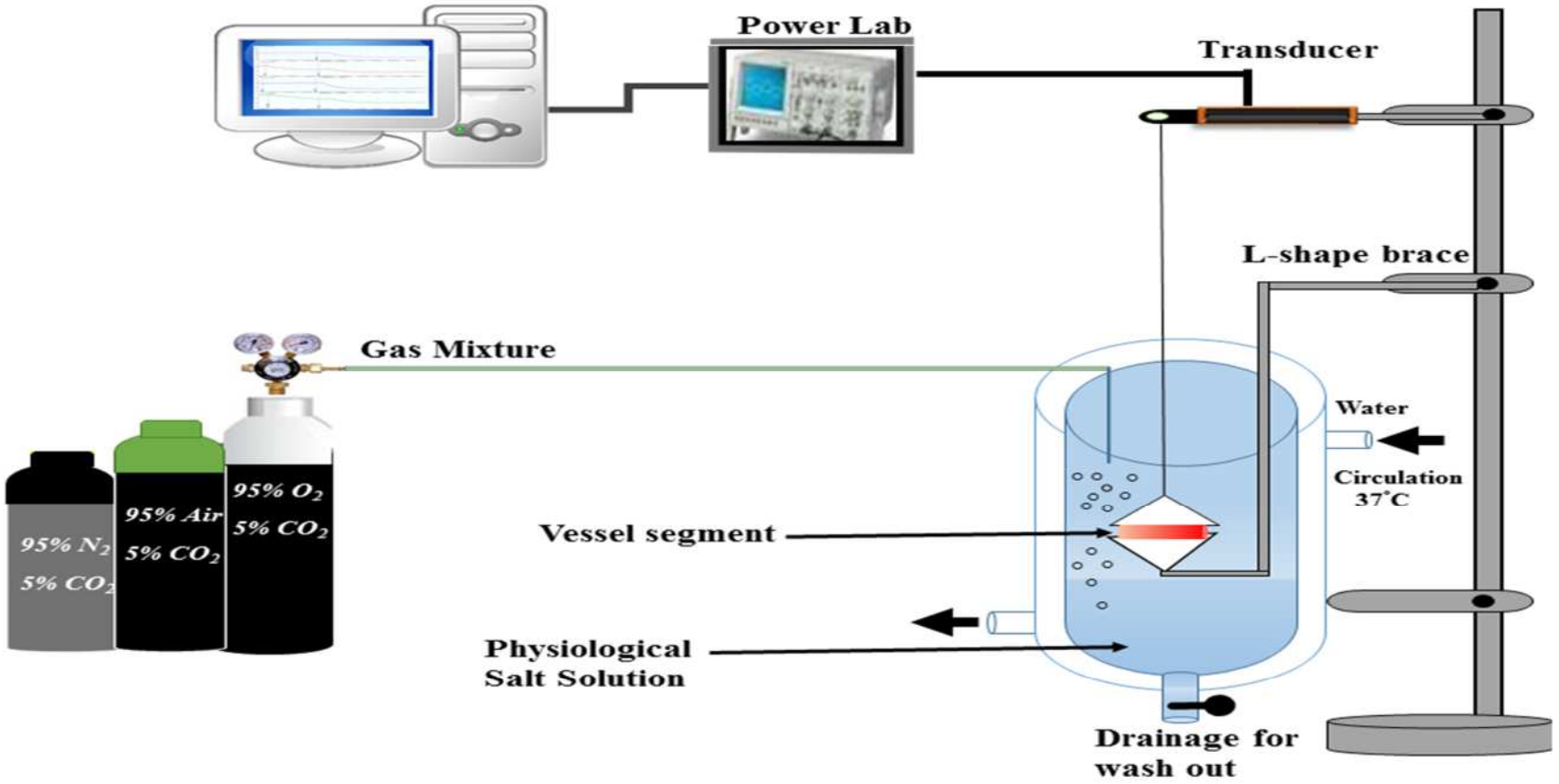


Figure 2.2 Isolated organ bath set-up for blood vessels contractility studies.

Table 2.1 List of reagents and chemicals used for contractility studies. Details on the concentrations used will be further mentioned in the following results chapters.

Reagents	**Source/Manufacture**
Krebs-Henseleit buffer in mM/L, pH 7.4, gassed for 15 minutes with 95% oxygen, 5% carbon dioxide	
Sodium chloride (NaCL) (120 mM)	Fisher Scientific, Loughborough, UK
Potassium chloride (KCl) (4.8 mM)	
Sodium hydrogen carbonate ($NaHCO_3$)(25 mM)	
Magnesium sulfate ($MgSO_4.7H_2O$) (1.1 mM)	
Calcium chloride ($CaCl_2$) (1.25 mM)	
Glucose (11 mM)	
Potassium dihydrogen orthophosphate (KH_2PO_4) (1.2 mM)	BDH, Poole, UK
Chemicals	
U46619 (9,11-didexoy-9α,11α-methanoepoxy prostaglandin F2α methyl acetate)	Acros Organics, USA.
Sodium hydrosulphide hydrate (NaHS)	
Endothelin	Sigma Aldrich, Poole, UK
L-NAME (N-nitro-L-arginine methyl ester)	
Substance P	
Ethanol	
DMSO (dimethylsulphoxide) (glibenclamide solvent)	
Glibenclamide	
L-cysteine	
DL-propargylglycine (PPG)	
Aminooxyacetic acid (AOAA)	
Aspartate	
Febuxstat	
Forskolin	
Copper Sulphate (CuSO4)	

2.2 Western blotting

2.2.1 Tissue preparation for Western blotting

Porcine spleens and lungs were collected from the abattoir and placed in in ice-cold Krebs-Henseleit buffer that had been gassed with 95% O_2: 5% CO_2 for 15 minutes. On arrival, in the dissection room, the porcine splenic and pulmonary arteries were dissected and placed in a dissection dish containing sufficient buffer where they were finely dissected to remove the remaining adipose and connective tissue. Segments of porcine arteries were extracted and stored at -80 ^{0}C. Samples were homogenized in lysis buffer (see table 2.2) containing 1 % (v/v) protease inhibitor cocktail EDTA free. For protein assay, samples were kept at -80°C. For Western blot experiments, samples were mixed with 6X solubilisation buffer (see table 2.2) in 1:5 ratio (buffer: sample) and kept at -20°C if not used immediately.

2.2.2 Lowry test

A modified Lowry assay method was used to determine the protein concentration of the supernatants (Lowry *et al.*, 1951). A stock bovine serum albumin (BSA, 1mg/ml) solution was prepared in distilled water; a serial dilution (from 0.05-0.45 mg/ml) was prepared from the stock to generate the standard curve (0.4, 0.35, 0.3, 0.25, 0.2, 0.15, 0.1, 0.05, and 0 µg/µl). Samples and BSA standards were mixed with Lowry AB solution (see table 2.2), and incubated for 10 minutes at room temperature. Subsequently, folin phenol reagent solution, prepared by diluting 1:1 in distilled water, was added to all Samples and BSA standards and incubated for 45 minutes at room temperature. The samples were added into a 96-well plate and the intensity of the absorbance was measured

using SpectraMAX 340 PC plate reader at 750 nm wavelength. The protein concentrations of the samples were calculated from the BSA standard curve.

2.2.3 Western blotting

2.2.3.1 Electrophoresis separation

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 4-20 % gradient gel (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). The gel was gently removed from the silver bag, the comb was removed from the gel and the green plastic tape at the bottom of the gel was removed. Next, the gel was put in the tank and electrophoresis buffer was added into the properly prepared reservoir. Western blot samples were heated at 95°C for 5 minutes followed by centrifugation for 1 minute at 13000x g. A molecular marker (Bio-Rad, precision plus protein) was loaded in the first well and the rest of the wells were loaded with heated samples by gel loading tips on the gel. Samples were separated by electrophoresis at 175-volt for 40 minutes using Bio-rad mini protean 3 electrophloresis tank.

2.2.3.2 Transferring phase

When the running phase was finished, the gel was gently removed and soaked in transfer buffer (TB). The gel sides were sliced down, and the gel side was placed down onto the filter paper. After that, Nitrocellulose membrane(GE Healthcare, Little Chalfont, Buckinghamshire, UK) was placed onto the gel, and the second filter paper was added to the sandwich, and, by the roller, the bubbles were removed gently. Next, the closed cassette was placed into the tank filled with TB and refrigerated with ice. The proteins transferred onto a nitrocellulose

membrane in a Western blot tank at 100-volt for 60 minutes at 4°C in Western transfer buffer. In order to check that the loaded protein had been successfully transferred, ponceau S solution (Sigma, UK) was added to stain the nitrocellulose membrane, the membrane was quickly washed with tris-buffered saline containing the detergent Tween 20 (TBST) to remove the stain. Following this, block of the nitrocellulose membranes was carried out by incubation with blocking solution (5% (w/v) non-fat milk solution in TBST) for one hour at room temperature, with shaking on a rocking platform.

2.2.3.3 Developing image

The primary antibody was prepared at 1:1000 dilution in 10 mL of 5% fat-free milk. The blots were incubated overnight at 4°C with the primary antibody Rabbit anti-CBS (1:1000 dilution), rabbit anti-CSE (1:1000 dilution), and mouse monoclonal anti-β-actin antibody (1:50 000) in blocking solution with shaking (see tables 2.2 and 2.3). The next day any unbound primary antibody were removed by washing the immunoblots with TBST for 5, 10 and 15 minutes. In the final step after washing in TBST, the blots were incubated with corresponding secondary antibodies IRDye®680LT Goat anti-mouse IgG (1:10000) and IRDye® 800CW Goat anti-rabbit IgG (1:10000) at room temperature with shaking for 1 hour. After that any unbound secondary antibody were removed by another washing the immunoblots as before. Immunoblots were analysed using the Li-Cor/Odyessy Imager Scanner and the densities of the bands were determined using Image studio (Application software version 3.1 LI-COR Biosciences, Cambridge, UK).

Table 2.2 List of materials and reagents used to prepare buffers necessary for Western blotting

Reagents	**Source/Manufacture**
Lysis buffer	
Tris (20 mM)	Invitrogen, Rockford, USA
Ethylene glycol tetraacetic acid (EGTA) (1 mM)	Sigma Aldrich, Poole, UK
Triton X100 (0.1% v/v)	
Sodium fluoride (1 mM)	
β-glycerophosphate (10 mM)	
Sucrose (320 mM)	BDH, Poole, UK
Protease inhibitor cocktail (1% (v/v))	Invitrogen, Rockford, USA Calbiochem, VWR International Ltd, Lutterworth, Leicestershire, UK
BSA blocking solution	
Bovine serum albumin (BSA, 1mg/ml) dissolved in distilled water	Sigma Aldrich, Poole, UK
Lowry A solution	
Sodium hydroxide (NaOH) (0.4 % (w/v)	Sigma Aldrich, Poole, UK
Sodium dodecyl sulphate (SDS) 0.2 % (w/v)	
Sodium carbonate (Na_2CO_3) (2% w/v)	
Lowry B solution	
Sodium potassium tartrate (2% w/v)	
Copper sulphate (2% w/v)	
Lowry AB solution consists of adding 100 µl sodium potassium tartrate and 100 µl copper sulphate to 20 ml of solution A.	
6X Concentrated solubilisation buffer	
β-mercaptoethanol (5% (v/v)	Sigma Aldrich, Poole, UK
Bromophenol blue (0.01% (v/v)	
Sodium dodecyl sulphate (SDS) (8.3 mM)	

Tris (62.5 mM)	Invitrogen, Rockford, USA
10X Electrophoresis buffer	
Tris (0.19 M)	Invitrogen, Rockford, USA
Glycine (1.9 M)	Sigma Aldrich, Poole, UK
Sodium dodecyl sulphate (SDS) (35 mM)	
Tris-buffered saline containing 0.1% Tween 20 (TBST) to PH 7.6	
Tris (25 mM)	Invitrogen, Rockford, USA
Sodium chloride (125 mM)	Fisher Scientific, Loughborough, UK
Tween 20 (0.1% (v/v)	Sigma Aldrich, Poole, UK
Hydrochloric acid (HCl) (1M) (sufficient drops until pH 7.6)	
Transfer buffer	
Tris (0.19 M)	Invitrogen, Rockford, USA
Glycine (1.9 M)	Sigma Aldrich, Poole, UK
Methanol (20% (v/v)	
Ponceau S buffer	
Acetic acid (5% (w/v)	Sigma Aldrich, Poole, UK
Ponceau S	
Folin and Ciocalteu's phenol reagent	
Milk blocking solution	
Milk 5% (w/v) dissolved in TBST buffer	Skimmed milk powder: Sainsburys foods (London, UK)
4-20% SDS-PAGE	Bio-Rad, Hemel Hempstead, Hertfordshire, UK
Nitrocellulose membrane	Thermo Fisher Scientific, Rockford, USA

Table 2.3 List primary and secondary antibodies used in Western blotting

Antibody	Host	Antibody type	Dilution	Source& Catalogue Number
The primary antibody				
Anti-CBS	Rabbit	Polyclonal	1:1000	Abnova, UK H00000875-D01P
Anti-CSE	Rabbit	Polyclonal	1:1000	Abcam Ab80643
Note: 10 µL of antibody stock + 10 mL of 5% TBST fat-free milk				
The secondary antibody				
Goat anti-rabbit, IR800 CW, Green	Rabbit	Polyclonal	1:10,000	LI-COR Biosciences, Cambridge, UK 925-32211
Goat anti-mouse GAPDH, IR680CW, Red	Rabbit	Polyclonal	1:10,000	LI-COR Biosciences, Cambridge, UK 925-32210
Note: 3 µL of antibody stock + 30 mL of 5% TBST fat-free milk				
β-Actin Red	Mouse	monoclonal	1:50,000	Sigma, UK A2228

2.3 Drugs and solutions

Drugs and chemicals are listed in tables 2.1, 2.2, 2.3.

2.4 Data analysis

Data analysis was carried out using Prism (Version 7, Graph Pad Software). All values were expressed as mean ± standard error of the mean (S.E.M). Where n = the number of observations. The difference between means was considered statistically significant at a value of $P < 0.05$. Differences between concentration response curves were assessed using two-way ANOVA in conjunction with the Bonferroni post-hoc test to assess possible difference at individual concentrations. Data were analysed by 2-tailed, paired Student's *t*-test to compare the concentrations and levels of induced tone of pre-constriction compounds.

Chapter 3:

Effect of Oxygen Levels on responses to NaHS in Blood Vessels

3.1 Introduction

In the last decade, the physiological importance of H_2S became recognised by the finding of endogenous H_2S formation in mammalian cells (Liu *et al*., 2012). H_2S has been shown to have many cardiovascular physiological functions, mediated through various molecular targets (Wang, 2012), including vascular reactivity (Ali *et al.,* 2006, Cheng *et al*., 2004, Kiss *et al*., 2008, Cindrova-Davies *et al*., 2013, Materazzi *et al*., 2017, Lim *et al*., 2008, Ping *et al*., 2015, Caprada *et al*., 2017), cardioprotection (Elrod *et al.*, 2007, Johansen *et al*., 2006) and modifying natriuresis and diuresis (Beltowski, 2010, Xia *et al*., 2009). In the vasculature, the response to H_2S differs according to the vessel origin (Liu *et al*., 2012) and the levels of oxygen (Koenitzer *et al.*, 2007).

H_2S is endogenously synthesized from L-cysteine by two pyridoxal-5'-phosphate-dependent enzymes, cystathionine-y-lyase (CSE) and cystathionine-β-synthase (CBS) (Li and Moore, 2007). CSE and CBS are expressed in many cells and a variety of tissues; CBS is highly expressed in nervous system tissue, while CSE is most prominent in the vasculature and peripheral tissue (Ishii *et al.*, 2004). More recent evidence has shown that CBS is also located within the vascular endothelium and smooth muscle cells (Baragatti *et al*., 2013). NaHS is usually applied as an H_2S donor since it dissociates to Na^+ and HS^-; the latter then partially binds H^+ to form un-dissociated H_2S (Lowicka and Beltowski, 2007), therefore, without having to apply the toxic gas of H_2S, we can use NaHS as an H_2S donor. NaHS has the advantage of boosting H_2S concentration rapidly (at the time the solution is prepared) (Zhao *et al*., 2014).

In the majority of vascular preparations, H_2S has been reported to cause vasorelaxation, such as, in rat aorta (Zhao *et al*., 2001), rat hepatic artery (Fiorucci *et al*., 2005b, Siebert *et al*., 2008), rat mesenteric resistance arteries (Cheng *et al*., 2004, Tang *et al*., 2005), rat coronary artery (Cheang *et al*., 2010), rat pulmonary artery (Wang *et al*., 2008), canine coronary arteries (Casalini *et al.*, 2013), human pulmonary arteries (Ariyaratnam *et al*., 2013), human mesenteric arteries (Materazzi *et al*., 2017), human chorionic arteries (Cindrova-Davies *et al*., 2013), human internal mammary artery (Webb *et al*., 2008), and piglet cerebral arterioles (Liang *et al*., 2011).

In addition to vasorelaxation, it has been shown to contract some blood vessels. For example, Ali *et al.,* (2006) demonstrated that, at low levels (10–100 μM), NaHS caused a contraction in rat aorta. Low levels of NaHS also reversed the NO-generated vasodilation response promoted by acetylcholine (Whiteman *et al.*, 2006). They also found that low doses of NaHS increased mean arterial blood pressure in anesthetized rats. Similarly, Kubo *et al*., (2007) demonstrated that, at low levels (100–300 μM in rat aorta and 30–100 μM in mouse aorta), NaHS caused contraction in both rat and mouse aorta. In rat aorta, Lim *et al*., (2008) demonstrated that low concentrations (10–100 μM) of H_2S caused contraction. In addition, NaHS induced vasoconstriction in rat coronary artery (Ping *et al*., 2015), in rabbit blood vessels (Aorta, carotid, renal and iliac) (Caprada *et al*., 2017), in rat cerebral arteries (Li *et al*., 2015) and in rat aortic rings (Orlov *et al*., 2017).

Thus the responses to H_2S in blood vessels are complex with either relaxation or contraction reported and the responses to H_2S may also be sensitive to the levels

of oxygen. Oxygen can have an antagonistic effect on H_2S, leading to its oxidation (Li and Lancaster, 2013) and consequently reducing its biological actions (Stein and Bailey 2013). The spontaneous reaction of H_2S with O_2 can cause a reduction in H_2S levels; tissues with relatively high O_2 tensions may have less H_2S compared with tissues with lower O_2 levels (Olson *et al.,* 2013, Whitfield *et al.,* 2008). In most experiments studying responses to exogenous donors of H_2S, the vascular preparations have been gassed at high pO_2 concentrations, usually 95% O_2: 5% CO_2 mixtures. However, Koenitzer *et al.,* (2007) investigated whether experimental conditions could contribute to the variability in responses to H_2S. They observed that the effect of H_2S was dependent upon the prevailing O_2 tension. In rat aorta pre-contracted with phenylephrine, H_2S promoted vasoconstriction at high O_2 levels and vasorelaxation was produced at lower concentrations of oxygen. This alteration in response was proposed to be due to oxidation of H_2S at high O_2 tension, decreasing H_2S availability or, to the formation of H_2S-derived oxidation compounds that had contractile activity. For example, an oxidation compound of H_2S, meta (bis) sulphate, induced vasoconstriction in rat aortic rings (Will *et al.,* 1989). Kiss *et al.,* (2008), also found an improved vasodilator response to H_2S under conditions of low O_2 (hypoxic condition).

The present study investigated the effect of O_2 levels on responses to NaHS in systemic arteries and vein, pulmonary arteries and vein and chorionic vessels. Vessels were gassed with 95% O_2:5% CO_2, 95% Air: 5% CO_2 or 95% N_2:5% CO_2.

3.2 Materials and Methods

3.2.1 Materials

Materials used for the experiments of this chapter were listed in table 2.1

3.2.2 Methods

3.2.2.1 Isometric tension recording

3.2.2.1.1 Set up and the general protocol for recording isometric tension

Porcine splenic, porcine pulmonary and human chorionic blood vessels were isolated and set up in an isometric tension recording system as described in the methods chapter. The cleaned vessels were set up in organ baths containing PSS at 37˚C and gassed with either 95% O_2:5% CO_2, 95% Air: 5% CO_2 or 95% N_2:5% CO_2. The PSS was pre-gassed for approximately 30 minutes to ensure gaseous equilibrium of the PSS. Following that, an initial resting tension was applied to vessels which were allowed to relax for approximately 30-45 minutes to equilibrate and achieve a stable baseline tone. Thereafter, vessels were exposed to KCl (60mM) for 15 minutes and allowed to contract to confirm their viability. Following two exposures of the vessels to KCl (60mM), vessels were pre-contracted to a level of 30-70% of the maximum KCl response. In most experiments, this was achieved using either, the thromboxane A2 receptor agonist, U46619 or the endothelin receptor agonist, endothelin-1. Once vessel tension plateaued, a concentration-response curve to NaHS was attained by applying it in a cumulative manner, in 3-fold increments between 1 μM and 1 mM, with a duration of 3 minutes between additions. In all experiments another paired segment (from the same tissue) was set up and used as a control. Only one concentration response curve to NaHS was attained in each preparation. In some experiments, a concentration-response curve to L-cysteine was obtained

by applying it in a cumulative method, in 3-fold additions between 10 µM and 3 mM, with a duration of 5 minutes between increments.

3.2.3 Data analysis

Data analysis was carried out using Prism (Graph Pad Software). All values were expressed as mean ± standard error of the mean (S.E.M). Where n = the number of observations. The difference between means was considered statistically significant at a value of $P < 0.05$. Differences between concentration response curves were assessed using two-way ANOVA in conjunction with the Bonferroni post-hoc test to assess possible difference at individual concentrations.

3.3 Results

3.3.1. Isometric tension recording

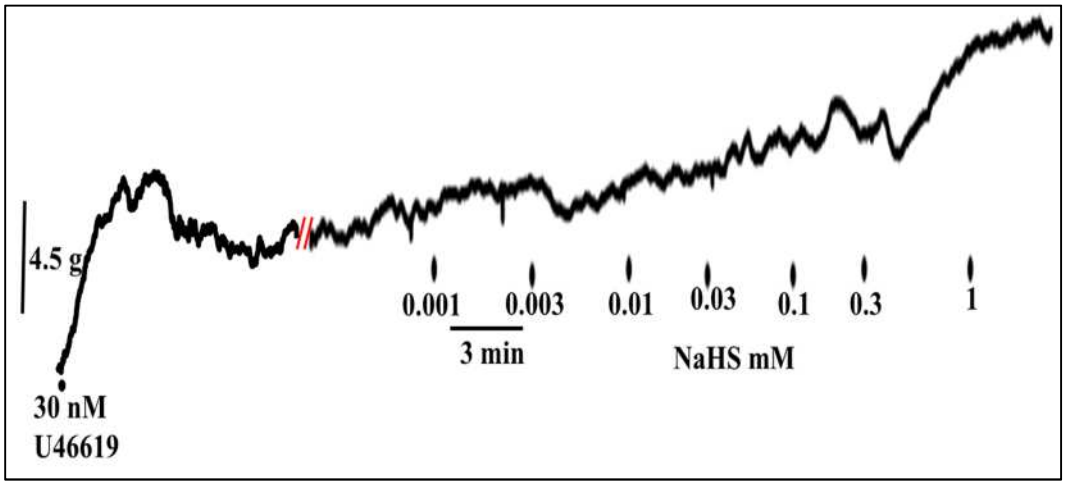
3.3.1.1. Response to NaHS under different oxygen conditions in Porcine Splenic Arteries

In the current experiment I examined responses to NaHS on pre-constricted splenic artery when the preparations were gassed with (a) 95% O_2: 5% CO_2, (b) 95% Air: 5% CO_2 and (c) 95% N_2: 5% CO_2. First, splenic artery samples were contracted with U46619 then a cumulative concentration response curve to NaHS was generated. A paired group of splenic artery segments had nothing added and acted as the control to monitor the stability of the U46619-contraction. KCl responses, U46619 responses, and U46619 concentrations under different gassing conditions are summarised in table 3.1. There was a significant increase in the concentrations of U46619 required to induce tone under 95% N_2: 5% CO_2 gassing conditions compared with 95% O_2: 5% CO_2 ($p>0.05$; Student's t-test). When gassed with 95% O_2: 5% CO_2 and 95% air: 5% CO_2, the splenic artery responded to NaHS with concentration dependent contractions which became prominent at concentrations above 30µM NaHS (figure 3.2a and 3.2b), but in 95% N_2:5% CO_2, NaHS caused a relaxation responses at 0.1mM and 0.3mM NaHS. At the highest concentration used (1mM) this reverted to a contraction of the splenic artery (figure 3.2c).

PSA			
	95%O_2:5% CO_2	**95%air: 5% CO_2**	**95%N_2:5% CO_2**
KCL Responses	10.3 ± 1.2 g	10.0 ± 1.5 g	7.4 ± 0.5 g
U46619 Responses	44.6 ± 3%	50.6 ± 5%	43.7 ± 5%
U46619 Concentration	-7.9 ± -8.4 M	-8.3 ± -8.9 M	-6.9 ± -7.3 M

Table 3.1 shows the average mean of response to KCL (60mM), the level of induced tone by U46619 and the U46619 concentration required to produce this.

a)



b)

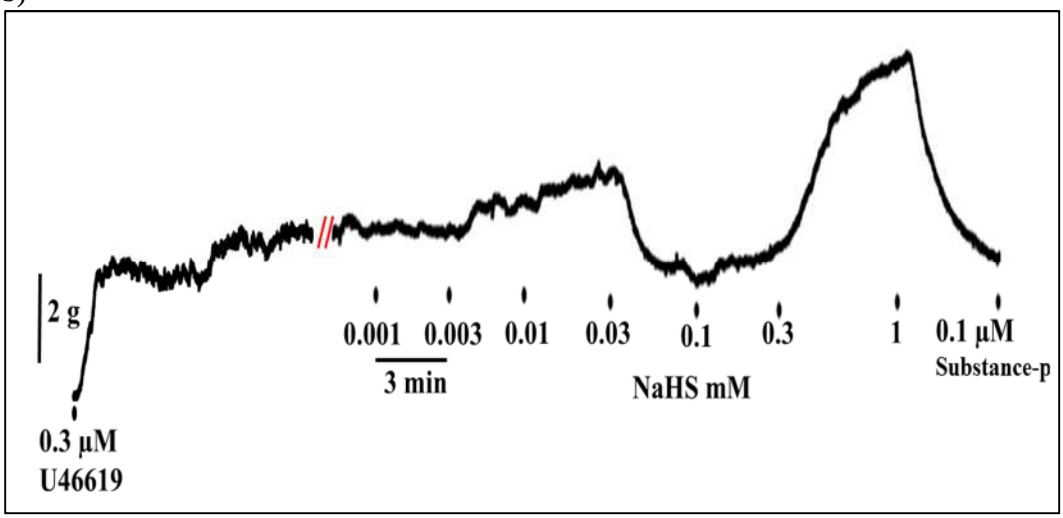


Figure 3.1 Isometric tension measurement trace showing the response of porcine splenic artery to U46619 and to cumulative concentration of NaHS in preparations gassed with (a) 95% O_2:5% CO_2, (b) 95%N_2: 5% CO_2.

