Cystathionine γ-lyase and hypoxia: Implicating hydrogen sulfide in the hypoxic stress response

by

Sean A. Bryan

Department of Biology

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Faculty of Graduate Studies
Lakehead University
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Abstract

Hydrogen sulfide (H_2S) has emerged as a novel and important gasotransmitter for the cardiovascular system, where it is generated mainly by cystathionine γ-lyase (CSE). Abnormal metabolism and functions of the H_2S/CSE pathway have been linked to cardiovascular diseases including hypertension and atherosclerosis. Hypoxic stress is a hallmark of these pathophysiological processes. Here we characterized the response of vascular smooth muscle cells (SMCs) derived from CSE-knockout (KO) mice to hypoxic stress. Under basal conditions we found that KO cells exhibited increased metabolic activity (+27 %), ROS levels (+127 %) and mitochondrial membrane potential (Ψ_m) (+62 %) than their wild-type (WT) counterparts. These data suggest an impaired ability of KO cells to regulate redox levels and/or increased by-production of ROS resulting from deficient H_2S-mediated regulation of mitochondrial activity in the KO cells. Hypoxic insult (12 h, 1 % O_2) caused a decrease (-61 %) in KO cell metabolism and a dramatic increase in apoptosis of KO versus WT cells (+85 % versus +23 %), indicating susceptibility of KO SMCs to hypoxic stress. We also found hypoxia-induced increases in the activity of antioxidant enzyme, superoxide dismutase, in both WT and KO cells, but markedly increased (+75 %) ROS levels in hypoxic KO cells, revealing a profound redox imbalance therein. Additionally, we noted: (i) a large Ψ_m increase (+118 %) in hypoxic KO versus WT cells; (ii) that KO SMCs featured altered inflammatory mediator expression both under basal and hypoxic conditions; (iii) blunted hypoxia inducible factor-1α expression in the CSE-deficient cells. Taken together, these data demonstrate that H_2S/CSE pathway is essential for SMC survival under hypoxic conditions, and suggest that endogenous H_2S modulates redox and inflammatory status and mitochondrial activity, deficiency of which may cause the observed vulnerability of KO cells to hypoxia.

In another series of experiments, we examined the effect of the hypoxia mimetic, cobalt chloride ($CoCl_2$), as well as hypoxia-reoxygenation (H-R) stress, on HL-1 murine cardiomyocytes. We demonstrated that both $CoCl_2$ and H-R caused decreased cardiomyocyte viability (-28 % and -37 %, respectively), and that CSE expression was concomitantly increased (+79 % and +94 %, respectively). D-propargylglycine-mediated inhibition of CSE was found to exacerbate $CoCl_2$-induced decreases in cardiomyocyte viability. Taken together, these data indicate that H_2S/CSE pathway is upregulated in response to both simulated hypoxia and H-R stress, and that endogenous H_2S/CSE pathway contributes to cardioprotection against $CoCl_2$-induced stress.

These findings suggest an important role for endogenous H_2S/CSE pathway in cytoprotection against hypoxia in murine SMCs, as well as against simulated hypoxia and H-R stress in HL-1 murine cardiomyocytes. Moreover, they are consistent with the concept of meaningful involvement of H_2S/CSE pathway in the hypoxia stress response and the recent suggestion of H_2S/HIF-1 interaction.

Abbreviations

ANX – Annexin V

AP – Activator protein

ATP – Adenosine triphosphate

BSA – Bovine serum albumin

cAMP – Cyclic adenosine monophosphate

CBS – Cystathionine beta-synthase

CD – Cluster of differentiation

cDNA – Complementary deoxyribonucleic acid

cGMP – Cyclic guanosine monophosphate

RNS – Reactive nitrogen species

CM-H_2DCFDA – 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester

CO – Carbon monoxide

$CoCl_2$ – Cobalt chloride

CSE (CTH, CGL) – Cystathionine gamma-lyase

ddH_2O – Double-distilled water

DNA – Deoxyribonucleic acid

ECL – Enhanced chemiluminescence

ET – Endothelin

eNOS – Endothelial nitric oxide synthase

ERK – Extracellular signal-regulated kinase

FBS – Fetal bovine serum

PPG (PAG) – D,L-propargylglycine

FLICA – Fluorochrome inhibitors of caspases

GSH – Glutathione

GSSG – Oxidized glutathione

GTP – Guanosine triphosphate

H_2O_2 – Hydrogen peroxide

H_2S – Hydrogen sulfide

HIF – Hypoxia-inducible factor

HO – Heme-oxygenase

HPH – Hypoxic pulmonary hypertension

HRP – Horseradish peroxidase

I-R – Ischemia-reperfusion

IFN – Interferon

IL – Interleukin

iNOS – Inducible nitric oxide synthase

JC-1 – 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide

JNK – Jun n-terminal kinase

K_{ATP} – Adenosine triphosphate-sensitive potassium channel

K_{Ca} – Calcium-sensitive potassium channel

kDa – Kilodalton

KO – Knockout

LPS – Lipopolysaccharide

LTP – Long-term potentiation

MAPK – Mitogen-activated protein kinase

MCP – Monocyte chemoattractant peptide

MDA – Malondialdehyde

MI – Myocardial infarction

MMP – Matrix metalloproteinases

mRNA – Messenger ribonucleic acid

mPTP – Mitochondrial permeability transition pore

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAD+/NADH – Nicotinamide adenine dinucleotide

NBT – Nitro-blue tetrazolium chloride

NF-kB – Nuclear factor-kappa B

NMDA – N-methyl D-aspartate

nNOS – Neuronal nitric oxide synthase

NO – Nitric oxide

NOS – Nitric oxide synthase

NYHA – New York Heart Association

O_2^- – Superoxide radical

$ONOO^-$ – Peroxynitrite

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PI – Propidium iodide

PI3K – Phophoinositide 3-kinase

PKC – Protein kinase C

PS – Phosphatidylserine

qPCR – Quantitative real-time polymerase chain reaction

RIPA – Radioimmunoprecipitation assay

ROS – Reactive oxygen species

NADPH – Nicotinamide adenine dinucleotide phosphate

SAC – S-allylcysteine

SDS-PAGE – Sodium docecyl sulfate-polyacrylamide gel electrophoresis

SEM – Standard error of the mean

SMase – Sphingomyelinase

SMC – Smooth muscle cell

SOD – Superoxide dismutase

SP – Substance P

T-AOC – Total antioxidant capacity

TBST – Tris-buffered saline containing 0.1% Tween-20

TGF – Transforming growth factor

TIMP – Tissue inhibitors of matrix metalloproteinases

HPH – Hypoxic pulmonary hypertension

TNF – Tumour necrosis factor

TNFR – Tumour necrosis factor receptor

VHL – Von Hippel Lindau

WT – Wild-type

Ψ_m – Mitochondrial membrane potential

Preface

Deep within the Hadean ocean, the first precursors of modern life may have consisted of a simple metabolism upon an iron sulfide surface, driven by its oxidation to pyrite and the formation of hydrogen from hydrogen sulfide (H_2S) [1]. When the unmanned submarine "Alvin" probed the depths of the Pacific Ocean near the Galapagos Islands in 1977, scientists came upon hydrothermal vents featuring a unique and previously unknown ecosystem of creatures that had developed alone in a harsh and sunless environment. The hot, metal sulfide-rich waters were enriched with H_2S that spilled out of the chimney-like structures, and this was providing a food source for these life forms. However, natural gases are not solely an alimentary aspect of microbial life; many archaea and bacteria produce large amounts of gas for their own use and for the maintenance of their immediate environment. Not unlike these microorganisms, the human body also produces many gases, the functions of many of which remain unclear.

Given H_2S's ancient essential role, one might think it unsurprising that over 3.8 billion years later, it appears that H_2S is critical to the machinations of human life. But it *is* surprising, for we have, until scantly 10 years ago, known H_2S – with its signature odour of rotten eggs – to be a notorious environmental and industrial toxicant that is roughly equivalent to cyanide. Still, a rich and rapidly growing body of evidence demonstrates that not only is H_2S endogenously produced within mammalian tissues, but also that it exerts significant physiological effects [2]. Thus, there exists a striking parallel: H_2S as primordial heart of a chemoautotrophic modality that may underlie the origins of life, and H_2S's increasingly clear physiological importance for mammalian biological homeostasis.

At the turn of the century, H_2S joined nitric oxide and carbon monoxide as the newest member of the new, unique class of gaseous signaling molecules called 'gasotransmitters' [3].

Their common defining feature permits them to move through membranes, free of the regimented, receptor-dependent movement typical of other signaling transmitters. And while functional and mechanistic similarities exist among these gasotransmitters, H_2S acts in some novel ways, including, for example, inducement of vascular and non-vascular smooth muscle relaxation via the direct activation of adenosine triphosphate-sensitive potassium channels [3, 4]. Perhaps unsurprising given its mobility, a plethora of other roles for H_2S have been discovered in wide-ranging biological systems including the cardiovascular, neuronal and gastrointestinal, affecting a variety of cell types in a multitude of ways.

H_2S has emerged as an important regulator of cardiovascular homeostasis, playing key roles in cardiac function, cell survival, maintenance of mitochondrial structure and function, oxidative stress, inflammation, ion channel modulation and metabolism. H_2S is cardioprotective against ischemia-reperfusion injury, is implicated in the phenomena of ischemic pre- and post-conditioning, and its biosynthesis is impaired in contributing to atherosclerosis, diabetes and hypertension. Recently, an oxygen-sensing role for H_2S has also been suggested, which, considering its ancient context, further underscores its base centrality to essential cellular functions such as metabolism and oxygen handling. Here we have endeavoured to investigate H_2S's role in the cellular responses to hypoxia and 'simulated' hypoxia (chemical inducement of hypoxia-responsive signaling) in two murine cell types, cardiomyocytes and smooth muscle cells. We have focused on alteration and measurement of cystathionine γ-lyase, the principal source of H_2S in cardiovascular tissues. These investigations are timely, relevant, and add new knowledge to the explosive, exciting field of gasotransmitter biology.

Introduction

"We shall not cease from exploration

And the end of all our exploring

Will be to arrive where we started

And know the place for the first time."

- T. S. Eliot

1 – The arrival of hydrogen sulfide

Vehicles for intracellular communication fall into two categories: electrical signal via gap junction and chemical substances, which include hormones, autocoids and transmitters [5]. Put simply, the triggering event for most signal transduction processes is the binding of ligand with receptor at the plasma membrane, causing a cascade of intracellular second messengers that relay and propagate the extracellular signal and modulate cellular activity [5]. The novel concept of endogenous gaseous signaling transmitters or gasotransmitters has been verified in a wide variety of biological systems, including the cardiovascular, neuronal and gastrointestinal. Despite their common function as signal transducers, these molecules are drastically different from classic neurotransmitters and humoral factors – such as acetylcholine, catecholamines, glutamate, and adenosine triphosphate (ATP) and its metabolites – in a number of important ways, including [5]:

1. Neurotransmitters are released via exocytotic vesicles; gasotransmitters are released in the cytoplasm.
2. Neurotransmitters are re-uptaken; gasotransmitters are not.
3. Neurotransmitter removal is enzyme-dependent; gasotransmitter removal is not.

4. Neurotransmitters are unidirectional (pre- to post-synaptic membrane); gasotransmitters are bidirectional.
5. Neurotransmitters are membrane receptor-dependent; gasotransmitters are not.

Thus, gasotransmitters are starkly distinguished from their signaling forebears and deserving of separate treatment. However, given the plethora of gases present in mammalian tissues, ground rules for their identification are needed. The following criteria have therefore been suggested for the classification of gasotransmitters [3]:

1. They are small molecules of gas.
2. They are freely permeable to membranes. As such, their effects do not rely on cognate membrane receptors.
3. They are endogenously and enzymatically generated and their production is regulated.
4. They have well-defined and specific functions at physiologically relevant concentrations.
5. Their functions can be mimicked by their exogenously applied counterparts.
6. Their cellular effects may or may not be mediated by second messengers but should have specific cellular and molecular targets.

To date, it is agreed that nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S) are proven gasotransmitters. Several other gases – including ammonia and acetaldehyde – have also been proposed, but a relative paucity of information prevents their classification as yet [5]. And while these three gases share common chemical features and biological modes of action, important differences exist, particularly in the case of H_2S, the most recent arrival on the gasotransmitter scene. Chief among their common modalities is the regulation of membrane ion channels, either via second messengers or direct interaction with ion channel proteins; a novel mechanism that has garnered much interest in far-ranging biomedical fields [5]. These include

interactions with calcium-sensitive potassium (K_{Ca}), ATP-sensitive potassium (K_{ATP}), voltage-gated Ca^{2+}, voltage-gated Na^{+} and cyclic nucleotide-gated ion channels, via mechanisms including phosphorylation, *S*-nitrosylation, carboxylation, sulfuration, and altered cellular redox status [5]. The following brief overview provides further context on H_2S's gasotransmitter forerunners, NO and CO.

The discovery that NO was the molecule responsible for the majority of the vasorelaxant effect of the so-called endothelium-derived relaxing factor led to the journal *Science* naming it the 1992 "Molecule of the Year". Indeed, the seminal discovery of NO's signaling properties by Ferid Murad, Robert Furchgott and Louis Ignarro earned them the Nobel Prize in Physiology or Medicine in 1998. NO is synthesized from L-arginine by nitric oxide synthase (NOS), of which there are three isoforms featuring different tissue distribution and functions: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) [6]. Put simply, both eNOS and nNOS are calcium-dependent enzymes that produce relatively small amounts of NO, while iNOS is calcium-independent and capable of producing relatively large amounts of NO that often exert toxic effects [6]. Each NOS isoform consists of a carboxy-terminal reductase section, an aminoterminal oxygenase section and a calmodulin section that is responsible for electron flow from the reductase to the oxygenase domain. In general, lower NO concentrations promote cell survival and proliferation, whereas higher levels favour cell cycle arrest, apoptosis and senescence [7]. The major cell types that produce NO are endothelial cells, neurons, smooth muscle cells (SMCs) and macrophages [6].

NO acts through several mechanisms, including oxidation of heme proteins, activation of soluble guanylate cyclase, adenosine diphosphate ribosylation of proteins, protein sulfhydryl group nitrosylation, iron regulatory factor activation and activation of nuclear factor (NF)-κB [8].

NO has important effects – both physiological and pathophysiological – in wide-ranging processes including vascular homeostasis [9], neurotransmission [10] and the immune response [11]. Its most studied action is the relaxation of smooth muscle, which it effects via activation of soluble guanylate cyclase to form cyclic guanylate monophosphate (cGMP), eventually inducing dephosphorylation of myosin light-chain [6]. Unsurprisingly, NO pathway deficiency has been linked to pathophysiological conditions such as atherosclerosis, diabetes and hypertension [12]. Moreover, excessive NO is an important contributor to oxidative stress, due to its reaction with superoxide (O_2^-) to produce the damaging oxidant, peroxynitrite ($ONOO^-$) [13], a major reactive nitrogen species (RNS). Briefly, NO's major cellular actions include relaxation of vascular and gastrointestinal smooth muscle, inhibition of platelet aggregation, retrograde neural modulation, and destruction of parasites, bacteria and tumour cells [6].

CO, which is acquired by organisms via inhalation, metabolic by-production and endogenous production via the action of three heme-containing enzymes, heme oxygenase (HO)-1, -2 and -3, succeeded NO as the second gasotransmitter [14]. While HO-2 and -3 are constitutive isoforms, HO-1 is an inducible stress protein associated with cytoprotection [14]. By virtue of CO's bodily redistribution via carboxyhemaglobin, the locations and mechanisms of its biological functions are closely linked to the respiratory and cardiovascular systems. While toxic at high concentrations, at low concentrations it is best characterized as an anti-inflammatory and cytoprotective agent, and has been studied – both as a causative factor and promising therapeutic – in the context of pathophysiological conditions including ischemia-reperfusion (I-R) injury, transplant rejection, atherosclerosis, sepsis and autoimmunity [15]. Vascular SMCs and neurons are major CO producers, and CO induces increased cGMP via activation of soluble guanylate cyclase (however, its efficacy is on the order of ~30-100 times lower than NO). CO can complex

with any heme-containing protein, including myoglobin, iNOS, cytochrome p450 and cytochrome c oxidase. CO's major cellular actions include relaxation of vascular and bronchial smooth muscle, anti-inflammatory and anti-apoptotic effects, and neurotransmission. It is metabolized via hemoglobin, with which it has an affinity on the order of ~250 times that of O_2, producing carboxyhemoglobin, which is circulated to the lungs and excreted by diffusion [15].

It is important to appreciate the development of H_2S research in the context of NO and CO because the investigations of this new gaseous transmitter have, in no small part, been influenced by the approaches and preconceptions of the past. Indeed, this has proven to be both helpful and hurtful, since while many similarities exist, many more and interesting differences do as well, and these have set the stage for the truly novel avenues of research that H_2S offers. The interrelationships between NO, CO and H_2S are discussed further at the end of this section.

1.1 – Chemical and physical properties

H_2S is a colorless, flammable gas known for its characteristic smell of rotten eggs at concentrations roughly 400-fold lower than its toxic levels [3, 16]. Soluble in both polar and nonpolar solvents, it is unstable in physiological (pH 7.4) aqueous solutions, with approximately two-thirds of a given amount of H_2S hydrolyzing to produce hydrosulfide ions and protons [2, 3, 16, 17]. At sufficiently high pH, these ions can subsequently decompose to produce sulfide ions and protons [2, 3]. H_2S is highly lipophilic, allowing it to penetrate any cell type [18].

1.2 – Toxicology

Sulfur, a ubiquitous, nonmetallic, greenish-yellow substance known in ancient times as 'brimstone', forms many compounds including H_2S [17]. This environmental and industrial

gaseous pollutant – considered to be one of the leading causes of sudden death in the workplace – is especially dangerous because it is colourless, heavier than air and undetectable by smell at higher concentrations [17]. Its danger is especially relevant to worker safety in Canadian industries such as sour gas processing – so-called due to the presence of significant concentrations of H_2S – wherein, according to H_2S toxicology expert, Dr. Sheldon Roth, H_2S exposure safety regulations likely fall far short of being sufficiently stringent (personal communication). A broad-spectrum toxicant by virtue of its effects on many different tissues and organs, including the respiratory tract, eye, brain and olfactory system, it is those tissues with high O_2 demand that are most susceptible to H_2S due its primary mechanism of toxicity, the inhibition of cytochrome c oxidase [17, 19]. However, despite decades of research, a relative paucity of literature on the subject exists. Indeed, Roth (2004) observed that "more research is required on susceptible populations such as developing organisms, children, the elderly, and those with cardiovascular and respiratory symptoms" [17].

1.3 – Endogenous production and metabolism

Mammalian tissues contain 'bound sulfur' that can be liberated via reducing agent or acidification as inorganic sulfide, present as H_2S, HS^- or S_2^- in aqueous phase, and as H_2S in gaseous phase [20]. The major mammalian source of H_2S is L-cysteine, which can be derived from the trans-sulfuration pathway, from alimentary sources or liberated from endogenous proteins **(Figure 1)** [21]. The trans-sulfuration pathway – a metabolic pathway involving the interconversion of cysteine and homocysteine via the intermediate cystathionine – is critical for creating cysteine from the essential amino acid, methionine, in eukaryotes. Here, methionine is first converted to homocysteine by demethylation, which is then converted to the amino acid,

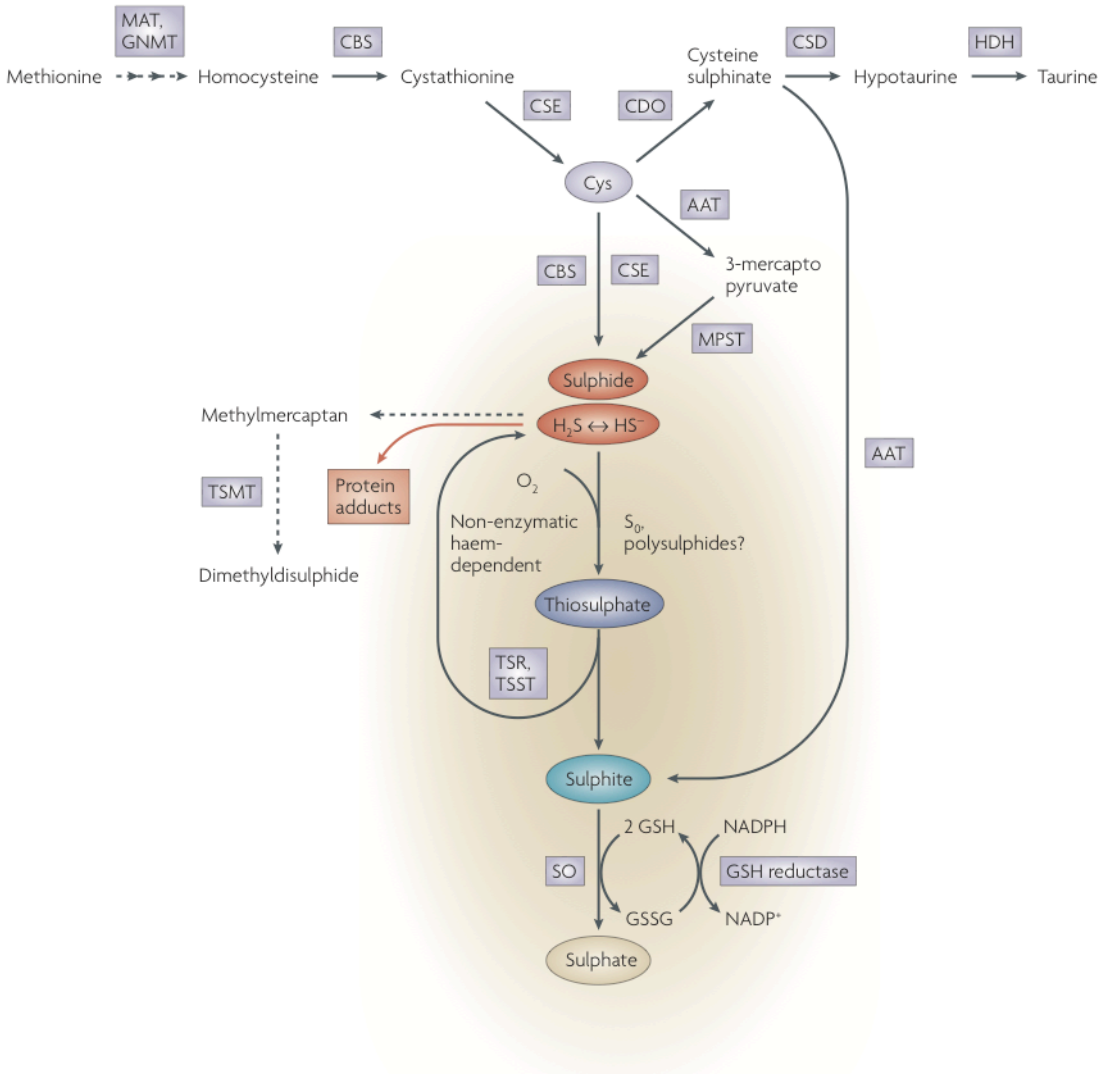

Figure 1. Enzymatic pathways of H_2S production in mammalian cells. Image and text from Szabó, C. *Nature reviews* (2007). Methionine, which is derived from alimentary sources, is converted to S-adenosylmethionine by methionine adenosyltransferase (MAT). S-adenosylmethionine is subsequently hydrolysed to homocysteine by glycine N-methyltransferase (GNMT). CBS catalyses the production of cystathionine by transferring serine to homocysteine. CSE subsequently converts cystathionine to cysteine (Cys). CSE catalyses a β-disulphide elimination reaction that results in the production of pyruvate, NH_4^+ and thiocysteine. Thiocysteine may react with cysteine or other thiols to form H_2S. The above reactions predominantly take place in the cytosol. In the mitochondria, cysteine can get converted to 3-mercaptopyruvate by aspartate aminotransferase (AAT), which can then be converted to H_2S by 3-mercaptopyruvate sulphur transferase (MPST).

cysteine, via the trans-sulfuration pathway. Pathway enzymes include cystathionine B-synthase (CBS) (EC 4.2.1.22), cystathionine y-lyase (CSE, CTH or CGL) (EC 4.4.1.1), cysteine aminotransferase (EC 2.6.1.3), mercaptopyruvate sulfurtransferase (EC 2.8.1.2), rhodanese (EC

2.8.1.1) and cysteine lyase (EC 4.2.1.10). Of these, only CBS and CSE have been shown to produce H_2S at physiologically relevant pH and substrate concentrations [20].

Both CBS and CSE are pyridoxal-5′-phosphate (vitamin B_6)-dependent enzymes, but differ in their specific mechanism of H_2S formation. CSE catalyzes the conversion of cystine (a cysteine disulfide) to thiocysteine, pyruvate and ammonia, and cystine and H_2S are then derived from the nonenzymatic decomposition of thiocysteine [2]. By contrast, the major mechanism of CBS-mediated H_2S production involves the condensation of homocysteine with cysteine to yield cystathionine, releasing H_2S in the process [22]. CBS and CSE are widely distributed in tissues, with large amounts of CBS occurring in mammalian brain and nervous system (especially Purkinje cells and hippocampus) [18, 23], while CSE is the major source of H_2S in the cardiovascular system [18, 24]. Both enzymes contribute to H_2S generation in the liver and kidney [2, 18]. Given its prominence in the cardiovascular system and pertinence to the present studies, only CSE is discussed further for the purposes of this review.

Mammalian CSE is a ~44 kDa homotetramer featuring one proteolipid protein coenzyme per monomer [20]. Two forms of messenger RNA (mRNA) for human CSE have been characterized, and likely represent splice variants, with the shorter form featuring an internal deletion of 132 base pairs [18, 25]. CSE expression has been demonstrated upon I-R stress [26, 27], high glucose [28, 29], exposure to lipopolysaccharide (LPS) [30] and NO [31, 32], as well as in animal models of diseases including type 1 diabetes mellitus [29] and pancreatitis [33]. Recently, several CSE mutations have been described in patients with cystathioninuria, a rare and poorly understood genetic disease [34, 35]. As well, a common CSE single nucleotide polymorphism that converts serine to isoleucine at position 403 has been linked to elevated plasma homocysteine levels [34].

H_2S is metabolized primarily via oxidation in mitochondria and methylation in the cytosol, but can also be scavenged by methemoglobin or metallo- or disulfide-containing molecules such as oxidized glutathione (GSSG). Ultimately, it is excreted mainly by the kidney as free or conjugated sulfate [2, 36].

1.4 – Molecular targets

The H_2S-synthesizing enzyme, CBS, is highly expressed in the hippocampus [37] and cerebellum [38], and likely represents the principal source of H_2S therein, as the gas was not detectable in CBS knockout mice [38]. A neuromodulatory role for H_2S has been suggested [37, 39] that centres on facilitation of long-term potentiation (LTP), a synaptic model of learning and memory thought to involve the N-methyl- D-aspartate (NMDA) receptor as well as the release of corticotrophin-releasing hormone from the hypothalamus [40]. Indeed, H_2S has been shown to induce cyclic adenosine monophosphate (cAMP) release that stimulates NMDA-receptor activity [39]. Furthermore, the involvement of H_2S in neurodegenerative disorders has been suggested [41] based on evidence that H_2S is decreased by 55 % in the brains of Alzheimer's disease patients [42], that exogenous H_2S protects neurons against amyloid-β [43], and that chronic exposure to H_2S impairs fetal neuronal development in rats [44].