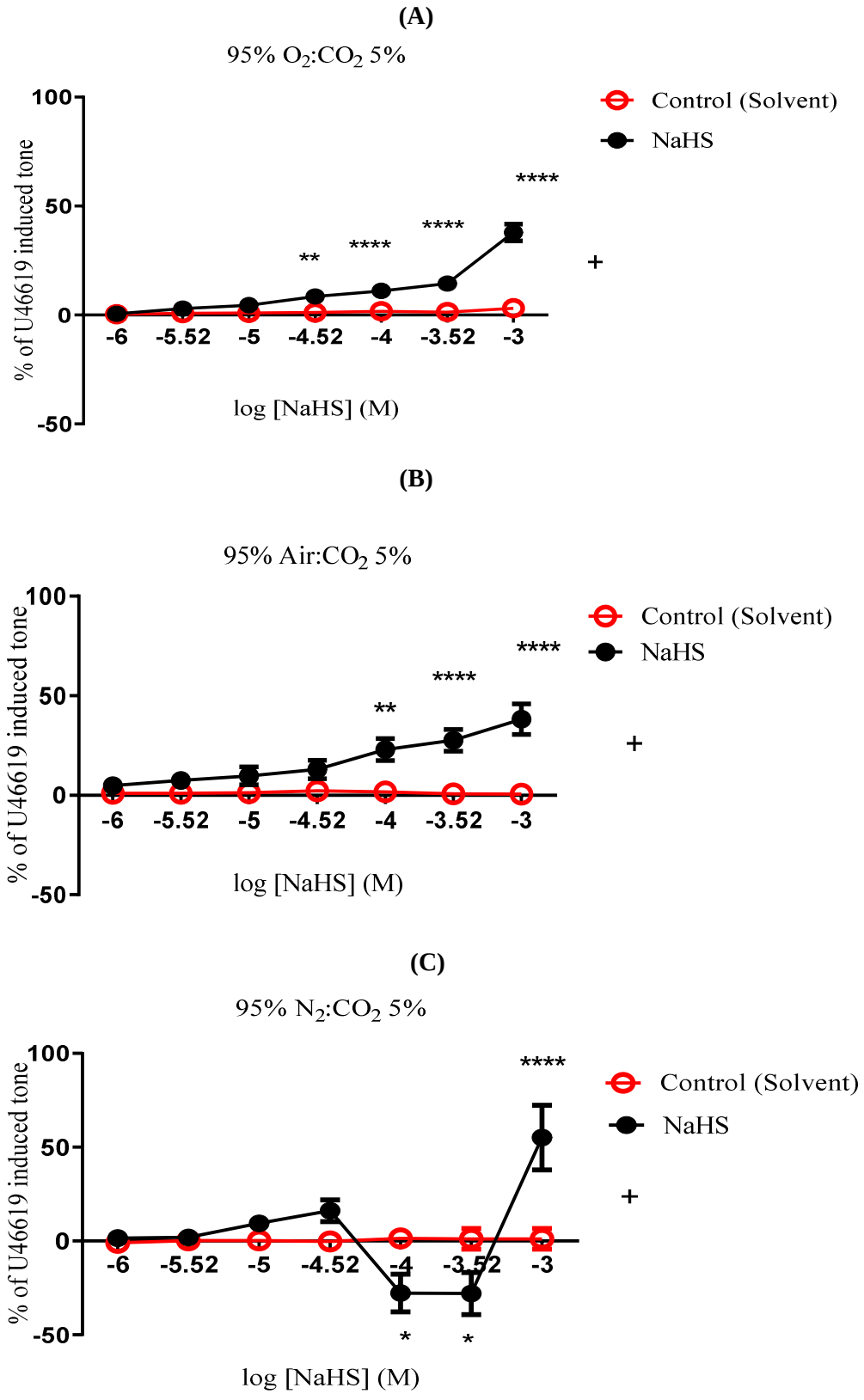


Figure 3.2 Concentration response curves to NaHS in porcine splenic arteries pre-constricted with U46619 and gassed with (a) 95% O_2:5% CO_2, (b) 95%Air: 5% CO_2, (c) 95%N_2: 5% CO_2, Each point represents the mean ± SEM (n=6-8). + indicates a significant difference between curves ($p<0.05$, ANOVA). *, **, **** represents significant difference in response at single concentration ($p<0.05$, $p<0.01$, $p<0.0001$ respectively, Bonferroni test).

3.3.1.2. Response to NaHS under different oxygen conditions in Porcine Splenic Veins.

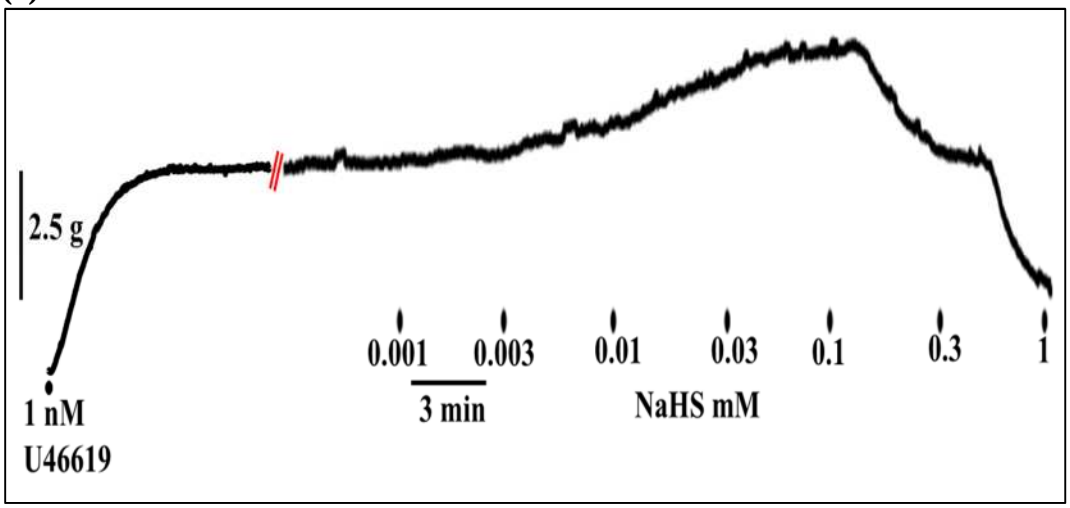
Splenic veins were contracted with U46619 before a cumulative concentration response curve to NaHS was generated. KCl responses, U46619 responses, and U46619 concentrations under different gassing conditions are summarised in the table 3.2. There was a significant decrease in the concentrations of U46619 required to induce tone under 95% N_2: 5% CO_2 gassing conditions compared with 95% O_2: 5% CO_2 and 95% Air: 5% CO_2 ($p>0.05$; Student's t-test).

Under 95%O_2:5% CO_2 and 95%air: 5% CO_2 gassing conditions, the splenic vein responded to NaHS with concentration dependent contractions starting at 3µM NaHS followed by relaxation at 0.3mM and 1mM NaHS (figure 3.4a and 3.4b). Under 95%N_2:5% CO_2 condition, NaHS caused concentration dependent relaxations which became prominent at 30µM NaHS (figure 3.4c).

PSV			
	95%O_2:5% CO_2	**95%air: 5% CO_2**	**95%N_2:5% CO_2**
KCL Responses	4.5 ± 1.2 g	5.6 ± 0.3 g	5.0 ± 1.0 g
U46619 responses	50.3 ± 9%	62 ± 4%	56.2 ± 3%
U46619 concentrations	-9 ± 0 M	-9 ± 0 M	-9.7 ± -10.2 M

Table 3.2 shows the average mean of response to KCL (60 mM), the level of induced tone by U46619 and the U46619 concentration required to produce this.

(a)



(b)

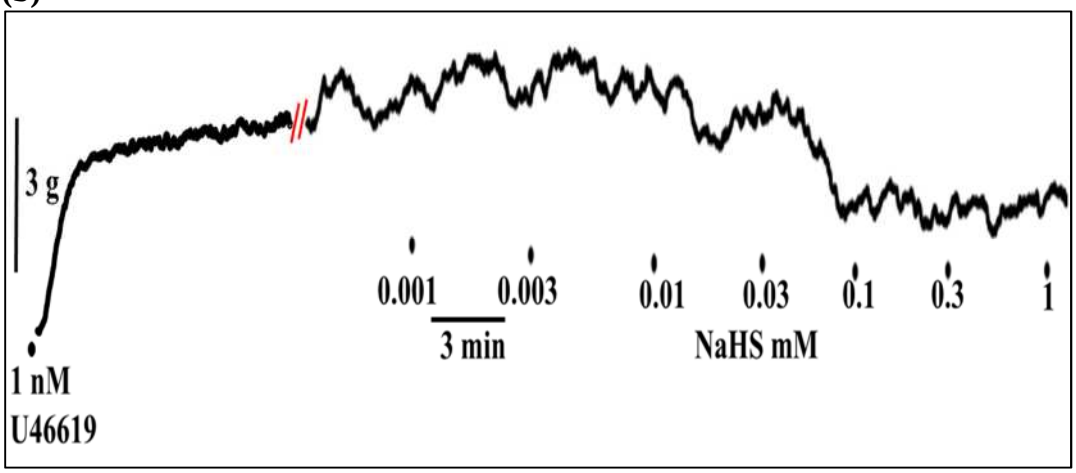


Figure 3.3 Isometric tension measurement trace showing the response of porcine splenic vein to U46619 and to cumulative concentration of NaHS in preparations gassed with (a) 95% O_2:5% CO_2, (b) 95%N_2: 5% CO_2.

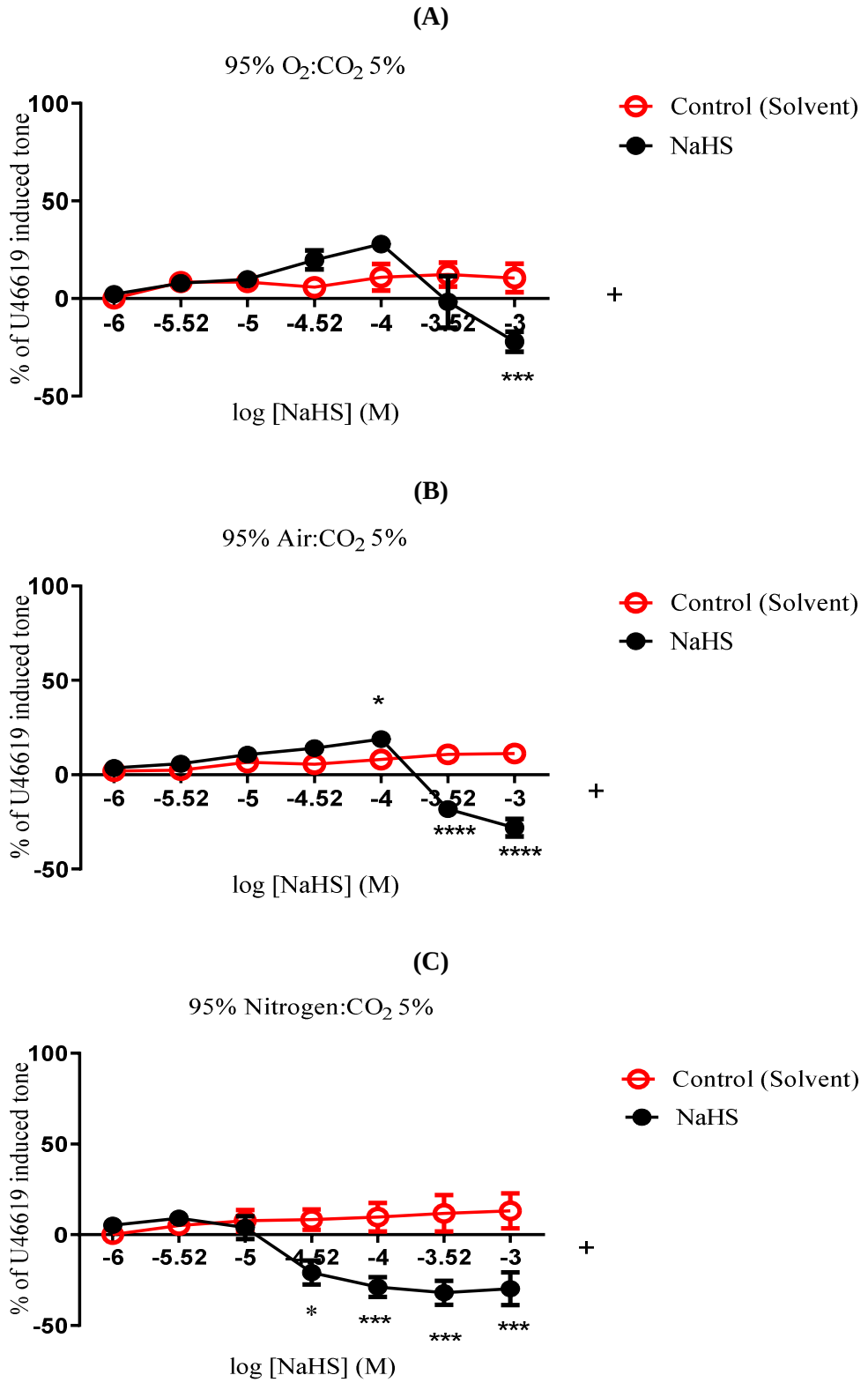


Figure 3.4 Concentration response curves to NaHS in porcine splenic veins pre-constricted with U46619 and gassed with (a) 95% O_2:5% CO_2, (b) 95%Air: 5% CO_2 and (c) 95%N_2: 5% CO_2, Each point represents the mean ± SEM (n=6). + indicates a significant difference between curves ($p<0.05$, ANOVA). *, ***, **** represents significant difference in response at single concentration ($p<0.05$, $p<0.001$, $p<0.0001$ respectively, Bonferroni test).

3.3.1.3. Response to NaHS under different oxygen conditions in Porcine Pulmonary Arteries

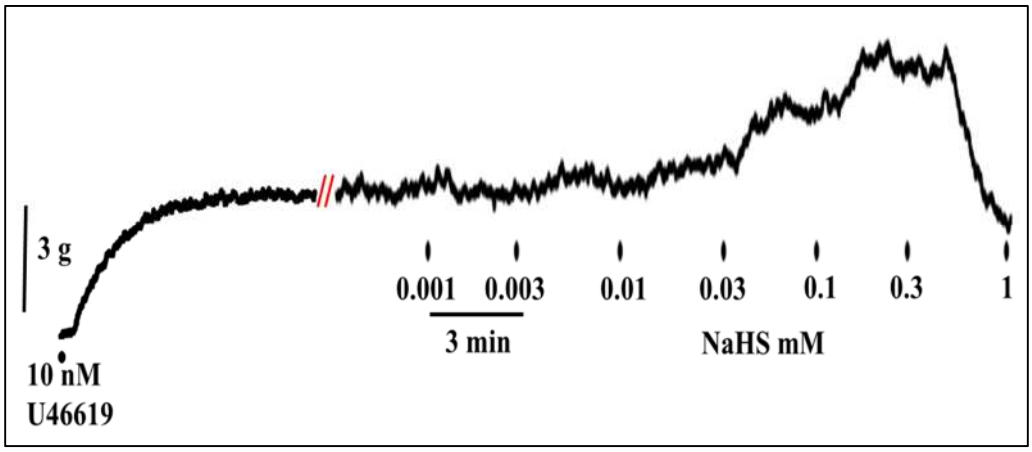
Experiments were carried out to examine NaHS responses in the porcine pulmonary artery when the preparations were gassed with gas mixtures containing different levels of O_2. Pulmonary artery samples were contracted with U46619 or Endothelin-1 then a cumulative concentration response curve to NaHS was generated. A paired pulmonary artery segment had nothing added and acted as the control to monitor the stability of the U46619 or Endothelin-1 contraction. The average KCl responses, U46619 or Endothelin-1 responses, and U46619 or Endothelin-1 concentrations under different gassing conditions are summarised in the table 3.3.

Under 95% O_2: 5% CO_2 and 95% air: 5% CO_2 gassing conditions, the pulmonary artery responded to NaHS with concentration dependent contractions starting at 3µM NaHS followed by relaxation at 1mM and at 0.3mM and 1mM NaHS respectively (figure 3.6a, b). Under 95% N_2: 5% CO_2 condition, NaHS caused concentration dependent relaxations which became prominent at 100µM NaHS (figure 3.6c).

PPA				
	95%O_2: 5% CO_2		**95%air: 5% CO_2**	**95%N_2: 5% CO_2**
KCL Responses	6.9 ± 1g	**KCL Responses**	7.5 ± 0.8g	7.1 ± 0.6g
U46619 responses	42.1 ± 2.8%	**Endothelin-1 responses**	66.5 ± 4.4%	48.4 ± 5.1%
U46619 concentration	-8 ± 0 M	**Endothelin-1 concentration**	-9 ± 0 M	-9 ± 0 M

Table 3.3 shows the average mean of response to KCL (60 mM), the level of induced tone by U46619 and Endothelin-1, and the U46619 and Endothelin-1 concentration required to produce this.

(a)



(b)

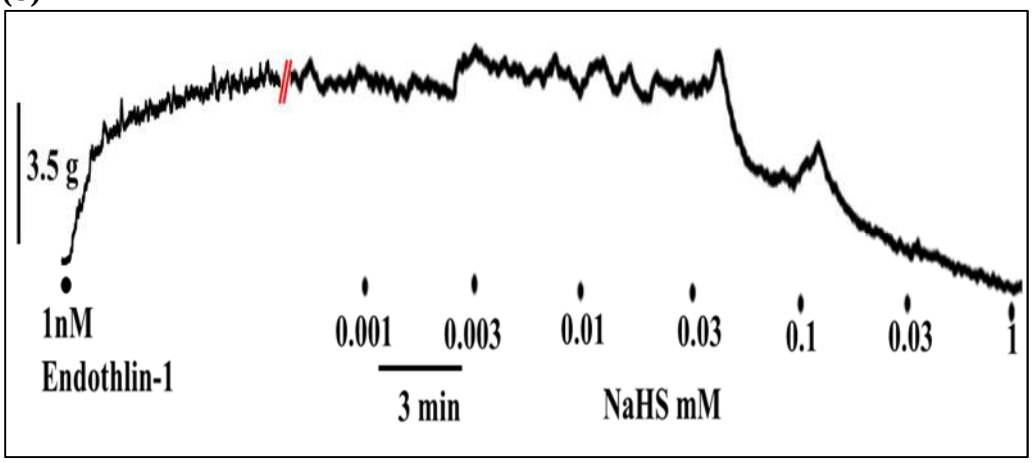


Figure 3.5 Isometric tension measurement trace showing the response of porcine pulmonary artery to U46619 or Endothelin-1 and to cumulative concentration of NaHS in preparations gassed with (a) 95% O_2:5% CO_2, (b) 95%N_2: 5% CO_2.

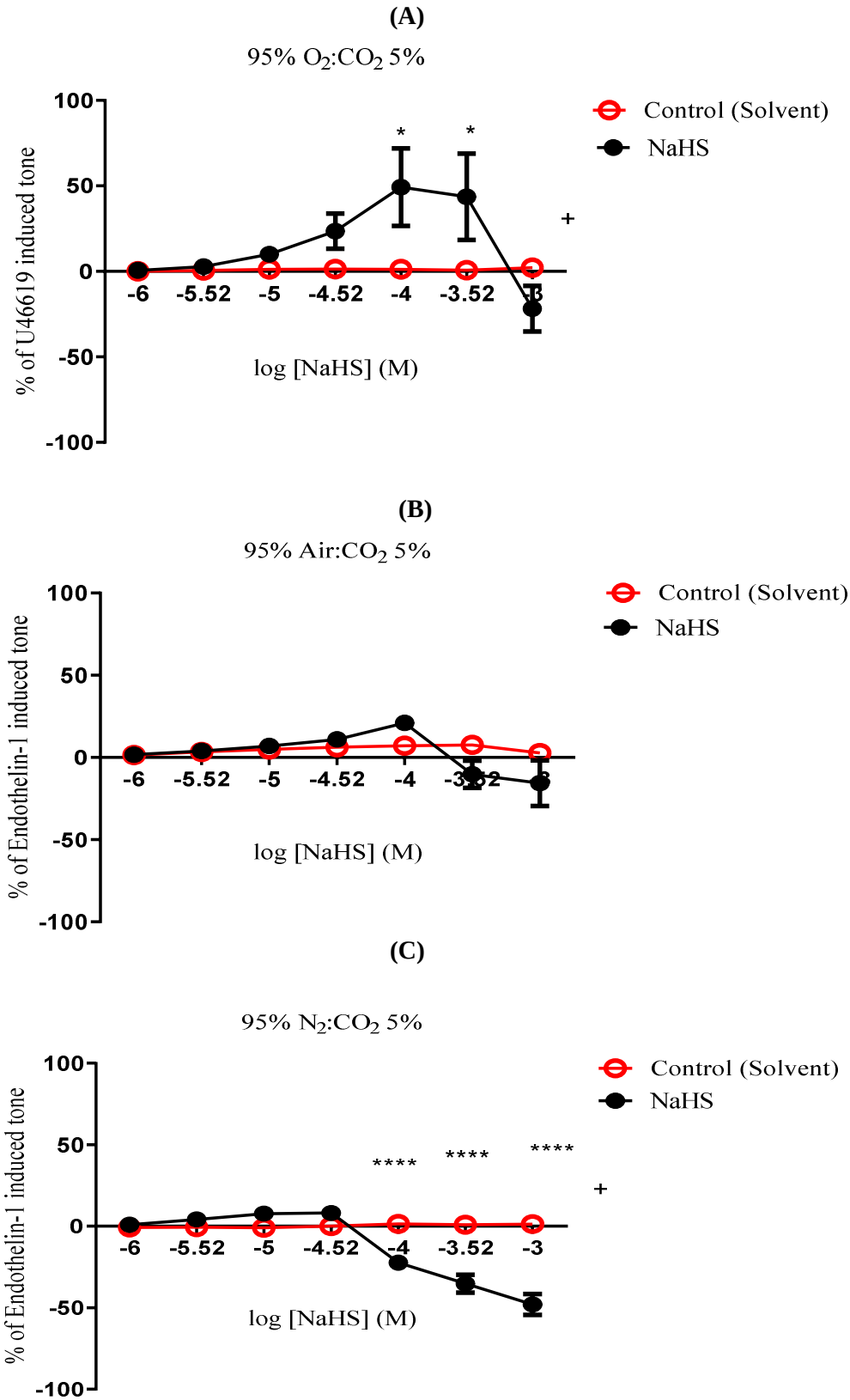
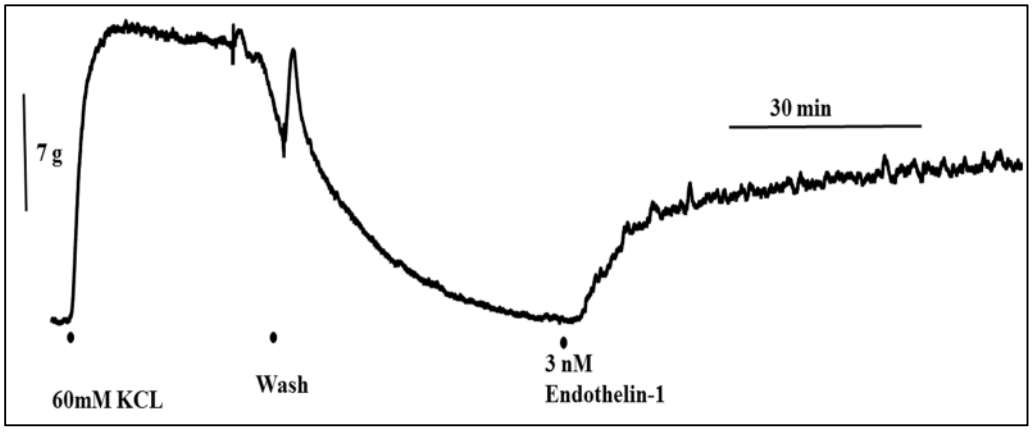


Figure 3.6 Concentration response curves to NaHS in porcine pulmonary arteries pre-constricted with (a) U46619 and gassed with 95% O_2:5% CO_2, (b) Endothelin-1 and gassed with 95%Air: 5% CO_2, (c)) Endothelin-1 and gassed with 95%N_2: 5% CO_2, Each point represents the mean ± SEM (n=6). + indicates a significant difference between curves ($p<0.05$, ANOVA). * represent significant difference in response at single concentration ($p<0.05$, Bonferroni test).

(a)



(b)

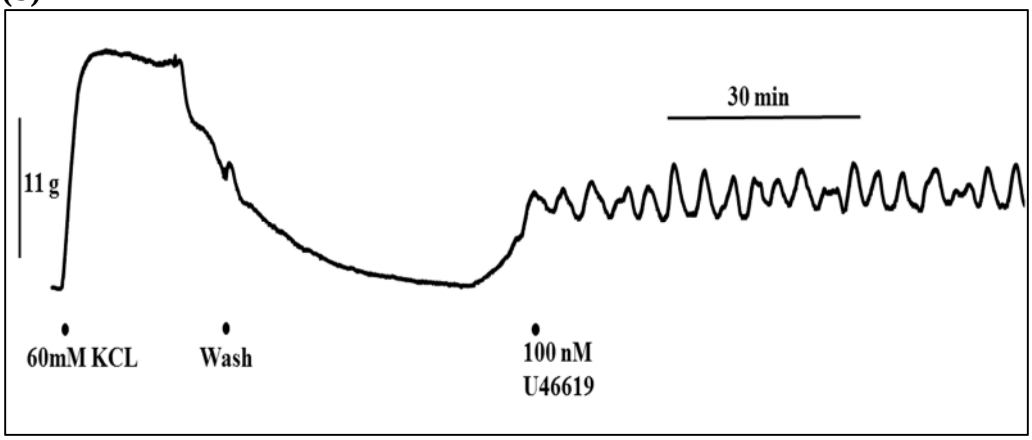


Figure 3.7 Isometric tension measurement trace showing the response of porcine pulmonary artery to (a) Endothelin-1 (stable tone) and to (b) U46619 (unstable tone) in preparations gassed with 95%N_2: 5% CO_2.

3.3.1.4. Response to NaHS under different oxygen conditions in Porcine Pulmonary Veins.

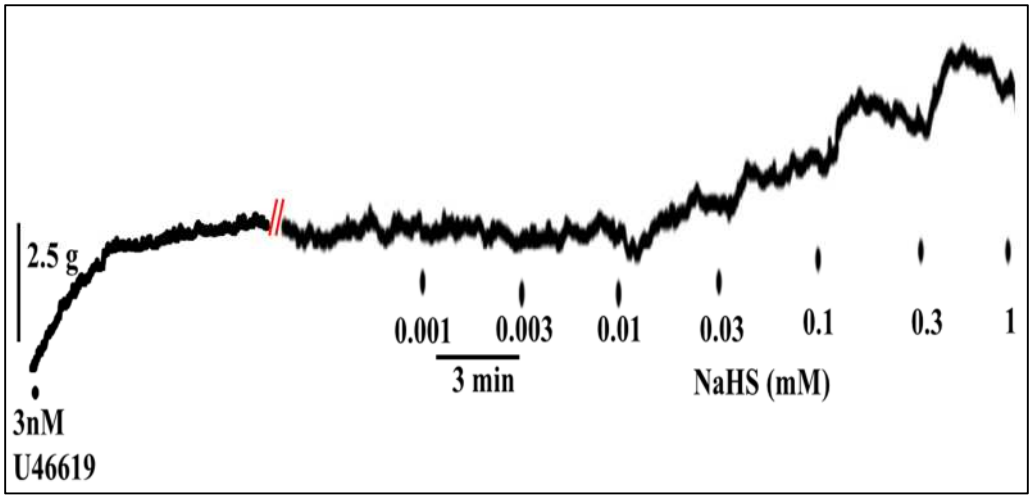
Pulmonary veins were contracted with U46619 before being exposed to increasing concentrations of NaHS. The average KCl responses, U46619 responses, and U46619 concentrations required under different gassing conditions were summarised in the table 3.4. There was a significant increase in the concentrations of U46619 required to induce tone under 95% N_2: 5% CO_2 and 95% Air: 5% CO_2 gassing conditions compared with 95% O_2: 5% CO_2 ($p>0.05$; Student's t-test).

Under the 95% O_2: 5% CO_2 gassing conditions, the pulmonary vein responded to NaHS with concentration dependent contractions which became prominent at 30µM NaHS (figure 3.9a). In 95%air: 5% CO_2, the pulmonary vein responded to NaHS with concentration dependent contractions starting at 100µM NaHS which reversed at 1mM NaHS (figure 3.9b). Under the 95% N_2: 5% CO_2 condition, NaHS caused a small concentration dependent contraction starting at 30µM NaHS which reversed at 0.3 mM and 1mM NaHS (figure 3.9c).

PPV			
	95%O_2: 5% CO_2	**95%air: 5% CO_2**	**95%N_2: 5% CO_2**
KCL Responses	5 ± 0.3 g	5.5 ± 0.5 g	4.5 ± 0.3 g
U46619 responses	46.8 ± 4.4%	52.6 ± 5%	45.3 ± 3.3%
U46619 concentration	-8.8 ± -9.4 M	-7.9 ± -8.5 M	-8 ± 0 M

Table 3.4 shows the average mean of response to KCL (60 mM), the level of induced tone by U46619 and the U46619 concentration required to produce this.

(a)



(b)

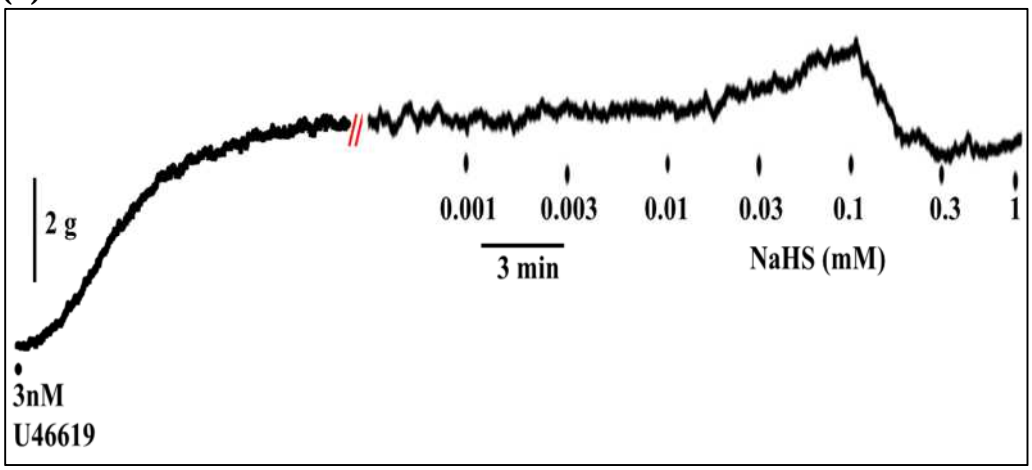


Figure 3.8 Isometric tension measurement trace showing the response of porcine pulmonary vein to U46619 and to cumulative concentration of NaHS in preparations gassed with (a) 95% O_2:5% CO_2, (b) 95%N_2: 5% CO_2

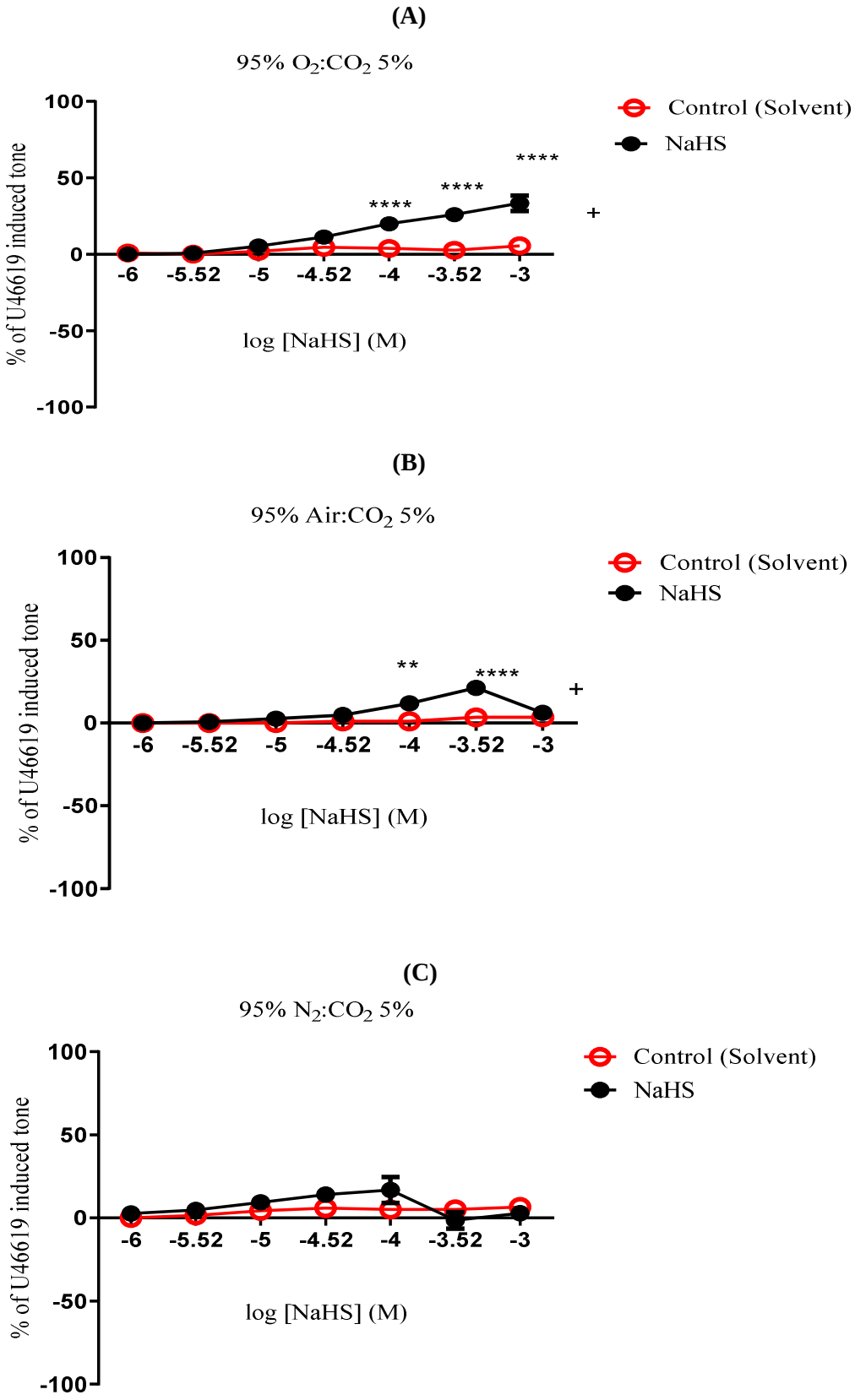


Figure 3.9 Concentration response curves to NaHS in porcine pulmonary veins pre-constricted with U46619 and gassed with (a) 95% O_2:5% CO_2, (b) 95%Air: 5% CO_2 and (c) 95%N_2: 5% CO_2, Each point represents the mean ± SEM (n=6). + indicates a significant difference between curves ($p<0.05$, ANOVA). **, **** represents significant difference in response at single concentration ($p<0.001$, $p<0.0001$, respectively, Bonferroni test).

3.3.1.5. Response to NaHS under different oxygen conditions in Human Chorionic Arteries (HchA)

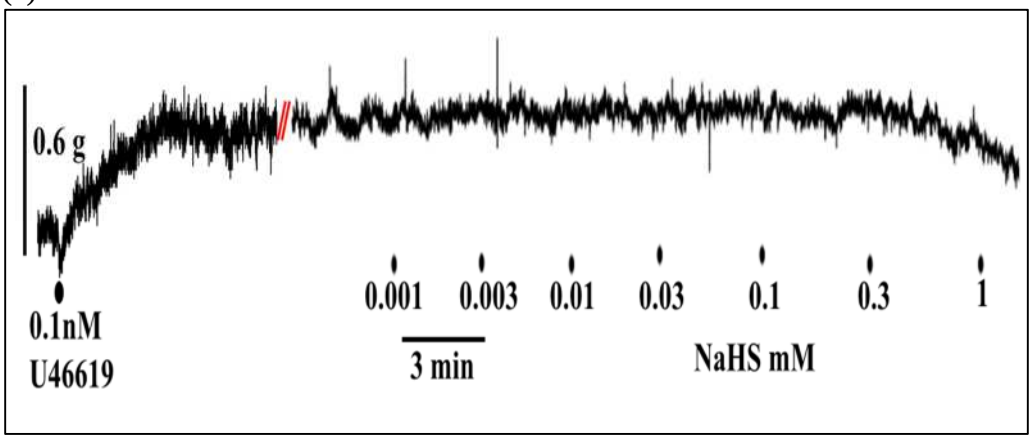
In the current experiment I examined responses to NaHS on pre-constricted human chorionic artery when the preparations were gassed with (a) 95% O_2: 5% CO_2, (b) 95% Air: 5% CO_2 and (c) 95% N_2: 5% CO_2. First, chorionic artery samples were constricted with U46619 then a cumulative concentration response to NaHS was generated. A paired group of chorionic artery segments had nothing added and acted as the control to monitor the stability of the U46619-contraction. KCl responses were 0.9 ± 0.1 g in 95%O_2:5% CO_2 (n = 5), 0.7 ± 0.2g in 95% air: 5% CO_2 (n = 6) and 1.4 ± 0.3 g in 95%N_2:5% CO_2 (n = 6). For this series of experiments, vessels were pre-constricted with U46619 to 68.3 ± 3% of the KCl responses (60 mM) in 95% O_2: 5% CO_2 using an average U46619 concentration of $1.2 \times 10^{-10} \pm 4.6 \times 10^{-11}$ M (n = 5), 60.6 ± 5% in 95% Air:5% CO_2 using an average U46619 concentration of $2.6 \times 10^{-10} \pm 3.3 \times 10^{-11}$ M (n = 6) and 55.4 ± 7% in 95% N_2:5% CO_2 using an average U46619 concentration of $3.5 \times 10^{-10} \pm 1.4 \times 10^{-10}$ M (n = 6).

In chorionic arteries, under conditions of 95% O_2:5% CO_2 and 95% Air: 5% CO_2 relaxation responses were observed at high concentration of NaHS (figure 3.11a, b), while under gassing conditions of 95% N_2:5% CO_2, NaHS produced a contraction at low concentrations. This waned at high concentrations (figure 3.11c).

HchA			
	95%O_2:5% CO_2	**95%air: 5% CO_2**	**95%N_2:5% CO_2**
Kcl responses	0.9 ± 0.1 g n=5	0.7 ± 0.2 g n=6	1.4 ± 0.3 g n=5
U46619 responses	68.3 ± 3%	60.6 ± 5%	55.4 ± 7%
U46619 concentration	-9.9 ± 10.3 M	-9.6 ± 10.5 M	-9.5 ± -9.9 M

Table 3.5 shows the average mean of response to KCL (60 mM), the level of induced tone by U46619 and the U46619 concentration required to produce this.

(a)



(b)

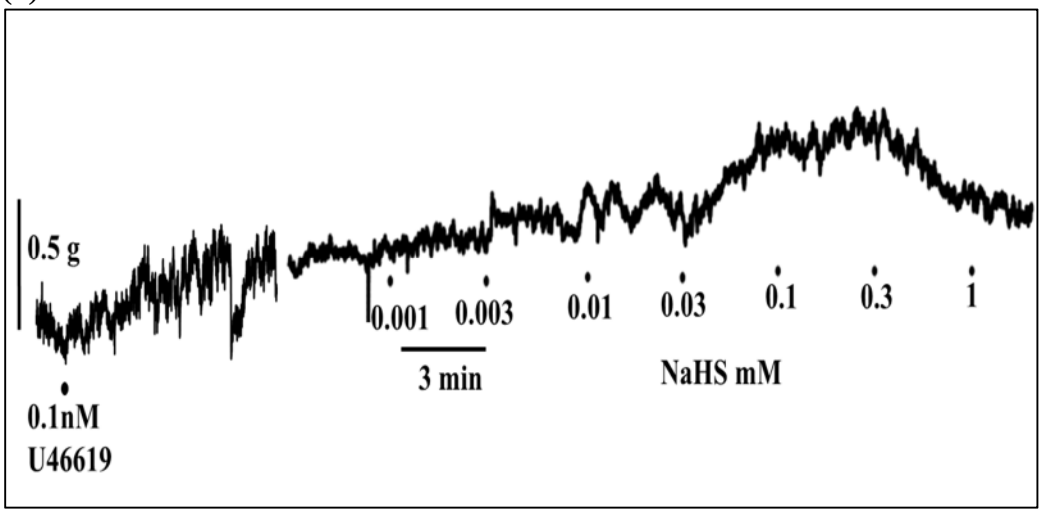


Figure 3.10 Isometric tension measurement trace showing the response of human chorionic artery to U46619 and to cumulative concentration of NaHS in preparations gassed with (a) 95% O_2:5% CO_2, (b) 95%N_2: 5% CO_2.

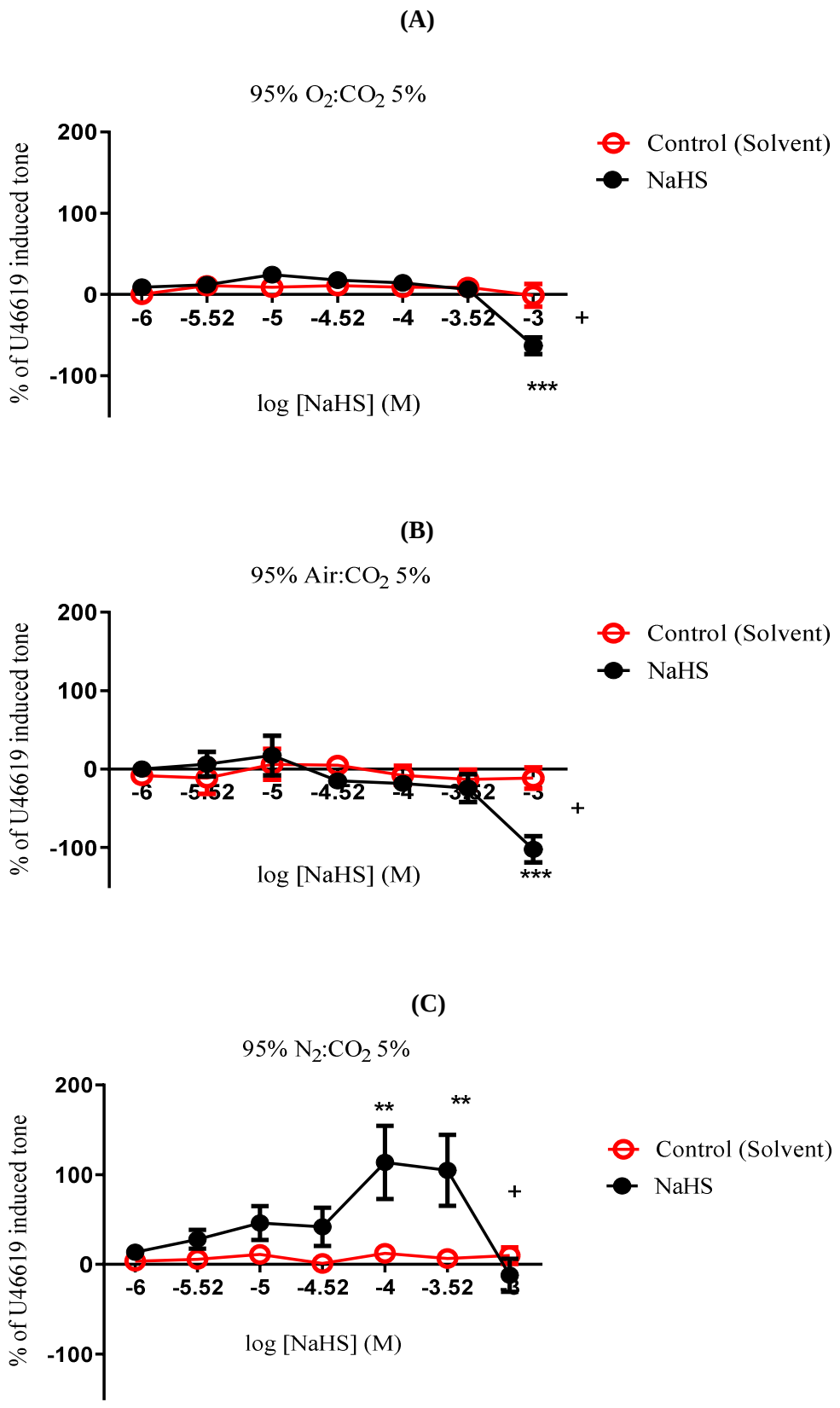


Figure 3.11 Concentration response curves to NaHS in human chorionic arteries pre-constricted with U46619 and gassed with (a) 95% O_2:5% CO_2, (b) 95%Air: 5% CO_2, (c) 95%N_2: 5% CO_2, Each point represents the mean ± SEM (n=5-6). + indicates a significant difference between curves ($p<0.05$, ANOVA). **, *** represents significant difference in response at single concentration ($p<0.01$, $p<0.001$ respectively, Bonferroni test).

3.3.1.6. Response to NaHS under different oxygen conditions in Human Chorionic Veins (HchV).

Human chorionic veins were contracted with U46619 before a cumulative concentration response curve to NaHS was generated. Average KCl responses were 2.1 ± 0.5 g in 95%O_2:5% CO_2 (n = 5), 1.3 ± 0.3 g in 95% air: 5% CO_2 (n= 5), 1.3 ± 0.2 g in 95%N_2:5% CO_2 (n= 5). The level of pre-constriction in the 95% O_2:5% CO_2 data was 56.0 ± 6.6% of the 60 mM KCl response using an average U46619 concentration of -9.5 ± 9.8 M (n=5), in the 95% air:5% CO_2 data the level of pre-constriction was 52.5 ± 6.3% using an average U46619 concentration of -9.3 ± -9.8 M (n=5) and in the 95% N_2:5% CO_2 it was 50.5 ± 8.3% using an average U46619 concentration of -9.8 ± -10.3 M (n=5).

In chorionic veins, under conditions of 95% O_2:5% CO_2 and 95% Air: 5% CO_2 relaxation responses were observed at high concentration of NaHS (figure 3.12a and 3.12b), while under gassing conditions of 95% N_2:5% CO_2, NaHS produced a contraction at low concentration which was reversed at high concentration (figure 3.12c).

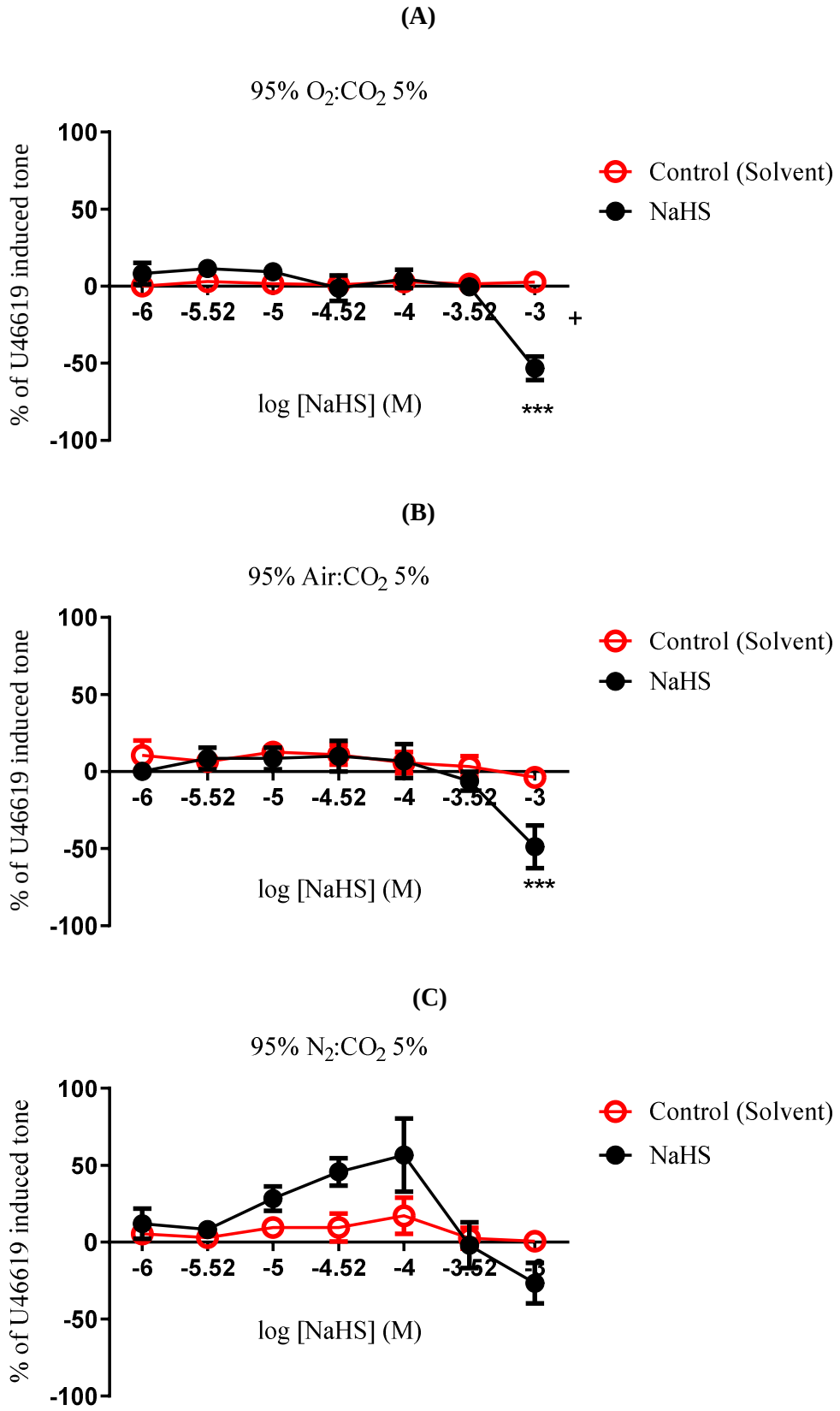


Figure 3.12 Concentration response curves to NaHS in Human chorionic veins pre-constricted with U46619 and gassed with (a) 95% O_2:5% CO_2, (b) 95%Air: 5% CO_2, (c) 95%N_2: 5% CO_2, Each point represents the mean ± SEM (n=5). + indicates a significant difference between curves ($p<0.05$, ANOVA). *** represents significant difference in response at single concentration ($p<0.001$, Bonferroni test).

3.4 Discussion

In the present study, the effect of changing the oxygen concentration on NaHS responses was investigated. NaHS caused a complex concentration–dependent response in systemic, pulmonary and placental blood vessels, depending upon the prevailing conditions. Under 95% air: 5% CO_2 (at a pO_2 of around 140 mmHg (White, 2012) which is closer to physiological levels for systemic arteries and pulmonary veins) a contractile response was evoked by NaHS in porcine splenic arteries and porcine pulmonary veins. In contrast, when gassing porcine splenic veins and porcine pulmonary arteries with 95% N_2: 5% CO_2, (at a pO_2 of around 33 mmHg (White, 2012) which is closer to physiological levels for systemic veins and pulmonary arteries) NaHS caused a relaxation response. In human chorionic arteries and veins, high concentrations of NaHS induced a vasorelaxation under super physiological oxygen tension for the fetoplacental circulation (95% Air: 5% CO_2), while under gassing conditions with a physiological oxygen tension for chorionic arteries and veins (95% N_2: 5% CO_2), NaHS produced a contraction at low concentrations.

The partial pressures of O_2 of PSS gassed with 95% O_2: 5% CO_2, 95% air: 5% CO_2 or 95% N_2: 5% CO_2 was shown to be 619 ± 17 mmHg, 140 ± 4 mmHg and 33 ± 6 mmHg, respectively (White, 2012). In mammals, the arterial pO_2 is normally around 100 mmHg and venous pO_2 is between 15-50 mmHg (Erecinska and Silver, 2001; Tsai *et al*., 2003). In normal pregnancy, the umbilical arterial pO_2 is normally between 16-28 mmHg and venous pO_2 is between 28-35 mmHg (Lackman *et al*., 2001, Nicolaides *et al*., 1989). This suggests that the most physiologically relevant conditions in the current study

were achieved by gassing with 95% air: 5% CO_2 for porcine splenic arteries (PSA) and porcine pulmonary veins (PPV) and 95% N_2: 5% CO_2 for porcine splenic veins (PSV), porcine pulmonary arteries (PPA) and human chorionic blood vessels.

In the majority of experiments studying responses to exogenous donors of H_2S, vascular preparations have been gassed at high pO_2 concentrations, usually 95% O_2: 5% CO_2 mixtures. For example, under high level of O_2, NaHS caused vasorelaxation in rat aortic (Zhao *et al*., 2001), in Human mammary artery (Webb *et al*., 2008) and in human chorionic arteries (Cindrova-Davies *et al*., 2013). Ali *et al.,* (2006) demonstrated that, at high oxygen levels, NaHS caused a contraction in rat aorta. The results of these studies may have less relevance to normal physiological conditions.

In PSA and PPV, the responses to NaHS were affected by the oxygen levels used to gas preparations with contractile response predominating under physiological gassing conditions (95% air: 5% CO_2) and vasorelaxtory responses evident at sub-physiological O_2 levels (95% N_2: 5% CO_2) in PSA. This is consistent with the observations in the rat aorta made by Koenitzer *et al*., (2007), where H_2S promoted vasoconstriction at high pO_2 levels. They demonstrated that H_2S was rapidly oxidized at high tension of O_2, thus decreasing its availability and lowering its capacity for vasorelaxation activity. However, they also suggested that formation of a H_2S oxidation compound at higher oxygen tension may evoke the contractile response. Similarly, Kiss *et al*., (2008) reported, in rat aortic rings, that H_2S-induced relaxation was more pronounced at a low oxygen tension,

whereas vasoconstriction was uncovered at a high oxygen concentration. They suggested that this was probably due to the competition between sulfide and oxygen at the level of cytochrome c, indicating that H_2S caused vasorelaxation through the inhibition of cytochrome c oxidase, causing depletion of ATP, since the vasorelaxant response of sulfide was less pronounced in the presence of higher oxygen level. Ali *et al.,* (2006) showed NaHS induced vasoconstriction and suggested this occurred by an interaction between NO and H_2S, such that NO vasorelaxantion was diminished. Supporting this suggestion, Whiteman *et al.,* (2006), under high oxygen levels, reported evidence for the creation of nitrosothiol product from the interaction between NO and H_2S. In addition, Kashiba *et al.,* (2002), showed that the higher oxygen tension induces free radicals oxygen and reactive species. Superoxide radicals react with NO creating peroxynitrite ($ONOO^-$) (Afanas, 2007). Synthetic peroxynitrite ($ONOO^-$) also reacts with H_2S to produce nitrosothiol (Whiteman *et al.,* 2006). Therefore, under high oxygen tension, the contraction induced by H_2S may occur due to the production of oxygen free radicals that mop up the basal NO and obviate the vasorelaxant responses of both NO and H_2S. While, under 95% N_2: 5% CO_2 gas condition, the production of oxygen free radicals would be fewer, then a vasorelaxatory response to H_2S may be evident.

In PSV and PPA, under lower O_2 levels (95% N_2: 5%CO_2) (at a pO_2 of around 33 mmHg (Tsai *et al*., 2003) which is more physiological for systemic veins and pulmonary arteries than traditional 95% O_2: 5%CO_2 experimental method) NaHS induced a vasorelaxation. This observation is similar to White, (2012) who showed that, in porcine splenic vein, the NaHS-induced vasorelaxation, at

low pO_2 levels. They suggesting that the veins exposed to a more physiological pO_2 did not mediate a contraction, being replaced by a NO-mediated vasorelaxation. Hosoki *et al*., (1997) demonstrated something similar, in rat portal vein, although the pO_2 was not reported. In addition, Eberhardat *et al.*, (2014) suggested that NO and H_2S acted co-operatively to produce nitroxyl (HNO) and that led to vasodilation through stimulation of TRPA1 channels on sensory nerves. Bir *et al*., (2012) showed that H_2S increased NO bioavailability at low oxygen tension.