H_2S's novel ability to activate K_{ATP} channels [3] is arguably its most distinctive property, setting it apart from its gasotransmitter brethren. It has been shown to activate K_{ATP} channels in smooth [31, 45-47] and cardiac muscle [48-51], pancreatic β cells [28, 52, 53] and the hypothalamus [54]. K_{ATP} channels feature Kir6.x-type and sulfonylurea receptor subunits, and can be identified by their position within the cell as either sarcolemmal, mitochondrial or nuclear [2]. Changes in the transcription of the four identified K_{ATP} family member genes, and thus the

production of K_{ATP} channels, are directly linked to changes in the metabolic environment, such as glucose level [55] and O_2 availability [56]. Briefly, the mechanism proposed for the cellular K_{ATP} reaction to hypoxia holds that low O_2 causes decreased metabolic rate via suppression of citric acid cycle activity that in turn causes decreased intracellular nicotinamide adenine dinucleotide (NAD+)/NADH ratio and subsequent activation of phosphatidylinositol-3-kinase (PI3K) and extracellular signal-regulated kinases (ERK), upregulation of c-jun transcription and production of sur2 promoter-binding proteins that activate the K_{ATP} genes [57]. A significant implication of this link between metabolism and K_{ATP} production is that metabolically compromised cells are hypersensitive to low O_2 injury, as evidenced by the diabetic susceptibility to cardiac ischemia [58]. Indeed, studies of both sarcolemmal and mitochondrial K_{ATP} channels indicate that they are critical for the cardioprotection afforded by ischemic pre-conditioning [59, 60]. Moreover, absence of sarcolemmal K_{ATP} channel function also impairs cardiac Ca^{2+} distribution [61] and vascular smooth muscle tone [62].

Importantly for the aforementioned processes, H_2S-induced cardioprotection against I-R injury is effected via H_2S's opening of K_{ATP} channels and regulation of Ca^{2+} handling [48-51, 63, 64]. H_2S-mediated K_{ATP} activation has also been implicated in H_2S's maintenance of vascular tone [31, 45-47] and insulin secretion [28, 52, 53, 65]; deficiency of which is thought to contribute to vascular pathologies including diabetes, hypertension and atherosclerosis. Here, other critical damaging processes exist, including elaboration of inflammation and oxidative stress, as well as cell over-proliferation and apoptosis. H_2S has important functions in regulating these processes as well, and these are reviewed briefly below.

1.5 – Cellular actions in the cardiovascular system

In this section, several of H_2S's most important cellular effects in the cardiovascular system are briefly outlined, with special focus on those relevant to the present study: redox and inflammatory regulation, cell proliferation and death, and mitochondrial function. Sections 3 & 4 of this introduction expound on these cellular effects in describing H_2S's larger roles in the heart and vasculature, respectively.

Lefer (2007) observed that H_2S "possesses all of the positive effects of NO without the capacity to form a toxic metabolite such as $ONOO^-$" [66]. Indeed, far from being a direct source of free radicals, H_2S has been shown to have considerable antioxidant effects. Findings from a variety of models support both a direct and an indirect antioxidant role for H_2S. In neurons, H_2S effects cytoprotection via induction of the important antioxidant, glutathione (GSH) [67, 68]. However, as GSH alone could not account for the observed antioxidant protection in these models, further investigation revealed that H_2S has other, direct antioxidant effects in addition to bolstering the cells' antioxidant defenses [68]. Interestingly, this effect of H_2S on GSH occurs in two ways: by enhancing the production of GSH via enhancement of cystine/cysteine transporters, and by redistributing GSH to mitochondria. In addition, mitochondrial H_2S may directly suppress oxidative stress [69]. Moreover, in human neuroblast cell model, exogenous H_2S scavenged $ONOO^-$ – the RNS believed to be a major contributor to neuronal damage in Alzheimer's and other neurodegenerative diseases – abrogating the redox damage and rescuing cells [70].

H_2S also exerts antioxidant effects in animal models of I-R stress [71, 72]. Here, H_2S-mediated cardioprotection was associated with improved GSH to GSSG ratio, reduced lipid hydroperoxide formation and increased expression of thioredoxin-1 [71], as well as increased superoxide dismutase (SOD) and GSH peroxidase (GSHPx) expression [72]. Various studies

have demonstrated similar effects of H_2S in vascular models. In carotid artery endothelial cell injury, H_2S mitigated the subsequent vascular remodeling by balancing redox stress and normalizing matrix metalloproteinase (MMP) and tissue inhibitor of MMP (TIMP) expression [73]. As well, in hypoxic pulmonary hypertension (HPH), endogenous H_2S levels were decreased – suggesting an essential homeostatic role – and exogenous H_2S decreased GSSG, improving total antioxidant capacity (T-AOC) and abrogating the HPH-induced oxidative stress [74].

A predominantly pro-inflammatory role for H_2S has been demonstrated in animal models of septic/endotoxic and haemorrhagic shock [75-77], pancreatitis [33, 78-81] and carrageenan-evoked hindpaw oedema [82], wherein enhanced H_2S production was present, and inhibition of which attenuated inflammation [83]. Indeed, much evidence exists for a detrimental effect of H_2S, whether as regulator or inducer of pro-inflammatory mediators such as the neuropeptide, substance P (SP) [84]. However, for the purposes on this review, only findings from cardiovascular system-specific studies will be addressed directly.

In contrast to the principally pro-inflammatory role of H_2S in models of shock, in I-R injury and vascular function it appears to be generally anti-inflammatory [82, 84-86], supporting the concept of H_2S as an endogenous cardioprotectant. Elrod et al. (2007) demonstrated that H_2S reduced neutrophilic infiltrate and myeloperoxidase activity, and prevented expression of the inflammatory cytokine, interleukin (IL)-1β, in the heart of I-R-injured mice [85]. As well, they observed H_2S to completely inhibit thrombin-induced leukocyte-endothelial cell interactions, which was consistent with reports of H_2S inhibiting aspirin-induced leukocyte rolling [86]. Zhang and Bhatia (2008) summarized the mechanisms by which H_2S regulates inflammatory mediators and modulates leukocyte rolling and adherence as follows: (a) stimulation of mitogen-activated protein kinase (MAPK)-NF-κB pathway and/or K_{ATP} channels; (b) promotion and release of

neuropeptides such as SP; (c) regulation of leukocyte survival and proliferation [87]. Further studies are required in order to clarify H_2S's inflammatory effects, particularly in cardiac tissues given the striking, but otherwise largely unsupported findings [85].

Vascular relaxation is absolutely dependent on proper coordination between endothelial and smooth muscle cells (SMCs), and arterial resistance increases with the proliferation of these cells [88]. Consistent with its role in regulating vascular tone and involvement in vascular pathophysiology, H_2S has been shown to have important effects on SMC relaxation [4, 89-93], proliferation [93-96] and death [93-95, 97, 98]. Indeed, taken together with its aforementioned redox and inflammatory effects, H_2S has rapidly been established as a critical regulator of vascular homeostasis [21, 99], a subject discussed in detail in Section 4 of this introduction

Finally, the mitochondrion has recently become a key area of H_2S research. Mitochondrial K_{ATP} channels have been implicated as molecular targets of H_2S, and their activation has been variously linked to anti-apoptotic [100] and pro-apoptotic [101] effects alike. Interestingly, H_2S was cardioprotective against myocardial I-R injury via stabilization of mitochondrial structure and function [85]. Consistent with H_2S's potent and reversible inhibition of cytochrome c oxidase, H_2S dose-dependently reduced oxidative phosphorylation in cardiac mitochondria, and that this prevented I-R damage subsequent to myocardial infarction (MI) [85]. Indeed, as per the recent demonstration that H_2S can induce a suspended animation-like state in mice that protects against hypoxic damage [102, 103], H_2S-mediated suppression of metabolism is likely protective for two related reasons: (a) reduced ROS by-production (and therefore reduced ROS-induced damage), and (b) reduced cellular O_2 demand (and thus entrance into an adaptive, pro-survival mode). Further to the importance of the mitochondrion to H_2S's cellular functions, Olson et al. (2009) demonstrated that the consumption of exogenous H_2S by cardiac mitochondria was O_2-

dependent, which is consistent with their newly proposed concept of O_2-sensing via H_2S metabolism [104]. These concepts are discussed in more detail in Section 5 of this introduction.

1.6 – Gasotransmitter interactions

Sufficient evidence for important gasotransmitter interaction across mammalian and non-mammalian systems led Li and Moore to recently remark that "it may now be necessary to view these gases not as separate entities but as a triumvirate of molecules working together to regulate cell function in health and disease" [18]. While important relationships are suspected among all of the recognized gasotransmitters, such interaction is most clearly evident between NO- and CO-generating systems, which share similar modes of production and effects, such as relaxation of blood vessels via augmentation of cGMP in vascular SMCs [32]. Interestingly, some of NO's vasorelaxant effect is imparted via its direct and indirect (via triggering of heme release) induction of HO-1 expression, and the resultant increase in CO [105]. Moreover, CO directly inhibits iNOS via binding to its heme moiety, thereby acting as a cytoprotective factor in limiting excessive NO synthesis, such as often exists during oxidative stress [32, 105]. Thus, this synergy may also serve an adaptive function, such as in atherosclerotic vessels, wherein stress-responsive HO-1 activity could compensate for deficient NO production and function, thereby maintaining vascular tone [106].

Similarly, evidence of synergy between NO and H_2S exists. For example, NO blockade was found to dampen H_2S-induced relaxation of aortic rings; an effect replicated upon removal of endothelium [31]. However, despite the indication that H_2S induces NO that contributes to its net vasorelaxant effect, researchers have subsequently presented contradictory findings for such an effect, likely stemming from differences in sample preparation and experimental approach.

Indeed, reports of both left- and right-ward shifts in dose response smooth muscle relaxation curves exist, as well as mechanisms that do and do not centre on cGMP [31, 107-109]. Interestingly, NO has been found to increase endogenous H_2S production in a dose-dependent manner, suggesting a direct stimulatory effect of NO on CSE that may originate downstream of cGMP and/or via NF-κB [31, 32]. The recent important finding by Yang et al. (2008) that H_2S shares a much more considerable amount of the vasorelaxation workload with NO than previously appreciated suggests that it be considered as an equal partner, opening new, parallel avenues for the treatment of hypertension [99].

As Li and Moore recently observed, the answer to the question of how a single molecule, H_2S, can exert such wide-ranging and often contradictory cellular effects may lie in its interaction with its fellow gasotransmitters. That is, much may depend, and in a very localized fashion, on the larger balance of NO, CO and H_2S synthesis, activity and/or stability [18]. The onus, then, is on gasotransmitter biologists to consider the potential contributions of this so-called "triumvirate" when designing experiments that seek to deconstruct these mysterious gaseous mediators.

2 - The redox and inflammatory bases of cardiovascular disease

Among the noncommunicable diseases, cardiovascular disease is the leading cause of death globally, accounting for 30 % of all deaths – or about 17.5 million people – in 2005 [110]. Although typically considered a disease of developed countries, its incidence is increasing in the developing world [110]. Cardiovascular disease usually stems from vascular dysfunction – for example, as a result of atherosclerosis, thrombosis or high blood pressure – which then compromises organ function. Most notably, the heart and brain can be affected, as occurs in myocardial infarction and stroke, respectively. For heart disease in particular, a wide range of

underlying pathologies can lead to defective functioning of the heart muscle. A wide variety of stimuli and mechanisms, many of which operate simultaneously, contribute to the cardiac remodeling that can ultimately lead to heart failure. Pronounced oxidative stress, immune activation and inflammation are increasingly recognized as key features within many of these processes. Indeed, elevated oxidative stress [111-115] and inflammation [116-125] that correlate

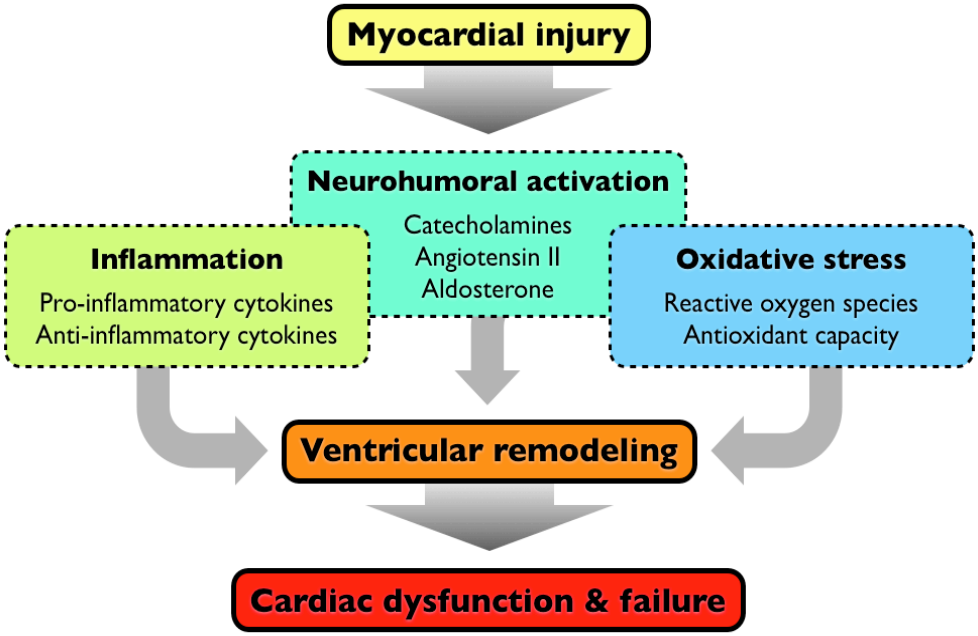

Figure 2. Oxidative, inflammatory and neurohumoral mediators initiated by myocardial stress, inform the development and progression of heart failure. Reactive oxygen species and inflammatory cytokines can contribute to the initial myocardial injury, act as or stimulate myriad secondary messengers such as neurohormones, and induce or exacerbate ventricular remodeling processes including hypertrophy, fibrosis, apoptosis, autophagy and necrosis. Thus, oxidative stress and inflammation are integral aspects of the pathogenesis of cardiac dysfunction and heart failure, with both their abundance and potency amplifying with the deterioration of the failing heart [13].

with the severity of disease have been reported in a variety of pathophysiological conditions including hypertension, atherosclerosis, coronary artery disease, cardiomyopathy and heart failure. Most of the components of neurohumoral and inflammatory activation such as catecholamines, angiotensin II, aldosterone and pro-inflammatory cytokines observed in heart failure induce oxidative stress through diverse mechanisms **(Figure 2)** [126-128]. In this regard, there has been special interest of late in the pathological relevance of the interrelation and

interaction of oxidative stress and inflammatory cytokines in cardiovascular disease.

2.1 – Oxidative stress

Oxidative stress refers to the total burden of potentially harmful ROS that form during cellular metabolism. Potential endogenous sources of free radicals include the mitochondrial electron transport chain, NADH/NADPH oxidase, the xanthine-xanthine oxidase system and many other redox reactions. Examples of ROS include O_2^- anion, hydrogen peroxide (H_2O_2), hydroxyl radical and hypochlorous acid. RNS include free radicals such as NO as well as non-radicals such as $ONOO^-$, which is generated via the ready reaction of NO and O_2^-. ROS and RNS trigger a cascade of harmful events including DNA damage, protein nitration, lipid peroxidation and activation of MMPs, contributing to cardiac remodeling via alteration in Ca^{2+} flux [129, 130]. As a defense mechanism, cells have evolved various enzymatic (i.e. SOD, catalase and GSHPx) and non-enzymatic antioxidants (i.e. GSH and antioxidant vitamins) that detoxify free radicals. When an abundance of ROS, from endogenous or exogenous influences, exceeds the capacity of the antioxidant defenses to detoxify them, oxidative stress results [114, 129]. However, despite these detrimental effects, it is essential to note that ROS generated under physiological conditions play important beneficial roles, including their acting as signaling molecules in a variety of cell signaling pathways, often as second messengers. Indeed, ROS act to modulate the activity of specific transcription factors including NF-κB and activator protein (AP)-1, and are also integral to defense mechanisms such as oxidative burst in phagocytosis, neutrophil function and shear stress-induced vasorelaxation [114, 129, 131].

Oxidative stress is involved in a variety of pathophysiological conditions, such as I-R injury, hypertrophy and heart failure, hypertension, catecholamine-induced cardiomyopathy,

diabetic cardiomyopathy and adriamycin-induced cardiomyopathy [111, 115, 131-133]. For example, in patients with dilated cardiomyopathy and congestive heart failure, plasma malondialdehyde (MDA) – a marker of oxidative stress – is increased, correlating with the severity of symptoms and inversely correlating with ejection fraction and exercise capacity [112, 128]. Similarly, an increase in oxidative stress and a deficit in antioxidant level have been reported in various animal studies related to cardiovascular complications [111, 114, 115, 132, 134].

Substantial evidence has implicated ROS signaling in cardiac hypertrophy. ROS stimulates myocardial growth and matrix remodeling in cardiac hypertrophy through a variety of signaling kinases and transcription factors such as tyrosine kinase, Src, guanosine triphosphate (GTP) binding proteins, Ras, protein kinase C (PKC) and ERK and jun n-terminal kinase (JNK) signaling [114, 130, 133, 135, 136], leading to altered signal transduction, calcium regulation and apoptosis [130, 132, 137, 138]. This modifies the collagen and overall matrix arrangement [139] resulting in further apoptosis, fibrosis or necrosis (referred to as ventricular remodeling), leading to cardiac dysfunction. ROS also play an important role in cardiac remodeling by angiotensin II and activation of AP-1 [140]. Moreover, it is increasingly recognized that ROS are both a contributor to and product of many cardiovascular disease processes, such that a positive feedback relationship can exist. It is for this reason that ROS level is generally observed to scale with the severity of disease [13].

2.2 – Inflammation

Like oxidative stress, inflammation represents an integral aspect of homeostatic regulation that can exert both beneficial effects and contribute to disease pathogenesis. Inflammation is a tightly regulated, complex tissue response to harmful stimuli that attempts to attenuate stressors and is also involved in the healing process [118, 119, 125, 141]. The inflammatory response is mediated by a variety of signaling molecules, including prostaglandins and C-reactive protein, soluble cluster of differentiation (CD)-40 ligand, adiponectin and inflammatory cytokines, such as tumour necrosis factor (TNF)-α. Many of these are increasingly used as biomarkers for the systemic inflammation associated with cardiac remodeling and heart failure [118, 141, 142]. Cytokine signaling is essential for the function of the innate and adaptive immune system, playing numerous roles in the host's inflammatory response including chemoattraction of neutrophils, monocytes and dendritic cells by chemokines, and triggering of fever [118, 119]. In short, inflammatory cytokines are ubiquitous, critical mediators of the protective inflammatory response to a wide variety of harmful or potentially harmful stimuli throughout the body. However, in pathophysiological conditions the dysregulation of various processes can lead to chronic inflammation, where the unchecked activity of inflammatory mediators and processes can be massively damaging [119, 141, 143].

Pro-inflammatory cytokines have been shown to contribute to cardiac dysfunction under various pathophysiological conditions associated with heart failure, including I-R injury, MI, atherosclerosis, hypertrophy, and acute viral myocarditis [116, 121, 122, 124, 125, 128, 144-149]. Inflammatory cytokines may modulate cardiovascular function by various mechanisms including altered adrenergic signaling, increases in NO or alteration of Ca^{2+} homeostasis and redox balance [134, 150, 151]. Several studies have shown that the inflammatory response resulting from MI

serves to further exacerbate myocardial injury, leading to deleterious remodeling of the heart and of the extracellular matrix. This can include MMP activation and excess collagen formation, increased apoptosis and hypertrophy, and, importantly, self-amplification of the inflammatory signal transduction pathways leading to depressed contractility [119, 143, 152-156].

2.3 – The 'vicious cycle' of redox-cytokine interaction

Besides its central role in challenging and aggravating myocardial injury directly, the oxidative stress pathway is demonstrably connected with key aspects of the inflammatory response. Recent findings suggest that oxidants promote inflammatory processes via the activation of downstream, redox-sensitive factors such as NF-κB, AP-1, and p38 MAPK, leading to the induction of various inflammatory cytokines [130, 137, 138, 143, 157-159]. In this way, oxidative stress contributes to the development of cardiovascular disease both directly and indirectly. Conversely, pro-inflammatory cytokines and chemokines have been implicated in the cascade of events leading to increased oxidative stress, which, in turn, contributes to disease progression via intensification of the inflammatory response [126, 134, 135, 151, 160]. Moreover, components of neurohormonal activation, such as catecholamines, angiotensin II, aldosterone, and endothelin (ET)-1 are potential contributors to the pro-inflammatory phenotype of heart failure and have been shown to enhance oxidative stress both directly and indirectly [127, 136, 161, 162].

Thus, current literature indicates that increased pro-inflammatory cytokine expression and ROS production are linked in a 'vicious', perpetuating cycle that may be of major significance in the development and progression of cardiovascular diseases leading to heart failure **(Figure 3)**. In the following sections, the pro-inflammatory cytokine, TNF-α, and the anti-inflammatory

cytokine, IL-10, are discussed in detail, as these mediators in particular have received the greatest attention in the literature with regard to their effects on oxidative stress.

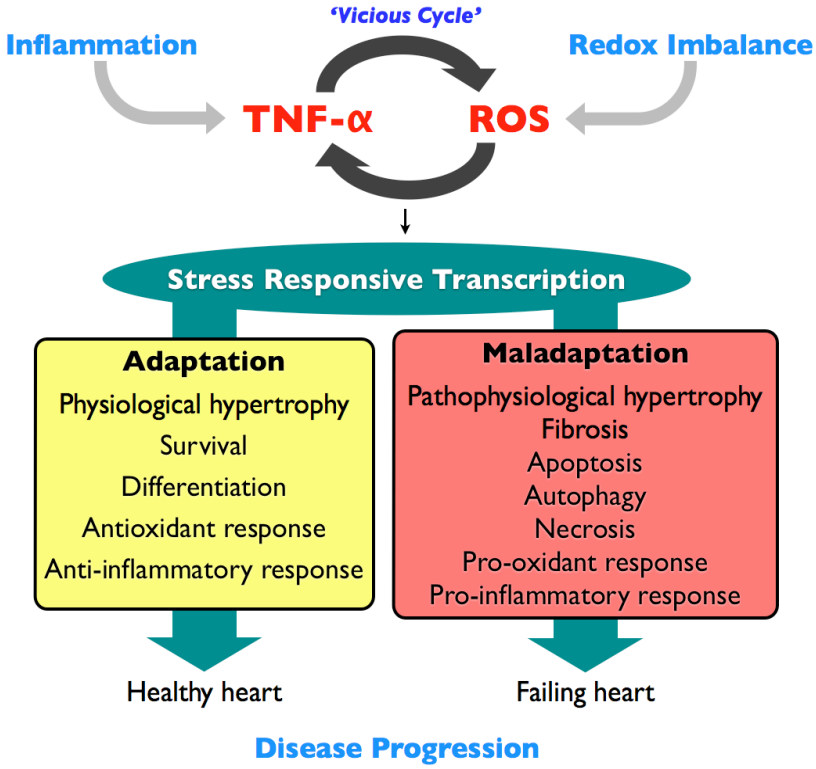

Figure 3. The 'vicious cycle' of inflammatory cytokine expression and oxidative stress and the stress response spectrum leading to heart failure. Linkages between oxidative stress and inflammatory cytokine/chemokine expression have been documented in the pathophysiology of heart failure. Both pathways exist in delicate balance between their respective pro-oxidant/pro-inflammatory and antioxidant/anti-inflammatory elements, and each has been shown to correlate with the severity of disease. These pathways also interact at a more global level, with TNF-α and other pro-inflammatory mediators contributing to the production of ROS, which in turn stimulates the expression of more such mediators, leading to a perpetuating, 'vicious cycle' of increasing systemic stress. Both pro-inflammatory cytokines such as TNF-α, and ROS activate transcription factors such as NF-κB and AP-1, which regulate the expression of a broad variety of stress response pathways. The extent of the response depends on the intensity and duration of the stimuli, and thus can range from acute to chronic in nature, producing phenotypic changes ranging from adaptive (hypertrophy, survival, differentiation, antioxidant response, anti-inflammatory response) to maladaptive (fibrosis, apoptosis, autophagy, necrosis, pro-oxidant response, pro-inflammatory response). This continuum is evident in the cardiac remodeling changes from normal to compensatory hypertrophy to heart failure. Importantly, inflammatory cytokines and ROS both stimulate the stress response and are regenerated by it, creating yet another common, positive feedback cycle that scales with disease progression [13].

2.4 – Tumour necrosis factor-α and oxidative stress

Although cross-promotion of inflammatory cytokines and oxidative stress may be manifested in many ways, the vast majority of research has specifically focused on the link

between oxidative stress and TNF-α. The most prominent of inflammatory cytokines**,** TNF-α, is expressed in numerous cell types, including macrophages, monocytes, T and B cells, mast cells, and cardiomyocytes. TNF-α exerts its effects via specific receptors (TNFR1 and TNFR2), and both the soluble and membrane bound TNF-α forms are capable of activating these receptors [163]. TNF-α acts as a master mediator of cytokine and other downstream pathways, and, consistent with its normal physiological role in the innate and adaptive immunity, it is a key factor in the host response to injury. However, this function can also be detrimental within a disease state such as that within the infarcted myocardium, where exaggerated TNF-α signaling via activation of other pro-inflammatory mediators such as IL-1β, IL-18 and the IL-6 family cytokines, can exacerbate an already highly stressed environment [117, 138, 141, 164].

Numerous studies have demonstrated a link between TNF-α and ROS. For example, increases in oxidative stress, cellular injury and apoptosis have been shown upon exposure to TNF-α [126, 135, 137]. TNF-α has been reported to contribute to progressive cardiac dysfunction in pacing-induced heart failure that is mediated by an increase in oxidative stress and apoptosis [165]. This is accompanied by impaired mitochondrial function, and *in vivo* TNF-α inhibition ameliorates cardiac mitochondrial dysfunction, oxidative stress and apoptosis in this model [165]. TNF-α enhanced the formation of NO, O_2^- and $ONOO^-$ via the stimulation of iNOS, xanthine oxidase and NADPH oxidase activities, contributing to depressed cardiac function in isolated perfused hearts of both rats and mice [150, 151, 166]. Increased formation of $ONOO^-$ by cytokines other than TNF-α, such as IL-1ß and IFN-γ, has also been reported in cardiomyocytes [167].

The potential for antioxidant-based regulation of TNF-α levels has been suggested in rodent models of MI and hypertension [152, 168]. In these studies, treatment with the GSH

precursor NAC was found to normalize serum and/or cardiac TNF-α, TNF-R1 and GSH levels, attenuate MMP expression and collagen deposition, and improve cardiac function. In other rodent studies of I-R injury, the antioxidant, probucol, was found to decrease levels of cardiac MDA and expression of IL-1β and IL-6, and to augment levels of GSHPx and catalase, contributing to cardioprotection and increased survival [169]. Importantly, therein was evidence of common TNF-α and redox-sensitive signaling pathway activation, as TNF-α was reported to activate multiple targets, including p38, JNK and ERK1/2 MAPK, as well as stimulating the phosphorylation of c-jun and activating transcription factor-2 [135, 153, 157, 164, 170, 171]. Activation of these factors can result in a diverse number of intracellular events including alterations in gene expression and apoptosis. Furthermore, both TNF-α and ROS can induce activation of transcription factors such as NF-κB and AP-1 [138]. In addition to TNF-α, there have only been a few studies on the interaction between chemokines and ROS. One such study demonstrated that MCP-1 enhanced the generation of O_2^- in monocytes from unstable angina patients and GSH modestly suppressed the production of IL-8 and MCP-1 in these cells, demonstrating an interaction between MCP-1 and ROS and its possible pathogenic role in plaque rupture [160]. Lipase activation and liberation of arachidonic acid and prostaglandins are also responsible for the production of ROS and oxidative stress by TNF-α [172].

2.5 – Interleukin-10 and oxidative stress

In contrast to TNF-α, IL-10 has been reported to have an antioxidant role, and has been shown to inhibit the formation of ROS as well as the production of NO [173, 174]. The initial suggestion that IL-10 might act as an antioxidant was reported by Bogdan et al. (1991), wherein recombinant IL-10 was found to suppress both TNF-α expression and ROS production in mouse

peritoneal macrophages [175]. Consistent with these findings, IL-10 was also suggested to modulate TNF-α mediated, oxidative stress-induced lung injury, a condition found to be augmented by the application of IL-10 antibody [176, 177]. In a murine renal model of I/R injury, IL-10 treatment reduced lipid peroxidation, while increasing the redox ratio and antioxidant enzyme activity [178]. IL-10 was previously shown to activate the ERK1/2 MAPK pathway, and evidence of p38 and ERK1/2 MAPK-mediated regulation of TNF-α/IL-10 interaction has recently been shown [135]. Here, it was also found that TNF-α significantly increased ROS levels and cardiomyocyte apoptosis, whereas these changes were prevented by the inclusion of IL-10 [135].

2.6 – Implications for interleukin-10/tumour necrosis factor-α ratio

Recently, it has been shown that in a rat model of MI, TNF-α exhibits a biphasic response, featuring decreased levels in the early stage and a return to higher levels during the chronic stages of heart failure. This response correlated with increased and decreased IL-10 levels in the early and chronic stages, respectively. Thus, increased IL-10:TNF-α in the early stages and decreased IL-10 to TNF-α ratio in the severe failure stages was observed [179]. A similar increase in TNF-α:IL-10 was also reported in patients with advanced congestive heart failure [180], and an imbalance between IL-10 and TNF-α has also been suggested to play a role in atherosclerotic lesions [181]. It has been further demonstrated that treatment with losartan, an angiotensin II type 1 receptor blocker, improved the ratio of membrane-bound as well as soluble fractions of IL-10:TNF-α protein in a rat model of MI [179]. Post-MI losartan-treated rats showed improved antioxidant status and hemodynamic function [111, 179]. Taken together, these studies strongly suggest that the two cytokines may interact in influencing oxidative stress and

that an appropriate balance between them may be of crucial importance for mitigating conditions leading to heart failure [179, 182, 183]. These findings are further supported by the recent finding of common regulatory mechanisms – p38 and ERK 1/2 MAP kinases as well as NF-κB pathway – that govern the interplay of TNF-α and IL-10 in regulating oxidative stress and cardiomyocyte apoptosis [135, 170]. Moreover, it has recently been reported that IL-10 prevents TNF-α-induced NF-κB activation and pro-apoptotic changes in cardiomyocytes by inhibiting IKK phosphorylation through the activation of ERK 1/2 MAPK [170].

3 – Hydrogen sulfide is a cardioprotective agent

Deficiencies resulting in reduced blood flow (and thus O_2 supply) or increased cardiac wall stress (and thus greater metabolic demand) produce conditions of low O_2, or hypoxia, in the heart. Indeed, tissue ischemia is the major pathophysiological end point of many disease processes, including cardiac arrest, shock, MI and stroke [184]. Cardiac ischemia results from progressive congestive cardiovascular processes of coronary artery disease, a leading cause of death in Canada (Heart and Stroke Foundation of Canada. The Growing Burden of Heart Disease and Stroke in Canada. Ottawa: Heart and Stroke Foundation of Canada, 2003). However, although an obligatory component, hypoxia represents only one aspect of the stress visited upon the ischemic heart. Indeed, while insufficient perfusion of cardiac tissues can be devastating, the return of blood, or reperfusion, is typically far more damaging [185]. These processes – collectively known as I-R injury – represent a major source of cardiac damage in many cardiovascular diseases, imparting massive oxidative stress to cardiovascular tissues by generating high levels of ROS by virtue of the profound influx of O_2 [185]. As described in Section 2 of this introduction, redox imbalance begets elaboration of inflammatory mediators,

which begets more ROS production, in a 'vicious cycle' of redox-cytokine interaction that underscores much of the pathophysiology of cardiovascular disease.

Two major strategies for abrogating the harmful effects of I-R are pre-conditioning and post-conditioning. While pre-conditioning has long been studied and is considered one of few powerful protective mechanisms against cardiac and cerebral ischemia, post-conditioning is a relatively novel concept [186]. Put simply, these are protective responses by the heart and other organs to sublethal levels of hypoxia or ischemia, resulting in the induction of adaptive physiological processes [185]. Both pre- and post-conditioning consist of one or more short ischemic/hypoxic events, differing mainly in their temporal application (i.e. either prior to or during I-R). Whereas pre-conditioning can render a naïve heart more resilient to subsequent I-R, post-conditioning can limit the reperfusion damage stemming from it [237].

Both pre- and post-conditioning elicit signaling cascades relating to reduced cell death and mitochondrial preservation, including those mediated by adenosine, eNOS and NO, the opening of K_{ATP} channels, and ERK1/2 and PI3K/Akt activation [187]. Importantly, H_2S's known biological activities include these targets/mediators, suggesting the potential for a role therein. However, given the contradictory evidence, the question remains open. Indeed, while endogenous H_2S has been implicated in ischemic [188, 189], metabolic inhibition [64] and endotoxin [51]-mediated pre-conditioning, other studies have demonstrated that inhibition of endogenous H_2S/CSE pathway did not affect ischemic pre-conditioning [51, 190]. In these studies, endogenous H_2S-mediated contributions to cardioprotection against I-R injury were associated with PKC and sarcolemmal K_{ATP} channel activation [51, 188] and provocation of NO release [64], but not protein kinase phosphorylation [190].

There is more coherence regarding the effect of sulfide pre-conditioning (typically via the H_2S donor, NaHS), which has been shown to produce effective cardioprotection from I-R injury in a manner similar to ischemic pre-conditioning [50, 189-194]. With the exception of one study [190], this cardioprotection was found to be mediated by PI3K/Akt and K_{ATP}/PKC/ERK1/2 pathway activation and NO, and was found to induce cyclooxygenase-2 expression/activation [50, 189, 191, 193, 194]. Furthermore, in a recent study of sulfide pre-treatment of I-R injury in small intestine, H_2S prevented leukocyte rolling and adhesion via an eNOS- and p38/MAPK-dependent mechanism [195].

Endogenous H_2S was also implicated in ischemic post-conditioning [196], and, as with sulfide pre-conditioning, sulfide post-conditioning was found to provide cardioprotective effects in a similar manner to ischemic post-conditioning [196, 197]. In these *ex vivo* rat models of I-R injury, H_2S given at the onset of reperfusion (sulfide post-conditioning) resulted in concentration-dependent decreases in infarct size and creatine kinase release. This effect was mediated by Akt, PKC and eNOS, and blockade of either CSE or K_{ATP} channels attenuated the observed cardioprotection [196, 197].

Although research on the processes underlying myocardial injury abounds, endogenous responses that protect cellular and subcellular functions – and especially mitochondria – during ischemic heart disease remain poorly understood and are not targeted in current therapeutic strategies [185]. As the centre of aerobic respiration, the mitochondrion is, ironically, both the primary source of [198] and the most susceptible cellular component affected by I-R-induced ROS [199, 200], as evidenced by the profound increase in oxidative stress [201] and decrease in aerobic energy production [202] upon reperfusion. Thus, as a key target of protective responses to hypoxia and the central integrator of O_2 and fuel substrate metabolism, as well as a central locus

for the regulation of cell death [85, 203, 204], the mitochondrion is the most important focal point of I-R injury. Recently, Elrod et al. (2007) noted that H_2S preserved cardiac function and limited infarct size via stabilization of mitochondrial structure and function. They found that H_2S dose-dependently reduced oxidative phosphorylation, preventing I-R damage subsequent to MI [85]. Moreover, in anesthetized and mechanically-ventilated mice, inhaled H_2S improved mitochondrial respiration by enhancing mitochondrial integrity, suggesting its potential benefit during therapeutic hypothermia [205].

While there is a certain level of ambiguity in terms of H_2S's role in pre- and post-conditioning, its role in the myocardium itself is clearer, despite only a few years of data. In multiple studies, cardiac injury was associated with reduced H_2S/CSE pathway expression [206-209]. Several studies have demonstrated that the cardioprotective effects of the garlic derivative, S-allylcysteine (SAC), are mediated by H_2S [192, 210]. Left ventricular CSE expression and plasma H_2S was increased by SAC administration in acute MI rats, and infarct size was increased upon inhibition of H_2S/CSE pathway [210]. In addition, the vasoactivity of garlic was similarly shown to operate via an H_2S-mediated mechanism [211]. In two mouse models of heart failure, exogenous H_2S preserved cardiac function and reduced fibrosis [206], as well as reducing cardiomyocyte apoptosis [212]. In another study, the H_2S-mediated reduction of cardiomyocyte apoptosis was shown to operate via induction of glycogen synthase kinase-3β and subsequent inhibition of mPTP opening [213]. As well, in two models of homocysteine-induced cardiac stress, exogenous H_2S attenuated endoplasmic reticulum stress and rescued cells [207], as well as improving redox balance [208]. Finally, in a model of adriamycin-induced cardiomyopathy – wherein endogenous H_2S/CSE pathway was compromised – exogenous H_2S markedly improved

cardiac function, ameliorated myocardial morphology, decreased thiobarbituric acid reactive substances and increased SOD and GSHPx [209].

Thus, having been shown to play key regulatory and/or protective roles in terms of preserving mitochondrial structure and function, regulating redox and inflammatory status, and modulating ion channels and metabolism, H_2S is certainly one answer to the question of a multi-faceted endogenous cellular regulator. Given the variability within the existing I-R models, such as the duration and extent of ischemic episodes and the method of heart failure induction, it will be important to carefully consider the timing of CSE inhibitor and H_2S donor application, as well as to monitor for temporal changes in channel activation and mRNA/protein expression alongside functional readouts. In particular, more attention should be paid to the redox and inflammatory aspects of H_2S's cardioprotection, since little, but promising data is currently available.

4 – Hydrogen sulfide in vascular pathophysiology

The roots of H_2S's arrival as an intriguing gasotransmitter lie in the discovery of its K_{ATP}-mediated vasorelaxant effect. Indeed, like its NO forebear, it is now recognized as both a critical regulator of vascular tone and an important factor in the pathophysiology of various vascular conditions, including hypertension, atherosclerosis and diabetes. Indeed, a now extensive literature supports the need for H_2S-based therapeutics to combat the modern scourge of vascular disease, and these hold great promise to someday rival the incredible impact that the discovery of NO has had over the past two decades.

In 2008, Yang et al. presented important new findings demonstrating that genetic deletion of the H_2S-synthesizing enzyme, CSE, markedly reduced serum and aorta and heart tissue H_2S levels [99]. Importantly, knockout mice displayed pronounced hypertension and decreased

endothelium-dependent vasorelaxation, providing direct, compelling evidence for H_2S's role as physiologic vasodilator and regulator of blood pressure. Furthermore, given that H_2S-deficiency (CSE knockout) caused elevated blood pressure and increased peripheral resistance to a similar extent as that observed in NO deficiency (eNOS knockout), the drastic impact of H_2S on regulation of the vasculature was realized, thereby opening novel avenues for treating hypertension [99]. This section summarizes much of the data that preceded these findings, and emphasizes the myriad effects that have already been attributed to H_2S in the pathophysiology of vascular diseases including HPH, atherosclerosis and diabetes.

Pulmonary hypertension is an important pathological process underlying many cardiovascular and pulmonary diseases that can ultimately lead to heart failure, but its mechanisms remain poorly understood [214]. Multiple groups have researched H_2S's role in pulmonary hypertension induced by hypoxia, high blood flow, and endotoxin, with the common pathological result of inducing pulmonary arterial remodeling and vasoconstriction. In rat models of HPH, significantly decreased CSE expression and activity in pulmonary artery and lung tissue of hypoxic animals was observed, indicating that endogenous H_2S was involved in the pathogenesis of the condition. By contrast, researchers noted that exogenous H_2S administration produced marked preservation/recovery of functional parameters, including decreased mean pulmonary arterial pressure [161, 215-224]. It was found that H_2S effected this protection through various mechanisms, including the abrogation of oxidative stress through decreased GSSG and enhanced T-AOC in lung tissues [161, 220], the inhibition of collagen types I and III expression, elastin, and TGF-β [217, 221], the inhibition of vascular SMC proliferation [217], the promotion of vascular SMC apoptosis via inhibition of Bcl-2 protein expression and activation of Fas and caspase-3 protein expression [215], and the upregulation of HO-1 expression and increased CO

production [219]. It was also found that a negative, but apparently essential, feedback relationship existed between H_2S and NO in HPH rats, such that inhibition of either CSE or NOS resulted in the worsening of the condition [222].

Other researchers used a rat model of LPS-induced pulmonary arterial hypertension, again implicating decreased H_2S/CSE in the impaired relaxation leading to maladaptive vessel constriction [218]. Similarly, in rat models of high blood flow-induced pulmonary hypertension, in which aorta-inferior vena cava-shunted animals developed pronounced pulmonary high blood flow and vascular structural remodeling, H_2S/CSE levels were also markedly downregulated [214, 225]. The NOS substrate, L-arginine, had previously been shown to attenuate pulmonary hypertension and vascular remodeling [220, 226], and was found to significantly upregulate H_2S/CSE pathway, indicating a H_2S-mediated mechanism of action [214]. Moreover, H_2S was subsequently shown to be protective against pulmonary hypertension and vascular remodeling, and correlated with suppressed NO/NOS and CO/HO-1 pathways, and proliferative cell nuclear antigen and ERK downregulation [227].

Taken together, these data indicate an important role for H_2S in hypoxic, high blood flow, and endotoxin-induced pulmonary hypertension and vascular remodeling, variously serving as an antioxidant, anti-proliferative and/or pro-apoptotic agent, as well as a potential regulator of NO and CO. The common finding of inter-regulation among the H_2S/CSE, NO/NOS and CO/HO-1 pathways in pulmonary hypertension is especially interesting given that all three gasotransmitters are potent vasodilators and modulate vascular remodeling. Indeed, the observed dysfunction of these pathways in the pathogenesis of this condition suggests the potential for intriguing combinational approaches to therapy.

Atherosclerosis is a complex, chronic pathological condition comprised of vascular inflammation, endothelial damage, SMC migration, foam cell accumulation and lipid and cholesterol deposition that begets plaque formation in blood vessels [228]. Evidence for an important role for H_2S in combating atherosclerosis includes the observations that it is anti-atherogenic [229-232], and inhibits SMC proliferation [93, 95] and homocysteine-induced vascular damage [88, 233]. Central to the present data is the question of basal H_2S level and or interaction of endogenous with exogenous H_2S, given that the latter had no effect in inducing apoptosis of SMCs without inhibition of the former [93, 95]. More recently, it was demonstrated that exogenous H_2S reduced atherosclerotic plaque while inhibition of endogenous H_2S exacerbated it, and that H_2S's anti-atherosclerotic effect was linked to reduced expression of the key inflammatory mediator, ICAM-1, in TNF-α-induced endothelial cells [228, 230]. Wang (2009) suggests that, because SMC-endothelial cell interaction is critical to atherosclerotic processes, the question of where H_2S is being produced and how its signaling affects these cells is an important one that, by virtue of recent advances in our understanding of CSE distribution in vascular tissues, may soon be answered [228].

Diabetes is properly understood to be included in the realm of cardiovascular disease, as its essential nature – compromised glucose metabolism – is so critically disruptive as to all but inevitably lead to cardiovascular complication. Perhaps unsurprisingly, given its clear roles in the pathophysiology of vascular disease and effects that centre on the mitochondrial regulation and metabolism, recent research has established the involvement of H_2S in processes underlying the pathogenesis of diabetes mellitus. Researchers observed abnormally high H_2S levels in the liver [29] and pancreas of diabetic rats [28, 29] and found H_2S to induce apoptosis therein via p38 MAPK-mediated ER stress [65]. H_2S levels were similarly decreased in another animal model of

diabetes, wherein vascular reactivity was also found to be impaired [234, 235]. Interestingly, as has been demonstrated in other models [93, 95], the effect of exogenous H_2S was most pronounced in the absence of endogenous H_2S [234], reinforcing the concept that basal H_2S level is essential in determining response. Consistent with these findings, recent evidence suggests that H_2S could be a novel insulin resistance regulator, as H_2S/CSE pathway was inversely correlated with glucose uptake in adipose tissue [236]. Interestingly, PI3K but not K_{ATP} channel pathway was involved in the observed inhibitory effect of H_2S on glucose uptake [236]. A significant recent publication suggests the generalizability of these concepts, as H_2S levels in both fasting blood of type 2 diabetes patients and diabetic rats was significantly lower than healthy controls [237]. Moreover, exogenous H_2S was found to prevent secretion of the inflammatory mediators, IL-8 and MCP-1, in high-glucose-treated human monocytes, suggesting that low H_2S levels may contribute to the vascular inflammation present in diabetes [237]. Further research is required, particularly in terms of the signaling mechanisms involved, but these data collectively indicate yet another cardiovascular disease wherein H_2S is a critical player.

5 – Is hydrogen sulfide a novel oxygen sensor?

H_2S has been implicated in the adaptive physiological responses to I-R injury as a powerful endogenous cardioprotectant, and has critical roles in maintaining vascular homeostasis, as evidenced by its role in vascular pathophysiology. In particular, the essential contribution H_2S/CSE pathway to ischemic pre- and post-conditioning and the parallel effects of sulfide conditioning strongly suggest that H_2S has a hypoxia-responsive capacity in the cardiovascular system. Importantly, new evidence has led to the arrival of the novel concept of H_2S metabolism as an O_2-sensing mechanism, further bolstering the concept of H_2S as a central cardiovascular

regulatory molecule. A discussion of this newly discovered role for H_2S begins with a brief description of our current knowledge of the mammalian O_2-sensing landscape.

Molecular O_2 serves as the primary electron acceptor in myriad cellular organic and inorganic reactions. It is especially important in mitochondria, where approximately 95 % of cellular O_2 is consumed by the ATP-generating process of oxidative phosphorylation [16, 238]. Mammalian cardiovascular systems have evolved to optimize O_2 and substrate delivery to all cells of the body, achieving exacting yet flexible maintenance of homeostasis [239]. A literal powerhouse, the mature heart is one of the most metabolically active organs of the body, and a mitochondria-rich environment of extreme O_2 handling. As such, it is especially vulnerable to conditions of low O_2, or hypoxia, which is a central component of pathologies including cerebral and myocardial ischemia, chronic heart and lung diseases, inflammation and cancer [238, 240, 241]. Hypoxia can result from both external and internal influences, including unbalanced ventilation/perfusion, over-paced metabolic demands, injury and immune responses [36]. It can also be highly localized – such as in a discrete cardiac injury from an occluded vessel; or can occur globally – such as in the system-wide impact of atherosclerosis. Thus, given its biological primacy and pathophysiological roles, it is unsurprising that tissue O_2 homeostasis is tightly regulated by numerous, highly conserved cellular and systemic processes, some of which remain incompletely understood or even unknown [238, 241].

The presence of a phyletically ancient gene-based system to defend against changes in O_2 availability in multicellular eukaryotic aerobic organisms underscores the evolutionary priority of regulating O_2 levels. Indeed, orthologues of the primary biochemical machinery for gene-based defenses against O_2 deprivation – the hypoxia-inducible factor (HIF) transcription factors and their associated regulators – has been identified throughout the animal kingdom, suggesting that

HIF-inducible transcription first evolved as a cell-autonomous system to promote cell survival through induction of stress-response genes and altered cellular metabolism [239]. In mammals, HIFs are likewise recognized as critical mediators of the response to hypoxia, activating over 200 genes encoding proteins that regulate cellular metabolism, proliferation, survival, motility and basement membrane integrity, as well as angiogenesis and hematopoiesis [185, 238, 242].

In mammals, hypoxia results in both acute and chronic adaptations. Rapid and reversible effects on cell signaling, contractility, ion flux and redox state balance energy supply and demand in the face of reduced capacity for oxidative metabolism. Whereas HIFs enable long-term cellular survival and vascular remodeling, a variety of HIF-independent pathways promote ATP conservation by limiting energy-consuming processes such as cell division, ribosome biogenesis, mRNA translation and ion channel activity. Indeed, it has been found that a rapid inhibition of protein synthesis, affected by means of a HIF-independent mechanism, is essential for cell survival during conditions of extreme hypoxia [16, 238].

Timely, appropriate responses to altered O_2 tension depend on O_2-sensing mechanisms at the 'global' (organism, organ) and 'local' (tissue, cell) levels, which monitor for very subtle changes in O_2 levels and signal various protective responses. Indeed, several non-cell-specific O_2-sensing strategies have since been suggested. For instance, by virtue of its central role as integrator of O_2 and fuel substrates, the mitochondrial electron transport chain may serve as O_2-sensing apparatus. During hypoxia, cytochrome c oxidase activity is suppressed due to substrate shortage, leading to electron leakage and the subsequent generation of ROS. These can then directly interact with effector proteins such as potassium channels and/or transcriptional factors such as HIF-1 [16]. As well, multiple O_2-sensitive K^+ channels have been identified that become inactivated upon hypoxia. These act opposite the voltage-gated, L-type calcium channels that,

when activated, serve to increase intracellular Ca^{2+}. These K^+ channels have since been found in virtually all O_2-sensing tissues, including chemoreceptive carotid and neuroepithelial bodies [243], adrenal chromaffin cells [244] and pulmonary artery [245]. Moreover, hypoxic vasodilation has been proposed to result from hypoxia-responsive activation of K_{ATP} channels, providing further support for a central role for K^+ channels in cardiovascular hypoxia. However, although these and other factors may be implicated in the response to low O_2, they generally appear to be downstream of some other factor that couples hypoxia to the response itself [36].

From among the plethora of these and other proposed candidates, the gasotransmitters NO, CO and H_2S have recently emerged as strong contenders. Such a role for NO and CO has previously been suggested due, in part, to the body's use of small amounts of O_2 as a substrate for their generation via NOS and HO, respectively. Put simply, this theory holds that as local O_2 concentrations decrease, so too does the activity of these reactionary pathways [16]. However plausible, these potential O_2-sensing mechanisms remain largely unexplored.

H_2S's inherent redox activities may alone have justified the exploration of this molecule as a putative O_2-sensor, but recent findings offer other, more specific lines of evidence for an important role for H_2S in O_2-sensing in vascular smooth muscle. Upon initially noticing that the *ex vivo* vascular responses to hypoxia and H_2S were similar, Olson et al. (2006) examined this potential relationship more closely and found that alteration of cellular H_2S level served to modulate the vascular hypoxic response in animal models [246]. This led him to formulate a model for O_2-sensing through the O_2-dependent regulation of H_2S metabolism. Here, H_2S produced in the cytoplasm is constitutively oxidized in the mitochondria so long as O_2 is readily available, and thus the cellular concentration of H_2S should depend on the balance between H_2S production and Po_2. As such, intracellular H_2S levels would remain comparatively low under

normoxic conditions, and increase proportionately to the severity of hypoxia. Indeed, in *ex vivo* lamprey, hagfish, rat and bovine vasotension models, hypoxia and H_2S produced identical contraction, relaxation or multiphasic tonal responses [36, 46, 247]. However, the observed effects were noted only at very low Po_2, and the type of tonal change, be it contraction or relaxation, was H_2S concentration-dependent. Olson (2008) suggests that this dynamic feature of H_2S may help to regulate pulmonary vasotension according to Po_2, which is an important cardiorespiratory mechanism [36]. Recently, Olson et al. (2008) strengthened the argument that H_2S metabolism might act as a general O_2-sensor upon finding that, as in vascular tissues, H_2S induced characteristic hypoxia responses in trout and zebrafish chemoreceptive tissues [248].

Olson (2008) cites other corroborating, as well as confounding, evidence for O_2-sensing through the O_2-dependent regulation of H_2S metabolism [36]. The evolutionary feasibility of a significant O_2:H_2S coupling is suggested by the molecules' ancient relationships, including the constitutive production of H_2S and its mitochondrial oxidation by O_2 in the ancestors of all eukaryotic cells [36, 249, 250]. As well, the H_2S-synthesizing enzymes CSE and CBS have been shown to be Po_2 and redox-sensitive [251-253], which Olson (2008) suggests may either "directly contribute to intracellular H_2S concentration or may provide a long-term mechanism to bias the rate of constitutive H_2S production" [36]. Indeed, the recent finding that myocardial H_2S production was inversely correlated with Po_2 [254] suggests an autocrine or paracrine mechanism for the involvement of H_2S/CSE in ischemic pre- [188, 189] and post-conditioning [196]. Moreover, this agrees with the recent evidence against appreciable circulating H_2S levels [254]. Finally, as previously discussed, H_2S is an established, ubiquitous signaling molecule with many known cytoprotective and modulatory roles in numerous systems, and certainly could, therefore, elicit physiological responses to hypoxia in a primary or secondary manner, such as via

stimulation of HIF-1-mediated signaling [255]. Taken together, these data strongly indicate that H_2S metabolism may represent a reliable O_2-sensing mechanism in many cells. If true, this further underscores the physiological primacy of H_2S, especially in the highly O_2-sensitive cardiovascular and neuronal systems, where H_2S's importance is already appreciated.

Objectives

Project I - Hypoxic stress in CSE-deficient smooth muscle cells

- **Brief Rationale**
  - Hypoxic stress is a ubiquitous feature of cardiovascular disease [13]. Recent findings suggest that H_2S may act as a novel O_2 sensor [256], and can regulate HIF-1 [255].
  - Vasculoprotective roles for H_2S have been well established in hypoxia-related vascular diseases including hypertension [99] and atherosclerosis [228].
  - Mice genetically deficient in CSE expression exhibited increased blood pressure, decreased H_2S level and compromised vasorelaxation [99]. Using SMCs isolated from this model, we have recently shown that over-proliferation of CSE-deficient SMCs contributes to the observed hypertension [94].