In the human chorionic arteries and veins, high concentration of NaHS induced a vasorelaxation under higher O_2 levels (95% O_2: 5% CO_2 and 95% Air: 5% CO_2) which is supra-physiological pO_2 in human chorionic vessels. This observation is similar to results in human chorionic arteries (Cindrova-Davies *et al*., 2013), where NaHS produced vasorelaxation under conditions of 95% O_2: 5% CO_2 through activation of ATP-sensitive K^+ channels and nitric oxide synthesis. Similar results were shown by Mohammed *et al*., (2017) where Na_2-S evoked a vasorelaxation in the human umbilical vessels under 95% O_2: 5% CO_2 gas condition through activation of ATP-sensitive K^+ channels, as shown since the specific K_{ATP} channel inhibitor, glibenclamide, attenuated responses. The effect of altering the O_2 level used to gas the PSS changed the NaHS response, under gassing condition of low O_2 level (95% N_2: 5% CO_2) which is closer to physiological levels for the fetoplacental circulation, NaHS caused vasoconstriction.

In contrast, Mohammed *et al*., (2017) showed that the responses of human umbilical vessels to another, H_2S donor, Na_2-S, were unaffected by changing the

oxygen level. The oxygen tension in the umbilical arterial blood is normally between 16-28 mmHg and venous blood is between 28-35 mmHg (Lackman *et al.*, 2001, Nicolaides *et al.*, 1989), this suggests that responses obtained under 95% N_2: 5% CO_2 are the most physiologically relevant, and thus a contraction may be the predominant effect of NaHS in human chorionic vessels.

In summary, the work outlined in this chapter found that responses to H_2S were affected by the oxygen concentration used to gas preparations. Under physiological oxygen tension, NaHS caused a contractile response in PSA and PPV, and produced vasorelaxation in PSV and PPA. Under gassing conditions with a more physiological oxygen tension for chorionic vessels, NaHS evoked a contractile response. Given the change in responses to H_2S, in vitro studies should take the oxygen concentration into account in designing experiments to assess the vascular biology of H_2S, particularly when using high O_2 levels, where results may have less relevance to normal physiological conditions.

The aim of the next chapter, was to examine the mechanism of H_2S action in porcine splenic and pulmonary arteries and veins and human chorionic arteries under different gassing conditions.

Chapter 4:

Mechanistic effects of vascular NaHS responses under different oxygen conditions

4 Mechanistic effects of vascular NaHS responses under different oxygen conditions

4.1 Introduction

In the previous chapter the effects of different gassing conditions were investigated on NaHS concentration response curves in a variety of blood vessels. It was shown that changing oxygen concentration altered the nature of NaHS-induced responses. In the main, in both porcine splenic arteries and pulmonary veins, NaHS caused a contraction response at high O_2 levels but at low O_2 levels caused a biphasic response, relaxation followed by contraction. In porcine splenic veins and pulmonary arteries, at high O_2 concentrations, NaHS caused a contraction which was converted to a relaxation at low O_2 levels. In human chorionic arteries and veins, under high oxygen condition relaxation responses were observed at a high concentration of NaHS, while under hypoxic condition, NaHS produced contraction at low concentration and relaxation at high concentrations.

In the vasculature, H_2S has been shown to produce complex actions, including both vasorelaxation and vasoconstriction (Dunn *et al.*, 2016) and multiple mechanisms have been proposed to underlie these effects. In the majority of vascular preparations, H_2S has been reported to cause vasorelaxation. In several studies the reported mechanism of NaHS-generated vasorelaxation has been shown to be through the activation of ATP-sensitive K channels (K_{ATP}) in vascular smooth muscle, since H_2S response was attenuated by the specific K_{ATP} channel blocker, glibenclamide (Zhao *et al*., 2001, Siebert *et al*., 2008, Cheng *et*

al., 2004, Sun *et al*., 2011, Liang *et al*., 2011, Casalini *et al*., 2013, Cindrova-Davies *et al*., 2013, Webb *et al*., 2008, Materazzi *et al*., 2017).

Another mechanism reported for H_2S-induced vasorelaxation is the production of NO, since H_2S relaxant response was attenuated by the nitric oxide synthase inhibitor, L-NAME (Hosoki *et al*., 1997, Zhao *et al*., 2001). To support this, Bir *et al*., (2012) demonstrated that H_2S activates NO production under hypoxia by stimulating nitrite reduction to NO via xanthine oxidase, since H_2S-mediated NO formation was blocked by xanthine oxidase inhibitor, febuxstat.

On the other hand, H_2S has also been shown to contract blood vessels via different mechanisms. For example, Kubo *et al*., (2007) demonstrated that NaHS produced dilation instead of contraction in the presence of L-NAME in the endothelium-intact aorta, suggesting that H_2S acted to inhibit NO produced in the vascular endothelium to cause vasoconstriction. Similarly, Ali *et al.*, (2006) demonstrated that NaHS-induced contractile response and the vasopressor response of H_2S in rats was abolished by L-NAME, they suggested that H_2S and NO react together to inactivate the latter and form a nitrosothiol product. They also found that low doses of NaHS increased mean arterial blood pressure in anesthetized rats, and this action was abolished in animals pre-treated with L-NAME. In support of this hypothesis, they subsequently found that H_2S could interact with NO to produce an inactive nitrosothiol compound (Whiteman *et al.*, 2006). In contrast, Filipovic *et al*., (2013) demonstrated that the reaction between H_2S and NO did not produce nitrosothiol but generated a nitroxyl (HNO) compound. In addition, NaHS reversed the vasodilation action produced by isoprenaline and salbutamol (β_2-adrenoreceptors) and forskolin (adenylyl

cyclase activator) suggesting that the H_2S may promote contraction by blocking cAMP accumulation (Lim *et al*., 2008, Li *et al*., 2015).

In this chapter, the underlying mechanisms of NaHS responses under different O_2 levels were examined in porcine splenic blood vessels and pulmonary arteries along with human chorionic arteries.

4.2 Materials and Methods

4.2.1 Materials

Materials used for the experiments of this chapter are listed in table 2.1.

4.2.2 Methods

4.2.2.1 Isolated tissue bath experiments

Collection of material and tissue preparation were described in chapter 2, section 2.1.1. Briefly, vessels were set up in organ baths containing PSS maintained at 37˚C and aerated with a gas mixture either, 95% O_2:5% CO_2, 95% Air: 5% CO_2 or 95% N_2:5% CO_2. Tissues were exposed to 60 mM KCl twice to assess their viability.

Thereafter, any test compounds used in the experiment were added and allowed at least 20-30 minutes incubation time. The following test compounds were used: the NOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME) (100 μM) (Zhao *et al.*, 2001); the K_{ATP} channel inhibitor, glibenclamide (10 μM) (Zhao *et al.*, 2001); the adenylyl cyclase activator, forskolin (0.1μM) (Lim *et al*., 2008, Li *et al*., 2015); the NO donor, sodium nitroprusside SNP (3μM) (Hosoki *et al*., 1997); the xanthine oxidase inhibitor, febuxstat (10nM) and sulphanilamide (1mM) (Bir *et al*., 2012); copper sulphate ($CuSO_4$ 200nM), which converts nitrosothiol to nitrite and nitrates (Ali *et al.*, 2006).

Cumulative additions of contractile agents, either the thromboxane A2 receptor agonist, U46619 or the endothelin receptor agonist, endothelin-1, were then used to achieve a vascular tone equivalent to 30 - 70% of the second KCl response in all tissues. Once stable tone was achieved, Segments were then exposed to increasing concentrations of NaHS (0.001 mM - 3 mM) raised in 3–fold

increments, with a period of 3 minutes between additions, to generate cumulative concentration-response curve to NaHS.

In all experiments a second segment from the same tissue was set up and used as a control.

4.2.3. Statistical analysis

As described in chapter 2, section 2.4.

4.3 Results

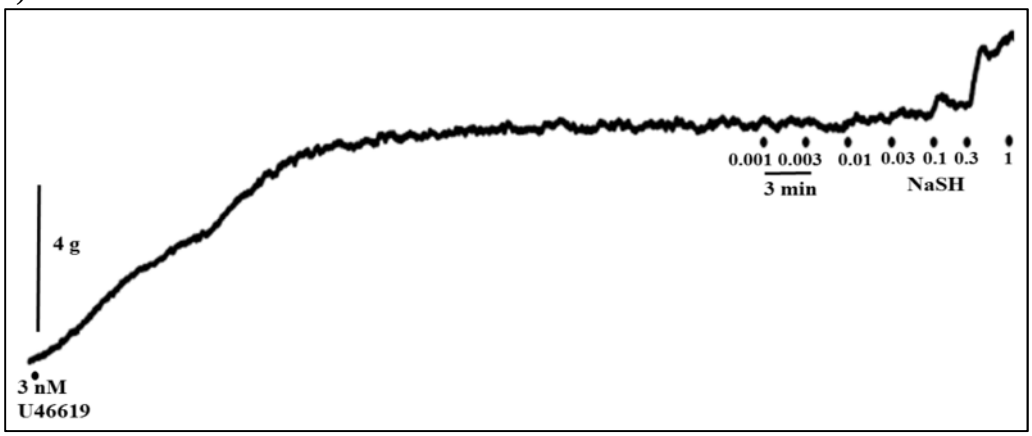
4.3.1 Mechanistic Effects of NaHS responses porcine splenic artery under 95% O_2: 5% CO_2.

4.3.1.1 Effect of L-NAME on the response of porcine splenic artery to NaHS under 95% O_2: 5% CO_2.

In 95% O_2:5% CO_2, The splenic artery responded to NaHS with concentration dependent contractions. This experiment was performed to study the involvement of nitric oxide (NO) in the responses of porcine splenic artery to NaHS. This experiment was carried out under 95% O_2: 5% CO_2 using nitic oxide synthase inhibitor (L-NAME, 100µM). Samples were incubated with L-NAME (100µM), which had no effect on tone itself, before being contracted with U46619. Vessels were pre-constricted by 53 ± 7.5% in the absence of L-NAME and 69 ± 3.6% in the presence of L-NAME (n = 6), the concentration required to raise tone with U46619 was less in the presence of L-NAME (U46619, 1nM) compared to the absence of L-NAME (U46619, 3nM). The paired control group was not exposed to L-NAME.

L-NAME had a significant effect on the NaHS responses, decreasing contractions, and uncovering a relaxation response to NaHS (0.1mM, 0.3mM and 1mM) (Figure 4.1b).

a)



b)

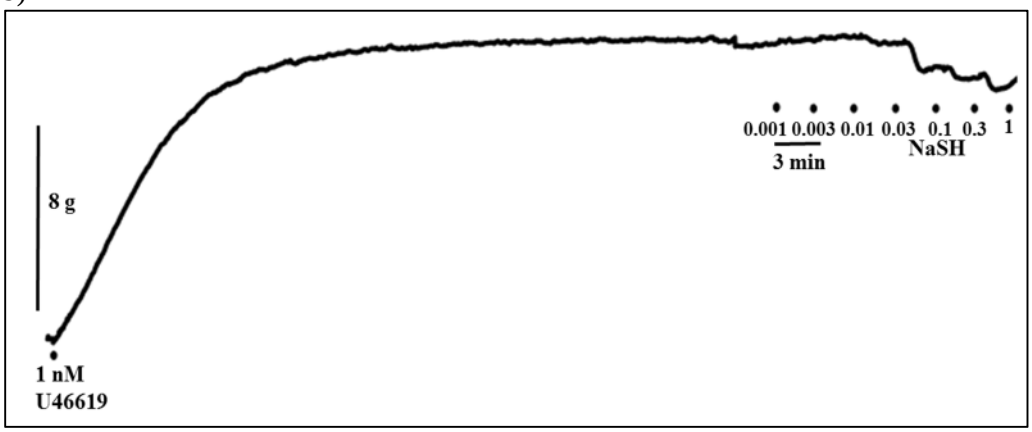


Figure 4.1a Isometric tension measurement trace showing the response of porcine splenic artery to U46619 and to cumulative concentration of NaHS in preparations gassed with 95% O_2:5% CO_2 in the absence (a) or in presence (b) of L-NAME (100µM).

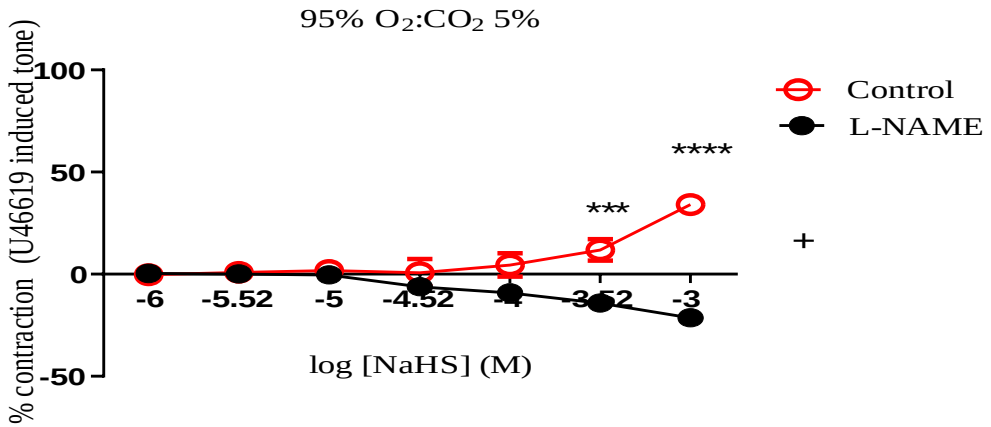


Figure 4.1b Responses of porcine splenic arteries to cumulative concentration of NaHS in the presence (●) or absence (○) of L-NAME (100µM) (a) under 95% O_2: 5% CO_2. The contraction caused by NaHS was expressed as a percentage of U46619-induced contraction. Each point represents the mean ± SEM (n= 6). + indicates a significant difference between the curves ($p<0.05$, ANOVA). ***, **** represents significant difference in response at single concentration ($p<0.001$, $p<0.0001$ respectively, Bonferroni test)

4.3.1.2 Effect of Copper Sulphate ($CuSO_4$) on the response of porcine splenic artery to NaHS under 95% O_2: 5% CO_2 condition.

Ali *et al.,* (2006) showed that L-NAME abolished contractile responses in the rat aorta. They also showed that it formed a nitrosothiol compound and the formation of which be prevented using $CuSO_4$. However, $CuSO_4$ (200nM) had no effect on the NaHS concentration response curve in the PSA (Figure 4.2).

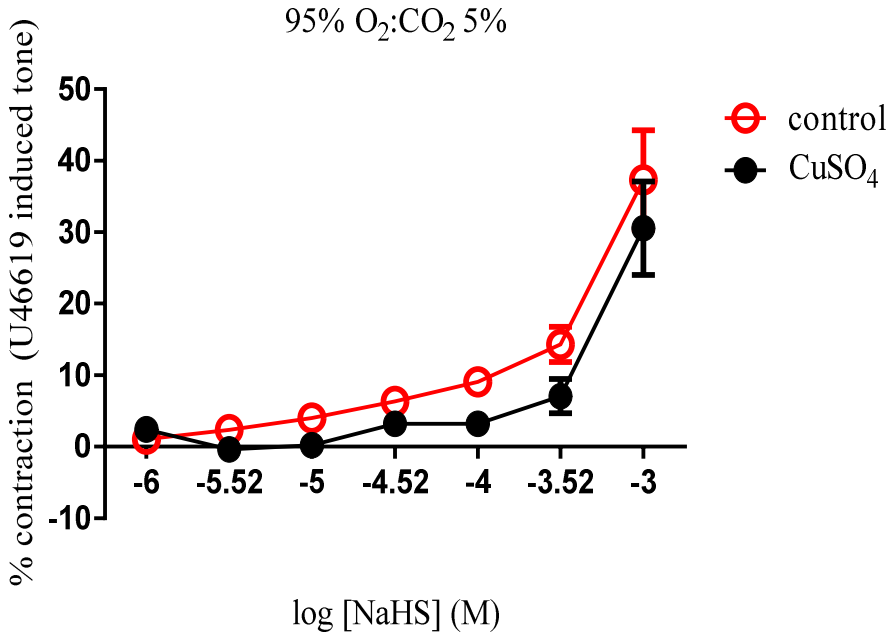


Figure 4.2 Concentration response curves to NaHS in the presence (⊖) or absence (⊖) of Copper Sulphate (200nM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% O_2:5% CO_2 Each point represents the mean ± SEM (n= 7). There was no statistical difference between the curves ($p>0.05$; ANOVA).

4.3.1.3 Effect of glibenclamide on the response of porcine splenic artery to NaHS under 95%O_2:5% CO_2 condition.

To assess whether NaHS activated K_{ATP} channels in the porcine splenic artery, preparations were incubated with glibenclamide (10µM), a specific K_{ATP} channels blocker, before being contracted with U46619 under 95%O_2:5% CO_2 condition.

Glibenclamide had no effect on the NaHS responses under 95%O_2:5% CO_2 conditions (Figure 4.3).

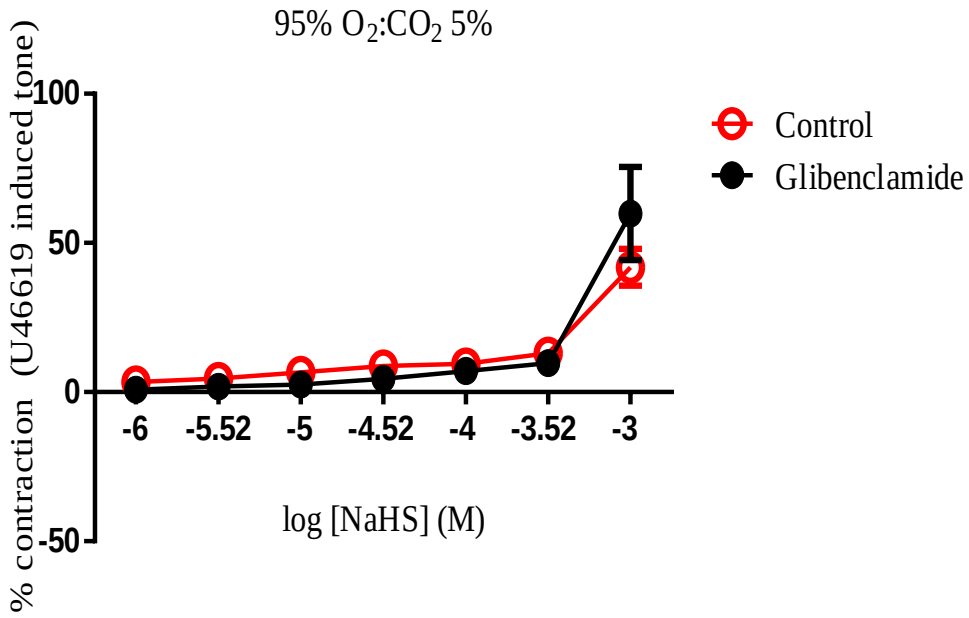


Figure 4.3 Concentration response curves to NaHS in the presence (⊷) or absence (⊖) of glibenclamide (10µM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% O_2:5% CO_2. Each point represents the mean ± SEM (n= 7). There was no statistical difference between the curves ($p>0.05$; ANOVA).

4.3.1.4 Effect of Forskolin on the response of porcine splenic artery to NaHS under 95% O_2: 5% CO_2 condition.

Lim *et al*., (2008) and Li *et al*., (2015) found that H_2S induced vasoconstriction by inhibiting the cAMP/adenylyl cyclase pathway. Under conditions where forskolin was used to increase cAMP levels prior to inducing tone with U46619, there was no change in responses to NaHS. (Figure 4.4).

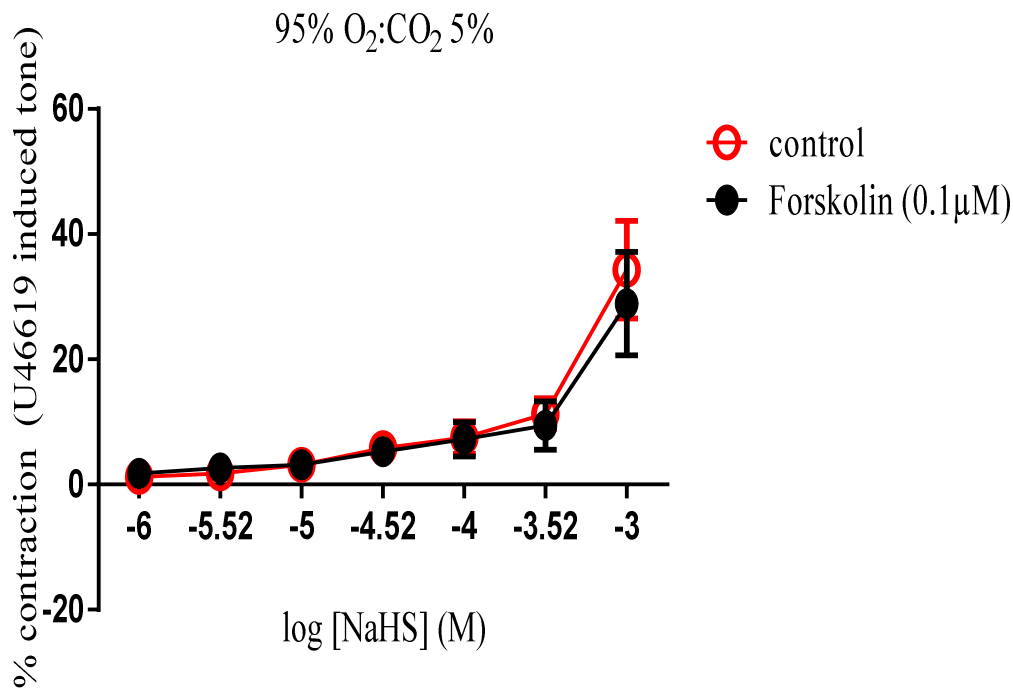


Figure 4.4 Concentration response curves to NaHS in the presence (⊸) or absence (⊖) of forskolin (0.1µM) porcine splenic arteries pre-constricted with U46619 and gassed with 95% O_2:5% CO_2 Each point represents the mean ± SEM (n= 7). There was no statistical difference between the curves ($p>0.05$; ANOVA).

4.3.2 Mechanistic Effects of NaHS responses porcine splenic artery under 95% Air: 5% CO_2 gassing conditions.

Under 95% air: 5% CO_2, a contractile response was evoked by NaHS in porcine splenic arteries similar to that seen in 95% O_2: 5% CO_2 (Figure 4.5).

4.3.2.1 Effect of L-NAME on the response of porcine splenic artery to NaHS under 95% Air: 5% CO_2.

Under 95% Air: 5% CO_2, L-NAME significantly decreased the contractile response to NaHS, although less of a vasorelaxation response was observed compared to the effect of L-NAME in splenic arteries gassed with 95% O_2: 5% CO_2 (Figure 4.5).

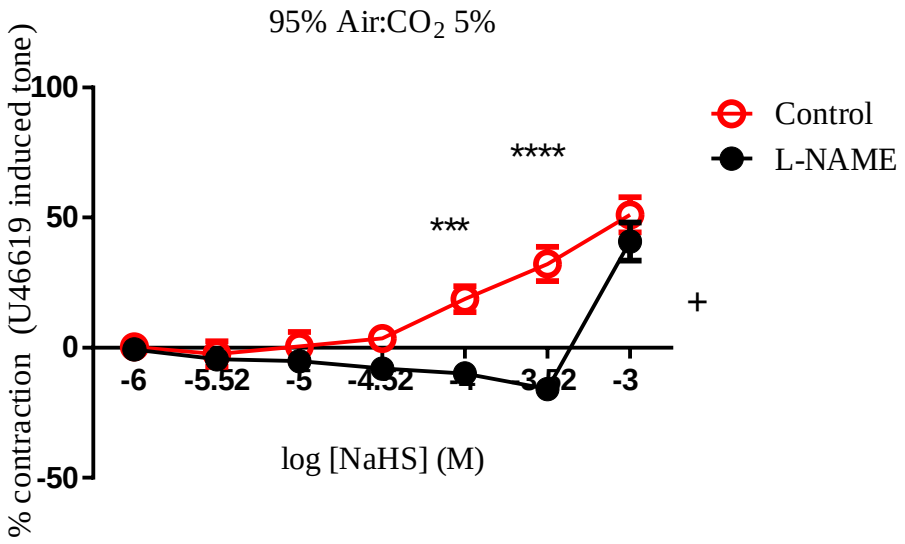


Figure 4.5 Responses of porcine splenic arteries to cumulative concentration of NaHS in the presence (●) or absence (⊖) of L-NAME (100µM) under 95% Air: 5% CO_2. The contraction caused by NaHS was expressed as a percentage of U46619-induced contraction. Each point represents the mean ± SEM (n= 8). + indicates a significant difference between the curves ($p<0.05$, ANOVA). ***, **** represents significant difference in response at single concentration ($p<0.001$, $p<0.0001$ respectively, Bonferroni test)

4.3.2.2 Effect of glibenclamide on the response of porcine splenic artery to NaHS under 95%Air: 5% CO_2 condition.

Glibenclamide had no effect on the NaHS responses under 95% Air: 5% CO_2 gassing conditions (Figure 4.6).

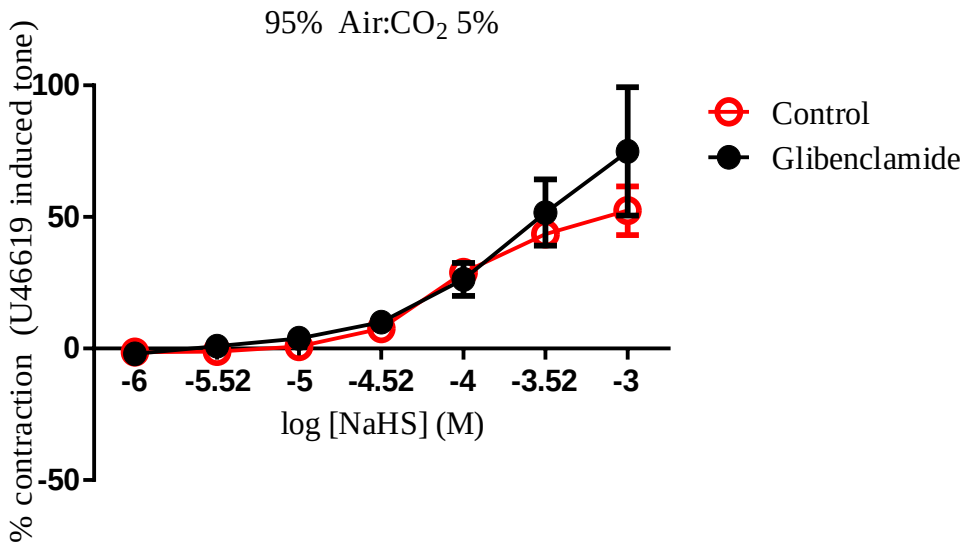


Figure 4.6 Concentration response curves to NaHS in the presence (⊷) or absence (⊶) of glibenclamide (10μM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% Air: 5% CO_2. Each point represents the mean ± SEM (n= 6). There was no statistical difference between the curves ($p>0.05$; ANOVA).

4.3.3 Mechanistic Effects of NaHS responses porcine splenic artery under 95% N_2: 5% CO_2.

The H_2S donor, NaHS, caused a contraction response at high O_2 levels but at 95% N_2: 5% CO_2 levels caused a biphasic response, a small relaxation followed by contraction at high concentrations in porcine splenic artery (Figure 4.7).

4.3.3.1 Effect of L-NAME on the response of porcine splenic artery to NaHS under 95% N_2: 5% CO_2.

Under these conditions, L-NAME (100µM) had no effect on responses to NaHS (Figure 4.7).