- **Hypotheses**
  - Given H_2S's roles in oxidative stress and inflammation in the vasculature, **CSE-deficient SMCs will exhibit increased mitochondrial activity, and unbalanced redox and inflammatory status versus their wild-type counterparts**.
  - Given that deficient H_2S/CSE pathway is implicated in the pathogenesis of hypertension and atherosclerosis, **CSE-deficient SMCs will have a compromised hypoxia stress responsive capacity than their wild-type counterparts.**

- **Objectives**
    - Measure and compare primary CSE-WT and -KO vascular SMCs under hypoxic stress in terms of:
        - cell viability (via mitochondrial dehydrogenase activity and apoptosis),
        - redox status (via superoxide dismutase activity and intracellular ROS),
        - mitochondrial activity (via mitochondrial membrane potential),
        - inflammatory status (via inflammatory cytokine expression),
        - stress-responsive transcription (via HIF-1α expression).

Project II - CSE in simulated hypoxia and hypoxia-reoxygenation cardiac stress

- **Brief Rationale**
    - H_2S is a potent cardioprotectant, and has been shown to regulate redox balance and abrogate damage by preserving mitochondrial integrity [85]. Recent findings suggest that H_2S has important relationships with O_2 homeostasis [256] and HIF-1 [255].
    - The hypoxia mimetic cobalt chloride ($CoCl_2$) induces so-called 'simulated hypoxia' by activating HIF-1 under normoxic conditions, thereby inducing hypoxia stress responsive transcription. It has previously been shown to induce ROS elaboration and mitochondrial impairment in HL-1 cardiomyocytes [257].
    - I-R injury is a major complicating feature of clinical disease entities including myocardial infarction and stroke [13]. Reperfusion imparts massive oxidative stress to cardiovascular tissues by generating high levels of ROS via the profound influx of O_2 [185].

- **Hypotheses**
    - Given the relevance of H_2S's cardioprotective effects and proposed HIF-1 interaction to the mechanisms of action in $CoCl_2$-induced 'simulated hypoxia', **$CoCl_2$ treatment of HL-1 cardiomyocytes is likely to engender H_2S/CSE pathway involvement; inhibition of which should be detrimental to cell survival under $CoCl_2$.**
    - Given H_2S's cardioprotective role against I-R injury *in vivo*, **hypoxia-reoxygenation (H-R) stress is likely to engender H_2S/CSE pathway**

involvement in HL-1 cardiomyocytes; inhibition of which should be detrimental to cell survival under H-R stress.

- **Objectives**
  - Establish HL-1 cardiomyocyte models of simulated hypoxic stress and H-R stress (via measurement of mitochondrial dehydrogenase activity and intracellular ROS levels).
  - Measure stress-induced CSE protein expression (via immunoblotting).
  - Inhibit CSE and measure viability (via mitochondrial dehydrogenase activity) to assess the contribution of H_2S/CSE pathway to hypoxic cell survival.

Methods

1.1 – CSE-deficient mouse model

The CSE-deficient mouse model was developed by Dr. Rui Wang's research group at the University of Saskatchewan. Readers are directed to their 2008 paper in the journal, *Science*, for a complete description of the methodology used in its generation [99].

1.2 – CSE-deficient vascular smooth muscle cells

Single SMCs from mesenteric artery of CSE WT and CSE KO mice were isolated and identified as described previously [258]. These SMCs were cultured in Dulbecco's modified Eagle's medium containing 10 % supplemented foetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. The SMC identity was confirmed by staining with SMC-specific a-actin monoclonal antibody (Sigma, Oakville, ON, Canada), and the cells had the usual growth characteristics and at confluence exhibited the typical 'hill-and-valley' pattern [258]. Cell counts and viabilities were assessed via automated Trypan Blue Exclusion assay using a Vi-Cell XR Cell Viability Analyzer (Beckman Coulter, Mississauga, ON, Canada; see below). Doubling time analysis was completed as per convention; generally, cells were maintained at a passage density of 1:3 to 1:6 depending on the intended usage. All experiments were completed when the cells reached 70-80 % confluence with matched (i.e. same passage number for both WT and KO) cultures between passage 6 and 12.

1.3 – Murine cardiomyocyte cell line

The murine atrial cardiomyocyte (HL-1) cell line, obtained from the laboratory of Dr. William C. Claycomb, was cultured in gelatin/fibronectin (0.02 %) (Sigma)-coated Costar 0.2

μM vent cap cell culture flasks (Corning, Corning, NY, USA) in a specially formulated growth media (Claycomb Media, JRH Biosciences, Lenexa, KS, USA) supplemented with 10 % fetal bovine serum (FBS) (JRH Biosciences), L-glutamine (2 mM) (Invitrogen, Carlsbad, CA, USA), norepinephrine (100 μM) (Sigma), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen, Burlington, ON, Canada). All cardiomyocytes were cultured at 37 °C, 5 % CO_2 and used for experimentation upon reaching approximately 95 % confluence. Cell counts and viabilities were assessed using a Vi-Cell XR Cell Viability Analyzer (Beckman Coulter; see below). Doubling time analysis was completed as per convention, and cultures were maintained according to this information in concert with the available instructions from Dr. Claycomb's laboratory. HL-1 cells are derived from the murine atrial cardiomyocyte primary culture, AT-1. In contrast to AT-1 cells, HL-1 cells can be serially passaged, yet retain many characteristics of mature cardiomyocytes, although they are less differentiated, with less organized myofibrillar structure [259, 260]. Indeed, in the present studies, spontaneous contractions of the cells were neither induced nor observed. All experiments were completed with cultures between passage 51 and 70.

1.4 – Hypoxia chamber

A humidified hypoxia glove box chamber with automatic controller (Coy, Grass Lake, MI, USA) was employed for the hypoxia phases of all treatment regimes, and set to 37 °C, 5 % CO_2 and 1 % O_2. A separate, interior, humidified tissue culture chamber was covered with aluminum foil to prevent photosensitive reactions with the cells and media. All interior surfaces were routinely sterilized via ethanol. Cell cultures were moved gently but rapidly to and from a second tissue culture incubator (~20 °C) via purgeable airlock during O_2 treatment regimes. O_2

levels did not fluctuate by more than 1 % O_2 for more than 30 seconds during addition or removal of cell cultures from the chamber.

1.5 – Cobalt chloride preparation

$CoCl_2$ (Sigma) was dissolved in ddH_2O to produce 10 mM stock solutions that were stored at -20 °C in the absence of light. Stock solutions were used within 1 month and not refrozen. Sterile aliquots were added to serum-free culture media to form specific working treatment concentrations ranging from 50-500 μM.

1.6 – D,L-propargylglycine preparation

D,L-propargylglycine (PPG or PAG, Sigma) was dissolved in ddH_2O to produce various stock solutions immediately prior to experiments. Sterile aliquots were added to serum-free culture media to form specific working treatment concentrations ranging from 100 μM to 10 mM.

1.7 – Trypan Blue Exclusion assay

The diazo dye, Trypan Blue ((3Z,3'Z)-3,3'-[(3,3'-dimethylbiphenyl-4,4'-diyl)di(1Z)hydrazin-2-yl-1-ylidene]bis(5-amino-4-oxo-3,4-dihydronaphthalene-2,7-disulfonic acid), is a vital stain used to discern living from dead cells or tissues. It is cell-impermeable, penetrating only damaged, non-viable cells with compromised membrane integrity. Viable cell counts were performed via automated Trypan Blue Exclusion assay using a Vi-Cell XR Cell Viability Analyzer (Beckman Coulter), wherein 0.5 mL of diluted, trypsinized cell suspensions were loaded, mixed with Trypan Blue reagent, and live/dead cell counts assessed via 50 individual sub-samples measured using the Vi-Cell XR Cell Viability Analyzer software. The

total viable cell count was used when passaging or standardizing cell samples for experimentation. Individual cell profiles for SMCs and HL-1 cardiomyocytes were created using the Vi-Cell XR Cell Viability Analyzer software to facilitate optimal cell counting accuracy.

1.8 – MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is used as a quantitative index of activity of mitochondrial and cytosolic dehydrogenases, which, in living cells, reduce the yellow tetrazolium salt to produce a purple formazan dye that can be measured spectrophotometrically [261]. While typically described as measuring cell viability – particularly in toxicology and pharmacology studies – this assay is, by virtue of its test mechanism, more accurately described as a cell metabolism assay. Indeed, in the present studies the MTT assay was used to measure both cell viability and cell metabolism. Cells were seeded onto sterile flat-bottom 96-well plates (Corning) and incubated overnight to achieve the desired confluence. Plated cells were subjected to chemical, hypoxic or normoxic pre- or co-treatment (various chemicals and conditions including $CoCl_2$, PPG, 1 % O_2, ~20 % O_2, etc. at various concentrations in serum- and antibiotic-free media). With t = 4 hours of treatment time remaining, a suitable volume of MTT reagent (Thiazolyl Blue Tetrazolium Bromide; Sigma, St. Louis, MO, USA) was added to wells to achieve a final concentration of 10 % (v/v), and cells were incubated at 37 °C for an additional 4 hours, during which time the MTT reagent was converted to purple formazan crystals in living cells according to their metabolic activity. Following this, the incubation media was aspirated and 50 μL of dimethylsulfoxide per well was added to solubilise the formazan crystals. Following 10 minutes of agitation on a Belly Dancer shaker (Stovall, Greensboro, NC, USA) at its highest setting, absorbance was measured spectrophotometrically at a wavelength of

490 nm (650 nm correction wavelength) using a PowerWave XS Microplate Spectrophotometer (BioTek, Winooski, VT, USA). Viability of treated wells was assessed relative to control wells, which were considered to represent 100 % viability. Note that in early HL-1 cell experiments, samples were cultured in all columns and rows of the 96-well plates. However, upon observing a distinct difference in absorbance in the wells around the perimeter of the plate (i.e. rows 1 and 8, columns 1 and 12) – the so-called 'edge effects' – this practice was stopped, and existing data was revisited to correct the operator error.

1.9 – Reactive oxygen species indicator assay

CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) is used as a cell-permeable indicator of ROS. This molecule remains non-fluorescent until the acetate groups are removed by intracellular esterases as oxidation occurs within the cell. In addition, esterase cleavage of the lipophilic blocking groups yields a charged form of the dye that is much better retained by cells than the parent compound. Moreover, the chloromethyl derivative of H_2DCFDA used here allows for covalent binding to intracellular components, permitting even longer retention within the cell. Cells were seeded onto sterile flat-bottom 6-well plates (Corning) and grown overnight to achieve the desired confluence. Plated cells were subjected to various time periods of chemical, hypoxic or normoxic pre- or co-treatment (various chemicals and conditions including $CoCl_2$, PPG, 1 % O_2, ~20 % O_2, etc. at various concentrations in serum- and antibiotic-free media). The previously optimized, so-called 'snapshot' method of CM-H_2DCFDA loading was employed, facilitating better insight into the present redox status, as opposed to the more cumulative measure afforded by pre-loading of cells with label. Following challenge, cells were washed with PBS and stained for 30 minutes with CM-H_2DCFDA

(Molecular Probes, Eugene, OR, USA) under standard incubation conditions (even for hypoxia-only treatment samples). Stained cells were washed with PBS and either detached from the plate surface using either trypsin or disposable sterile cell scrapers (Fisher Scientific) and suspended in PBS for flow cytometric analysis using the FL1-H channel of a BD FACSCalibur Flow Cytometer (BD Biosciences) supported by BD CellQuest Pro Software. A minimum of 1 x 10^4 gated events were acquired per trial. Mean fluorescence (specifically: geometric mean fluorescence) was understood to be directly proportional to levels of intracellular ROS. In early experiments, a H_2O_2 control was also analyzed to aid in calibrating the flow cytometer for this assay.

1.10 – Phosphatidylserine residue-based apoptosis detection assay

In normal, viable cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane, but becomes translocated from the inner to the outer leaflet in during apoptosis [262]. The human vascular anticoagulant, annexin V, is a 35-36 kD Ca^{2+}-dependent phospholipid-binding protein that possesses a high affinity for PS. Thus, fluorophore-labeled annexin V can be used to identify apoptotic cells by virtue of its binding to PS on the outer leaflet. However, because annexin V conjugates are able to pass through the compromised membranes of necrotic cells and bind to PS in the interior of the cell, propidium iodide (PI), a live and early apoptotic cell-impermeable (necrotic cell) stain that binds to DNA, is used in combination with annexin V staining to distinguish necrotic from apoptotic cells.

PS translocation was assessed via flow cytometric analysis of control and treated cells that were dually stained with annexin V and PI using the Annexin V-FITC Apoptosis Detection kit (Sigma). Cells were seeded onto sterile flat-bottom 25 cm^2 culture flasks (Corning) and grown

overnight to achieve the desired confluence. Plated cells were subjected to various time periods of chemical, hypoxic or normoxic pre- or co-treatment (various chemicals and conditions including $CoCl_2$, PPG, 1 % O_2, ~20 % O_2, etc. at various concentrations in serum- and antibiotic-free media). Following challenge, cells were washed with PBS and suspended via trypsinization in 100 µL annexin-binding buffer at 1 x 10^6 cells / mL. Cells were then incubated at room temperature with annexin V-FITC and/or PI stain in the absence of light. Following 15 minute incubation, single- and dual-stained samples were diluted with 400 µL annexin-binding buffer and immediately analyzed via flow cytometry with a BD FACSCalibur Flow Cytometer (BD Biosciences) supported by BD CellQuest Pro Software on the FL1-H (ANX) and FL2-H (PI) channels, acquiring a minimum of 1 x 10^4 gated events per trial. ANX-/ PI- cells were considered be to living, ANX+/ PI- cells apoptotic, and ANX+/-/ PI+ cells necrotic.

1.11 – Active caspases 3/7-based apoptosis detection assay

The central component of the apoptotic process is a cascade of proteolytic enzymes called caspases, which participate in a series of reactions triggered in response to pro-apoptotic signals that result in the cleavage of protein substrates, causing the disassembly of the cell [263]. A cell-permeable, carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor, fluorochrome inhibitors of caspases (FLICA), covalently binds to a reactive cysteine residue that resides on the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity. The bound labeled reagent is retained within the cell, while any unbound reagent will diffuse out of the cell and is washed away. The green fluorescent signal is a direct measure of the amount of active caspase-3 or caspase-7 present in the cell at the time the reagent was added. Active caspase-3/7 activity was assessed via flow cytometric analysis of control and treated cells that

were dually stained with FLICA and PI using the CaspaTag Caspase-3/7 In Situ Assay kit (Chemicon International, Temecula, CA, USA). Cells were seeded onto sterile flat-bottom 25 cm^2 culture flasks (Corning) and grown overnight to achieve the desired confluence. Plated cells were subjected to various time periods of chemical, hypoxic or normoxic pre- or co-treatment (various chemicals and conditions including $CoCl_2$, PPG, 1 % O_2, ~20 % O_2, etc. at various concentrations in serum- and antibiotic-free media). Following challenge, cells were washed with wash buffer and suspended via trypsinization in PBS to achieve 1 x 10^7 cells / mL. 10 µL of freshly prepared FLICA reagent was added to 290 µL of cell suspension, mixed gently and incubated at 37 $^{\circ}C$ for 1 hour in the absence of light (mixing gently twice during this incubation). Following several wash and count steps, samples were immediately analyzed via flow cytometry with a BD FACSCalibur Flow Cytometer (BD Biosciences) supported by BD CellQuest Pro Software on the FL1-H (FLICA) and FL2-H (PI) channels, acquiring a minimum of 1 x 10^4 gated events per trial. FLICA-/PI- cells were considered be to living, FLICA+/ PI- cells apoptotic, and FLICA+/PI+ cells necrotic.

1.12 – Mitochondrial membrane potential assay

The heart is dependent upon adequate O_2 delivery for normal function, as it derives most of its metabolic energy from oxidative phosphorylation. The mitochondrial proton gradient, of which mitochondrial membrane potential (Ψ_m) forms by far the largest part, is the most direct measure of the state of energization of the mitochondria [264]. Indeed, although often approximated via measurement of adenine phosphorylation potential (i.e. adenosine triphosphate ATP), creatinine, or inorganic phosphate), this can be a poor surrogate given that ATP content and mitochondrial respiration have been shown to be clearly out of step during ischemia-

reperfusion [265]. The carbocyanine derivative, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanineiodide), is a cationic dye that exhibits potential-dependent accumulation in mitochondria and a corresponding conversion from monomer to J-aggregate evidenced by a shift in emitted light upon excitation at 490 nm [266], thereby facilitating measurement of Ψ_m.

Ψ_m was assessed using the MitoProbe JC-1 Assay Kit for Flow Cytometry (Molecular Probes). Cells were seeded onto sterile flat-bottom 6-well plates (Corning) and grown overnight to achieve the desired confluence. Plated cells were subjected to various time periods of chemical, hypoxic or normoxic pre- or co-treatment (various chemicals and conditions including $CoCl_2$, PPG, 1 % O_2, ~20 % O_2, etc. at various concentrations in serum- and antibiotic-free media). Following challenge, cells were washed with PBS and stained for 30 minutes with JC-1. Stained cells were then detached from the plate surface via trypsinization and suspended in PBS for flow cytometric analysis using the FL1-H (monomer) and FL2-H (aggregate) channels of a BD FACSCalibur Flow Cytometer (BD Biosciences) supported by BD CellQuest Pro Software. A minimum of 1 x 10^4 gated events were acquired per trial. The ratio of red fluorescence (potential-dependent generation of J-aggregates) to green fluorescence was used, as per convention, to describe the relative status of mitochondrial electric potential, wherein high ratio indicated high potential and, thus, mitochondrial activity, and low ratio indicated low potential and, thus, compromised and/or low mitochondrial activity.

1.13 – Superoxide dismutase activity assay

The production of superoxide radicals is a substantial contributor, if not the primary cause, of ischemia reperfusion injury [267]. Superoxide dismutases (SODs) catalyze the

dismutation of the superoxide radical (O_2^-) into H_2O_2 and elemental oxygen (O_2) which diffuses into the intermembrane space or mitochondrial matrix, providing an important defense against the toxicity of superoxide radicals [268]. Indeed, given its major contribution to total antioxidant capacity, measurement of SOD provides excellent general insight into cellular redox status. In the Superoxide Dismutase Assay (Trevigen, Helgerman Ct. Gaithersburg, MD, USA), ions generated from the conversion of xanthine to uric acid and H_2O_2 by xanthine oxidase, converts nitro-blue tetrazolium chloride (NBT) to NBT-diformazan, which absorbs light at 550 nm. SOD reduces superoxide ion concentrations, thereby lowering the rate of NBT-diformazan formation, which can thus be used to measure the SOD activity present in an experimental sample [269].

Cells were seeded onto sterile 150 cm^2 culture flasks (Corning) and grown overnight to achieve the desired confluence. Plated cells were subjected to various time periods of chemical, hypoxic or normoxic pre- or co-treatment (various chemicals and conditions including $CoCl_2$, PPG, 1 % O_2, ~20 % O_2, etc. at various concentrations in serum- and antibiotic-free media). Immediately following challenge, pelleted cell samples were lysed and their total protein quantitated via DC Protein Assay (Bio-Rad), being careful to keep protein samples cold to prevent degradation. Samples were assayed as per the kit's instructions and absorbance read at 550 nm using a PharmaSpec UV-1700 Visible Spectrophotometer (Shimadzu, Columbia, MD, USA). The extent of SOD activity was gleaned from calculating absorbance at 330 seconds subtracted by absorbance at 30 seconds, where greater SOD activity would be evidenced by less change in absorbance over time. These values were converted to units SOD per volume by reference to an SOD inhibition curve, generated as per the kit's instructions.

1.14 – Radioimmunoprecipitation assay

Cell samples for use in immunoblotting were lysed via modified radioimmunoprecipitation assay (RIPA), a strongly denaturing lysis buffer due to its ionic detergents, sodium dodecyl sulfate (SDS) and sodium deoxycholate. This lysis buffer and protocol was based on that previously used for HL-1 cardiomyocytes [270], but included also the protease inhibitors, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, pepstatin, and phosphatase inhibitors, sodium fluoride and activated sodium orthovanadate. These inhibitors were prepared in ddH_2O or ethanol and stored at -20 $^{\circ}$C. Inhibitors were added immediately prior to RIPA buffer application, and aliquots were not refrozen to ensure their activity. Upon addition of ice-cold lysis buffer to pelleted cell samples, the crude lysate was pipetted up and down repeatedly using a chilled pipette tip, then passed through a chilled syringe to further degrade the cellular structures. Lysates were vortexed, incubated on ice for 10 minutes, vortexed again and incubated on ice for a further 10 minutes. Lysates were then centrifuged for 12 minutes at 1 x 10^4 rpm to remove cellular debris. Supernatant was collected and stored at - 80 °C and/or kept on ice for immediate quantitation. All protein lysis and sample handling was completed at a cold-room bench at 4 °C, and all tubes and equipment were pre-cooled.

1.15 – Total protein assay

Protein lysates were quantitated via a detergent-compatible, colourimetric, Lowry-based protein assay. Using the DC Protein Assay (Bio-Rad)'s 'microplate' method, which requires only small (5 µL) quantities of protein lysate, full-strength and 1/10-strength samples were measured alongside a range of bovine serum albumin (BSA; Fisher) standard concentrations from 0.2-1.5 mg / mL. Following a 15-minute colour-development period, standard and sample absorbance

was measured spectrophotometrically at a wavelength of 750 nm using a PowerWave XS Microplate Spectrophotometer (BioTek). Microsoft Excel for Macintosh was used to create a standard curve from the BSA standards, and the equation of the line of best fit (minimum accepted $R^2 = 0.99$) was used to calculate the sample protein concentrations. Stock sample lysates were kept on ice to minimize degradation during the course of the assay.

1.16 – Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Immediately following quantitation, 6x Laemmli sample loading buffer containing ß-mercaptoethanol (Sigma-Aldrich) or dithiothreitol (Sigma) was added to the sample lysates, which were then placed in a 100 °C water bath for 5 minutes (with punctured tube lids to alleviate pressure). Boiled lysates were quick-spun and either stored at -80 °C for future use or loaded into 12 % sodium dodecyl sulfate-polyacrylamide gels at ~10-50 µg per well (Bio-Rad Mini PROTEAN 3 Cell System apparatus was used). 10-15 µL (equivalent to 10-15 µg) of HiMark™ Pre-stained protein standard (Invitrogen) or Precision Plus Kaleidoscope (Bio-Rad) protein standard was loaded alongside the sample wells. Empty wells were partially filled with 1x Laemmli sample loading buffer to ensure even migration across the gel (first and last columns left empty save for sample loading buffer due to poorer polymerization). The electrophoresis apparatus was filled with 1x Running buffer (standard Laemmli 1x Running buffer was prepared from a 10x stock). Gels were run at 100 V for 20 minutes or until the sample front cleared the stacking gel, followed by 150 V until the appropriate kDa range had migrated to the centre of the resolving gel, as indicated by reference to the protein standard indicator.

1.17 – Electrophoretic transfer

Immediately upon completion of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (to prevent protein band dissociation), gels were removed from the electrophoresis assembly, their edges trimmed and orientation marks added (i.e. diagonal edge cut at top-left), and then were soaked in ice-cold 1x Transfer buffer for ~15 minutes for equilibration (standard Laemmli 1x transfer buffer was prepared from a 10x stock). Polyvinylidene fluoride transfer membrane (Pierce, Rockford, IL, USA) was soaked in methanol for no more than 30 seconds before rinsing with ddH_2O. The transfer cassette was assembled as per the manufacturer's instructions, filled with ice-cold 1x Transfer buffer, and the transfer was performed at either 100 V for 1 hour or at 25-30 V for 8-16 hours at 4 °C, with ddH_2O ice blocks and rapid, magnetic-stirrer buffer agitation.

1.18 – Protein immunoblot and antibodies

Following electrophoretic transfer, gels were soaked in Coomassie Blue dye (Sigma) on a rocker for at least 1 hour and then soaked in de-stain solution while actively monitored to verify successful protein transfer (i.e. insubstantial Coomassie Blue staining of the gels). Membranes were stained with Ponceau S solution (Bio-Rad) for 5-10 minutes to further verify transfer efficiency (i.e. substantial Ponceau S staining of the membranes), and Ponceau S stain was removed by successive washes in ddH_2O. The membranes were then soaked in Tris-buffered saline containing 0.1 % Tween-20 detergent (TBST) (Sigma) for ~15 minutes (protein side up) to wash and equilibrate them before being blocked with 5 % milk in TBST solution for 2 hours at room temperature or 8-16 hours at 4 °C.

Blocked membranes were briefly rinsed in TBST before incubation with 1:1000 CSE polyclonal primary antibody in 5 % milk / TBST for 2 hours at room temperature on a Belly Dancer shaker (Stovall) set to low agitation. The CSE antibody was produced by Dr. Rui Wang's University of Saskatchewan laboratory and directly provided by Dr. Guangdong Yang). Membranes were then washed in TBST on a vigorous rocker for 1 hour, changing the wash solution every 10 minutes. Following this step, 1:5000 HRP-conjugated goat anti-rabbit IgG secondary antibodies (Pierce) in 5 % milk / TBST solution were used to probe the membrane on a rocker for 45-60 minutes at room temperature. Following this step, membranes were again washed with TBST on a vigorous rocker for 1 hour, changing the wash solution every 10 minutes.

Following chemiluminescent imaging (below), membranes were stripped for re-blotting with the protein standard, 1:2000 β-actin monoclonal primary antibody (Novus, Littleton, CO, USA). A 'medium' strength stripping buffer, containing SDS, was required to sufficiently remove antibodies from the membranes. In initial experiments, the CSE primary antibody detected a ~100 kDa, non-specific product. However, via a combination of increasing the protein sample concentration, cutting gels below the 100 kDa range (via the visible, multi-colourimetric protein ladder) and overexposing the chemiluminescent blot images, the appropriate, ~ 44.5 kDa CSE product could be clearly discerned in HL-1 cardiomyocyte lysates.

1.19 – Chemiluminescent imaging and densitometry

Enhanced chemiluminescence (ECL) utilizes horseradish peroxidase enzyme (HRP) tethered to the molecule of interest (usually through labeling an immunoglobulin that specifically recognizes the molecule). This enzyme complex then catalyzes the conversion of the ECL

substrate into a sensitized reagent in the vicinity of the molecule of interest, which on further oxidation by H_2O_2, produces a triplet (excited) carbonyl which emits light when it decays to the singlet carbonyl. Standard ECL was performed to detect protein-banding patterns on the immunoblots by virtue of the HRP-conjugated secondary antibodies (above). Chemiluminescent immunoblots were detected via 10-minute High-Sensitivity Chemiluminescent exposures using a Chemidoc XRS imager (Bio-Rad) supported by Quantity One software for Windows (Bio-Rad). Brief Epi-White exposures were also collected in order to interpret chemiluminescent banding patterns vis-à-vis the membranes' visible protein ladders. Blots were analyzed via densitometry using the Quanity One software, with reference to the re-probed β-actin control.

1.20 – RNA isolation

RNA isolation was performed using the Aurum Total RNA Mini kit (Bio-Rad) in accordance with the manufacturer's instructions using certified RNase-free barrier tips (Ambion, Foster City, CA, USA). Cells were seeded onto sterile 25 cm^2 culture flasks (Corning) and grown overnight to achieve the desired confluence. Plated cells were subjected to various time periods of chemical, hypoxic or normoxic pre- or co-treatment (various chemicals and conditions including $CoCl_2$, PPG, 1 % O_2, ~20 % O_2, etc. at various concentrations in serum- and antibiotic-free media). Adherent cells were washed once with PBS and detached via trypsinization. Following centrifugation (500 × g for 5 min at 4 ˚C), cells were washed with PBS and lysed using the provided lysis and binding buffer containing chaotropic components for stabilization of RNA and inhibition of RNase activity. Lysates were placed in RNAse-free microfuge tubes (Ambion) and RNA was extracted using a silica membrane spin column. Upon loading the RNA onto the column, genomic DNA was digested with an RNase-free DNase enzyme and, following washes

to remove degraded genomic DNA, salts, and other cellular components, an elution buffer of low ionic strength was used to collect the pure RNA from the column. Aliquots were stored at -80 ˚C unless being used immediately, in which case they were kept on ice and separated into stock and sample aliquots, the latter used for RNA quantitation.

1.21 – Automated electrophoretic RNA analysis

The concentration and integrity of extracted RNA was assessed using the Experion RNA StdSens kit (Bio-Rad) on an Experion Automated Electrophoresis Station (Bio-Rad) supported by Experion software for Windows (Bio-Rad), in accordance with the manufacturer's instructions. Briefly, 1 µL aliquots of denatured RNA samples and ladder were loaded onto a microfluidic chip comprised of channels that, once primed with a gel matrix, allowed for the separation, staining, detection, and data analysis of samples by via measurement of the 18 and 28 S rRNA peaks. Only high-quality (i.e. 9+ RNA quality index, as per automatic Experion software calculation) RNA samples were used for subsequent gene expression analysis.

1.22 – cDNA synthesis

First strand complementary DNA (cDNA) synthesis reactions were performed using a RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, Flamborough, ON, Canada) in accordance with the manufacturer's instructions. This kit features reverse transcriptase with a point mutation that completely eliminates RNase H activity and, in concert with the kit's RNase inhibitor, helps prevent RNA degradation during cDNA synthesis. Briefly, RNA samples were mixed with oligo$(dT)_{18}$ primers and the other kit components and incubated at 42 ˚C for 1 hour

before terminating the reaction by heating at 70 ˚C for 5 minutes. The resultant products were stored at -20 ˚C unless being used immediately, in which case they were kept on ice.

1.23 – Quantitative real-time polymerase chain reaction

Quantitative real-time PCR (qPCR) was performed using primers for various genes (SABiosciences, Frederick, MD, USA) and SYBR Green RT^2 qPCR Master Mix (SABiosciences) via iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) in accordance with the manufacturer's instructions. Briefly, 10 µL of 2x Master Mix was pipetted into the wells of a sterile 96-well PCR plate (Bio-Rad). Primer was added to nuclease-free ddH_2O at a ratio of 1 µL primer to 8 µL ddH_2O, and then 9 µL of the mix was pipetted into the wells of the PCR plate, followed by 1 µL cDNA template. The PCR plate was sealed with optical film (Bio-Rad). Following iQ5 calibration, qPCR analysis of triplicate samples was performed using a two-step cycling program involving an initial single cycle of 95 ˚C for 10 min (required to activate the DNA polymerase), followed by 40 cycles of 95 ˚C for 15 seconds and 60 ˚C for 1 minute. Following the qPCR reaction, a first derivative dissociation curve was performed as a quality control measure. Briefly, the reaction was heated to 95 ˚C for 1 min, cooled to 65 ˚C for 2 min, then ramped from 65 to 95 ˚C at a rate of 2 ˚C per minute. The formation of a single peak at temperatures greater than 80 ˚C indicated the presence of a single PCR product in the reaction mixture. Gene of interest expression was normalized to the housekeeping gene, β2-microglobulin, which was found to be most consistent as compared to either β-actin or GAPDH.

Gene name	UniGene #	RefSeq Accession #	Band Size (bp)	Reference Position	Source
Tumour necrosis factor-α	Mm.1293	NM_013693	93	879	SABiosciences
Interferon-γ	Mm.240327	NM_008337	95	330	SABiosciences
Interleukin-10	Mm.874	NM_010548	110	1005	SABiosciences
Transforming growth factor-β	Mm.248380	NM_011577	162	267	SABiosciences
Hypoxia inducible factor-α	Mm.3879	NM_010431	151	3633	SABiosciences
B2-microglobulin	Mm.163	NM_009735	192	304	SABiosciences

Table 1. Quantitative real-time PCR primers. These primer sets were used as per manufacturer's instructions, as described above.

1.24 – Statistics

Data were presented as mean ± standard error of the mean (SEM), and all data presented here represents $n \geq 3$ independent experiments. Statistical analyses were performed using GraphPad Prism software. One-way ANOVA with post hoc Tukey's test were utilized when possible, and the Student's t-test used otherwise, with $p < 0.05$ considered significant. Asterisks are used herein to denote significance according to the following scheme: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Results

Project I - Hypoxic stress in CSE-deficient smooth muscle cells

Basal metabolic activity, intracellular ROS and Ψ_m in CSE WT and KO SMCs

Given the significant differences we had previously observed between the CSE-deficient mouse model-derived wild-type (WT) and knockout (KO) SMCs [94], their further characterization was of great interest and relevance. Thus were undertaken comparative assessments of cellular metabolism, ROS levels and Ψ_m, via the colourimetric MTT assay and the fluorescent CM-H_2DCFDA (using the 'snapshot' method) and JC-1 assays, respectively. Under basal conditions (24 hours serum- and antiobiotic-free media, but no additional stressor), KO cells featured significantly higher metabolic activity (+27 %, $p < 0.001$; **Figure 4**), intracellular ROS levels (+127 %, $p < 0.001$; **Figure 5**) and Ψ_m (+62 %, $p < 0.05$; **Figure 6**) versus their WT counterparts.

Effect of hypoxia on CSE WT and KO SMC cell viability and apoptosis

Following consideration of the available literature, and in an effort to produce conditions that would approximate the *in vivo* situation for SMCs experiencing pathophysiologically relevant hypoxia, SMCs were subjected to a hypoxia regimen of 12 hours at 1 % O_2 (via hypoxic tissue culture chamber). Cell viability/metabolic activity was assessed via the colourimetric MTT assay, wherein MTT reagent was added to cultures at t = 8 hours and incubated for the remaining 4 hours of hypoxia before analysis at t = 12 hours. Whereas 12-hour hypoxia-induced KO cell viability was significantly decreased (-61 %, $p < 0.001$; **Figure 7**), WT viability was not. Apoptosis was assessed via the fluorescent CaspaTag 3/7 assay, with analysis via flow cytometry.

Hypoxia induced greater apoptosis in KO (+85 %, $p < 0.001$; **Figure 8**) than in WT cells (+23 %, $p < 0.05$; **Figure 8**). Given that the extent of the hypoxia-induced changes in apoptosis was deemed to be within a reasonable, physiologically relevant range, the present hypoxia protocol was retained for subsequent experiments.

Effect of hypoxia on CSE WT and KO SMC redox balance and Ψ_m

In order to investigate the underlying factors to the observed hypoxia-induced SMC death, and to ascertain whether the redox and Ψ_m differences between WT and KO cells noted under basal conditions were also present upon hypoxic insult, redox status and mitochondrial membrane potential were assessed via the colourimetric SOD activity assay and the fluorescent CM-H_2DCFDA and JC-1 assays, with analysis via flow cytometry. 12-hour hypoxia induced similarly modest but significant increases in SOD content in WT (+9 %, $p < 0.05$) and KO cells (+9 %, $p < 0.05$; **Figure 9**). Despite this parity, intracellular ROS was significantly increased in hypoxic KO (+75 %, $p < 0.001$; **Figure 10**) but not WT cells. Ψ_m was not significantly impacted by hypoxia, but, as was the case under basal conditions, the absolute Ψ_m values remained significantly higher in KO (+118 %, $p < 0.01$; **Figure 11**) versus WT cells.

Effect of hypoxia on CSE WT and KO SMC inflammatory mediator expression

Given the observed redox imbalance, and the known relationship of pro-oxidant and pro-inflammatory status in cardiovascular disease, assessment of the mRNA expression of several inflammatory mediators – TNF-α, IFN-γ, IL-10 and TGF-β – was undertaken via qPCR, with normalization via β2M. Under basal conditions, KO cells featured significantly lower expression of TNF-α (-80 %, $p < 0.001$; **Figure 12**) and IFN-γ (-43 %, $p < 0.001$; **Figure 13**), and

significantly higher expression of IL-10 (+483 %, $p < 0.001$; **Figure 14**) and TGF-β (+194 %, $p < 0.001$; **Figure 15**).

Upon 12-hour hypoxic insult, TNF-α expression was significantly decreased in WT (-94 %, $p < 0.001$; **Figure 12**) but not KO cells. IFN-γ expression was decreased in WT (-28 %, $p < 0.05$; **Figure 13**) but substantially increased in KO cells (+259 %, $p < 0.001$; **Figure 13**). IL-10 expression was massively increased in WT (+412 %, $p < 0.001$; **Figure 14**) but substantially decreased in KO cells (-73 %, $p < 0.001$; **Figure 14**). TGF-β expression was increased in KO cells (+64 %, $p < 0.001$) but massively increased in WT cells (+319 %, $p < 0.001$; **Figure 15**).

Effect of hypoxia on CSE WT and KO SMC HIF-1α expression

Given the patently poorer ability of the CSE-deficient SMCs to respond to hypoxic stress, and in light of the established role of master transcriptional regulator, hypoxia inducible factor (HIF)-1, to promote the cellular hypoxic stress response, an assessment of the mRNA expression of its O_2-sensitive alpha subunit, HIF-1α, was undertaken via qPCR, with normalization via β2M. 12-hour hypoxia induced increased HIF-1α expression in KO cells (+138 %, $p < 0.001$), but a greater increase in expression in WT cells (+258 %, $p < 0.001$; **Figure 16**).

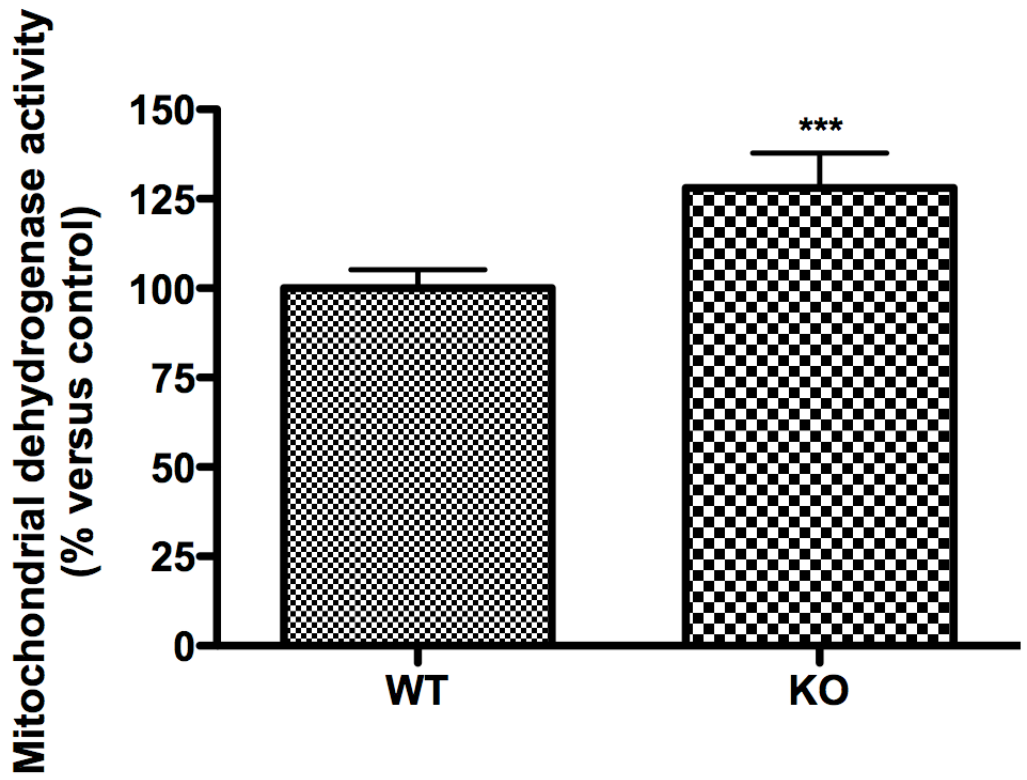

Figure 4. Basal metabolic activity in CSE-WT and -KO SMCs. Basal metabolic activity of CSE-WT and -KO SMCs was assessed via colourimetric measurement of mitochondrial dehydrogenase activity (MTT assay). Matched passage WT and KO cells were seeded onto 96-well plates, grown overnight to 70-80 % confluence, and incubated in serum- and antibiotic-free medium for 12 hours. MTT reagent was added at t = 8 hours and absorbance was measured spectrophotometrically at 490 nm (650 nm correction wavelength) at t = 12 hours. Normal viability was standardized to the normoxic WT condition. Bars represent mean ± SEM of 3 independent experiments. *** denotes significance of $p < 0.001$.

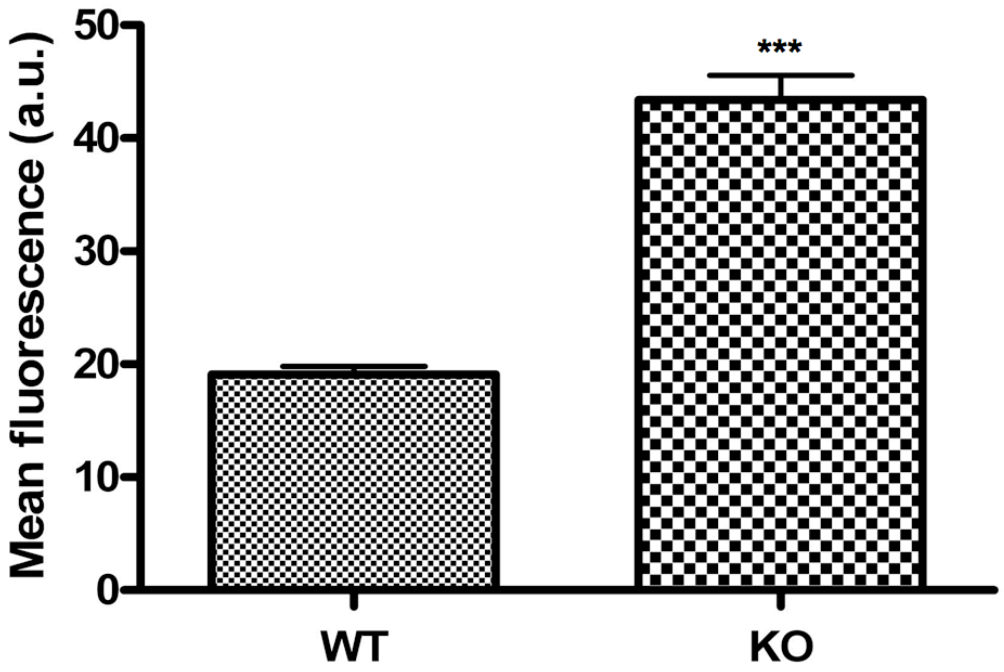

Figure 5. Basal intracellular ROS levels in CSE-WT and -KO SMCs. Basal intracellular ROS levels in CSE-WT and -KO SMCs was assessed via fluorescent detection of oxidation-induced esterase activity (CM-H_2DCFDA assay), and analyzed via flow cytometry. Matched passage WT and KO cells were seeded onto 6-well plates, grown overnight to 70-80 % confluence, and incubated in serum- and antibiotic-free medium for 12 hours. The 'snapshot' method was utilized, whereby cells were incubated with fluorescent label for 30 minutes post hypoxic stress in order to ascertain the final redox environment of the cells, and a minimum of 1 x 10^4 gated events were analyzed via flow cytometry. Bars represent mean ± SEM of 3 independent experiments. *** denotes significance of $p < 0.001$.

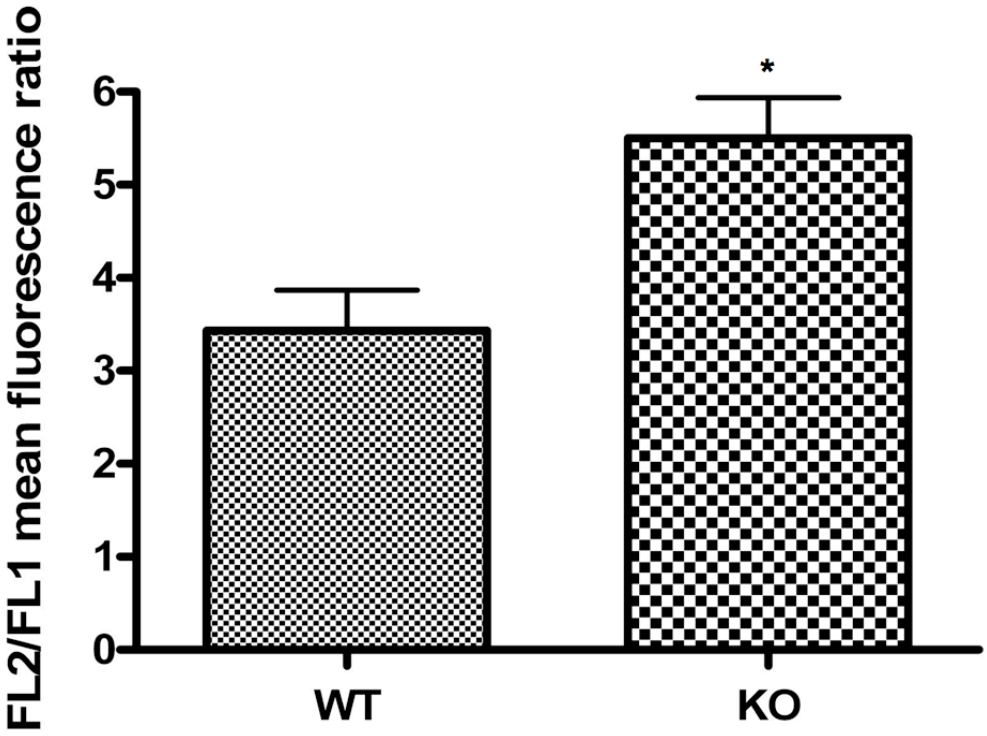

Figure 6. Basal Ψ_m in CSE-WT and -KO SMCs. Basal Ψ_m of CSE-WT and -KO SMCs was assessed via the fluorescent detection of potential-dependent incorporation and conversion of a cationic dye (JC-1 assay), and analyzed via flow cytometry. Matched passage WT and KO cells were seeded onto 6-well plates, grown overnight to 70-80 % confluence, and incubated in serum- and antibiotic-free medium for 12 hours. Cells were incubated with fluorescent label for 30 minutes, and a minimum of 1 x 10^4 gated events were analyzed via flow cytometry, wherein the ratio of red to green fluorescence indicated mitochondrial polarization status (where increased conversion of the green fluorescent label to red, and thus a higher fluorescence ratio, indicated greater depolarization). Bars represent mean ± SEM of 3 independent experiments. * denotes significance of $p < 0.05$.

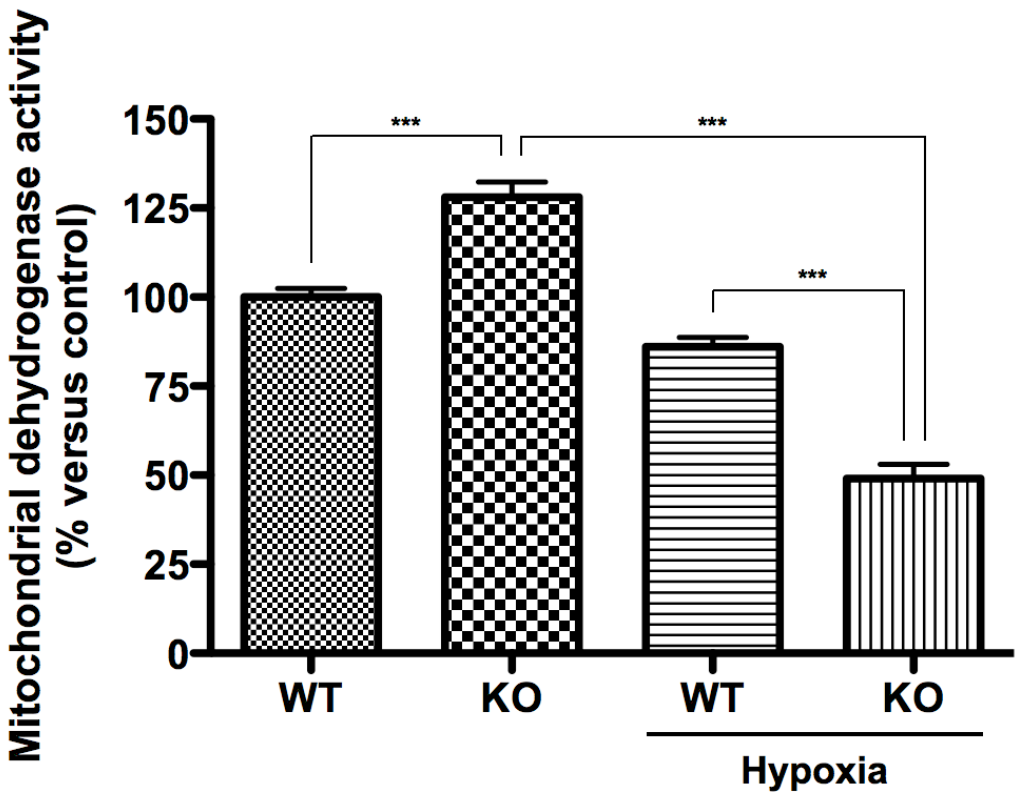

Figure 7. Effect of hypoxic stress on viability of CSE-WT and -KO SMCs. The effect of hypoxic stress on the viability of CSE-WT and -KO SMCs was assessed via measurement of mitochondrial dehydrogenase activity (MTT assay). Matched passage WT and KO cells were seeded onto 96-well plates, grown overnight to 70-80 % confluence, and incubated in serum- and antibiotic-free medium in a hypoxia chamber at 1 % O_2 for 12 hours. MTT reagent was added at t = 8 hours and absorbance was measured spectrophotometrically at 490 nm (650 nm correction wavelength) at t = 12 hours. Normal viability was standardized to the normoxic WT condition. Bars represent mean ± SEM of 3 independent experiments. Connected lines indicate relationship; * denotes significance of $p < 0.05$; *** denotes significance of $p < 0.001$.

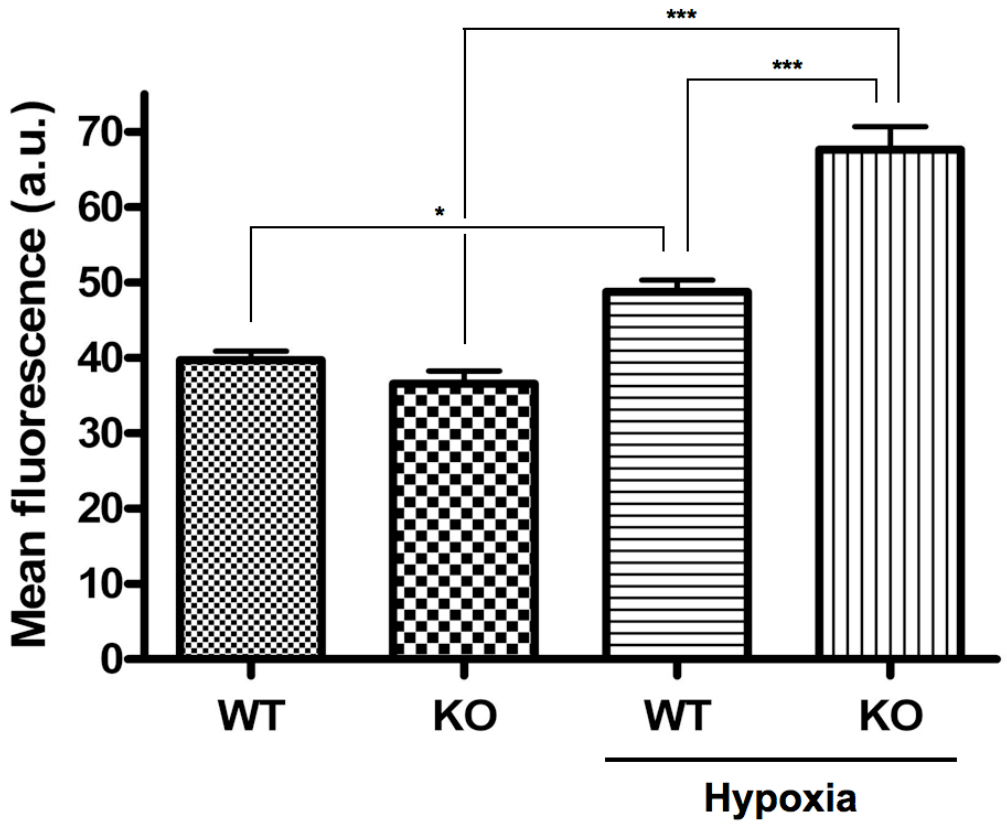

Figure 8. Effect of hypoxic stress on CSE-WT and -KO SMC apoptosis. The effect of hypoxic stress on CSE-WT and -KO SMC apoptosis was assessed via the fluorescent detection of active caspase-3 and -7 (CaspaTag 3/7 assay), and analyzed via flow cytometry. Matched passage WT and KO cells were seeded onto 25 cm^2 tissue culture flasks, grown overnight to 70-80 % confluence, and incubated in serum- and antibiotic-free medium in a hypoxia chamber at 1 % O_2 for 12 hours. Cells were incubated with fluorescent FLuorochrome Inhibitors of CAspases (FLICA) and/or propidium iodide (PI) labels for 15 minutes, and a minimum of 1 x 10^4 gated events were analyzed via flow cytometry, with PI-positive staining subtracted from FLICA-positive staining to control for necrosis, thereby specifying for apoptotic cells. Bars represent mean ± SEM of 3 independent experiments. Connected lines indicate relationship; * denotes significance of $p < 0.05$; *** denotes significance of $p < 0.001$.