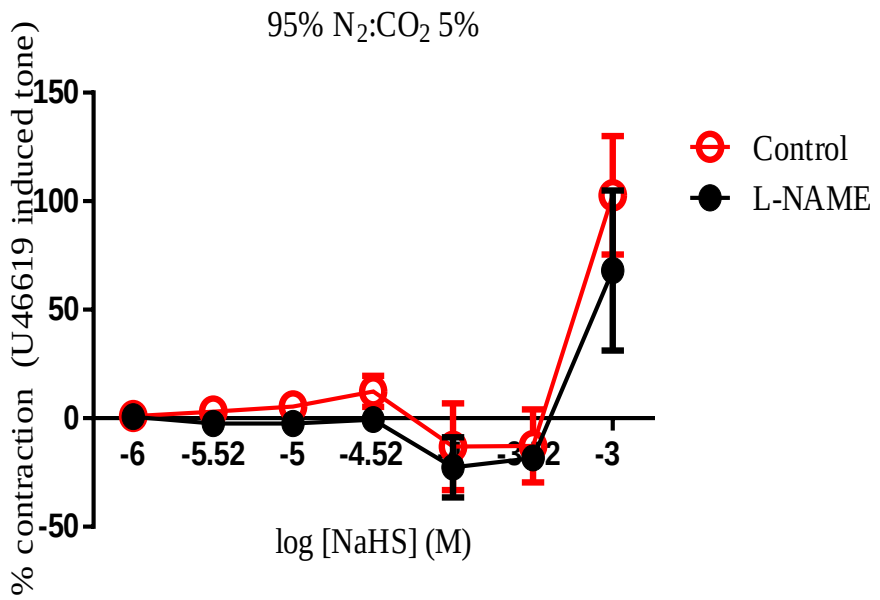


Figure 4.7 Concentration response curves to NaHS in the presence (⊸) or absence (⊸) of L-NAME (100µM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% N_2:5% CO_2. Each point represents the mean ± SEM (n=5). There was no statistical difference between the curves ($p>0.05$; ANOVA).

4.3.3.2 Effect of glibenclamide on the response of porcine splenic artery to NaHS under 95%N_2: 5% CO_2 condition.

Glibenclamide had no effect on the NaHS responses under 95% N_2: 5% CO_2 conditions and while there was some reduction relaxation observed at 0.1mM and 0.3mM concentration of NaHS this was not significant (Figure 4.8).

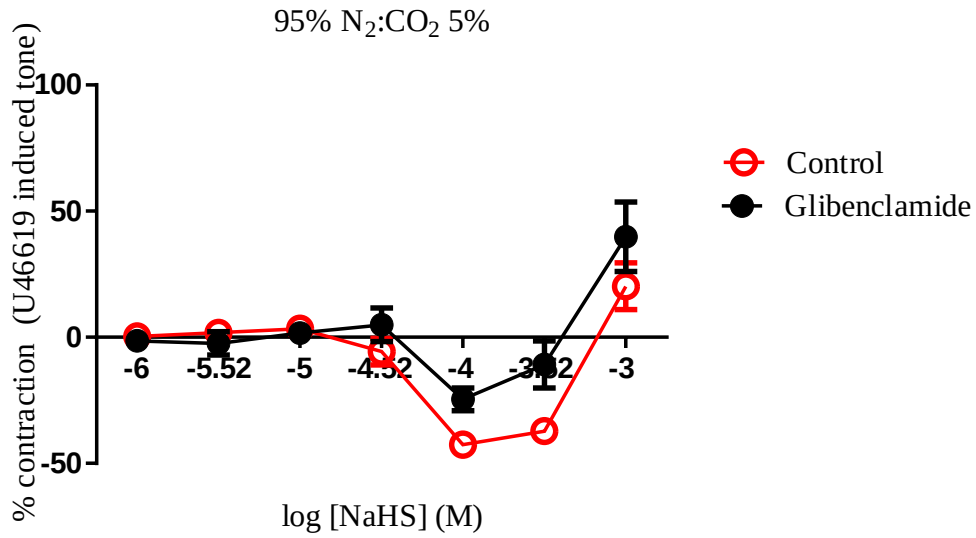


Figure 4.8 Concentration response curves to NaHS in the presence (⊷) or absence (⊷) of glibenclamide (10µM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% N_2:5% CO_2. Each point represents the mean ± SEM (n= 5). There was no statistical difference between the curves ($p>0.05$; ANOVA).

4.3.3.3 Effect of Febuxstat on the response of porcine splenic artery to NaHS under 95% N_2: 5% CO_2 condition.

Bir *et al.*, (2012) found that, under hypoxia, H_2S increases NO levels by stimulating nitrite reduction to NO via xanthine oxidase, and H_2S-mediated NO formation was blocked using xanthine oxidase inhibitor, febuxstat. While there was no evidence for the involvement of NOS-dependent nitric oxide in mediating NaHS-induced relaxation under 95% N_2: 5% CO_2 condition (Figure 4.7), the possible role of a non NOS mechanism, was assessed in the presence and absence of febuxstat (10nM). In this series of experiments the vasorelaxation response to NaHS was less robust. Nonetheless febuxstat had no effect on responses to NaHS (Figure 4.9).

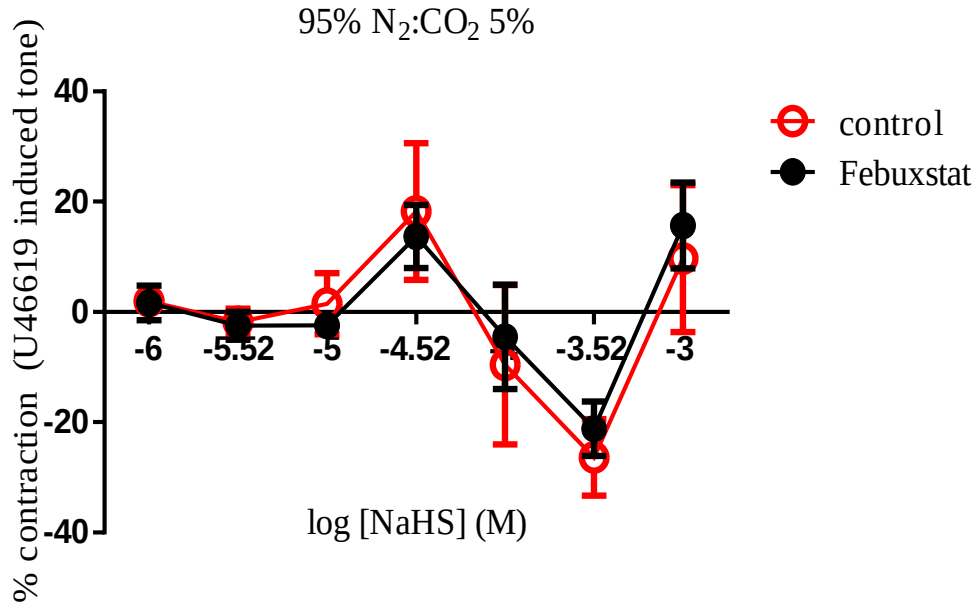


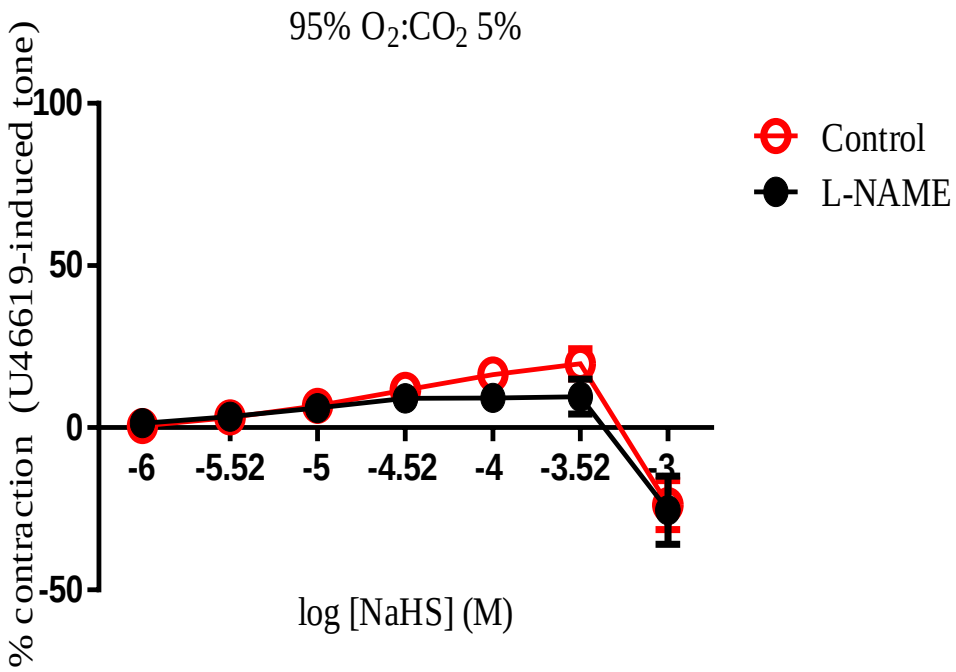
Figure 4.9 Concentration response curves to NaHS in the presence (⊖) or absence (⊖) of febuxstat (10 nM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95%N_2: 5% CO_2, Each point represents the mean ± SEM (n=7). There was no statistical difference between the curves ($p>0.05$; ANOVA).

4.3.4 Mechanistic Effects of NaHS responses porcine splenic vein

4.3.4.1 Effect of L-NAME and glibenclamide on the response of porcine splenic vein to NaHS under 95% O_2: 5% CO_2 and 95% air: 5% CO_2

In PSV under 95% O_2: 5% CO_2 and 95% air: 5% CO_2 NaHS produced a small contractile response at low concentration with a larger relaxation response at high concentrations. Glibenclamide did not affect responses to NaHS under either gassing conditions (Figure 4.10b & 4.11b) however, L-NAME abolished the contractile response when preparations were gassed with 95% air: 5% CO_2 (Figure 4.10a) but not in 95% O_2: 5% CO_2 (Figure 4.11a).

(a)



(b)

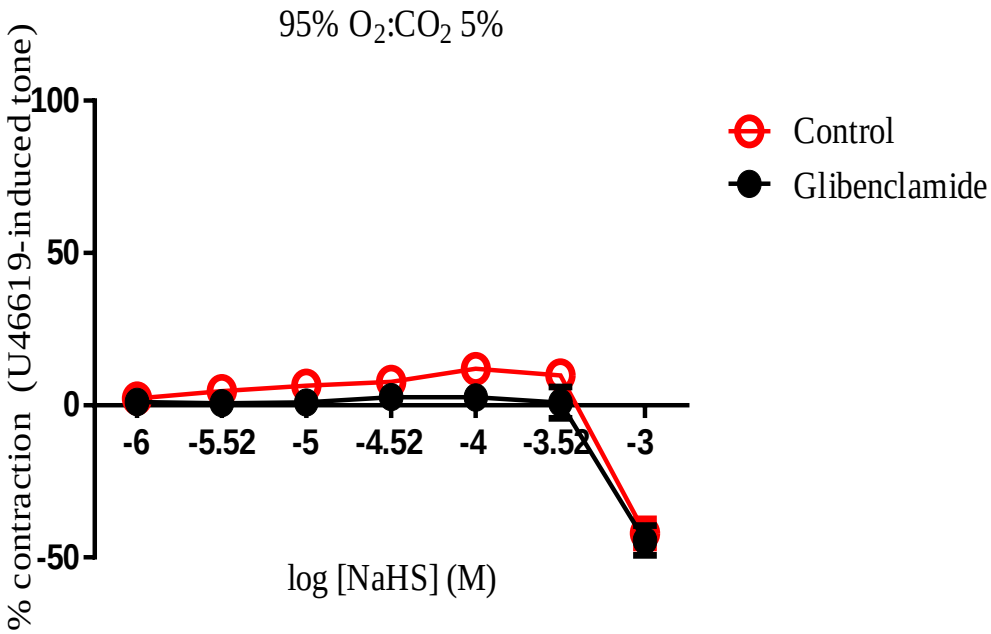
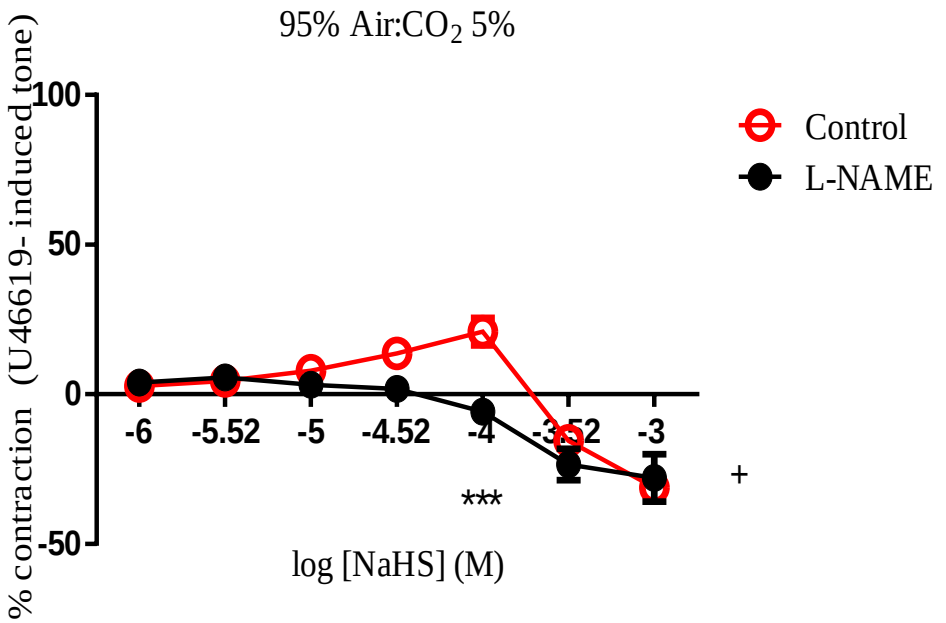


Figure 4.10 Responses of porcine splenic veins to cumulative concentration of NaHS in the presence (●) or absence (⊖) of L-NAME (100µM) (a) and in the presence (●) or absence (⊖) of glibenclamide (10µM) (b) under 95% O_2: 5% CO_2 condition. Each point represents the mean ± SEM (n= 5-6). There was no statistical difference between the curves ($p>0.05$; ANOVA).

(a)



(b)

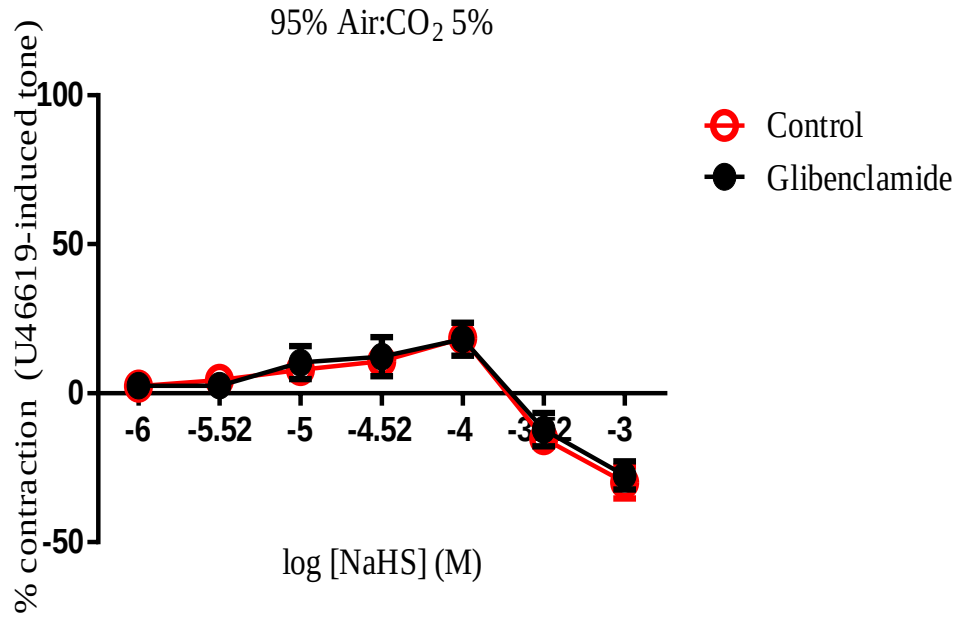


Figure 4.11 Responses of porcine splenic veins to cumulative concentration of NaHS in the presence (●) or absence (⊖) of L-NAME (100 µM) (a) and in the presence (●) or absence (⊖) of glibenclamide (10 µM) (b) under gas condition of 95% Air: 5% CO_2. Each point represents the mean ± SEM (n= 5-6). + indicates a significant difference between the curves ($p<0.05$, ANOVA). *** represents significant difference in response at single concentration ($p<0.001$, Bonferroni test).

4.3.4.2 Effect of L-NAME on the response of porcine splenic vein to NaHS under 95% N_2: 5% CO_2.

In mammals, the pO_2 is normally around 100mmHg in arterial blood and varies considerably in venous blood, between 15 and 50 mmHg (Erecinska and Silver, 2001, Tsai *et al*., 2003). The environment created by gassing with 95% N_2:5% CO_2 (33 ± 6 mmHg) is thus of more relevant to physiological condition in venous preparations. L-NAME abolished the relaxation response produced at concentration above 30µM NaHS with a small contractile response subsequently evident (Figure 4.12).

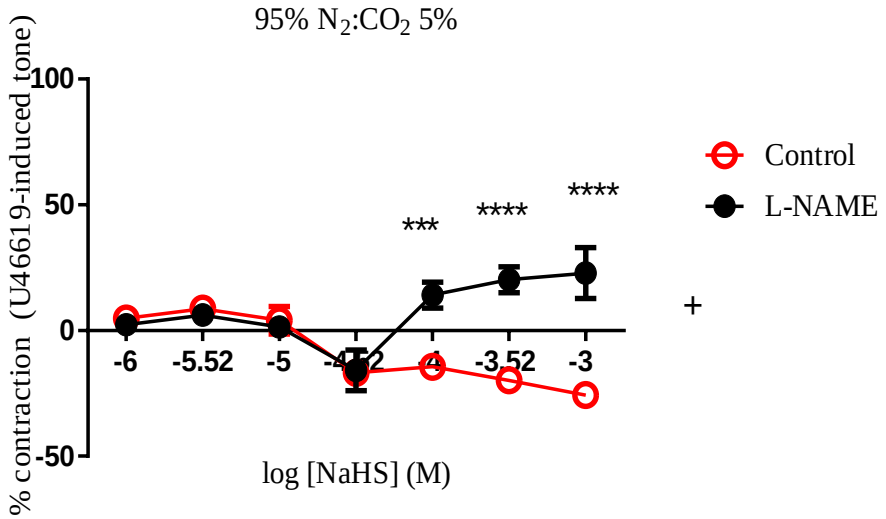


Figure 4.12 Responses of porcine splenic veins to cumulative concentration of NaHS in the presence (⊖) or absence (⊖) of L-NAME (100µM) under 95% N_2: 5% CO_2 condition. Each point represents the mean ± SEM (n= 5). + indicates a significant difference between the curves ($p<0.05$, ANOVA). ***, **** represents significant difference in response at single concentration ($p<0.001$, $p<0.0001$ respectively, Bonferroni test).

4.3.4.3 Effect of Glibenclamide on the response of porcine splenic vein to NaHS under 95% N_2: 5% CO_2.

The relaxation response was not affected by glibenclamide (Figure 4.13).

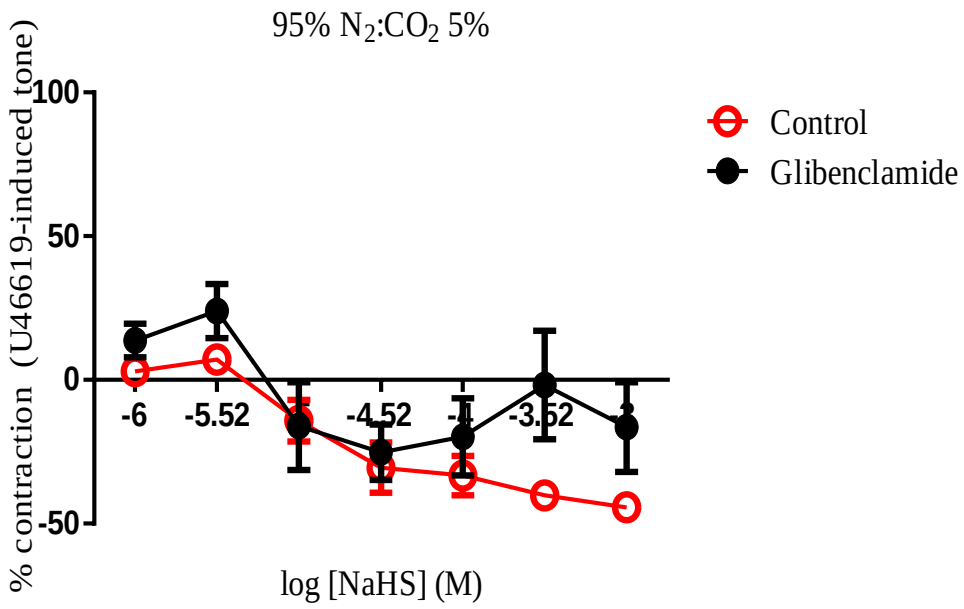


Figure 4.13 Responses of porcine splenic veins to cumulative concentration of NaHS in the presence (⊖) or absence (⊖) of glibenclamide (10µM) under 95% N_2: 5% CO_2 condition. Each point represents the mean ± SEM (n= 5). There was no statistical difference between the curves ($p>0.05$; ANOVA).

4.3.5 Mechanistic Effects of NaHS responses porcine pulmonary artery

4.3.5.1 Effect of L-NAME on the response of porcine pulmonary artery to NaHS under different oxygen conditions.

Pulmonary arteries differ from systemic arteries in oxygen tension. The pO_2 of systemic arteries is normally around 100 mmHg while the pO_2 of pulmonary arteries is between 15-50 mmHg. In systemic and pulmonary arteries, altering oxygen tension cause changed the nature of the response to NaHS. Under 95% O_2: 5%CO_2 conditions, L-NAME significantly decreased the small contractile effect generated by NaHS at 0.1mM and 0.3mM (Figure 4.14a). L-NAME had no effect on NaHS responses under 95% air: 5% CO_2 conditions (Figure 4.14b). In preparations gassed with 95% N_2: 5% CO_2, L-NAME abolished the relaxation effect generated at concentration of NaHS above 30μM with a contractile response exposed (Figure 4.14c).

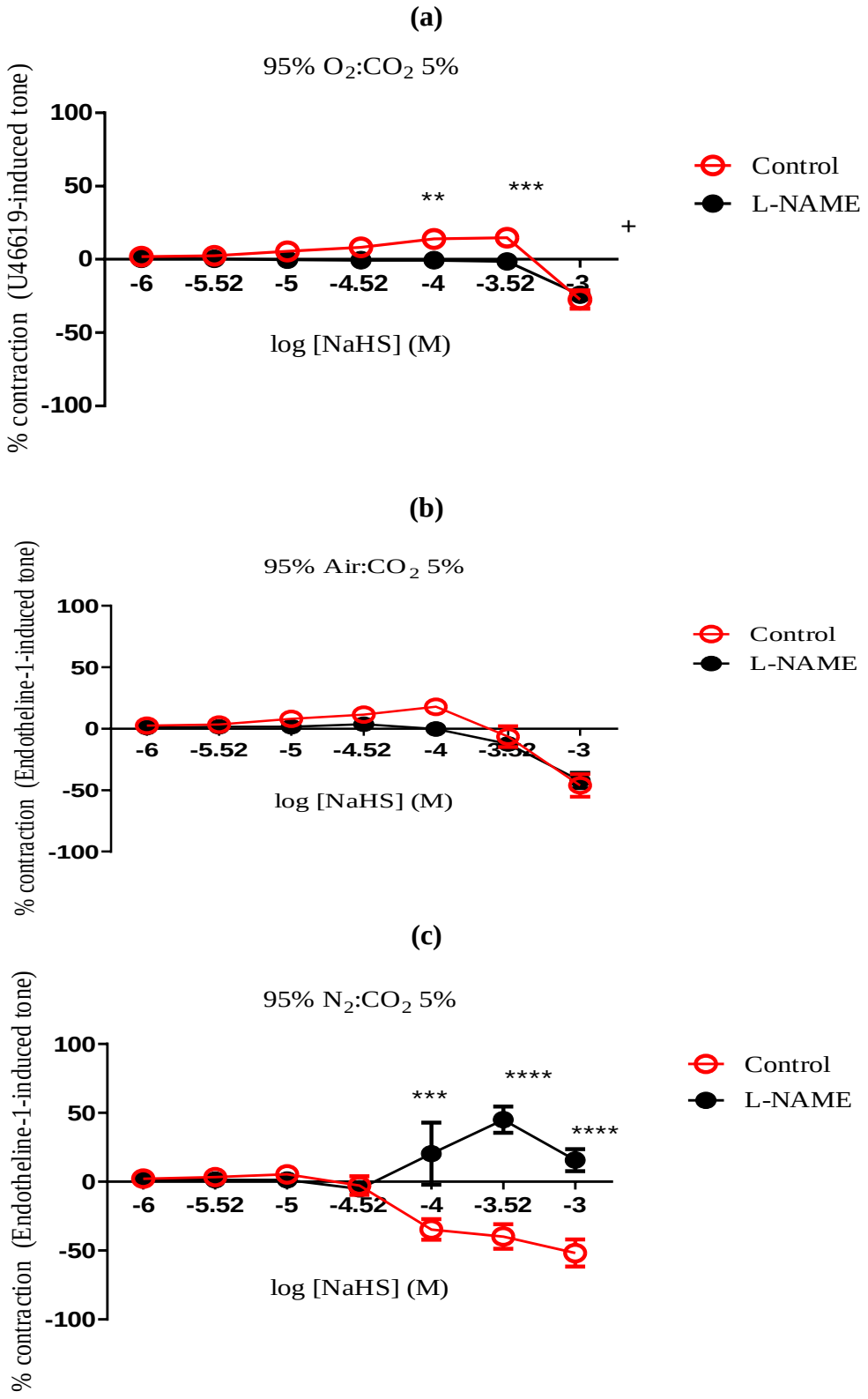


Figure 4.14 Concentration response curves to NaHS in the presence ( ) or absence ( ) of L-NAME (100M) porcine pulmonary arteries pre-constricted with (a) U46619 and gassed with 95% O_2:5% CO_2, (b) Endotheline-1 and gassed with 95% air: 5% CO_2 and (c) Endothelin-1 and gassed with 95%N_2: 5% CO_2 Each point represents the mean ± SEM (n= 5-6). + indicates a significant difference between the curves ($p<0.05$, ANOVA). **, ***, **** represents significant difference in response at single concentration ($p<0.01$, $p<0.001$, $p<0.0001$ respectively, Bonferroni test).

4.3.6 Mechanistic Effects of NaHS responses human chorionic artery

4.3.6.1 Effect of L-NAME on the response of human chorionic artery to NaHS under 95% N_2:5% CO_2 condition.

In normal pregnancy, the umbilical arterial pO_2 is between 16-28 mmHg (Lackman *et al*., 2001, Nicolaides *et al*., 1989), this suggest that responses obtained under 95% N_2: 5% CO_2 (pO_2 = 33 ± 6 mmHg) are the most physiological relevant. At these O_2 levels, NaHS caused vasoconstriction. The contractile response under hypoxia conditions was not affected in the presence of L-NAME (figure 4.15), suggesting that the contractile responses to NaHS are not by removal of interactions with NO.