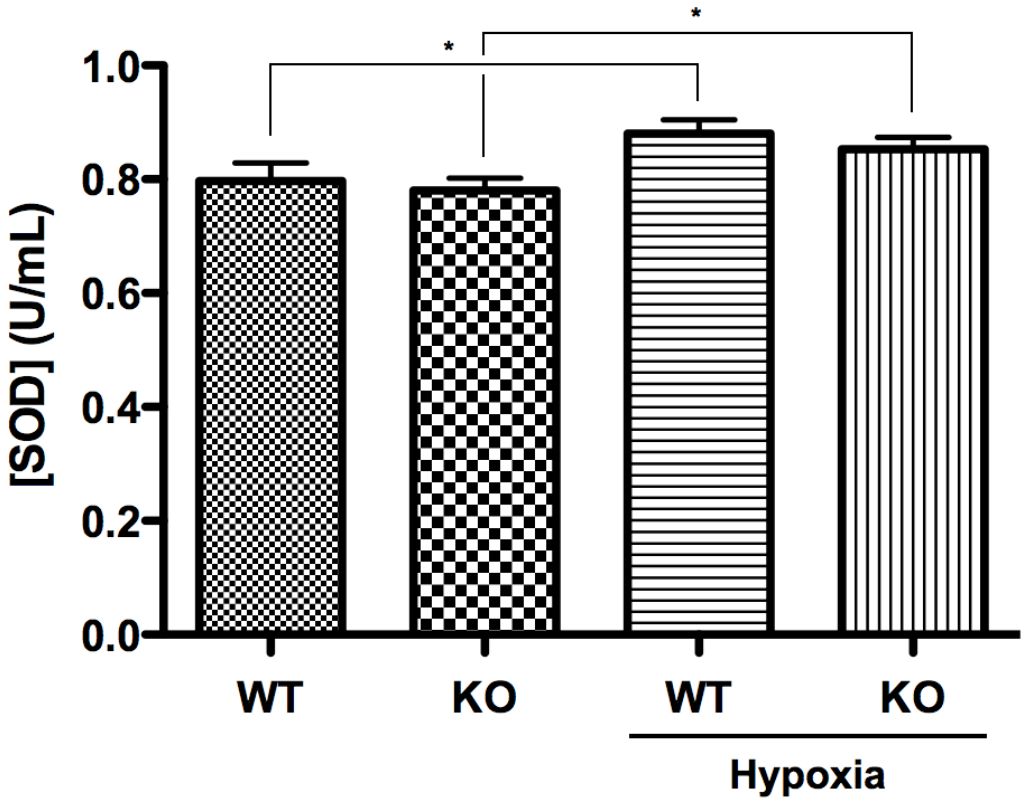

Figure 9. Effect of hypoxic stress on SOD activity in CSE-WT and -KO SMCs. The effect of hypoxic stress on SOD activity in CSE-WT and -KO SMCs was assessed via a colourimetric SOD activity assay. Matched passage WT and KO cells were seeded onto 150 cm^2 tissue culture flasks, grown overnight to 70-80 % confluence, and incubated in serum- and antibiotic-free medium in a hypoxia chamber at 1 % O_2 for 12 hours. Sample cell lysates were quantitated and assayed via measurement over time of absorbance at 550 nm. SOD activity was converted to units SOD per volume by reference to an SOD inhibition curve. Bars represent mean ± SEM of 3 independent experiments. Connected lines indicate relationship; * denotes significance of $p < 0.05$.

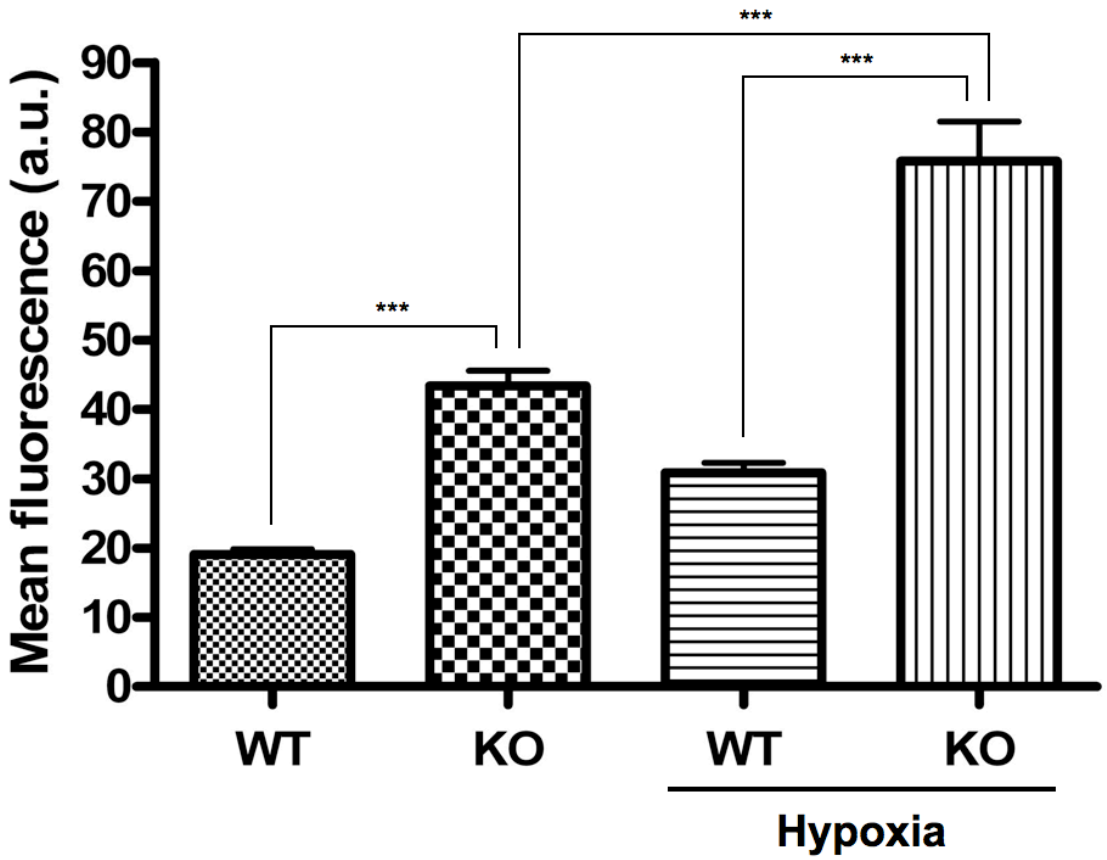

Figure 10. Effect of hypoxic stress on intracellular ROS levels in CSE-WT and -KO SMCs. The effect of hypoxic stress on intracellular ROS levels in CSE-WT and -KO SMCs was assessed via fluorescent detection of oxidation-induced esterase activity (CM-H_2DCFDA assay), and analyzed via flow cytometry. Matched passage WT and KO cells were seeded onto 6-well plates, grown overnight to 70-80 % confluence, and incubated in serum- and antibiotic-free medium in a hypoxia chamber at 1 % O_2 for 12 hours. The 'snapshot' method was utilized, whereby cells were incubated with fluorescent label for 30 minutes post hypoxic stress in order to ascertain the final redox environment of the cells, and a minimum of 1 x 10^4 gated events were analyzed via flow cytometry. Bars represent mean ± SEM of 3 independent experiments. Connected lines indicate relationship; *** denotes significance of $p < 0.001$.

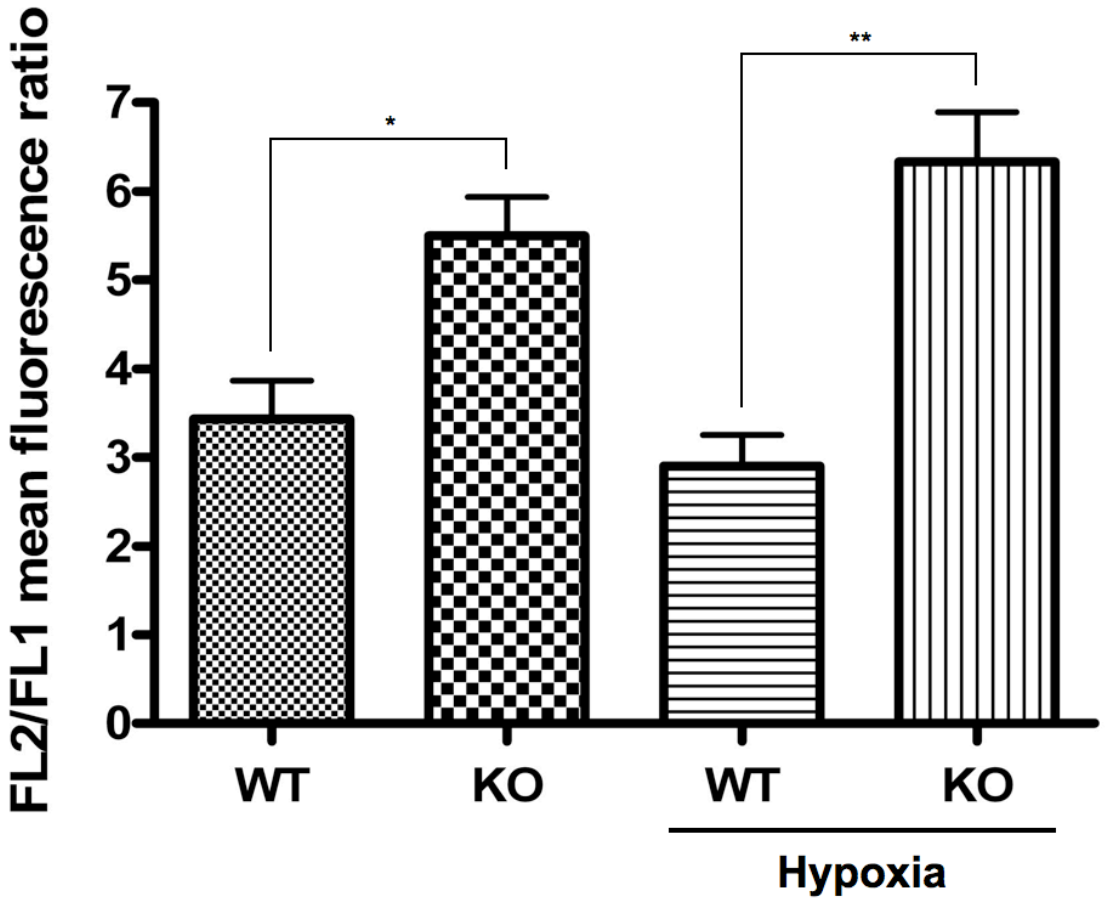

Figure 11. Effect of hypoxic stress on Ψ_m in CSE-WT and -KO SMCs. The effect of hypoxic stress on Ψ_m in CSE-WT and -KO SMCs was assessed via the fluorescent detection of potential-dependent incorporation and conversion of cationic dye (JC-1 assay), and analyzed via flow cytometry. Matched passage WT and KO cells were seeded onto 6-well plates, grown overnight to 70-80 % confluence, and incubated in serum- and antibiotic-free medium in a hypoxia chamber at 1 % O_2 for 12 hours. Cells were incubated with fluorescent label for 30 minutes, and a minimum of 1 x 10^4 gated events were analyzed via flow cytometry, wherein the ratio of red to green fluorescence indicated mitochondrial polarization status (where increased conversion of the green fluorescent label to red, and thus a higher fluorescence ratio, indicated greater depolarization). Bars represent mean ± SEM of 3 independent experiments. Connected lines indicate relationship; * denotes significance of $p < 0.05$; ** denotes significance of $p < 0.01$.

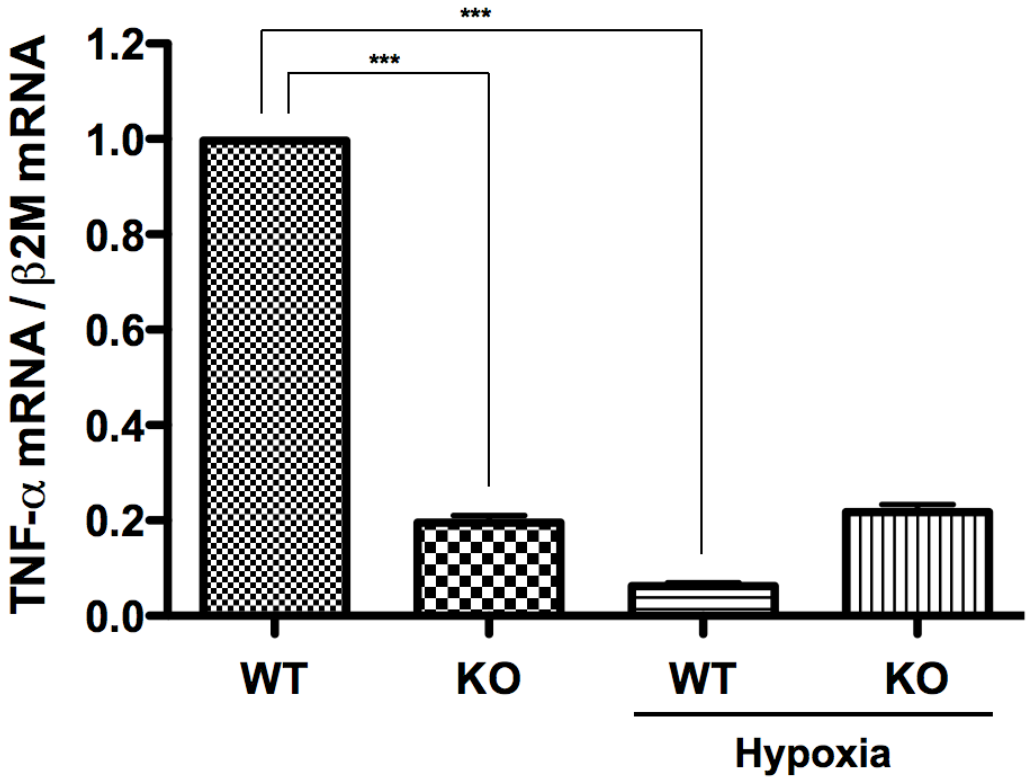

Figure 12. Effect of hypoxic stress on TNF-α mRNA expression in CSE-WT and -KO SMCs. The effect of hypoxic stress on TNF-α mRNA expression in CSE-WT and -KO SMCs was assessed via qPCR. Matched passage WT and KO cells were seeded onto 75 cm^2 tissue culture flasks, grown overnight to 70-80 % confluence, and incubated in serum- and antibiotic-free medium in a hypoxia chamber at 1 % O_2 for 12 hours. Cells were lysed and their RNA isolated via silica membrane spin column. RNA quantity and integrity was assessed via automated electrophoresis. cDNA was generated via oligo-dT-primed reverse transcription. Real-time PCR was performed via a two-step cycling program in accordance with the DNA polymerase and primer specifications. Results were normalized to β2M and normal expression standardized to the normoxic WT condition. Bars represent mean ± SEM of 3 independent experiments. Connected lines indicate relationship; *** denotes significance of $p < 0.001$.

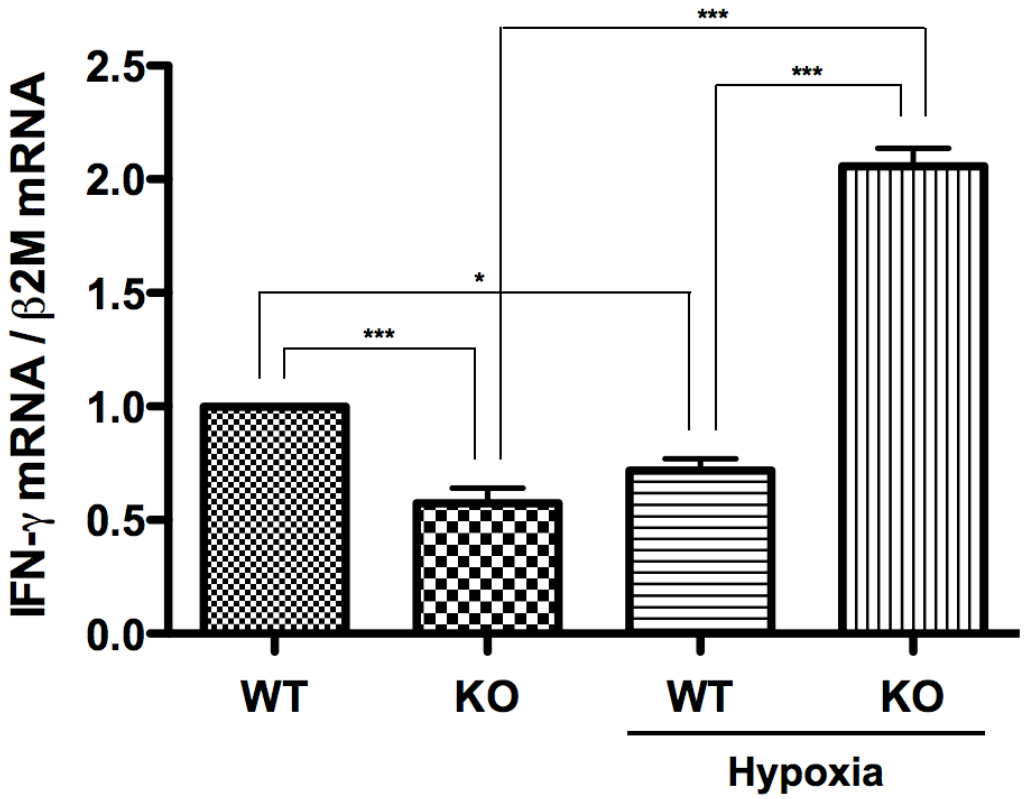

Figure 13. Effect of hypoxic stress on IFN-γ mRNA expression in CSE-WT and -KO SMCs. The effect of hypoxic stress on IFN-γ mRNA expression in CSE-WT and -KO SMCs was assessed via qPCR. Matched passage WT and KO cells were seeded onto 75 cm^2 tissue culture flasks, grown overnight to 70-80 % confluence, and incubated in serum- and antibiotic-free medium in a hypoxia chamber at 1 % O_2 for 12 hours. Cells were lysed and their RNA isolated via silica membrane spin column. RNA quantity and integrity was assessed via automated electrophoresis. cDNA was generated via oligo-dT-primed reverse transcription. Real-time PCR was performed via a two-step cycling program in accordance with the DNA polymerase and primer specifications. Results were normalized to β2M and normal expression standardized to the normoxic WT condition. Bars represent mean ± SEM of 3 independent experiments. Connected lines indicate relationship; * denotes significance of $p < 0.05$; *** denotes significance of $p < 0.001$.

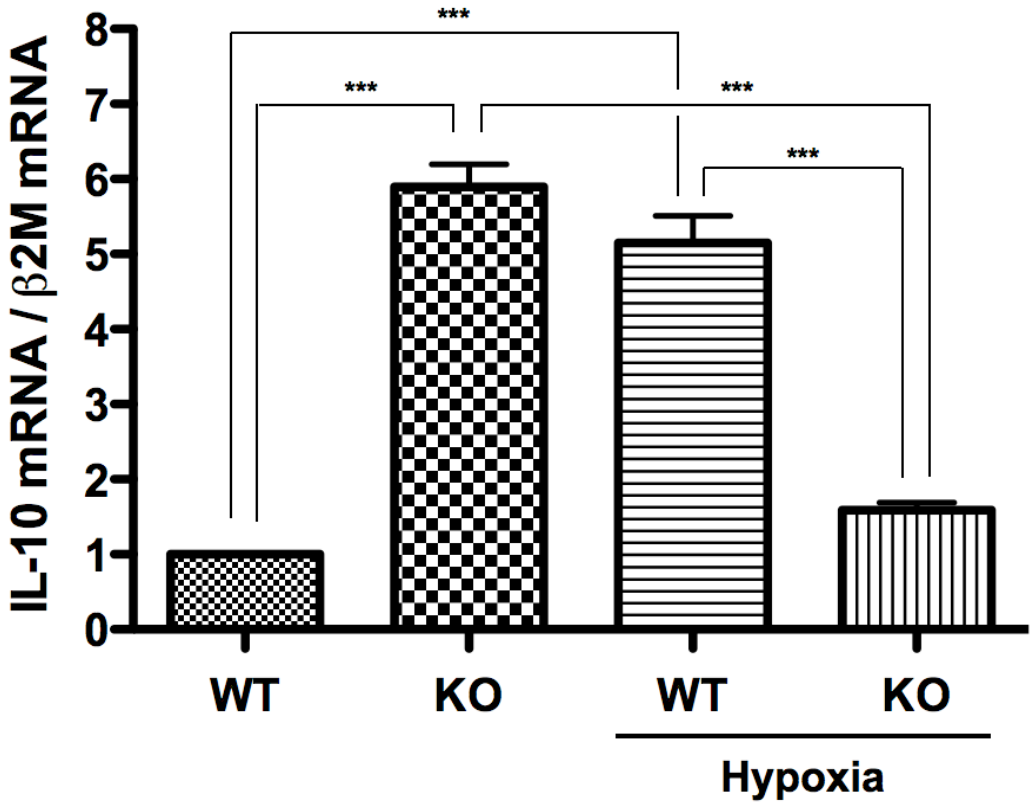

Figure 14. Effect of hypoxic stress on IL-10 mRNA expression in CSE-WT and -KO SMCs. The effect of hypoxic stress on IL-10 mRNA expression in CSE-WT and -KO SMCs was assessed via qPCR. Matched passage WT and KO cells were seeded onto 75 cm^2 tissue culture flasks, grown overnight to 70-80 % confluence, and incubated in serum- and antibiotic-free medium in a hypoxia chamber at 1 % O_2 for 12 hours. Cells were lysed and their RNA isolated via silica membrane spin column. RNA quantity and integrity was assessed via automated electrophoresis. cDNA was generated via oligo-dT-primed reverse transcription. Real-time PCR was performed via a two-step cycling program in accordance with the DNA polymerase and primer specifications. Results were normalized to β2M and normal expression standardized to the normoxic WT condition. Bars represent mean ± SEM of 3 independent experiments. Connected lines indicate relationship; * denotes significance of $p < 0.05$; *** denotes significance of $p < 0.001$.

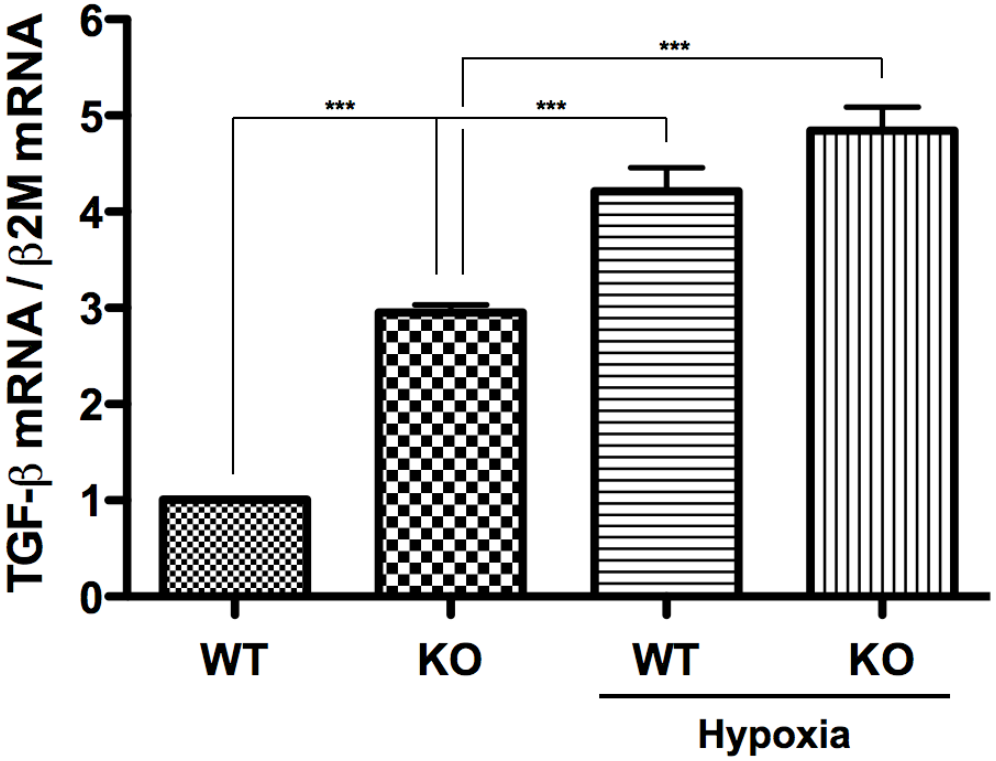

Figure 15. Effect of hypoxic stress on TGF-β mRNA expression in CSE-WT and -KO SMCs. The effect of hypoxic stress on TGF-β mRNA expression in CSE-WT and -KO SMCs was assessed via qPCR. Matched passage WT and KO cells were seeded onto 75 cm^2 tissue culture flasks, grown overnight to 70-80 % confluence, and incubated in serum- and antibiotic-free medium in a hypoxia chamber at 1 % O_2 for 12 hours. Cells were lysed and their RNA isolated via silica membrane spin column. RNA quantity and integrity was assessed via automated electrophoresis. cDNA was generated via oligo-dT-primed reverse transcription. Real-time PCR was performed via a two-step cycling program in accordance with the DNA polymerase and primer specifications. Results were normalized to β2M and normal expression standardized to the normoxic WT condition. Bars represent mean ± SEM of 3 independent experiments. Connected lines indicate relationship; * denotes significance of $p < 0.05$; *** denotes significance of $p < 0.001$.

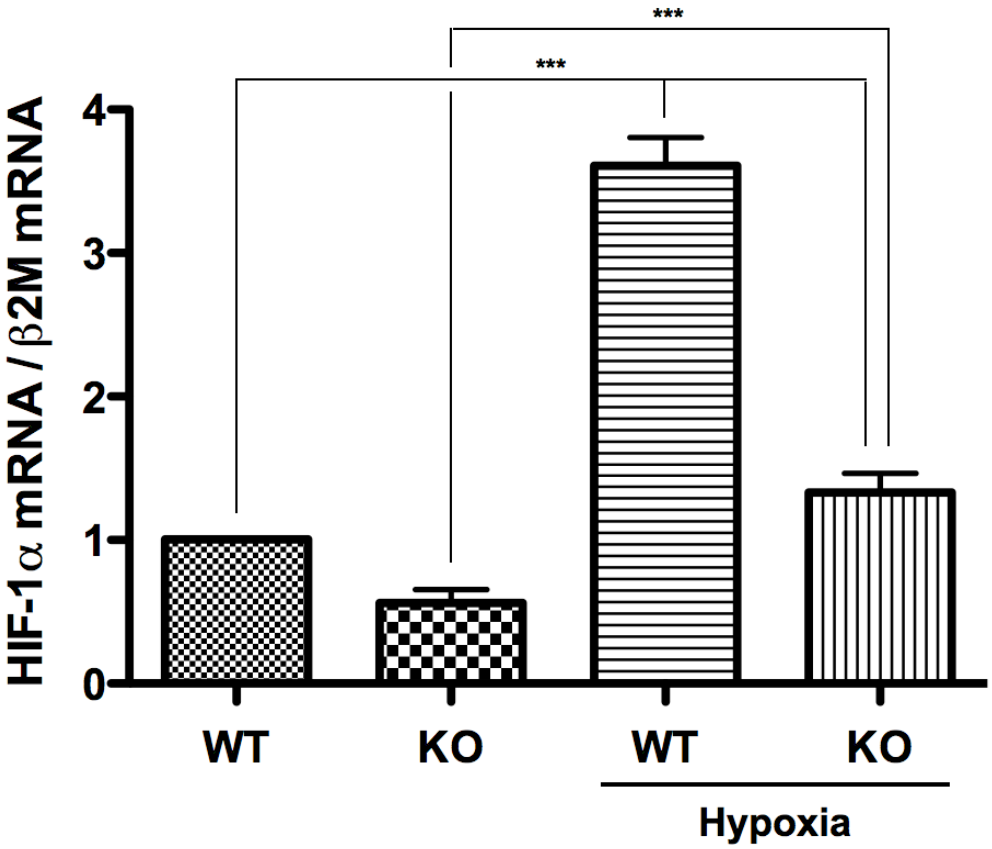

Figure 16. Effect of hypoxic stress on HIF-1α mRNA expression in CSE-WT and -KO SMCs. The effect of hypoxic stress on HIF-1α mRNA expression in CSE-WT and -KO SMCs was assessed via qPCR. Matched passage WT and KO cells were seeded onto 75 cm^2 tissue culture flasks, grown overnight to 70-80 % confluence, and incubated in serum- and antibiotic-free medium in a hypoxia chamber at 1 % O_2 for 12 hours. Cells were lysed and their RNA isolated via silica membrane spin column. RNA quantity and integrity was assessed via automated electrophoresis. cDNA was generated via oligo-dT-primed reverse transcription. Real-time PCR was performed via a two-step cycling program in accordance with the DNA polymerase and primer specifications. Results were normalized to β2M and normal expression standardized to the normoxic WT condition. Bars represent mean ± SEM of 3 independent experiments. Connected lines indicate relationship; * denotes significance of $p < 0.05$; *** denotes significance of $p < 0.001$.

Project II - CSE in simulated hypoxia and hypoxia-reoxygenation cardiac stress

Effect of $CoCl_2$ on HL-1 cardiomyocyte viability

$CoCl_2$ induces so-called 'simulated' hypoxia by specifically activating HIF-1-mediated transcription, even under normoxic conditions. Following consideration of the available literature, and in an effort to reproduce, in part, the $CoCl_2$ stress model of simulated hypoxia described previously [257], a variety of concentrations and treatment durations were evaluated via colourimetric MTT assay (data not shown), and 24-hour treatment with 200 μM $CoCl_2$ was chosen for study. Cell viability was significantly decreased in 200 μM $CoCl_2$-treated HL-1 cardiomyocytes (-28 %, $p < 0.001$; **Figure 17**), which was similar to the extent of change previously described by Vassilopoulos et al. in this model [257].

Effect of $CoCl_2$ on HL-1 cardiomyocyte ROS levels

Given the observed $CoCl_2$-induced decreases in cardiomyocyte survival, and given that ROS elaboration was previously shown to be the major contributory factor to $CoCl_2$-induced HL-1 cardiomyocyte death [257], cells were subjected to 24 hours of 200 μM $CoCl_2$, and intracellular ROS levels were assessed via the fluorescent CM-H_2DCFDA assay (using 'snapshot' method), with analysis via flow cytometry. 200 μM $CoCl_2$ induced significantly increased ROS levels (+121 %, $p < 0.05$; **Figure 18**).

Effect of $CoCl_2$ on HL-1 cardiomyocyte CSE protein expression

In order to investigate the potential for changes in CSE expression in response to simulated hypoxia, given that the endogenous H_2S/CSE pathway had previously been shown to

be cardioprotective against ischemic insult [66, 85, 271], HL-1 cardiomyocytes were subjected to 24 hours of 200 μM $CoCl_2$, and CSE protein expression assessed via immunoblotting, with analysis via densitometry. CSE expression was significantly increased in 24-hour, 200 μM $CoCl_2$-treated HL-1 cardiomyocytes (+79 %, $p < 0.05$; **Figure 19**).

Effect of CSE inhibition on $CoCl_2$-induced decrease in HL-1 cardiomyocyte viability

Given the observed $CoCl_2$-induced CSE expression, inhibition of CSE via the irreversible chemical inhibitor, D,L-propargylglycine (PPG) was undertaken in order to investigate the potential contributions of H_2S/CSE pathway to cardiomyocyte function under $CoCl_2$ stress. Based on the available literature, a 24-hour concentration course of PPG concentrations including 100 μM, 1 mM, and 10 mM was performed, and cell viability was assessed via the colourimetric MTT assay (wherein MTT reagent was added at t = 20 hours). None of the evaluated PPG concentrations induced significant change in cell viability from control (**Figure 20**). As a result, the highest PPG concentration, 10 mM, was selected for future experiments; the thinking being that it should have the greatest CSE-inhibitory effect. The effect of PPG-mediated CSE inhibition in $CoCl_2$-stressed HL-1 cardiomyocytes was investigated via MTT assay, wherein cells were pre-treated with the previously optimized PPG concentration, 10 mM, and subjected to 200 μM $CoCl_2$ as previously described. While $CoCl_2$ significantly reduced cell viability (-27 %, $p < 0.01$; **Figure 21**) and PPG control had no effect, echoing the existing results (**Figure 17** and **20**, respectively), PPG + $CoCl_2$ resulted in much lower viability versus control (-46 %, $p < 0.001$; **Figure 21**) than $CoCl_2$ alone.

Effect of H-R on HL-1 cardiomyocyte viability

Following consideration of the available literature, and in an effort to produce conditions that would approximate the *in vivo* situation for cardiomyocytes experiencing I-R injury, HL-1 cardiomyocytes were subjected to an H-R regimen as follows: 12 hours of hypoxia (1 % O_2) followed by 4 hours of normoxia (~20 % O_2). Cell viability was assessed via the colourimetric MTT assay, wherein MTT reagent was added to cultures at t = 12 hours (upon completion of the hypoxia phase) and incubated for the remaining duration of the H-R regimen (4 hours normoxia), before analysis at t = 16 hours. Cell viability was significantly decreased in H-R-challenged HL-1 cardiomyocytes (-37 %, $p < 0.001$; **Figure 22**).

Effect of H-R on HL-1 cardiomyocyte CSE expression

Given that the endogenous H_2S/CSE pathway had previously been shown to be cardioprotective against ischemic insult [66, 85, 271], given our finding that CSE expression was increased in response to simulated hypoxia (**Figure 19**), CSE protein expression was assessed via immunoblot in H-R-challenged HL-1 cardiomyocytes, with analysis via densitometry. H-R induced significantly increased CSE expression (+94 %, $p < 0.05$; **Figure 23**) in the HL-1 cardiomyocytes, on the order of that observed upon $CoCl_2$ stress (**Figure 19**).

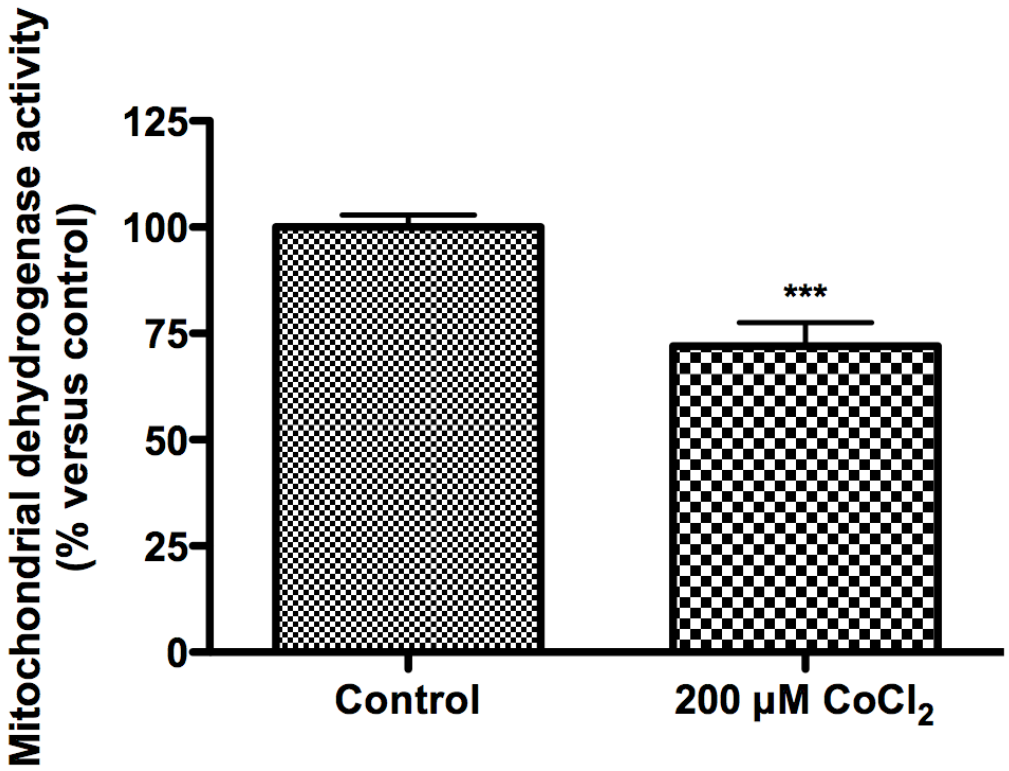

Figure 17. Effect of $CoCl_2$ on HL-1 cardiomyocyte viability. The effect of $CoCl_2$ on HL-1 cardiomyocyte viability was assessed via colourimetric measurement of mitochondrial dehydrogenase activity (MTT assay). Cells were seeded onto 96-well plates, grown overnight to approximately 95 % confluence, and in incubated serum- and antibiotic-free medium containing 200 µM $CoCl_2$ for 24 hours. MTT reagent was added at t = 20 hours and absorbance was measured spectrophotometrically at 490 nm (650 nm correction wavelength) at t = 24 hours. Normal viability was standardized to the unstressed control condition. Bars represent mean ± SEM of 3 independent experiments. *** denotes significance of $p < 0.001$.

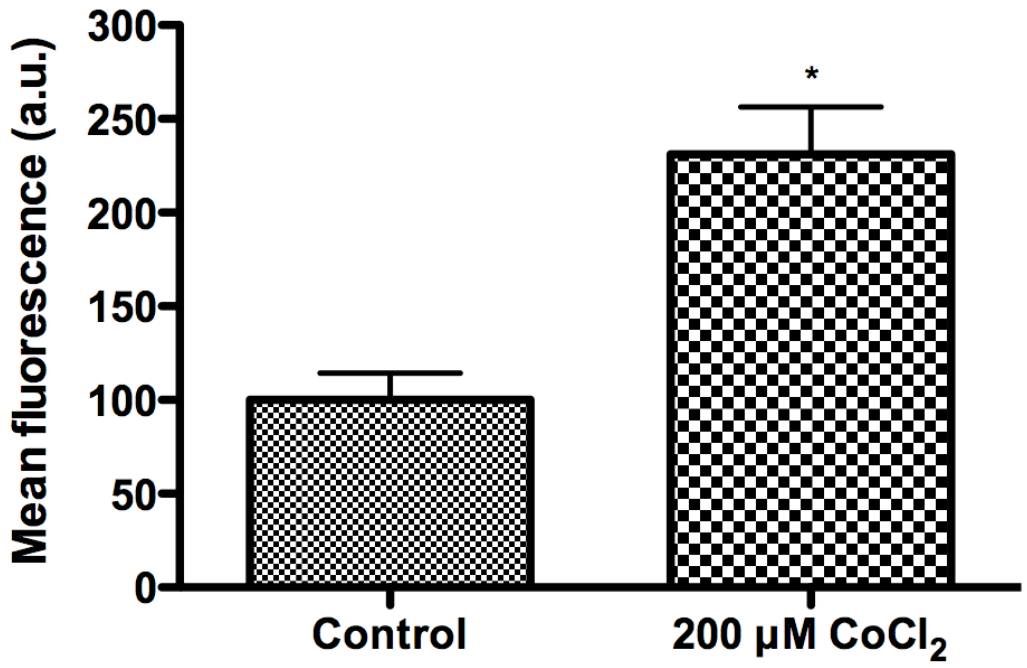

Figure 18. Effect of $CoCl_2$ on intracellular ROS levels in HL-1 cardiomyocytes. The effect of $CoCl_2$ on intracellular ROS levels in HL-1 cardiomyocytes was assessed via fluorescent detection of oxidation-induced esterase activity (CM-H_2DCFDA assay), and analyzed via flow cytometry. Cells were seeded onto 96-well plates, grown overnight to approximately 95 % confluence, and incubated in serum- and antibiotic-free medium containing 200 µM $CoCl_2$ for 24 hours. The 'snapshot' method was utilized, whereby cells were incubated with fluorescent label for 30 minutes post hypoxic stress in order to ascertain the final redox environment of the cells, and a minimum of 1 x 10^4 gated events were analyzed via flow cytometry. Bars represent mean ± SEM of 3 independent experiments. * denotes significance of $p < 0.05$.

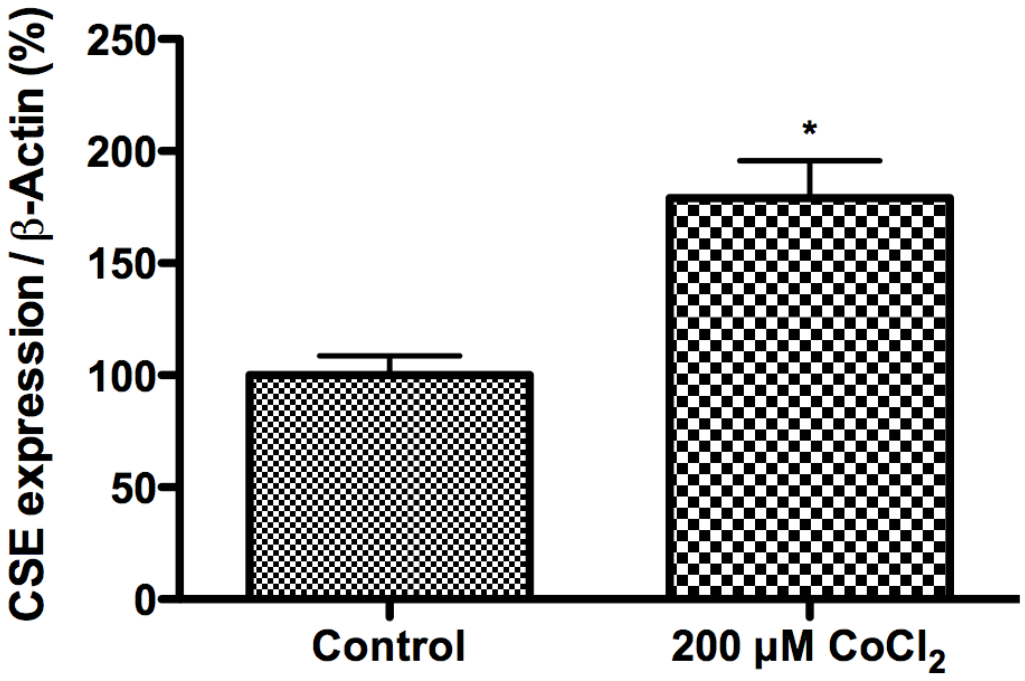

Figure 19. Effect of $CoCl_2$ on CSE protein expression in HL-1 cardiomyocytes. The effect of $CoCl_2$ on CSE protein expression in HL-1 cardiomyocytes was assessed via immunoblotting. Cells were seeded onto 75 cm^2 tissue culture flasks, grown overnight to approximately 95 % confluence, and incubated in serum- and antibiotic-free medium containing 200 μM $CoCl_2$ for 24 hours. Cells were lysed via RIPA buffer and their protein levels quantitated via colourimetric, Lowry-based assay. Total proteins were separated via SDS-PAGE (verified via Coomassie Blue stain), and transferred electrophoretically to PVDF membranes (verified via Ponceau S stain). Membranes were blocked in milk solution and incubated with polyclonal primary antibody, then washed repeatedly, incubated with secondary antibody, and washed again. Membranes were analyzed via enhanced chemiluminescent detection imager and software-assisted densitometry. Bars represent mean ± SEM of 3 independent experiments. * denotes significance of $p < 0.05$.

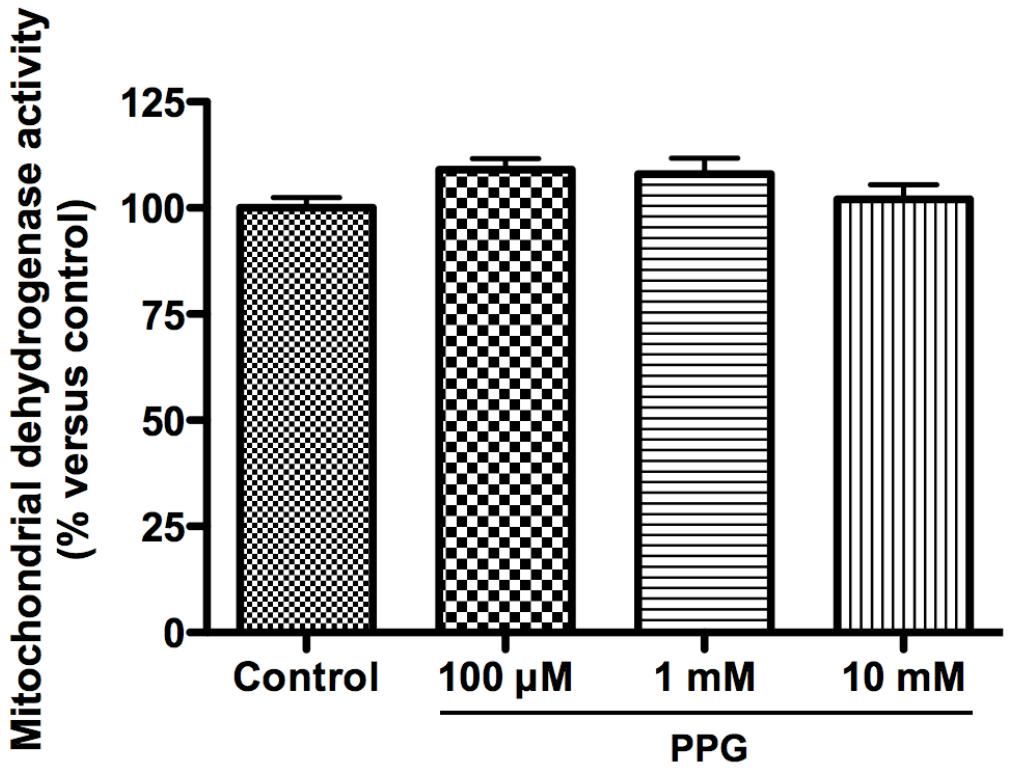

Figure 20. Effect of PPG on HL-1 cardiomyocyte viability. The effect of PPG on HL-1 cardiomyocyte viability was assessed via colourimetric measurement of mitochondrial dehydrogenase activity (MTT assay). Cells were seeded onto 96-well plates, grown overnight to approximately 95 % confluence, and incubated in serum- and antibiotic-free medium containing 100 µM, 1 mM and 10 mM PPG for 24 hours. MTT reagent was added at t = 20 hours and absorbance was measured spectrophotometrically at 490 nm (650 nm correction wavelength) at t = 24 hours. Normal viability was standardized to the unstressed control condition. Bars represent mean ± SEM of 3 independent experiments.

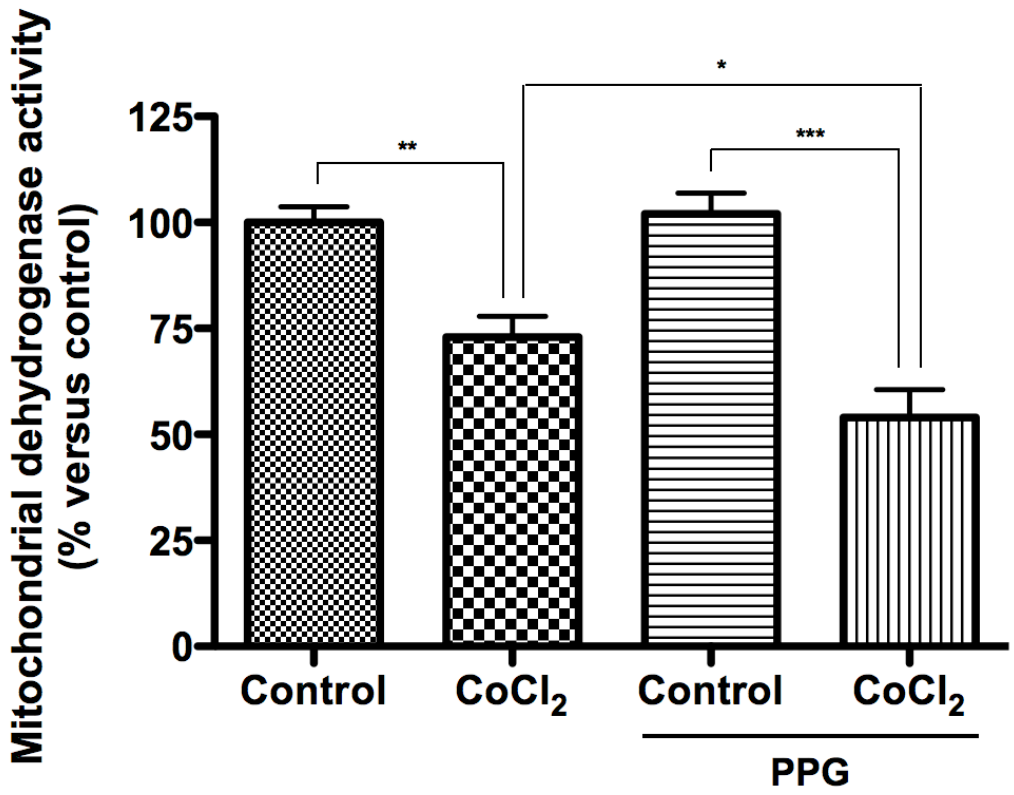

Figure 21. Effect of PPG pre-treatment on $CoCl_2$-mediated changes in HL-1 cardiomyocyte viability. The effect of PPG pre-treatment on $CoCl_2$-mediated changes in HL-1 cardiomyocyte viability was assessed via colourimetric measurement of mitochondrial dehydrogenase activity (MTT assay). Cells were seeded onto 96-well plates, grown overnight to approximately 95 % confluence, and pre-treated with serum- and antibiotic-free medium containing 10 mM PPG for 24 hours prior to incubation in serum- and antibiotic-free medium containing 200 μM $CoCl_2$ for 24 hours. MTT reagent was added at t = 20 hours ($CoCl_2$ phase) and absorbance was measured spectrophotometrically at 490 nm (650 nm correction wavelength) at t = 24 hours. Normal viability was standardized to the unstressed control condition. Bars represent mean ± SEM of 3 independent experiments. Connected lines indicate relationship; * denotes significance of $p < 0.05$; *** denotes significance of $p < 0.01$; *** denotes significance of $p < 0.001$.

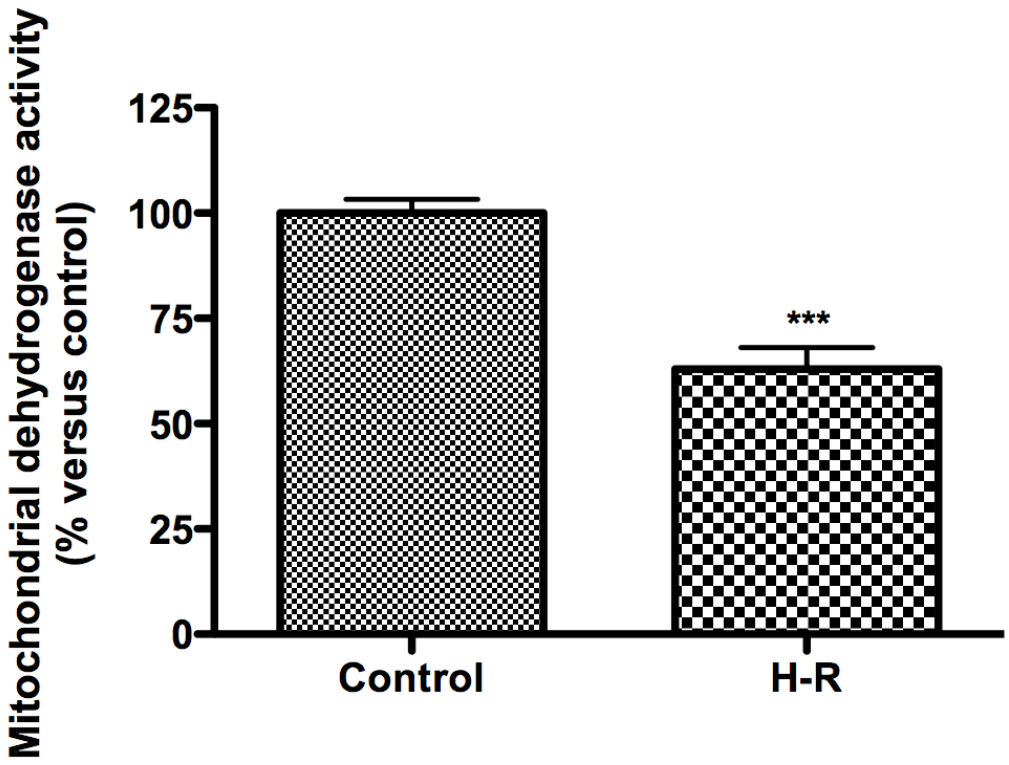

Figure 22. Effect of H-R on HL-1 cardiomyocyte viability. The effect of H-R on HL-1 cardiomyocyte viability was assessed via colourimetric measurement of mitochondrial dehydrogenase activity (MTT assay). Cells were seeded onto 96-well plates, grown overnight to approximately 95 % confluence, and incubated in serum- and antibiotic-free medium for 12 hours of hypoxia at 1 % O_2 followed by 4 hours of normoxia at ~20 % O_2. MTT reagent was added at t = 12 hours and absorbance was measured spectrophotometrically at 490 nm (650 nm correction wavelength) at t = 16 hours. Normal viability was standardized to the unstressed control condition. Bars represent mean ± SEM of 3 independent experiments. *** denotes significance of $p < 0.001$.

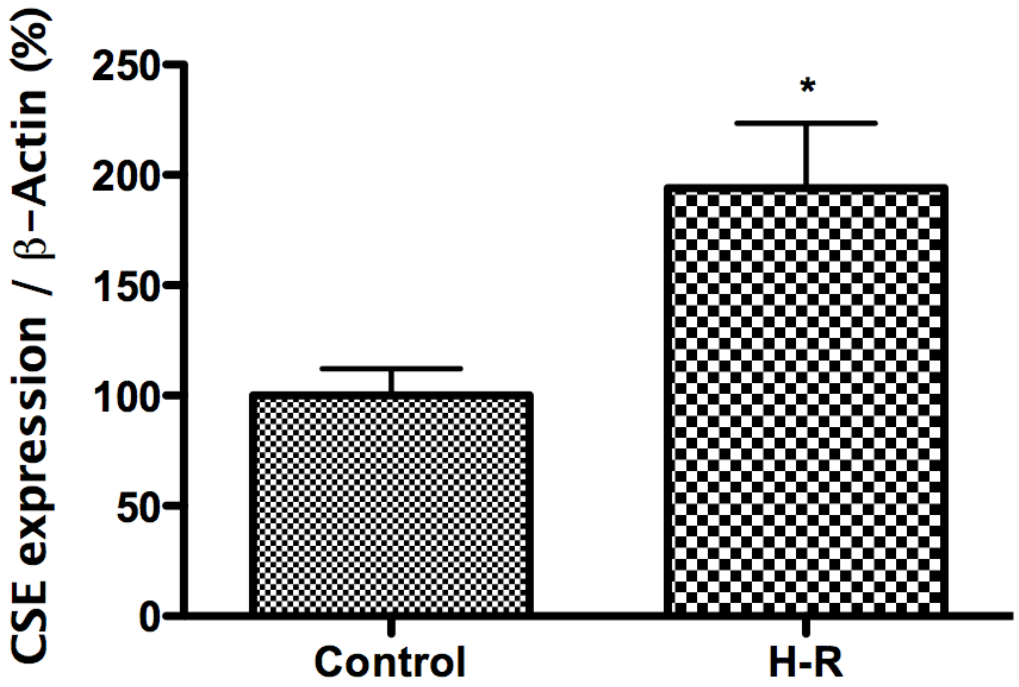

Figure 23. Effect of H-R on CSE protein expression in HL-1 cardiomyocytes. The effect of H-R on CSE protein expression in HL-1 cardiomyocytes was assessed via immunoblotting. Cells were seeded onto 75 cm^2 tissue culture flasks, grown overnight to approximately 95 % confluence, and incubated in serum- and antibiotic-free medium for 12 hours of hypoxia at 1 % O_2 followed by 4 hours of normoxia at ~20 % O_2. Cells were lysed via RIPA buffer and their protein levels quantitated via colourimetric, Lowry-based assay. Total proteins were separated via SDS-PAGE (verified via Coomassie Blue stain), and transferred electrophoretically to PVDF membranes (verified via Ponceau S stain). Membranes were blocked in milk solution and incubated with polyclonal primary antibody, then washed repeatedly, incubated with secondary antibody, and washed again. Membranes were analyzed via enhanced chemiluminescent detection imager and software-assisted densitometry. Bars represent mean ± SEM of 3 independent experiments. * denotes significance of $p < 0.05$.