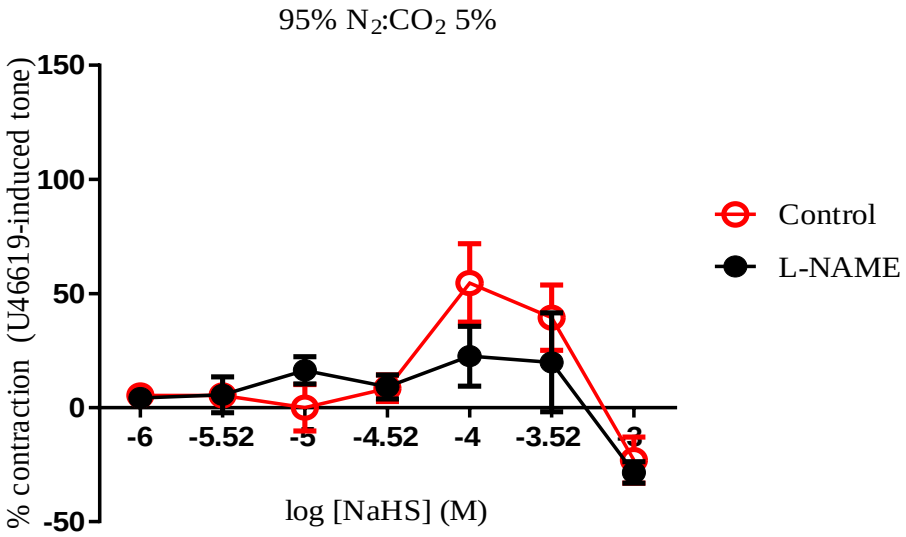


Figure 4.15 Concentration response curves to NaHS in the presence (⊷) or absence (⊶) of L-NAME (100 µM) Human chorionic arteries pre-constricted with U46619 and gassed with 95% N_2:5% CO_2 Each point represents the mean ± SEM (n= 5). There was no statistical difference between the curves ($p>0.05$; ANOVA).

4.4 Discussion

The object of the present study was to examine the mechanism of H_2S-mediated responses in porcine splenic blood vessels and pulmonary arteries along with human chorionic arteries. The major findings of this study were that the contractile response in PSA and PPA, under physiological gassing condition (95% air: 5% CO_2 for PSA and 95% N_2: 5% CO_2 for PPA) was abolished in the presence of L-NAME suggesting that removing the vasorelaxatory actions of NO contributed to the contractile response produced by NaHS. In contrast, in PSV when gassing with lower O_2 levels which is more physiological for systemic veins, L-NAME abolished the relaxation response of H_2S.

Under physiological gassing condition, in PSA and PPA, the contractile response was abolished in presence of L-NAME, whereupon a relaxation response was seen, suggesting that the contraction likely involved 'mopping up' endogenous nitric oxide. These observations are similar to those made by Ali *et al.* (2006), in rat aortic rings where H_2S produced vasoconstriction when endogenous NO is present. They also found that low concentrations of NaHS increased mean arterial blood pressure in anesthetized rat and the vasopressor effect of H_2S was abolished by L-NAME, suggesting that H_2S may quench endogenous NO *in vivo* as well as *in vitro*. Similarly, Webb *et al*., (2008) suggested that H_2S-induced contractions are due either to inactivation of NO or inhibition of NO production. Kubo *et al*., (2007) suggested that direct inhibition of eNOS by H_2S, was responsible for its contractile action. Furthermore, Geng *et al*., (2007) suggested that H_2S could directly inhibit eNOS.

The mechanism of H_2S-induced contraction has been suggested to involve an interaction with NO, producing an inactive nitrosothiol product, leading to the loss of NO-derived vasorelaxant action (Ali *et al*., 2006). This observation was supported by Whiteman *et al*., (2006) who showed H_2S could interact with NO to generate an inactive nitrosothiol product, lowering biologically available NO levels. Ali *et al*., (2006) showed that $CuSO_4$ (200 nM) (which is known to convert nitrosothiols to nitrite and nitrate; Stubauer *et al*., 1999) blocked the contractile response of NaHS thus providing further evidence for the production of nitrosothiol compound within the organ bath. However, in porcine splenic arteries, $CuSO_4$ had no effect on NaHS-induced contractile responses under high O_2 levels, suggesting no role of a nitrosothiol compound in the contractile effect of H_2S. Similarly, Filipovic *et al*., (2013) and Yong *et al*., (2010) demonstrated that the reaction between H_2S and NO did not produce nitrosothiol but generated a nitroxyl (HNO) compound.

Zhao *et al*. (2001) and Hosoki *et al*., (1997) demonstrated NaHS-mediated vasorelaxation in rat aorta were significantly attenuated in the presence of the L-NAME, suggesting the involvement of NO. However, in PSA, at 95% N_2: 5% CO_2 (sub-physiological O_2 level), L-NAME had no effect on the vasorelaxation responses produced by NaHS, showing that NO was not responsible for generating the vasorelaxation response in this preparation. It is possible that NO, generated without the involvement of eNOS, contributes to the vasorelaxation since, Bir *et al*., (2012) showed that under hypoxic conditions H_2S causes NO production by stimulating nitrite reduction to NO, via xanthine oxidase (XO). The H_2S-mediated NO formation was blocked by xanthine oxidase inhibitor,

febuxstat. Similarly, Millar *et al*., (1998) showed that XO is capable of catalysing the reduction of nitrate and nitrite to NO under hypoxic conditions. However, febuxostat had no effect on the NaHS-mediated vasorelaxation, showing that NO was not responsible for generating this response under low oxygen levels (sub-physiological O_2 level) in the PSA. Therefore, it is possible that vasorelaxation is mediated through another mechanism such as K^+ channels (Jackson-Weaver *et al*., 2011, 2013, Martelli *et al*., 2013), or alterations in intracellular PH (Lee *et al*., 2007).

In the rat aorta, it has been shown that NaHS reversed the vasorelaxation action produced by forskolin (adenylyl cyclase activator) suggesting that the contractile activity of H_2S occurred by blocking cAMP accumulation (Lim *et al*., 2008, Li *et al*., 2015). However, in porcine splenic arteries, forskolin raising cAMP levels with made no difference to NaHS-induced vasoconstriction.

In porcine splenic veins (PSV) and porcine pulmonary artery (PPA), NaHS induced a vasorelaxation under the physiological gassing conditions (95% N_2:5%CO_2) most relevant for systemic veins and pulmonary arteries. L-NAME abolished the relaxation response, whereafter a contraction response was seen. This observation is similar to Zhao *et al*., (2001) and Zhao and Wang, (2002) who reported that, in rat aorta, the NaHS-induced vasorelaxation was attenuated by the removal of endothelium or blocking of NO synthase

Many initial studies reported the mechanism of NaHS-generated vasorelaxation to be through the activation of ATP-sensitive K channels (K_{ATP}) in vascular

smooth muscle, since H_2S response was attenuated by the specific K_{ATP} channel blocker, glibenclamide, (Zhao *et al*., 2001, Siebert *et al*., 2008, Cheng *et al*., 2004, Sun *et al*., 2011, Liang *et al*., 2011, Casalini *et al*., 2013, Cindrova-Davies *et al*., 2013, Webb *et al*., 2008, Materazzi *et al*., 2017, Drobna *et al*., 2015). However, in porcine splenic arteries, The K_{ATP} channel blocker glibenclamide did not affect the vasorelaxation or vasoconstrictor responses induced by NaHS with preparations gassed with high or low oxygen levels, suggesting that K_{ATP} channels do not contribute to responses to H_2S. Other studies have also shown that K_{ATP} activation is not a ubiquitous mechanism for NaHS induced vasorelaxation (Drobna *et al*., 2015, Cheang *et al*., 2010).

In summary, the contractile response caused by NaHS in systemic arteries gassed under physiologically relevant O_2 levels were abolished in the presence of L-NAME, suggesting that the H_2S-response is due to an interaction between NaHS and NO, such that the latter is made inactive. In contrast, the response in systemic veins and pulmonary arteries under relevant O_2 tension was a NO-mediated vasorelaxation.

Chapter 5:

Does H_2S play a role in the response of porcine blood vessels to hypoxia

5 Does H_2S play a role in the response of porcine blood vessels to hypoxia

5.1. Introduction

In previous chapters altering oxygen levels in the gassing mixture changed the nature of the response to exogenous H_2S. In general, contractile responses were shown in systemic arteries when gassing with high oxygen level that were abolished by the NOS inhibitor L-NAME, indicating an interaction with NO underlay the contractile response. As oxygen concentrations were reduced, relaxation responses were generally caused by NaHS. The pulmonary artery responded to NaHS with concentration dependent contractions followed by relaxation under high oxygen concentration whereas under low levels of oxygen, NaHS caused a relaxation that was abolished by L-NAME.

In cells, H_2S is mainly generated by desulfhydration of L-cysteine by the pyridoxal-5'-phosphate-dependent enzymes, cystathionine-y-lyase (CSE) and / or cystathionine-β-synthase (CBS) (Li and Moore, 2007). Production of endogenous H_2S has been demonstrated to principally involve CBS in the brain (Abe and Kimura, 1996) while CSE is responsible for H_2S formation in the vasculature (Hosoki *et al*., 1997). However, more recent result have shown that CBS is also present in vascular endothelium and smooth muscle (Baragatti *et al*., 2013).

Oxygen tension is one of the key factors required for cellular signalling and physiological functions. A low O_2 level leads to inadequate tissue oxygenation, tissue hypoxia, and the subsequent production of molecules that attempt to

address this (Solaini *et al.,* 2010). Hypoxia is an important regulator of vascular tone promoting vasoconstriction in the pulmonary circulation and vasodilation in the systemic vasculature (Wier and Archer 1995). Hypoxic pulmonary vasoconstriction (HPV) is an adaptive effect that diverts blood flow from hypoxic parts in the lungs towards better-oxygenated lungs segments and thereby maintains appropriate ventilation-perfusion matching (Hampl and Jakoubek 2009). In the pulmonary circulation, hypoxia has been reported to cause vasoconstriction including, in the cat pulmonary artery (Madden *et al.,* 1992), human and rat pulmonary arteries (Savineau *et al.,* 1995), rabbit pulmonary arteries (Talbot *et al.,* 2003), and mouse intrapulmonary arteries (Strielkov *et al.,* 2018). Most systemic vascular beds respond to hypoxia with vasodilation to encourage delivery of more blood (oxygen and nutrients) to tissues. In the systemic circulation, hypoxia has been demonstrated to cause vasodilation in porcine coronary arteries (Throne *et al.,* 2002, Hedegaard *et al.,* 2014, Donovan *et al.,* 2017), rat aorta (Olson *et al.,* 2006), cat cerebral arteries (Madden *et al.,* 1992), and mice cerebral arteries (Morikawa *et al.,* 2012).

Several mechanisms have been proposed to clarify hypoxic vascular response for systemic vasorelaxation or pulmonary vasoconstriction. Several mechanisms for hypoxia induced vasorelaxation have been proposed. For example, Daut *et al*., (1990) showed that hypoxic dilation of guinea pig coronary arteries involved activation of ATP-sensitive potassium channels, since glibenclamide, a blocker K_{ATP} channels, blocked the response. Hedegaard *et al*., (2014) showed that Hypoxia causes vasodilatation of porcine coronary arteries through activation of KV_7 channels. Hypoxia evoked pulmonary vasoconstriction occurs through

inhibiting redox sensitive K^+- channels, causing a depolarization of pulmonary arterial smooth muscle cells resulting in activation of L-type calcium channels, and contraction as Ca^{2+} levels in the cells rise (Archer and Michelakis 2002, Waypa *et al*., 2006). In support of this hypothesis, Savineau *et al*., (1995) showed that L-type calcium channels blockers, verapamil and nifedipine, abolished hypoxia-induced contraction in human and rat pulmonary arteries. Hypoxia can also act on the endothelium to release the potent vasoconstrictor peptide endothelin (ET)-1, which evokes HPV through stimulating ETA receptors on smooth muscle (Oparil *et al*., 1995, Liu *et al*., 2001). Lee *et al*., (1995) and Terraz *et al*., (1999) demonstrated that NOS inhibitors decrease HPV, suggesting that the mechanism involved may be a reduction of basal NO formation.

Recently, it has been proposed that the gasotransmitter hydrogen sulphide (H_2S) may act as an oxygen sensor/transducer of the hypoxic response in the vasculature. Hypoxia induces the expression of CBS and increases generation of H_2S resulting cerebral vasodilation in mouse (Morikawa *et al*., 2012) and in rat (Takano *et al*., 2014). Wang *et al*., (2014) showed that hypoxia increased CSE expression and increased the production and level of H_2S in mammalian cells. Olson *et al*., (2006) showed that hypoxia induced vasodilation in rat aorta occurred through generating H_2S, since PPG, a H_2S synthesis inhibitor, blocked the response. Similarly, Donovan *et al*., (2017) reported that AOAA (300µM) reduced hypoxic vasorelaxation in porcine coronary arteries, suggesting a role for H_2S in the hypoxic response in this vessel. The bioavailability of H_2S whether endogenous or exogenous concentrations, is dictated by O_2 levels. O_2 has an antagonistic effect on H_2S, leading to its

oxidation (Li and Lancaster, 2013). Olson et al., (2006) suggested that H_2S metabolism may serves as an oxygen-sensing mechanism to produce the hypoxic vascular response. The proposed mechanism is that a decline in tissue O_2 concentrations reduces the rate of H_2S oxidation, thus increasing the level of biologically active of H_2S which in many vessels increases relaxation to encourage blood flow to the affected area (Olson *et al.*, 2008).

The aim of this study was to determine (i) the presence of H_2S synthesis enzymes (CBS and CSE) in PSA and PPA, (ii) whether they were capable of generating endogenous H_2S production upon exposure to the precursor, L-cysteine, (iii) the influence of O_2 levels on responses to L-cysteine in porcine splenic, and pulmonary arteries, and (iv) whether endogenous H_2S was involved in contributing to hypoxia-induced systemic vasorelaxation and pulmonary vasoconstriction in PSA and PPA respectively.

5.2 Materials and Methods

5.2.1 Materials

Materials used for the experiments of this chapter were listed in table 2.1, 2.2 and 2.3.

5.2.2 Methods

5.2.2.1 Western Blotting analysis

Porcine spleens and lungs were collected from the abattoir and processed as described in chapter 2, section 2.3. Samples were run on SDS-PAGE gels using a Biorad Mini-protein 3 system. Subsequently, the nitrocellulose membrane was incubated with the primary antibody Rabbit anti-CBS (1:1000 dilution; Abnova #H00000875-D01P), rabbit anti-CSE (1:1000 dilution; abcam ab80643), and mouse monoclonal anti-β-actin antibody (A2228 Sigma, UK) (1:50 000)) diluted in 5%(w/v) non-fat milk solution at 4 C° overnight. After washing in TBST, the blots were incubated with corresponding secondary antibodies IRDye®680LT Goat anti-mouse IgG (1:10000) (LI-COR Biosciences, Cambridge, UK) and IRDye® 800CW Goat anti-rabbit IgG (1:10000) (LI-COR Biosciences, Cambridge, UK) at room temperature for 1 hour. After that the secondary antibodies were removed and the blots were washed in TBST. Immunoblots were visualized using the Li-Cor/Odyessy infrared imaging system.

5.2.2.2 Isometric tension recording

5.2.2.2.1 Set up and the general protocol for recording isometric tension

Porcine splenic and porcine pulmonary blood vessels were isolated and set up in an isometric tension recording system as described in the methods

chapter. The cleaned vessels were set up in organ baths containing PSS at 37˚C and gassed with either 95% O_2:5% CO_2, 95% Air: 5% CO_2 or 95% N_2:5% CO_2. The PSS was pre-gassed for approximately 30 minutes to ensure gaseous equilibrium of the PSS. Following that, an initial resting tension was applied to vessels and allowed to relax for approximately 30-45 minutes to equilibrate and achieve a stable baseline tone. Thereafter, vessels were exposed to KCl (60mM) for 15 minutes and allowed to contract to confirm their viability. Following two exposures of the vessels to KCl (60mM), test substances (the CSE inhibitor DL-propargylglycine (PPG 10 μM, 100 μM), CBS inhibitor aminooxyacetate (AOAA 100 μM, 300 μM), and/or MPST pathway inhibition (aspartate 1 mM)) were added for at least 30 minutes incubation time. After this time arteries and veins were pre-contracted to a level of 30-70% of the maximum KCl response, using the thromboxane A_2 receptor agonist, U46619. Once vessel tension plateaued, a concentration-response curve to L-cysteine was attained by applying it in a cumulative manner, in 3-fold increments between 10 μM and 3 mM, with a duration of 5 minutes between additions. Only one concentration response curve to L-cysteine was attained in each preparation. In other experiments, U46619 contracted vessels were exposed to hypoxia (95% N_2/5% CO_2) for 60 min after initially being gassed with 95% O_2:5% CO_2. The effects of the CSE inhibitor DL-propargylglycine (PPG 100 μM), The CBS inhibitor aminooxyacetate (AOAA 300 μM), or the nitric oxide synthase inhibitor (L-NAME) were investigated by application prior to the addition of U46619. Hypoxic responses were measured in grams (g) after 30 min. For each of the different experimental conditions another paired segment (from the same tissue) was set up and used as a control.

5.2.3 Data analysis

Data analysis was carried out using Prism (Graph Pad Software). All values were expressed as mean ± standard error of the mean (S.E.M). Where n = the number of observations. The difference between means was considered statistically significant at a value of $P < 0.05$. Differences between concentration response curves were assessed using two-way ANOVA in conjunction with the Bonferroni post-hoc test to assess possible difference at individual concentrations. Student's paired *t*-test for single data comparison against the control was performed, whilst one-way ANOVA was carried out on the results for multiple comparison tests against the control.

5.3 Results

5.3.1. Western Blotting Analysis

Western blot analysis was carried out to determine Cystathionine β synthase (CBS) and Cystathionine γ lyase (CSE) expression in PSA and PPA using anti-CBS and anti-CSE specific antibodies.

5.3.1.1. CBS and CSE expression in porcine splenic artery

The expression of CBS and CSE were shown as a 63 kDa and 42 kDa bands in all samples respectively. The lanes show splenic artery samples from 5 different pigs. The 63 kDa bands observed during western blotting correspond to the CBS enzyme (http://www.abcam.com/cystathionase-antibody-ab80643.html) (Figure 5.1). A band was obtained at the presence of the CSE antibody at around 45 kDa, corresponding with the predicted molecular weight in porcine splenic artery (Figure 5.2)

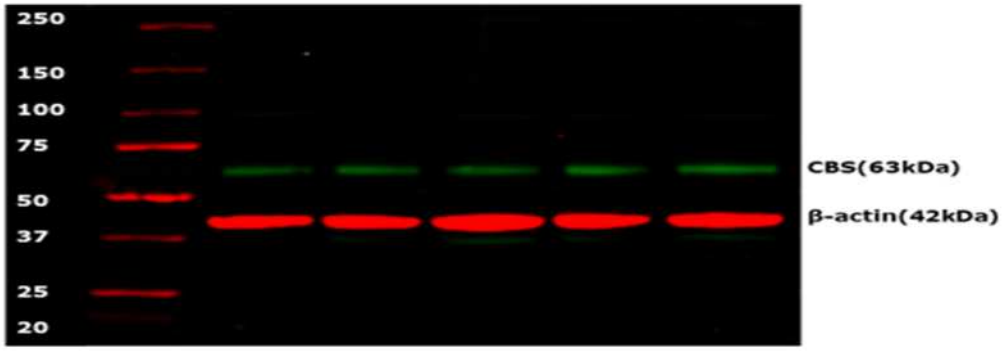


Figure 5.1 Western blot analysis to determine CBS expression in porcine splenic artery (n=5).

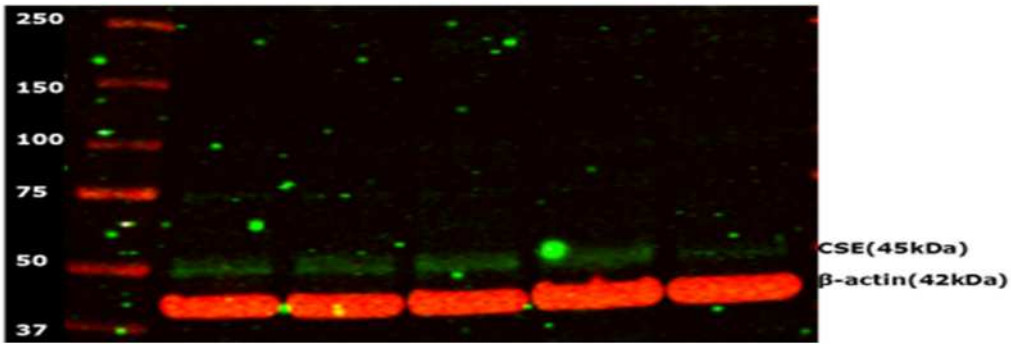


Figure 5.2 Western blot analysis to determine CSE expression in porcine splenic artery (n=5).

5.3.1.2. CBS and CSE expression in porcine pulmonary artery

The expression of CBS and CSE were shown as a 63 kDa and 42 kDa bands in all samples respectively. The lanes show pulmonary artery samples from 5 different pigs. The 63 kDa bands observed during western blotting correspond to the CBS enzyme (Figure 5.3). A band was obtained at the presence of the CSE antibody at around 45 kDa, corresponding with the predicted molecular weight in porcine splenic artery (Figure 5.4).

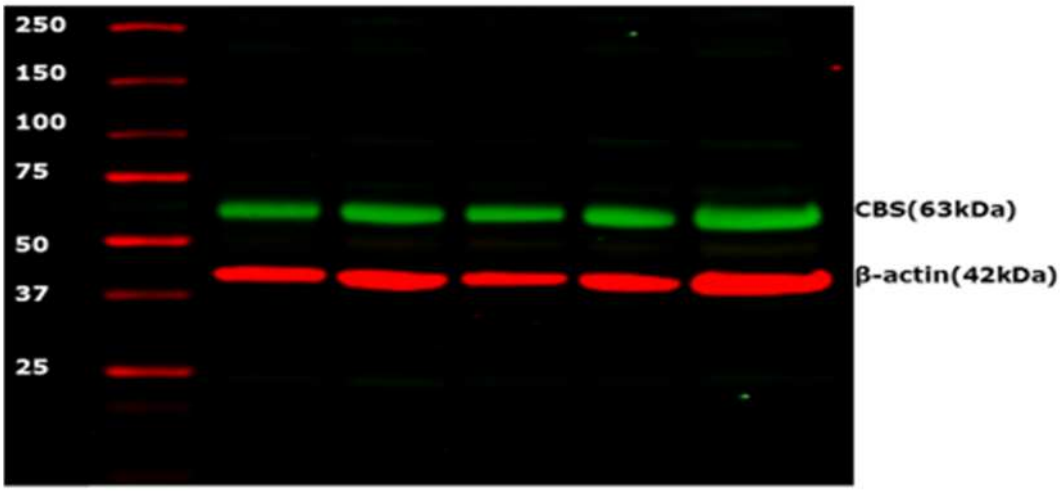


Figure 5.3 Western blot analysis to determine CBS expression in porcine pulmonary artery (n=5).

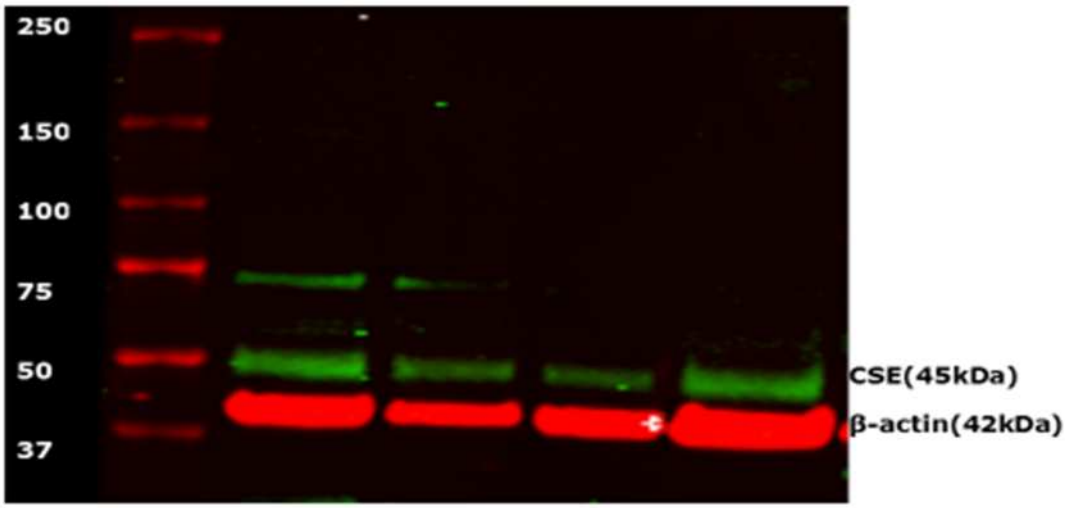


Figure 5.4 Western blot analysis to determine CSE expression in porcine pulmonary artery (n=5).

5.3.2. Isometric tension recording

5.3.2.1. Response to L-cysteine under different oxygen conditions in Porcine Splenic Arteries

Concentration response curves to L-cysteine were carried out under different gassing condition after pre-constricting the vessels with U46619. Average KCl responses were 6.5 ± 1.0 g in 95% O_2:5% CO_2 (n = 6), 7.1 ± 0.7 g in 95% air: 5% CO_2 (n = 6) and 5.6 ± 0.8 g in 95%N_2:5% CO_2 (n = 6). In these experiments, the level of pre-constriction in 95% O_2:5% CO_2 data was 59.2 ± 6% of the KCl responses (60 mM) using an average U46619 concentration of -8.3 ± -8.8× 10^{-9} M (n = 6), in the 95% air:5% CO_2 data the level of pre-constriction was 55.2 ± 4.8% using an average U46619 concentration of -8.3 ± -8.8 M and in the 95% N_2:5% CO_2 it was 50.3 ± 3.2% using an average U46619 concentration of -8.2 ± -8.7 M.

Under 95% O_2:5% CO_2, 95% air: 5% CO_2 and 95% N_2:5% CO_2 gassing conditions, the splenic artery responded to L-cysteine with large concentration dependent contraction starting at 30µM.

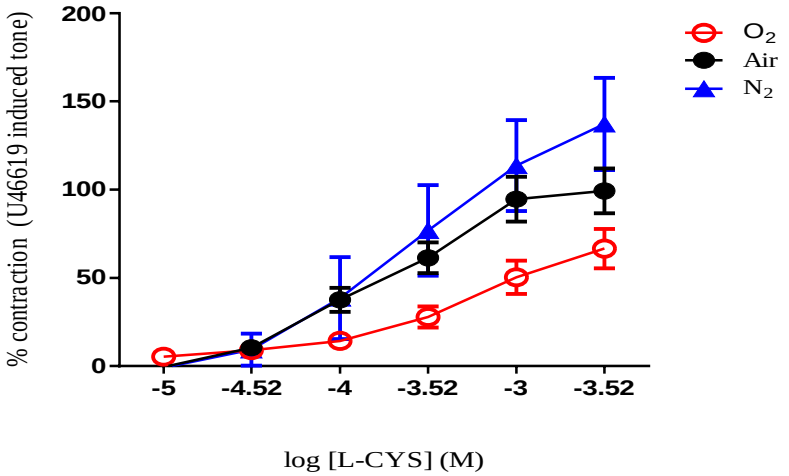
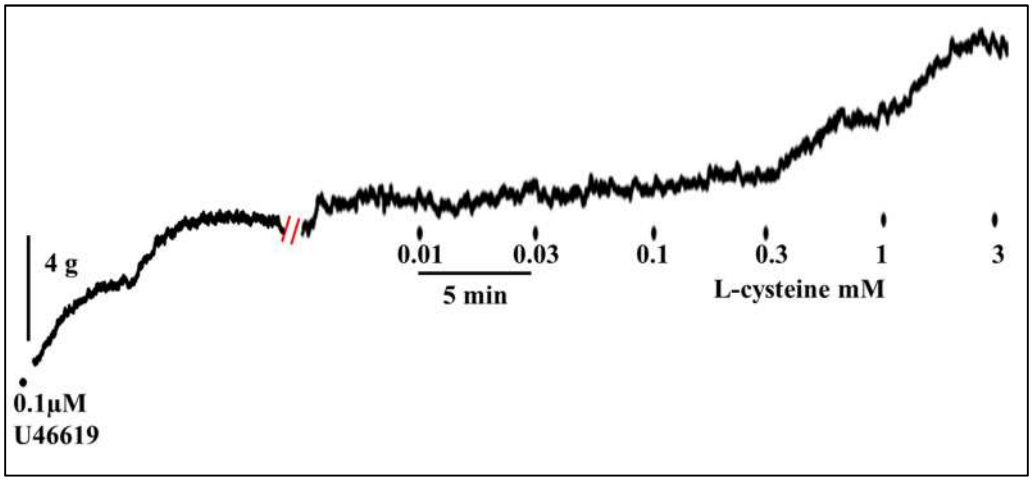


Figure 5.5 Concentration response curves to L-cysteine in porcine splenic arteries pre-constricted with U46619 and gassed with (a) 95% O_2:5% CO_2, (b) 95%Air: 5% CO_2, (c) 95%N_2: 5% CO_2, Each point represents the mean ± SEM (n=6).

(a)



(b)

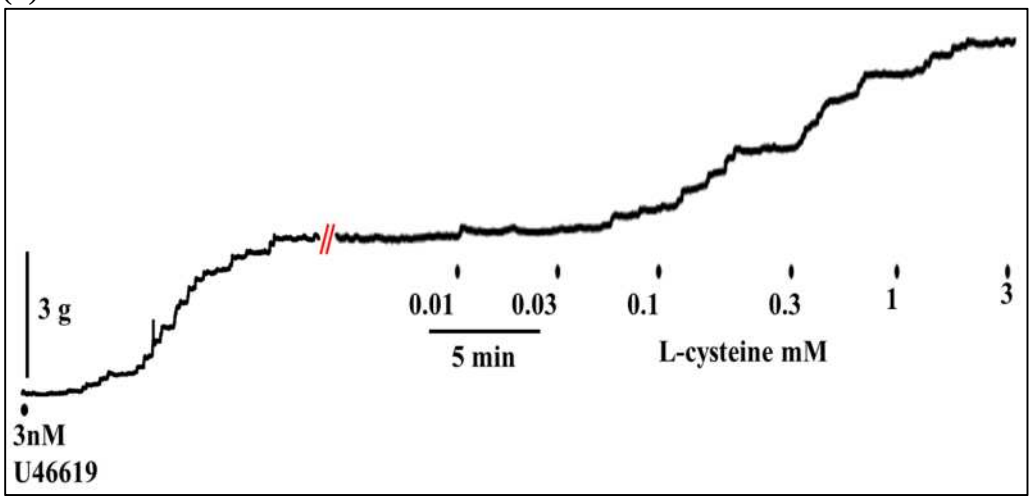


Figure 5.6 Isometric tension measurement trace showing the response of porcine splenic artery to U46619 and to cumulative concentration of L-cysteine in preparations gassed with (a) 95% O_2:5% CO_2, (b) 95%N_2: 5% CO_2.

5.3.2.2. Response to L-cysteine under different oxygen conditions in Porcine Pulmonary Arteries

Pulmonary arteries were contracted with U46619 before exposure to a cumulative addition of L-cysteine. Average KCl responses were 9.7 ± 1.1 g in 95% O_2:5% CO_2 (n = 5), 8.5 ± 0.5 g in 95% air: 5% CO_2 (n= 6), 12.3 ± 0.9 g in 95% N_2:5% CO_2 (n= 5). For this series of experiments vessels were pre-constricted with U46619 by 38.1 ± 2% of the KCl responses (60 mM) response in 95%O_2:5% CO_2 using an average U46619 concentration of $2.6 \times 10^{-8} \pm 4 \times 10^{-9}$ M (n = 6), 50.1 ± 5.8% in 95% Air:5% CO_2 using an average U46619 concentration of $2 \times 10^{-8} \pm 4.5 \times 10^{-9}$ M (n = 6) and 50.3 ± 5.6% in 95% N_2:5% CO_2 using an average U46619 concentration of $1.5 \times 10^{-8} \pm 5 \times 10^{-9}$ M (n = 5).

In 95%O_2:5% CO_2 and 95%air: 5% CO_2 gassing conditions, the pulmonary artery responded to L-cysteine with concentration dependent contractions which, although smaller than in the PSA, became prominent at 300µM L-cysteine. Under 95%N_2:5% CO_2 condition, the pulmonary artery did not respond consistently to L-cysteine.

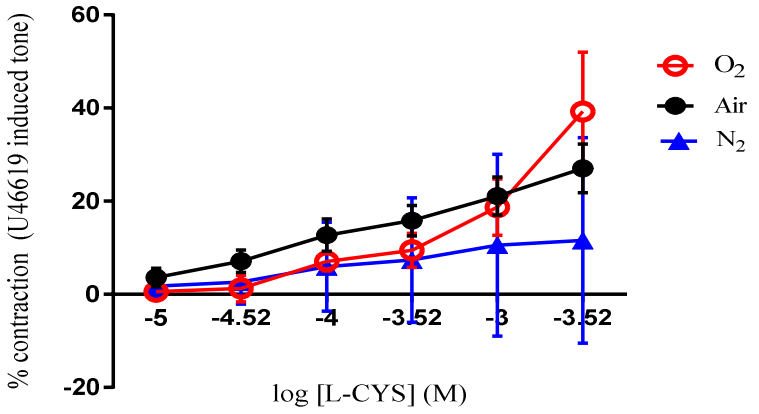
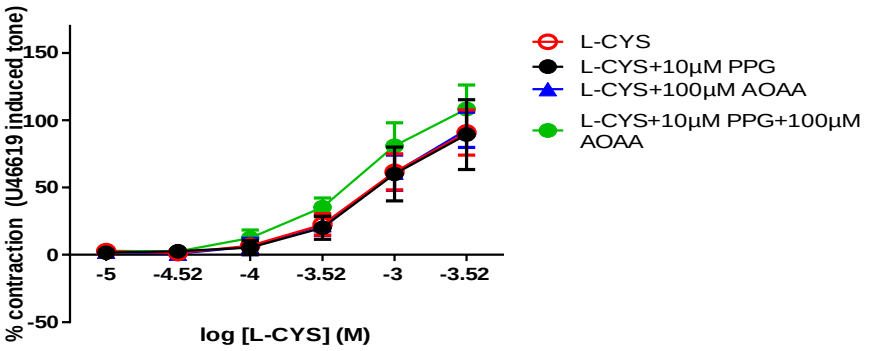


Figure 5.7 Concentration response curves to L-cysteine in porcine pulmonary arteries pre-constricted with U46619 and gassed with (a) 95% O_2:5% CO_2, (b) 95%Air: 5% CO_2, (c) 95%N_2: 5% CO_2, Each point represents the mean ± SEM (n=6).

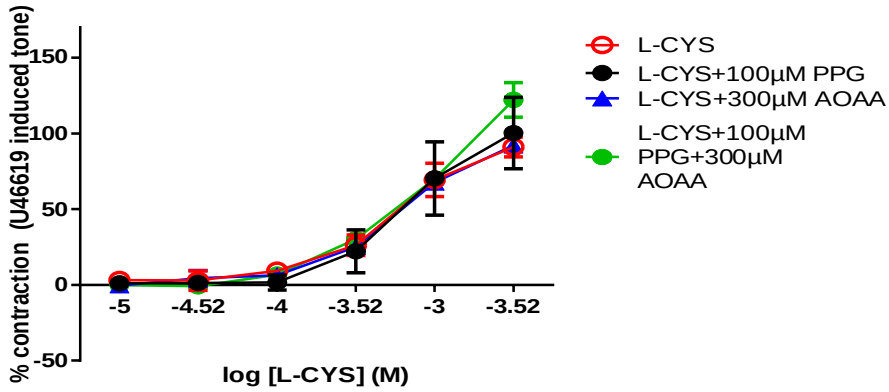
5.3.2.3. Effect of CSE inhibitor propargylglycine (PPG), CBS inhibitor amino-oxyacetate (AOAA) and MPST inhibitors (aspartate) on the response of porcine splenic artery to L-cysteine under conditions of 95% O_2:5% CO_2.

To assess whether the L-cysteine-induced contractile response was due to the endogenous production of H_2S, concentration response curves to L-cysteine were carried out in the presence and absence of the CSE inhibitor, PPG (10 μM, 100 μM), CBS inhibitor, AOAA (100 μM, 300 μM) and MPST inhibitor, aspartate (1mM) (Figure 5.8). PPG, AOAA and aspartate, even at high concentrations, did not significantly affect the contractile response to L-cysteine.

(A)



(B)



(C)

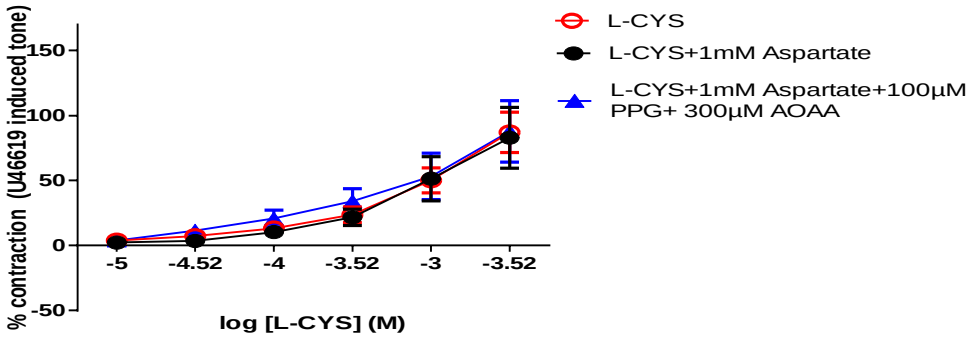


Figure 5.8 Response of porcine splenic artery to cumulative concentrations of L-cysteine in the absence (⊖) or presence of PPG (10 µM) (●), AOAA (100 µM) (▲) and a combination of PPG (10 µM) and AOAA (100 µM) (●) [A] , in the absence (⊖) or presence of PPG (100 µM) (●), AOAA (300 µM) (▲) and a combination of PPG (100 µM) and AOAA (300 µM) (●) [B] and in the absence (⊖) or presence of aspartate (1mM) (●),and a combination of aspartate (1mM), PPG (100 µM) and AOAA (300 µM) (▲) [C]. Each point represents the mean ± SEM (n= 5-7). There was no statistical difference between the curves ($p>0.05$; ANOVA).

5.3.2.4 Response of porcine splenic artery to hypoxia.

In the current experiment I examined responses of splenic artery to hypoxia, in samples pre-contracted with U46619, Once a stable tone (~30-70% of KCl response) was achieved, hypoxia was induced by switching the gas to 95% N_2: 5% CO_2 from 95% O_2: 5% CO_2 for 60 min. Hypoxic responses were measured in grams (g) after 30 min.

Hypoxia caused a biphasic vascular response; an initial transient contraction followed by prolonged relaxation. (Figure 5.9).

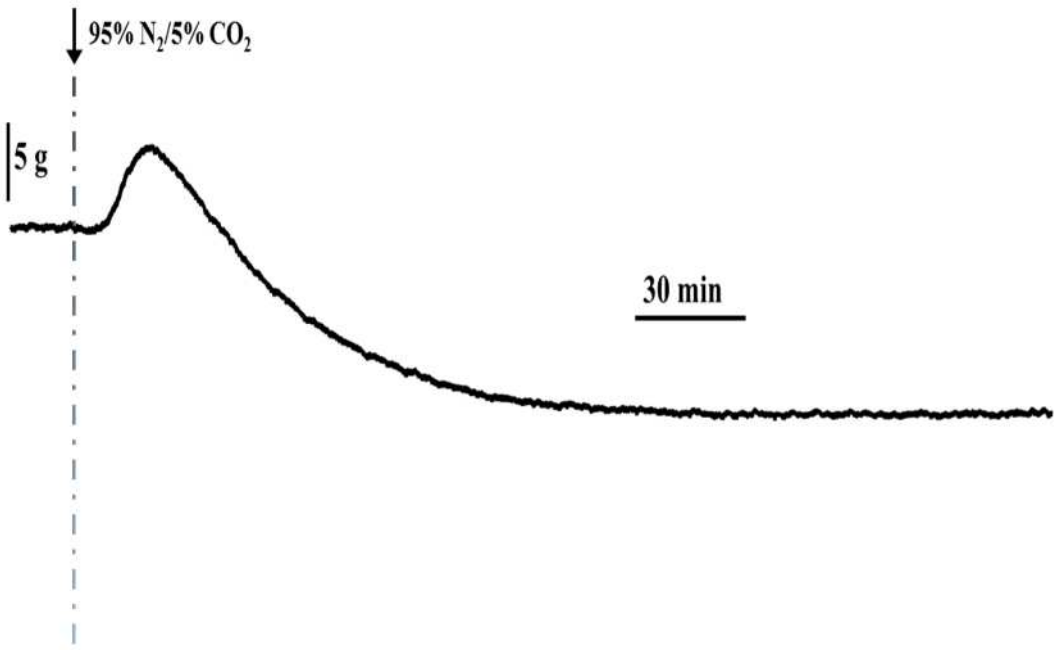


Figure 5.9 Hypoxia caused a biphasic vascular response; a transient contraction followed by prolonged relaxation in the porcine splenic artery.

5.3.2.5 Response of porcine splenic artery to hypoxia in the absence and presence of inhibitors of CSE and/or CBS.

Experiments were carried out to investigate responses to hypoxia on the pre-constricted splenic artery in the absence and presence of inhibitors of CSE and/or CBS when the preparations were gassed with 95% O_2: 5% CO_2. First, splenic artery samples were incubated with the CBS inhibitor amino-oxyacetate (AOAA 300μM) and/or the CSE inhibitor D, L-propargylgylcine (PPG 100μM) before being constricted with U46619. Once a stable tone (~30-70% of KCl response) was achieved, hypoxia was by switching the gas to 95% N_2: 5% CO_2 from 95% O_2: 5% CO_2 for 60 min.

Exposing splenic arteries to hypoxia caused a biphasic response; a transient contraction followed by a prolonged relaxation. Hypoxia-induced contraction was not significantly different in the presence of AOAA (300μM) and/or PPG (100μM) (Figure 5.10a). The CBS inhibitor amino-oxyacetate (AOAA 300 μM) alone or in combination with PPG (100μM) caused a significant reduction in the relaxation phase of the hypoxic response (control -92.38 ± 11%, +AOAA -55.2 ± 13%, P = 0.04, control -92.38 ± 11%, +AOAA/PPG -55.8 ± 7.8 %, P = 0.04). The CSE inhibitor D, L-propargylgylcine (PPG 100μM) had no effect on the relaxation response to hypoxia (Figure 5.10b).

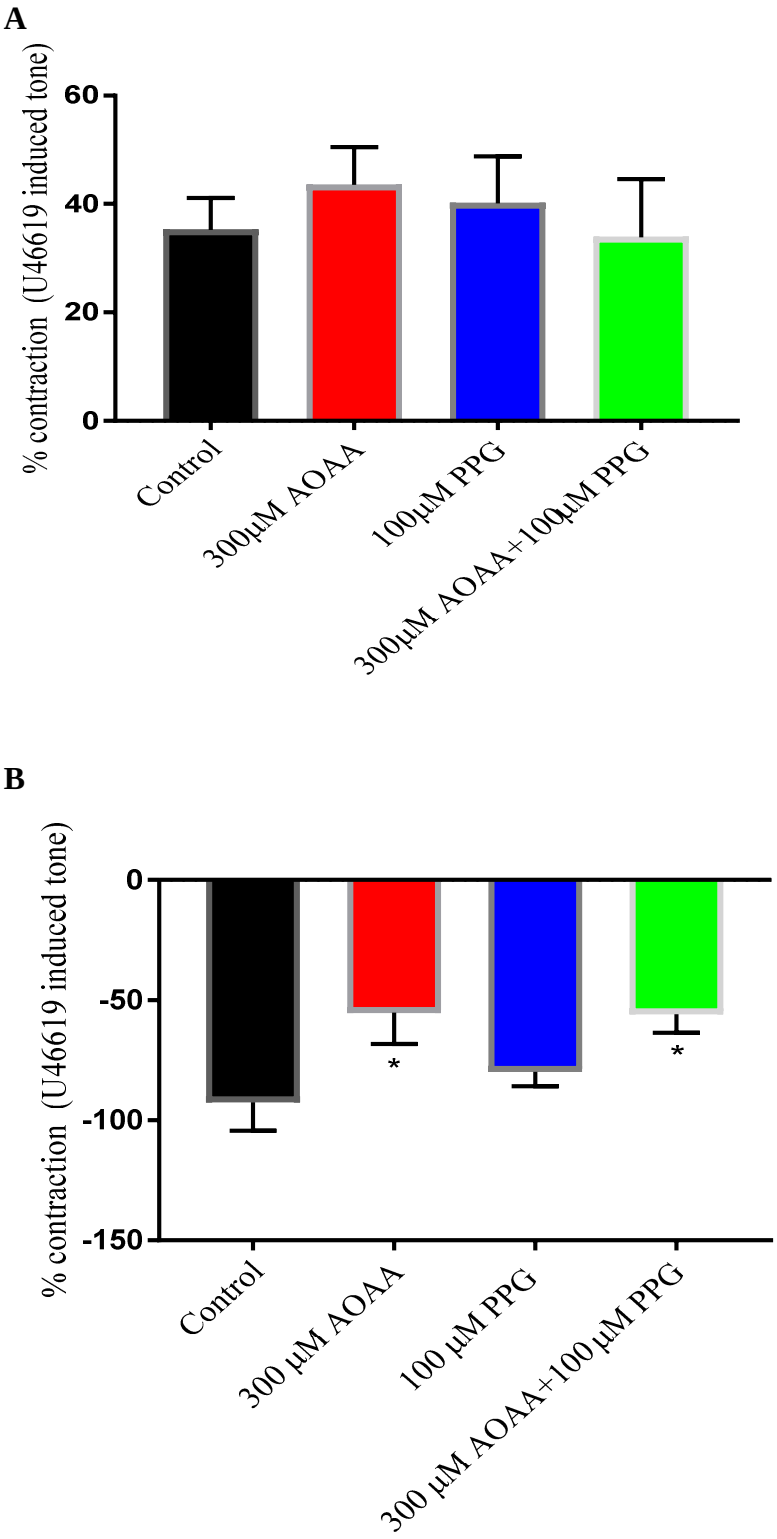


Figure 5.10 Hypoxia-induced contraction was unaffected in the presence of the CBS inhibitor amino-oxyacetate (AOAA 300µM) and the CSE inhibitor D,L-propargylglycine (PPG 100µM) (A). Hypoxia-induced relaxation was significantly reduced in the presence of the CBS inhibitor amino-oxyacetate (AOAA 300µM) and unaltered in the presence of CSE inhibitor D, L-propargylglycine (PPG 100µM) (B). *P < 0.05 (mean ± SEM) (n = 6).

5.3.2.6 Response of porcine splenic artery to hypoxia in the absence and presence of L-NAME.

To assess the involvement NO in the responses of splenic artery to hypoxia. Samples were incubated with L-NAME (100µM), before being contracted with U46619. Vessels were pre-constricted by 46 ± 5% in the absence of L-NAME and 63 ± 3% in the presence of L-NAME (n = 7), using an average U46619 concentration of $1.3 \times 10^{-8} \pm 2.9 \times 10^{-9}$ M in the absence of L-NAME and 3×10^{-8} M in its presence. Once a stable tone was achieved, hypoxia was induced using 95% N_2/5% CO_2 for 60 min. Hypoxic responses were measured in grams (g) after 30 min. The paired control group was not exposed to L-NAME before the U46619 contraction.

The NO synthase inhibitor, L-NAME, significantly abolished the contraction induced by hypoxia and caused a significant increase in the relaxation phase (Figure 5.11).

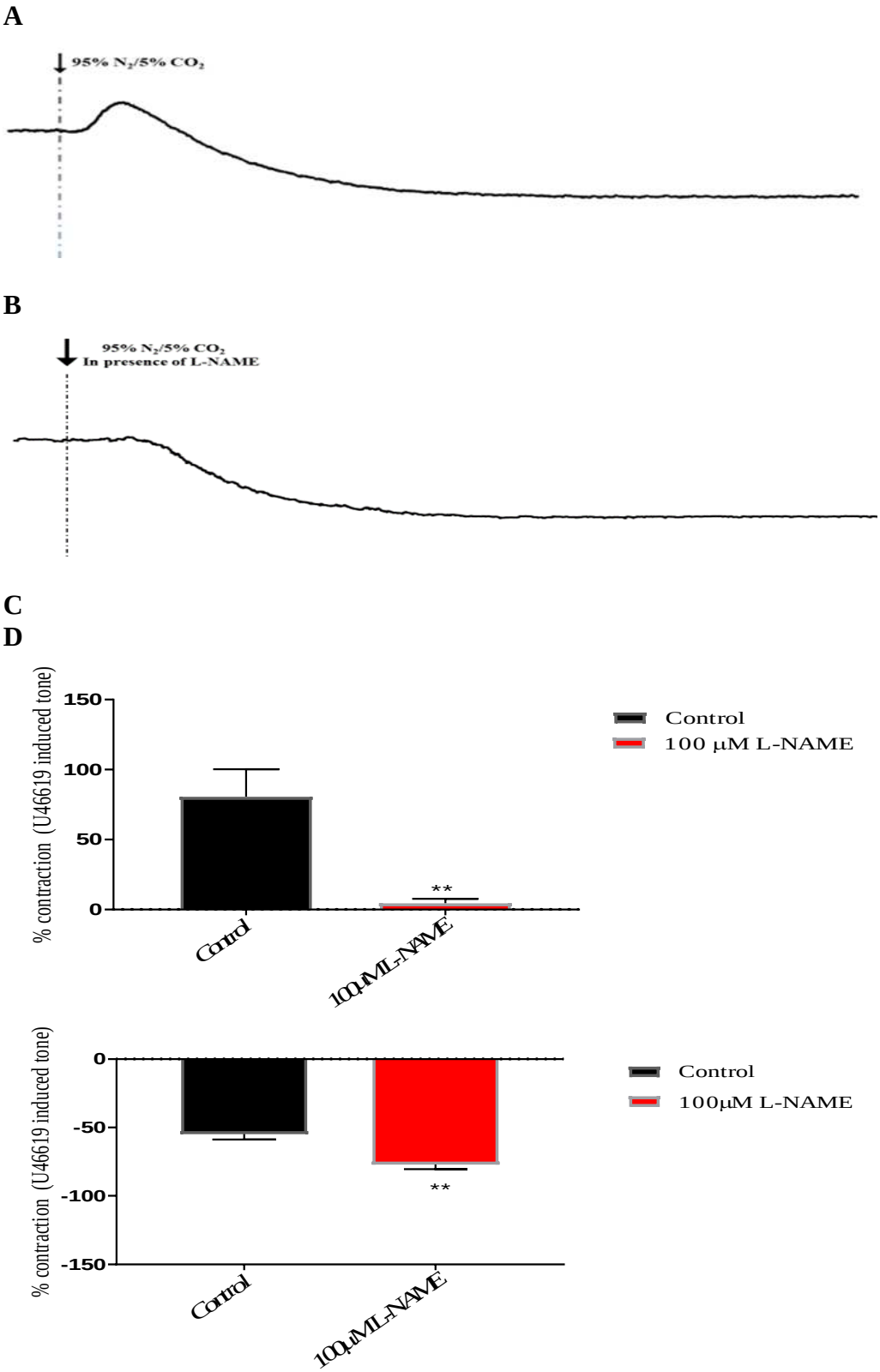


Figure 5.11 Hypoxia caused a biphasic vascular response; transient contraction followed by prolonged relaxation in the porcine splenic artery. (A) Representative trace to illustrate the effect of hypoxia, induced using 95% N_2: 5% CO_2, on the porcine splenic artery. (B) Representative trace to illustrate the effect of L-NAME (100 μM) on hypoxic response on the PSA. (C) Hypoxia-induced contraction was significantly reduced in the presence of L-NAME (100 μM). (D) Hypoxia-induced relaxation was significantly increased in the presence of L-NAME (100 μM). **P < 0.01 (mean ± SEM) (n = 7).

5.3.2.7 Effect of L-NAME on response of porcine splenic artery to hypoxia in the absence and presence of inhibitors of CSE and/or CBS.

To test the involvement NO in the responses of splenic artery to hypoxia in the absence and presence of inhibitors of CSE and/or CBS, samples were incubated with L-NAME (100 μM), AOAA (300 μM) and PPG (100 μM) before being contracted with U46619. Once a stable tone (~30-70% of KCl response) was achieved, hypoxia was induced by switching the gas to 95% N_2: 5% CO_2 from 95% O_2: 5% CO_2 for 60 min. Hypoxic responses were measured in grams (g) after 30 min.

In the presence of L-NAME, AOAA still had an effect but no bigger in effect than had been in the control condition when there is no L-NAME. (Figure 5.12).

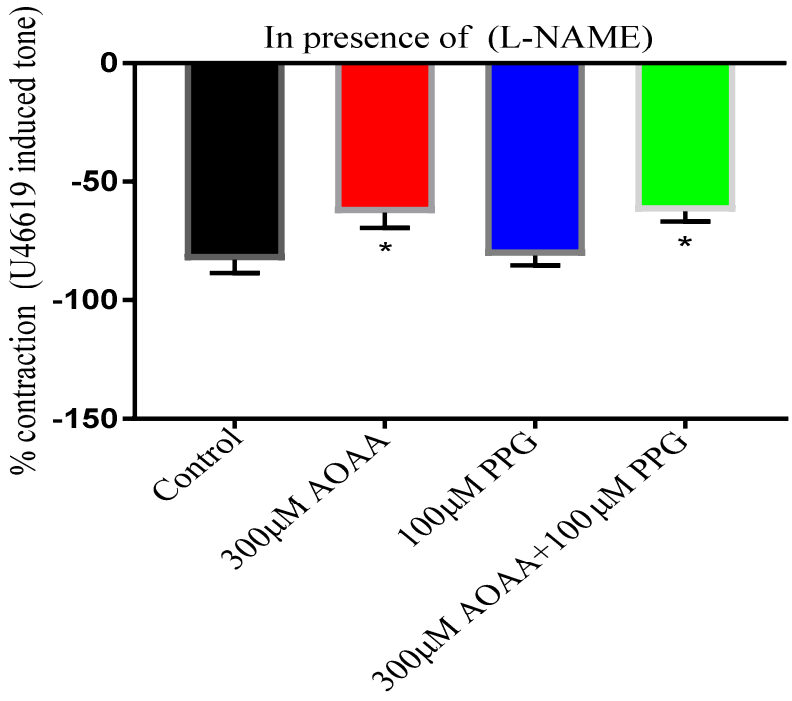


Fig. 5.12 L-NAME was not effect on hypoxia-induced relaxation in the presence of the CBS inhibitor amino-oxyacetate (AOAA 300μM) and the CSE inhibitor D, L-propargylgylcine (PPG 100μM). (mean ± SEM) (n = 7).

5.3.2.8 Response of porcine pulmonary artery to hypoxia

The present experiment investigated responses to hypoxia on pre-constricted porcine pulmonary artery. Tissues were contracted with U46619. Once a stable tone (~30-70% of KCl response) was achieved, hypoxia was induced by switching the gas to 95% N_2: 5% CO_2 from 95% O_2: 5% CO_2 for 60 min. Hypoxia caused a contraction in the porcine pulmonary artery. (Figure 5.13).

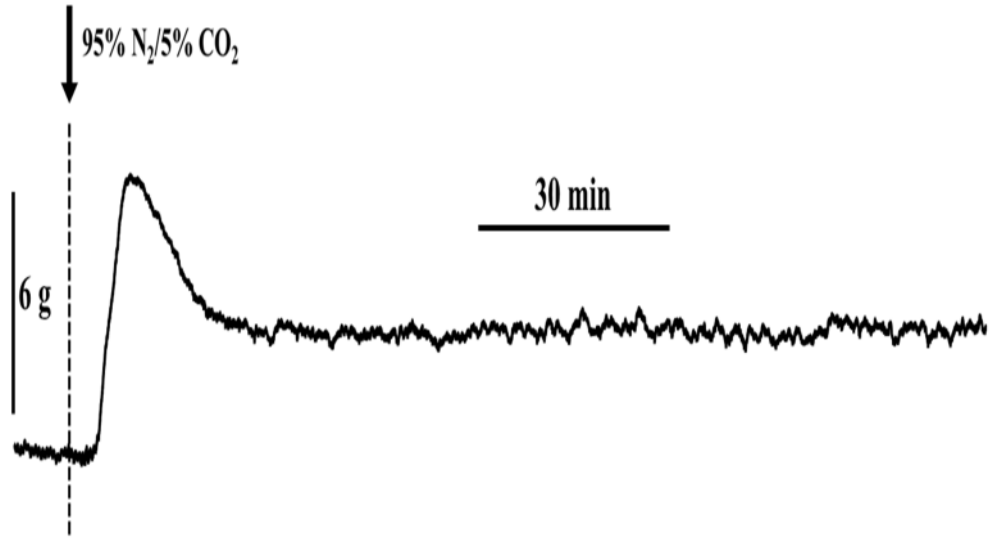


Fig 5.13 Hypoxia caused a contraction in the porcine pulmonary artery.

5.3.2.9 Response of porcine pulmonary artery to hypoxia in the absence and presence of inhibitors of CSE and/or CBS.

In the current experiment I examined responses to hypoxia on pre-constricted pulmonary artery in the absence and presence of inhibitors of CSE and/or CBS. First, pulmonary artery samples were incubated with the CBS inhibitor amino-oxyacetate (AOAA 300µM) and the CSE inhibitor D, L-propargylgylcine (PPG 100µM) before being constricted with U46619. Once a stable tone (~30-70% of KCl response) was achieved, hypoxia was induced by switching the gas to 95% N_2: 5% CO_2 from 95% O_2: 5% CO_2 for 60 min.

Hypoxia-induced contraction was not significantly different in the presence of the combination of AOAA (300µM) and PPG (100µM) (Figure 5.14).

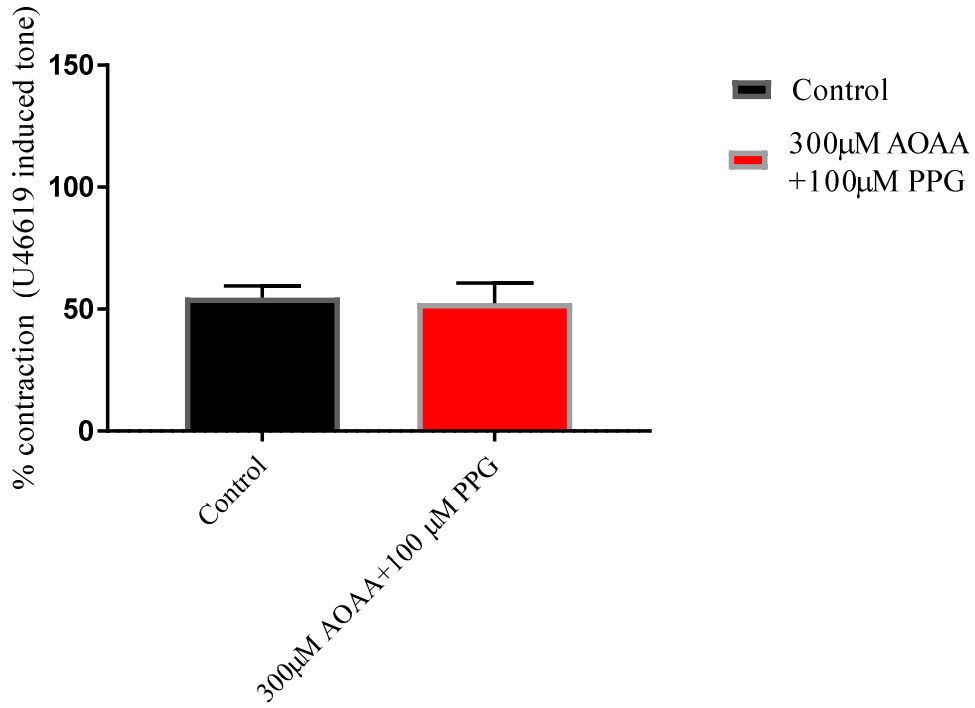


Figure 5.14 Hypoxia-induced contraction was unaffected in the presence of the CBS inhibitor amino-oxyacetate (AOAA 300µM) and the CSE inhibitor D, L-propargylgylcine (PPG 100µM).

5.3.2.10 Response of porcine pulmonary artery to hypoxia and in the absence and presence of L-NAME.

The present experiment investigated the involvement of NO in the responses of pulmonary artery to hypoxia. Tissues were incubated with L-NAME (100μM), before being contracted with U46619. Vessels were pre-constricted by 45.3 ± 3.5 % in the absence of L-NAME and 43 ± 3.5 % in the presence of L-NAME (n = 8), using an average U46619 concentration of $2.1 \times 10^{-8} \pm 1.1 \times 10^{-8}$ M in the absence of L-NAME and $3.3 \times 10^{-9} \pm 1.5 \times 10^{-9}$ M in its presence. Once a stable tone was achieved, hypoxia was induced by switching the gas to 95% N_2: 5% CO_2 from 95% O_2: 5% CO_2 for 60 min. The paired control group was not exposed to L-NAME before the U46619 contraction.

Hypoxia caused a contraction in the porcine pulmonary artery. The NO synthase inhibitor, L-NAME, abolished hypoxia induced contraction uncovering a relaxation response (Figure 5.15).

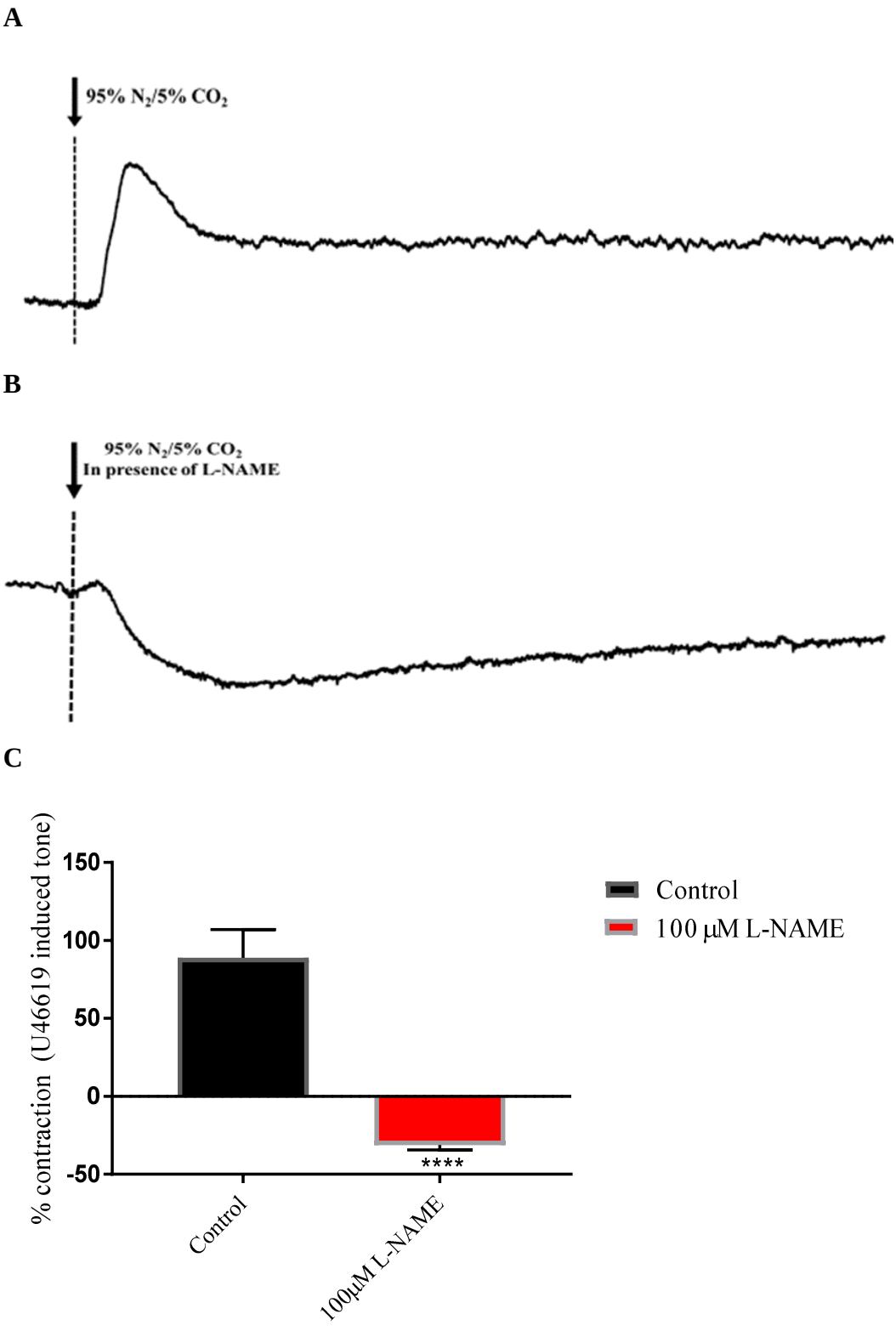


Figure 5.15 Hypoxia caused a contraction in the porcine pulmonary artery. (A) Representative trace to illustrate the effect of hypoxia, induced using 95% N_2/5% CO_2, on the porcine pulmonary artery. (B) Representative trace to illustrate the effect of L-NAME (100µM) on hypoxic response in the PPA. (C) Hypoxia-induced contraction was abolished uncovering a relaxation response in the presence of L-NAME (100µM). ****P < 0.0001 (mean ± SEM) (n = 8).

5.4 Discussion

In the present study it has been shown that both CBS and CSE are expressed in porcine splenic and pulmonary arteries. L-cysteine induced a contractile response in PSA and PPA but this was not blocked by H_2S synthesis- enzymes inhibitors, indicating that L-cysteine-elicited contraction was not through a H_2S-dependent mechanism. Hypoxia caused a biphasic vascular response; transient contraction followed by prolonged relaxation in PSA. The NO synthase inhibitor, L-NAME, significantly blocked the contraction response in PSA and increased the relaxation phase, indicating that hypoxia decreases NO production. Similarly, in PPA, hypoxia caused a contraction response that was decreased by L-NAME, uncovering a relaxation response to hypoxia. Inhibition of CBS caused a significant reduction in the relaxation phase of the hypoxic response, suggesting that there is some contribution to the hypoxic response from H_2S production by this enzyme.

The generation of endogenous H_2S has been demonstrated to principally involve CBS in the brain (Abe and Kimura, 1996) while CSE is responsible for H_2S formation in the vasculature (Hosoki *et al*., 1997). More recent data has shown that CBS is also present in vascular endothelium and smooth muscle of mouse ductus arteriosus (Baragatti *et al*., 2013). The present study showed that both CBS and CSE are present in both the PSA and PPA, although it was not determined in which cellular layer they are expressed.

Experiments were carried out to determine whether H_2S could be produced upon exposure of arteries to the precursor, L-cysteine. L-cysteine produced a

concentration-dependent contraction in PSA and PPA. This observation is similar to results in the rat aortic rings (Ali *et al.,* 2006) where L-cysteine caused a contractile response and reversed acetylcholine-induced vasorelaxation. By contrast, high concentrations of L-cysteine evoked vasorelaxation in mouse aorta (Al-Magableh and Hart 2011) and in rat mesentery arterial beds (Cheng *et al.*, 2004). In addition, Kohn *et al.,* (2012) showed that L-cysteine caused vasorelaxation in rat and mouse aortas.

In rat mesenteric arterial beds, it has been shown that a relaxation response generated by L-cysteine was blocked using PPG (10 μM) (Cheng *et al.*, 2004). L-cysteine caused a vasorelaxation in the mouse aorta through H_2S generation and this response was attenuated by PPG, although they used a much higher concentration of PPG (20mM) (Al-Magableh and Hart 2011). Ali *et al.,* (2006) showed that the L-cysteine responses in the rat aorta were partially blocked by PPG (1 mM). However, in the present study, there was no evidence for endogenous H_2S production from its biochemical substrate, L-cysteine, since neither PPG (10μM, 100μM) nor AOAA (100μM, 300μM) or Aspartate (1mM) blocked the contractile responses to L-cysteine. Similarly, Donovan *et al.*, (2017) showed that vasorelaxation of porcine coronary artery segments which evoked by L-cysteine, was not inhibited by PPG. It is possible that the concentration of PPG in the present study was not high enough it is more likely that. L-cysteine is likely to be generating vasoconstriction largely through a non H_2S dependent mechanism. Takemoto *et al.,* (1995), indicated that vasoconstrictor effects of L-cysteine probably due to automatic nervous activation combined with vasopressin release in superior mesenteric artery.

In PSA, the response to hypoxia was biphasic, consisting of a transient contraction followed by relaxation. The CBS inhibitor, aminooxyacetic acid (AOAA 300µM), caused a significant decrease in splenic hypoxic vasorelaxation, whereas The CSE inhibitor, propargylglycine (PPG 100µM) had no effect. These data are consistent with the idea that H_2S participates in vascular responses to hypoxia with CBS the likely source of H_2S production. Donovan *et al*., (2017) reported that AOAA (300µM) also reduced hypoxic vasorelaxation in coronary arteries. In addition, AOAA, inhibited hypoxic relaxation of rainbow trout urinary bladder (Dombkowski *et al.*, 2006) and rainbow trout and Coho salmon intestine (Dombkowski *et al.*, 2011). AOAA was also shown to decrease hypoxic contraction of bovine pulmonary arteries and hagfish aorta, with PPG having no effect, indicating the involvement of CBS (Olson *et al.*, 2006, 2008). In contrast, PPG abolished hypoxic vasorelaxation of the rat aorta (Olson *et al.*, 2006). Overall, these data indicate that the vascular response to hypoxia is mediated through H_2S and there are difference between tissues and / or species with respect to the possible involvement of CBS and CSE in the hypoxic response and production of H_2S.

L-NAME abolished the contractile response observed after exposure to hypoxia and increased the relaxation phase in PSA, This indicates that hypoxia decreases NO availability and suppresses a relaxating response. This observation is similar to results by Donovan *et al*., (2017) who showed that L-NAME blocked the contractile effect of hypoxia in coronary arteries whereas the relaxation phase was increased. Similarly, Graser & Vanhoutte 1991 showed that L-NAME

reversed the vasoconstrictor response to hypoxia into relaxation in canine coronary arteries.

In PPA, L-NAME abolished the contractile response and uncovered a relaxation response to hypoxia, suggesting again that hypoxia decreases the sustained release of NO from the vascular endothelium. Thus, blockage of NO release prior to hypoxia decreases the extent for reduction in NO formation by hypoxia, thereby decreasing the vasoconstrictor response to hypoxia. Similarly, MacEachern *et al*., (2004) showed that L-NAME effectively abolished the HPV in equine pulmonary arteries and partially depressed the HPV in bovine pulmonary arteries. Jin *et al*., (1992) also demonstrated that L-NAME abolished hypoxic contraction in rat pulmonary arteries. In contrast, a vasoconstrictor response to hypoxia seen in several studies was augmented by the inhibition of NO production (Grimminger *et al*., 1995, Nelin *et al*., 1996, Oh and Kim, 2001).

In summary, L-cysteine produced a concentration-dependent contraction in PSA and PPA in different gassing conditions. Since these responses were not blocked by H_2S synthetic enzyme inhibitors, this indicates that L-cysteine caused vasoconstriction through a non H_2S dependent mechanisms. Hypoxia caused a biphasic response in the PSA; a transient contraction followed by prolonged relaxation. The relaxation response were attenuated by AOAA, suggesting that, at least in part, H_2S mediates the hypoxic response. Hypoxia caused a contraction in the PPA. L-NAME, abolished contractions, and uncovered a relaxation response to hypoxia, indicating NO production is reduced under low O_2

conditions. There is no evidence of H_2S involvement in vascular response to hypoxia in PPA.

Chapter 6:

General discussion

6 General discussion

The object of this thesis was to examine the influence of O_2 levels on responses to H_2S and the mechanism of H_2S-mediated responses, using porcine splenic and pulmonary vessels and human chorionic blood vessels. In addition, since hypoxia is an important regulator of vascular tone, this study also examined whether H_2S contributed to the hypoxic vascular response. Vessels were gassed with 95% O_2: 5% CO_2, 95% air: 5% CO_2 or 95% N_2: 5% CO_2, to determine the influence of O_2 on H_2S responses. The partial pressures of O_2 of PSS gassed with 95% O_2: 5% CO_2, 95% air: 5% CO_2 or 95% N_2: 5% CO_2 was shown to be 619 ± 17 mmHg, 140 ± 4 mmHg and 33 ± 6 mmHg, respectively (White, 2012). In mammals, the arterial pO_2 is normally around 100 mmHg and venous pO_2 is between 15-50 mmHg (Erecinska and Silver, 2001, Tsai *et al.*, 2003). This suggests that the most physiologically relevant conditions in the current study were achieved by gassing with 95% air: 5% CO_2 for porcine splenic arteries (PSA) and porcine pulmonary veins (PPV) and 95% N_2: 5% CO_2 for porcine splenic veins (PSV), porcine pulmonary arteries (PPA). However, most studies examining the effects of H_2S on blood vessels have been performed gassing with 95% O_2: 5% CO_2 and this may have produced results that have limited physiological relevance (Zhao and Wang, 2002, Cheng *et al*., 2004).

In the current study, the responses to NaHS in both PSA and PPV were affected by the O_2 concentration of the gassing mixture. Generally, in both PSA and PPV, gassing with physiological levels of O_2 (95% air: 5% CO_2), was associated with NaHS causing a contractile response, while, gassing with sub-physiological levels (95% N_2: 5% CO_2), NaHS caused a relaxant response in

PSA. Koenitzer *et al*., (2007), proposed that H_2S was rapidly oxidized at high tension of O_2, thus decreasing its availability. The oxidation theory would argue that oxygen at high levels causes the oxidation of H_2S and lowers free H_2S level and its capacity for vasorelaxation activity. With low O_2, oxidation of H_2S is decreased, and more free H_2S is available to induce vasorelaxation.

The NOS inhibitor, L-NAME, had a significant effect on the NaHS responses, abolishing contractions, and uncovering a relaxation response to NaHS in PSA under physiological gassing conditions. This suggests that the contraction caused by NaHS likely involved quenching of endogenous NO. It has been shown that NO and H_2S interact to form an inactive nitrosothiol compound (Ali *et al.,* 2006 and Whiteman *et al*., 2006). In their hands, $CuSO_4$ (200 nM) blocked the contractile response of NaHS through the destruction of nitrosothiol compound (Ali *et al.,* 2006). The present study did not confirm a role for a nitrosothiol compound in the contractile response of H_2S such that the 'mopping up' of endogenous NO by exogenous NaHS seems to be the predominant mechanism for producing the NaHS-induced vasoconstriction in PSA. Under sub-physiological O_2 levels (95% N_2: 5% CO_2), L-NAME and febuxstat had no effect on the vasorelaxation responses produced by NaHS, showing that NO was not responsible for generating the vasorelaxation response in this preparation. Nor did glibenclamide and so K_{ATP} channels are not involved. Therefore, it is possible that vasorelaxation is mediated through another mechanism perhaps involving other K^+ channels (Jackson-Weaver *et al*., 2011, 2013, Martelli *et al.*, 2013), or alterations in intracellular PH (Lee *et al*., 2007), but this requires further study.

NaHS-induced vasorelaxation was observed in both of PSV and PPA under physiological gassing condition (95% N_2: 5% CO_2) (at a pO_2 of around 33 mmHg which is more physiological for systemic veins and pulmonary arteries). The vasorelaxatory responses were abolished by L-NAME, showing that NaHS is activating NOS to generate NO and cause vasorelaxation. In PSV, under physiological gassing condition, the vasorelaxation response to NaHS were not sensitive to K_{ATP} channel blockers. In PSV, under conditions where the O_2 levels were high, a contractile response was shown. L-NAME did not reduce this response, indicating NO was not involved for generating this response in contrast to the PSA.

This study also examined whether the hydrogen sulphide –synthesizing enzymes (CBS and CSE) were present in PSA and PPA, and investigated the endogenous formation of H_2S from exogenously applied L-Cysteine in these vessels. The present study identified that CBS and CSE are present in both the PSA and PPA, although it was not determined in which cellular layer they are expressed. L-cysteine induced a contractile response under different gassing conditions but there was no evidence of endogenous H_2S production from its biochemical substrate, L-cysteine, through the CSE, CBS and 3-MST, since neither PPG (100μM) nor AOAA (300μM) or Aspartate (1mM) blocked the contractile responses to L-cysteine. Thus, L-cysteine is likely to be generating vasoconstriction largely through a non H_2S dependent mechanism.

The present study also examined whether endogenous H_2S was involved in contributing to hypoxia-induced systemic vasodilation and pulmonary

vasoconstriction in PSA and PPA respectively. Hypoxia caused a biphasic response in the PSA; a transient contraction followed by prolonged relaxation. The relaxation response was attenuated by AOAA. Suggesting that H_2S participates in vascular responses to hypoxia with CBS the likely source of H_2S production. The CSE inhibitor, propargylglycine (PPG 100µM) had no effect on responses.

Hypoxia-induced vasoconstriction was observed in both PSA and PPA. The vasocontractile responses were abolished by L-NAME, showing that hypoxia decreases the sustained release of NO from the vascular endothelium. The data presented in this thesis suggests that the blockage of NO release prior to hypoxia decreases the extent for reduction in NO formation by hypoxia, thereby decreasing the vasoconstrictor response to hypoxia.

The systemic circulation dilates in response to hypoxia with the aim of encouraging blood flow and oxygen delivery to hypoxic tissues, in contrast to the pulmonary circulation, which vasoconstricts. This response of the pulmonary circulation is known as hypoxic pulmonary vasoconstriction and is a physiological reflex that aims to reduce perfusion of under-ventilated hypoxic regions and thus acts as a protective physiological process for maintaining oxygenation. Several mechanisms for hypoxia induced vasorelaxation have been proposed, such as K^+ channel activation in addition to a role for H_2S in the hypoxic response. Recently, it has been proposed that (H_2S) may act as the oxygen sensor/transducer of the hypoxic response in the vasculature. The proposed mechanism is that a decline in tissue O_2 levels decreases the rate of H_2S oxidation, thus increasing the level of biologically active H_2S, which in many vessels increases relaxation to encourage blood flow to the affected area.

The present study indicates that H_2S participates in vascular responses to hypoxia and supports the hypothesis that hydrogen sulfide (H_2S) may act as an oxygen sensor by an increase in endogenous levels of H_2S under hypoxia inducing relaxation of blood vessels, thus increasing the supply of oxygenated blood to tissue.

The responses to H_2S in human chorionic vessels were affected by the O_2 concentration used to gas preparations, with relaxant response predominating under high O_2 levels and vasoconstriction evident at low O_2 levels (95% N_2: 5% CO_2). The oxygen tension in the umbilical arterial blood is normally between 16-28 mmHg and venous blood is between 28-35 mmHg (Lackman *et al.*, 2001, Nicolaides *et al.*, 1989), this suggests that responses obtained under 95% N_2: 5% CO_2 are the most physiologically relevant, and thus a contraction may be the predominant effect of NaHS in human chorionic vessels. L-NAME was without effect on the contraction produced by NaHS in chorionic arteries under low O_2 gassing conditions, and so NO is not involved in this human blood vessel. As an alternative, Lim *et al.*, (2008) suggested that the contractile action of H_2S in the rat aorta may occur by preventing cAMP accumulation. Whether this happens in chorionic blood vessels remains to be determined. This study indicate that the predominant vasoactive mechanism of H_2S is O_2 dependent, with vasoconstriction being the response at physiological O_2 level (95% N_2: 5% CO_2) in human chorionic blood vessels.

Summary and recommendation

This thesis increases the understanding of the responses to H_2S in blood vessels affected by the oxygen concentration used to gas preparations. It showed that under physiological oxygen tension, NaHS caused a contractile response in PSA and PPV, and produced vasorelaxation in PSV and PPA. Under gassing conditions with a more physiological oxygen tension for chorionic vessels, NaHS evoked a contractile response. Given the change in responses to H_2S, in vitro studies should take the oxygen concentration into account in designing experiments to assess the vascular biology of H_2S, particularly when using high O_2 levels, where results may have less relevance to normal physiological conditions. Further mechanistic investigations are required to assess NaHS mechanism under physiological relevant gassing conditions.

Another important outcome of the present study is that, at least in part, H_2S mediates the hypoxic response in PSA. This work showed that hypoxia caused a biphasic response in the PSA; a transient contraction followed by prolonged relaxation, the relaxation response were attenuated by CBS enzyme inhibitor. These results indicate that H_2S participates in vascular responses to hypoxia and it is the metabolism of H_2S that serves as the 'O_2 sensor/transducer' in vascular smooth muscle.

Furthermore, in human chorionic blood vessels, NaHS caused vasoconstriction under gassing condition of low O_2 level (95% N_2: 5% CO_2) which is closer to physiological levels for the fetoplacental circulation. In perfused placenta a lower concentration of NaHS produce a vasorelaxation

response when gassed with 95% O_2: 5% CO_2 (Cindrova-Davies *et al.*, 2013). More studies are warranted in the future to test whether H_2S causes a contraction in the perfused human placenta when gassed with more physiologically relevant levels of oxygen.

BV	95% O_2: 5% CO_2	95% air: 5% CO_2	95% N_2: 5% CO_2
PSA	Vasocontraction	Vasocontraction	Biphasic response
PSV	Biphasic response	Biphasic response	Vasorelaxation
PPA	Biphasic response	Biphasic response	Vasorelaxation
PPV	Vasocontraction	Vasocontraction	Biphasic response
ChA	Vasorelaxation	Vasorelaxation	Vasocontraction
ChV	Vasorelaxation	Vasorelaxation	Biphasic response

Figure 6.1 Summary of H_2S responses on blood vessels under different gassing conditions.

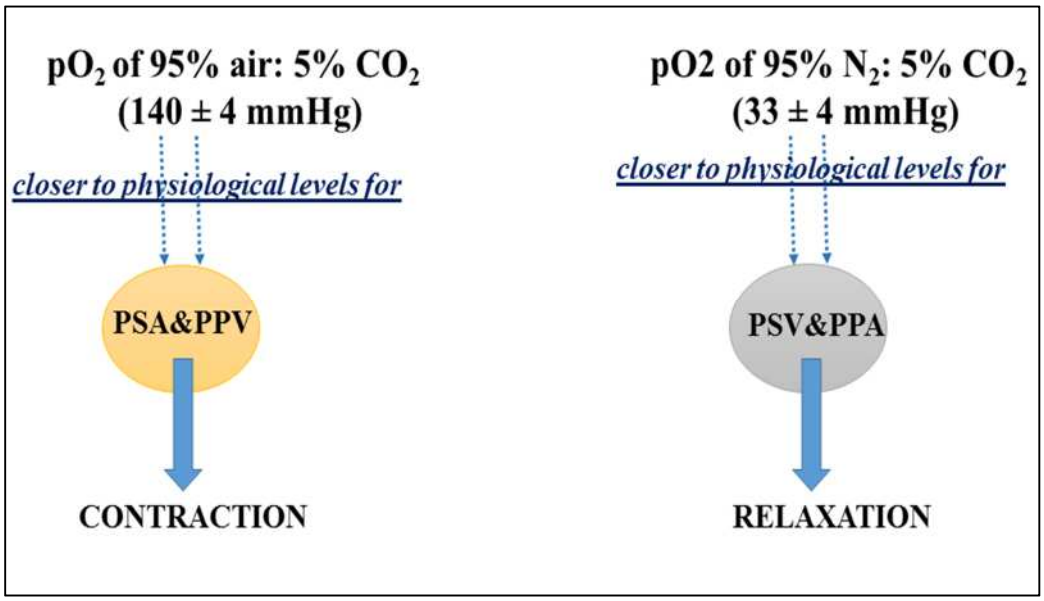


Figure 6.2 Summary of H_2S responses on blood vessels under physiological oxygen tension.

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