Discussion

Project I - Hypoxic stress in CSE-deficient smooth muscle cells

CSE WT and KO vascular SMCs were subjected to hypoxia in order to discern potential H_2S/CSE pathway-related contributions to the SMC hypoxic stress response. The likelihood of differences in the two cell lines was predicated on evidence for an important relationship between O_2 tension and H_2S's cellular functions [256], as well as essential roles for H_2S in hypoxia-related vascular diseases such as hypoxic pulmonary hypertension [161, 215-224] and atherosclerosis [85, 90, 92, 272-276]. CSE-deficiency had previously been demonstrated to result in hypertension and impaired blood pressure regulation in the very mouse model from which the present SMCs were derived [99]. Moreover, we previously described markedly enhanced proliferation of CSE KO versus WT SMCs under basal conditions that was primary to the observed hypertension *in vivo*, as evidenced by a lack of change in cell proliferation upon CSE KO animal blood pressure normalization via captopril [94]. We also found the CSE-deficient SMCs to be more susceptible to exogenous H_2S-induced apoptosis, which was mediated by phosphorylation of ERK1/2 and altered expression of the cell cycle regulators, cyclin D1 and $p21^{(Cip/WAF-1)}$ [94]. This sensitivity of endogenous H_2S/CSE pathway-inhibited cells to exogenously-applied H_2S has previously been demonstrated to effectively inhibit SMC proliferation [93, 95], deficiency of which is thought to contribute to atherosclerosis [228]. Taken together, these data add considerable weight to the concept of a critical role for H_2S/CSE pathway in the regulation of vascular homeostasis.

Given that hypoxia is a ubiquitous feature of the pathogenesis of atherosclerosis and hypertension, and given the aforementioned importance of H_2S therein, here we endeavoured to pursue the hypothesis that CSE-deficient SMCs should exhibit compromised hypoxia stress

response and poorer survivability. To this end, we measured cell viability and apoptosis, as well as the expression of the master transcriptional regulator of the hypoxic stress response, HIF-1, which had recently been implicated in H_2S/CSE pathway signaling [300]. Given the important contributions of oxidative stress [114, 131] and inflammation [131, 181] to vascular disease, we sought also to evaluate differences in CSE WT and KO SMC redox and inflammatory status. Finally, due to the recent fervent interest in, and evidence for, the cellular effects of H_2S that centre on the mitochondrion [102], we compared mitochondrial membrane potential (Ψ_m) in the two SMC lines. We had previously demonstrated that the CSE KO SMCs did not feature detectable CSE mRNA or protein, and that their H_2S production was only a small fraction of their WT counterparts [91]. These investigations were undertaken with the knowledge that no published data existed on the SMC response to hypoxia in CSE genetic knockout animals or cell models, and were thus timely and relevant to the state of the art.

Even under basal conditions – that is, 24-hour incubation is serum- and antibiotic-free medium, but no specific stressor – stark differences in CSE WT and KO SMCs were observed. KO cells featured significantly higher metabolic activity than their WT counterparts, as measured by mitochondrial dehydrogenase activity via the MTT assay (**Figure 4**). This data indicates a substantially higher rate of oxidative phosphorylation in the KO cells, suggesting greater ATP production and mitochondrial activity therein. The higher relative metabolic activity is consistent with our previous finding that (serum-supplemented) KO cells exhibited greater proliferation than WT cells [94], in that proliferating SMCs have greater energy requirements that must be met by ramped up glycolysis and mitochondrial respiration.

Commensurate with just such a scenario of increased cellular respiration and/or mitochondrial function – and thus increased by-production of ROS – both intracellular ROS

levels and Ψ_m were significantly higher in CSE KO versus WT SMCs (**Figures 5 & 6**). Importantly, these data are consistent with the previously described protective roles for H_2S/CSE pathway in regulating redox balance [64-71] and suppressing mitochondrial activity [82, 99, 100]. Indeed, Blackstone & Roth (2007) suggested that the mitochondria-suppressive effect of exogenous H_2S could be employed therapeutically to protect against hypoxia. They discovered that, while mice could not survive longer than 20 minutes at 5 % O_2, H_2S pre-treatment afforded their survival for 6.5 hours, with no apparent detrimental effects [102]. In light of this, then, it is perhaps unsurprising that CSE deficiency was presently found to have the opposite effect; unbridled cellular metabolism (**Figures 4 & 7**) and growth [91], with concomitant increases in intracellular ROS (**Figure 5**). Taken together, these findings suggest a compromised ability of KO cells to regulate redox levels and/or the by-production of ROS resulting from deficient homeostatic H_2S-mediated mitochondrial regulation.

In order to test to the relative responses of CSE WT and KO SMCs to hypoxic stress, cells were incubated for 12 hours in a hypoxic chamber set to 1 % O_2. While the literature offers many and varied *in vitro* hypoxia regimes, the present protocol was chosen in order to approximate the chronic situation typical of the prolonged, attritional pathology of vascular conditions such as atherosclerosis and hypertension [228]. While CSE WT cell viability was not significantly impacted by hypoxia, KO cell viability was drastically reduced relative to control, as measured via MTT assay (**Figure 7**). Similarly, although both cell lines experienced significant apoptosis versus their respective controls, the rate of KO SMC apoptosis was considerably greater than that of the WT cells, as evidenced by positive staining for active caspases-3 and -7 measured via flow cytometry (**Figure 8**). Taken together, these data clearly indicate that CSE KO cells were more susceptible to hypoxia-induced death than their WT counterparts, suggesting an essential

contribution of H_2S/CSE pathway to some aspect(s) of the protective hypoxic stress response. This is consistent with the previous findings that impaired endogenous H_2S/CSE pathway contributes to the pathogenesis of hypertension [163, 257-268] and diabetes [277, 278], wherein hypoxic stress is an important factor. As well, these data point to the importance of specific H_2S/CSE levels in exerting a specific homeostatic effect. That is, the present findings indicate that deficient H_2S/CSE compromises SMC survivability, while other studies have demonstrated similarly pro-apoptotic effects resulting from exogenous H_2S administration [65, 93, 95, 98, 272] and/or CSE over-expression [65, 95, 272]. Given that the extent of the hypoxia-induced changes in apoptosis was deemed to be within a reasonable, physiologically relevant range, the present hypoxia protocol was retained for subsequent experiments.

Keenly in step with the concepts that H_2S protects against hypoxia in part by regulating mitochondrial function [87, 99] via stabilization of its structure [85], and as well as modulation of redox balance via direct [65-69] and indirect [64, 65, 68-71] antioxidant effects, an investigation of the factors underlying the observed hypoxia-induced SMC apoptosis was undertaken, with redox status and Ψ_m assessed via the colourimetric SOD activity assay and the fluorescent CM-H_2DCFDA and JC-1 assays, respectively. Hypoxia caused identical increases in SOD activity in CSE WT and KO cells, revealing enhanced antioxidant capacity in response to the low O_2 stress that was not visibly affected by H_2S/CSE pathway status (**Figure 9**). Despite this parity, however, intracellular ROS levels were significantly increased in hypoxic KO SMCs while unchanged in their stressed WT counterparts (**Figure 10**). Thus, given no differences in antioxidant capacity between hypoxic WT and KO cells despite substantially greater ROS elaboration in the latter, a pronounced hypoxia-induced redox imbalance was present in the CSE-deficient SMCs. These findings are in agreement with the concept that decreased H_2S/CSE pathway in the pathogenesis

of vascular disease [74, 218, 224, 273-276] contributed to the redox imbalance typical therein [114, 131]. Moreover, these findings are consistent with the previous study by Wei et al. (2008), where, in hypoxia-stressed lung tissues, H_2S increased T-AOC and decreased GSSG, but did not affect SOD [74]. Indeed, they noted that, even in the control group, wherein hypoxia caused decreased H_2S/CSE pathway, no changes in SOD were observed [74], as per our present findings.

Hypoxic insult of the SMCs also did not significantly affect Ψ_m in either cell line, but, as was the case under basal conditions, the absolute Ψ_m values remained significantly higher in CSE KO versus WT SMCs (**Figure 11**). Taken together, these data indicate a hypoxic stress-induced exaggeration of the redox and Ψ_m discrepancies between WT and KO cells observed under basal conditions, underscoring the substantial impact of CSE-deficiency in SMCs. Moreover, these data indicate a pronounced hypoxia-induced redox imbalance that may explain the significantly increased apoptosis and decreased viability of hypoxic CSE KO SMCs versus their WT counterparts.

Inflammation is an important contributory factor in atherogenesis [131, 181], and an anti-inflammatory role for H_2S has been demonstrated in several cardiovascular models [86] [277, 278]. Moreover, a significant relationship exists between oxidative stress and inflammation in cardiovascular diseases leading to heart failure, such that increased ROS has been shown to promote pro-inflammatory mediator expression [13, 135, 170, 179, 182, 183]. Given this context, and the present observation of a pronounced redox imbalance in CSE KO SMCs by virtue of hypoxia-induced ROS elaboration, assessment of the mRNA expression of several inflammatory mediators – TNF-α, IFN-γ, IL-10 and TGF-β – was undertaken via qPCR, with normalization via β2M.

Under basal conditions, CSE KO SMCs featured significantly lower expression of TNF-α (**Figure 12**) and IFN-γ (**Figure 13**), and significantly higher expression of IL-10 (**Figure 14**) and TGF-β (**Figure 15**) versus their WT counterparts. Taken together, these data indicate that the CSE-deficient cells featured inherently perturbed inflammatory mediator expression, as evidenced by their substantially lower pro-inflammatory cytokine/chemokine (TNF-α & IFN-γ) and massively higher anti-inflammatory cytokine (IL-10 & TGF-β) expression. However, it is unclear why this was the case. Indeed, the opposite scenario would be easier to explain, given the generally anti-inflammatory role described for H_2S [2, 26, 30, 86, 100]. That said, few studies have reported on inflammatory mediator expression vis-à-vis endogenous H_2S/CSE pathway status, so these findings are not necessarily out of step with any established trend. In particular, Li et al. (2005) found plasma TNF-α to be increased upon administration of exogenous H_2S, as well as decreased inflammatory mediator expression upon blockade of endogenous H_2S/CSE pathway in a mouse model of endotoxin-induced shock [30]. Here, one positive is consistency; both of the pro-inflammatory and both of the anti-inflammatory cytokines were decreased and increased, respectively, perhaps lending some credence to these observations. However, further experiments are certainly warranted, as is cautiousness regarding the interpretation of the present results in the interim.

Interesting, albeit similarly perplexing results were obtained upon 12-hour hypoxic insult of the SMC lines. Here, TNF-α expression was significantly decreased in WT but not KO cells (**Figure 12**). IFN-γ expression was decreased in WT but substantially increased in KO cells (**Figure 13**). IL-10 expression was massively increased in WT but substantially decreased in KO cells (**Figure 14**). TGF-β expression was increased in KO cells but massively increased in WT cells (**Figure 15**). Taken together, these data indicate that hypoxic stress elicited a more pro-

inflammatory environment in the KO cells despite their lower relative inflammatory environment versus WT cells under basal conditions, suggesting an altered, CSE-deficiency-related inflammatory response to hypoxic stress. We have previously reviewed the importance and redox-relevance of inflammatory imbalance – in particular, of high IL-10:TNF-α – in the progression to heart failure [13]. Consistent with this concept, here we observed much higher IL-10:TNF-α in basal KO versus WT cells that diminished greatly upon hypoxic insult. Moreover, the observed change toward a more pro-inflammatory intracellular environment in the hypoxic CSE KO cells was accompanied by a concomitant change toward a more oxidized intracellular environment, as per the SOD (**Figure 9**) and ROS level (**Figure 10**) data above. However, several specific issues with these findings are additionally noteworthy.

First, given that an antioxidant effect of IL-10 in cardiovascular tissues has been described [13, 174, 182], the observation that its CSE KO SMC expression was much higher than that of WT cells under basal conditions (**Figure 14**) despite higher ROS (**Figure 5**) is incompatible with this concept. That said, given that deficient homeostatic H_2S-mediated mitochondrial regulation, as evidenced by increased Ψ_m (**Figure 6**), was proposed as a potential reason for the increased ROS in basal KO cells, and given that it did not significantly change upon hypoxic stress (**Figure 11**), it is possible that Ψ_m-associated ROS production explains these differences. However, this interpretation is largely unsupported and highly conjectural, and thus is provided only because due analysis is required. Second, the stark decrease in CSE WT SMC TNF-α expression upon hypoxia is not supported by the literature, and the near-equivalent TNF-α expression in all other conditions (**Figure 12**) begs the question of artifact. Thus, despite their promise, these findings require further validation before any real conclusions should be drawn. Ideally, a well-established inflammatory control, such as LPS, should be employed in order to demonstrate clearly that the

CSE WT SMCs respond in a manner that can be clearly supported by the literature. Failure to provide these assurances undermines any KO cell findings, however interesting and accurate they might be.

The hypoxia stress response is essential to cellular survival in low O_2 conditions, which are ubiquitous in disease, and play major roles in the pathogenesis of atherosclerosis [129, 131] and hypertension [16, 114, 131, 241]. HIF-1 is recognized as a critical mediator of the response to hypoxia, activating over 200 genes encoding proteins that regulate cellular metabolism, proliferation, survival, motility and basement membrane integrity, as well as angiogenesis and hematopoiesis [185, 238, 242]. Given the patently poorer ability of the CSE-deficient SMCs to respond to hypoxic stress, and in light of new research suggesting an important interaction of H_2S and HIF-1 [255] and potential role in O_2 sensing [256], an assessment of the mRNA expression of HIF-1's O_2-sensitive alpha subunit, HIF-1α, was undertaken via qPCR, with normalization via β2M. 12-hour hypoxia induced increased HIF-1α expression in CSE KO SMCs, but a significantly greater increase in HIF-1α expression in WT cells (**Figure 16**). These data indicate a H_2S/CSE pathway-related impact on HIF-1α expression, which may have contributed to the inability of KO cells to respond to hypoxic stress as effectively as their WT counterparts, as evidenced by redox (**Figures 9 & 10**) and inflammatory (**Figures 12-15**) imbalance, and decreased survival (**Figures 7 & 8**).

Budde & Roth (2010) discovered that *hif-1* was required for *Caenorhabditis elegans* survival upon H_2S exposure, and that elevated HIF-1 activity dramatically increased the maximum tolerable concentration of H_2S. They also demonstrated that both H_2S and hypoxia induced HIF-1 protein expression, but in different tissue patterns [255]. These findings are consistent with the present data indicating that hypoxic CSE KO SMCs feature blunted HIF-1α

mRNA expression versus their WT counterparts (**Figure 16**). That is, if endogenous H_2S is greatly reduced (we observed ~7 % H_2S production in the CSE KO cells [91]), and H_2S acts as a regulator of *hif-1* by virtue of cells' requiring its expression to deal with H_2S [255], then HIF-1α is likely also to be reduced in H_2S/CSE pathway-deficient cells. However, no discernable difference was evident under basal conditions, as hypoxia was required to reveal a significant difference in HIF-1α expression between the CSE WT and KO SMCs (**Figure 16**).

Taken together, the results of these experiments indicate that CSE-deficiency predisposes SMCs to decreased survivability upon hypoxic insult. The hypoxia-induced decrease in CSE KO cell viability (**Figure 7**) and increase in apoptosis (**Figure 8**) was associated with significant elaboration of intracellular ROS (**Figure 10**), pro-inflammatory climate (**Figure 12-15**) and compromised HIF-1α expression (**Figure 16**). The latter finding begs the questions of whether deficient H_2S directly or indirectly caused decreased HIF-1α, which in turn led to a generally poorer hypoxia stress response, or whether KO cells were more susceptible to hypoxia because the HIF-1-mediated stress response involves H_2S/CSE pathway function. The latter possibility is encouraged by the present finding that the hypoxia mimetic, $CoCl_2$, which is known to activate HIF-1 via stabilization of its O_2-sensitive alpha subunit, induced increased CSE protein expression in HL-1 cardiomyocytes (**Figure 19**; discussed below). Regardless, both potential situations are remarkable, as they would represent new knowledge and novel downstream targets of HIF-1 and/or H_2S. Indeed, whatever the 'chicken or egg' relationship between H_2S and HIF-1, it is clear that H_2S is essential to the SMC response to hypoxia. Such discoveries would surely find purchase in many areas, including H_2S's role in hypoxic pulmonary hypertension, atherosclerosis and O_2-sensing, as well as the myriad processes wherein HIF-1 is already implicated, such as cancer biology [279] and immune function [280]. Moreover, the present

findings provide a possible link between the phenomena of endogenous H_2S-mediated protection against hypoxia [102], haemorrhage [281] and I-R injury [85], as well as hypoxic and sulfide pre- [64, 190] and post-conditioning [191, 197], and O_2-sensing [36].

Project II - CSE in simulated hypoxia and hypoxia-reoxygenation cardiac stress

Hypoxia is a ubiquitous feature of pathologies including myocardial ischemia, stroke, inflammation and cancer [238]. By limiting O_2 availability for the electron transport chain and/or inhibiting its activity, hypoxia enhances electron leak, leading to oxidative stress. By virtue of their continuous exposure to ROS, mitochondria accumulate oxidative damage rapidly, and their dysfunction has been identified in many studies of ROS-induced cell death [257]. Increasing evidence indicates that hypoxia-induced ROS play a key role in promoting cytochrome c release in the mitochondria, leading to apoptosis [308, [282]. A cardioprotective role for H_2S has been described, and, in addition to its antioxidant [67-74] and anti-inflammatory [82, 84-87] effects, an important aspect of its cytoprotection appears to be stabilization of mitochondrial structure and function [85]. Indeed, in multiple models, H_2S has been shown to suppress metabolic activity via inhibition of cytochrome c release [102, 103], leading to the proposal for therapies based on this function [102]. Thus, H_2S is an attractive candidate for combating hypoxic stress, lending to the increasingly clear importance of its homeostatic role in the cardiovascular system, wherein it has been implicated in a variety of processes including ischemic pre- [50, 247-253] and post-conditioning [255-256], pulmonary hypertension [161, 215-224] and atherosclerosis [288-292]. However, the specifics of hypoxic regulation of H_2S/CSE pathway remain largely unexplored, and progress in old and new avenues of H_2S research alike, such as its newly proposed role in O_2 sensing [104], will depend heavily on an improved understanding of these processes.

To deal with hypoxic stress, cells employ adaptive mechanisms including activation of genes essential for cell survival, a process that is generally mediated by hypoxia inducible factor-1 (HIF-1), a transcriptional complex that binds to the specific hypoxia response element of target

genes [241]. A heterodimer, HIF-1 is composed of the rate limiting factor, Hif-1α, and the constitutively expressed Hif-1β (also called the aryl hydrocarbon receptor nuclear translocator). Hif-1α is expressed during normoxia, but is unstable due to constant rapid, proteasome-mediated degradation via ubiqitination of the hydroxylated, O_2-dependent degradation domain of the α-subunit by the product of the Von Hippel Lindau (VHL) tumor suppressor gene. Under hypoxic conditions, however, the VHL protein cannot recognize Hif-1a, leading to HIF-1 accumulation [289, 308]. In the ischemic myocardium, for example, HIF-1 mediates the transcription of the vascular endothelial growth factor (VEGF), adrenomedullin, ET-1 and atrial natriuretic peptide and other stress-responsive genes [308]. Uniquely, cobalt chloride ($CoCl_2$) acts as a hypoxia mimetic via stabilization of HIF-1 via inhibition of the Hif-1α-inhibiting prolyl hydroxylases, thereby inducing HIF-1's downstream genetic program [283, 284].

Vassilopoulos et al. (2005) employed $CoCl_2$ to activate HIF-1 and simulate hypoxia in the cardiomyocyte cell line, HL-1. Briefly, they found that $CoCl_2$ caused ROS elaboration, leading to decreased cardiomyocyte viability. Implicit in this process was ATP depletion, mPTP opening, Bax translocation to the mitochondria and cytochrome c release. They also discovered that prolonged and/or severe simulated hypoxia caused destabilization of Hif-1α. The $CoCl_2$-induced mitochondrial changes leading to decreased cell survival, as well as Hif-1α destabilization were attenuated by pre-treatment with various antioxidants, indicating that ROS was the principal mechanism of $CoCl_2$'s effect [308]. Given the similarity of $CoCl_2$-induced simulated hypoxia to the hypoxia-induced processes in the ischemic myocardium [13, 226], the specificity of $CoCl_2$ for HIF-1 activation [283, 284], and the previously described role for H_2S/CSE pathway in hypoxic conditions (REF), here we endeavoured to explore the potential involvement of H_2S/CSE pathway in $CoCl_2$-induced simulated hypoxia in HL-1 cardiomyocytes.

The mouse atrial cardiomyocyte cell line, HL-1, is an excellent choice for *in vitro* modelng of ischemic damage because it retains its differentiated phenotype indefinitely [259, 260], and I-R injury only ever occurs in mature cardiomyocytes [13]. Moreover, given that Vassilopoulos et al. (2005) used this very cell line for their own $CoCl_2$ studies [308], ready validation vis-à-vis the literature was possible. In an effort to reproduce, in part, their simulated hypoxia model, a variety of $CoCl_2$ concentrations and treatment durations were evaluated via colourimetric MTT assay (data not shown). Of these, the 24-hour treatment with 200 μM $CoCl_2$ was chosen for continued study. HL-1 cardiomyocyte viability was significantly decreased under these conditions (**Figure 17**), and to a very similar extent as previously reported by Vassilopoulos et al. [257]. As ROS elaboration had previously been shown to underlie decreased HL-1 cell viability, intracellular ROS level was assessed via the fluorescent CM-H_2DCFDA assay (using 'snapshot' method), with analysis via flow cytometry. 24 hours of 200 μM $CoCl_2$ induced significantly increased ROS elaboration in the HL-1 cardiomyocytes (**Figure 18**), thereby agreeing with the previously described finding that ROS elaboration was the principal mechanism for decreased HL-1 viability [257]. As Vassilopoulos et al. (2005) did not actually measure ROS production in their studies, but rather used antioxidant pre-treatment to obtain their findings, the present data was deemed to be a sufficient basic recreation of the previous model, and similar use of antioxidants was not undertaken.

Given that the endogenous H_2S/CSE pathway had previously been shown to be cardioprotective against ischemic insult [66, 85, 271], and in an effort to implicate its activity downstream of the HIF-1-mediated signaling cascade, HL-1 cardiomyocytes were subjected to 24 hours of 200 μM $CoCl_2$, and CSE protein expression assessed via immunoblotting, with analysis via densitometry. CSE expression was significantly increased (**Figure 19**), indicating that

H_2S/CSE pathway is increased upon $CoCl_2$-induced HIF-1 activation. These data suggest that CSE may be a novel downstream target of HIF-1 and/or that H_2S/CSE pathway may be involved in the hypoxic stress response. Moreover, these findings are consistent with the widely supported cardioprotective role for H_2S/CSE pathway, and could inform the recent evidence for an O_2-sensing role for H_2S [36], as well as the recent evidence for H_2S/HIF-1 interaction in *C. elegans* [255].

Given the observed $CoCl_2$-induced CSE expression, inhibition of CSE via the irreversible chemical inhibitor, PPG was undertaken in order to investigate the potential contributions of H_2S/CSE pathway to cardiomyocyte function upon $CoCl_2$ stress. Based on the available literature, a 24-hour concentration course of PPG concentrations including 100 μM, 1 mM and 10 mM was performed, and cell viability was assessed via the colourimetric MTT assay (wherein MTT reagent was added at t = 20 hours). None of the evaluated PPG concentrations induced significant change in cell viability from control (**Figure 20**). As a result, the highest PPG concentration, 10 mM, was selected for future experiments; the thinking being that it should have the greatest CSE-inhibitory effect. A variety of PPG concentrations have been used in different *in vitro* models, ranging from 200 μM [48] to 1-5 mM [47, 53, 285] to 10 mM [285]. In the latter study, while no detrimental effects of 10 mM PPG were reported, it did cause increased HO-1 expression in aortic SMCs at 12 hours, and increased carboxyhemoglobin levels at 18 hours [334]. This raises the possibility that CO/HO-1 pathway could have impacted the HL-1 cardiomyocytes in the presently described experiments. A recent study observed CO to increase HIF-1α and VEGF-β expression in cardiomyocytes [286]; theoretically, this would have little effect in a model whereby HIF-1α is already activated, and by a very specific and potent inducer, $CoCl_2$. However, ischemic insult has been shown increase CO/HO-1 pathway cardiac tissues [287], and hypoxia-

inducible CO has been shown to have cardioprotective effects that include an antioxidant element [288, 289]. Thus, as CO/HO-1 pathway was not evaluated in the following experiments, the possibility of their involvement via PPG-mediated inducement cannot be discounted.

The effect of PPG-mediated CSE inhibition in $CoCl_2$-stressed HL-1 cardiomyocytes was investigated via MTT assay, wherein cells were pre-treated with the previously optimized PPG concentration, 10 mM, and subjected to 200 μM $CoCl_2$ as previously described. While $CoCl_2$ significantly reduced cell viability (**Figure 21**) and PPG control had no effect, echoing the existing results (**Figure 17** and **20**, respectively), PPG + $CoCl_2$ resulted in much lower viability versus control (**Figure 21**) than $CoCl_2$ alone. These data indicate that PPG pre-treatment of $CoCl_2$-stressed cardiomyocytes was significantly detrimental to cardiomyocyte survival versus the stressor alone, strongly indicating a protective contribution of endogenous H_2S/CSE pathway and/or an essential stress-responsive role.

Taken together, these data suggest that H_2S/CSE pathway is stimulated downstream of HIF-1, and that endogenous H_2S/CSE pathway has an essential, cardioprotective role in abrogating (simulated) hypoxia-induced damage. The present data also lend credence to the novel findings of Budde & Roth (2010), who discovered that *hif-1* was required for *C. elegans* survival upon H_2S exposure, and that elevated HIF-1 activity dramatically increased the maximum tolerable concentration of H_2S [305]. Here, Budde & Roth found that H_2S induced HIF-1, which is consistent with our present data from the CSE WT and KO SMC model, wherein blunted HIF-1α expression was present upon hypoxic insult (**Figure 16**). It is important to note that the present proposal – that CSE is a novel downstream target of HIF-1 – is distinct from these findings, and, if verified, would represent new knowledge. Indeed, given the recent revelations regarding H_2S's integral relationship with O_2 homeostasis [256], the meta-concept of inter-

regulation between H_2S and HIF-1 is plausible, and certainly worthy of directed study. As mentioned above, whatever the 'chicken or egg' relationship between H_2S and HIF-1, it is clear that H_2S is essential to both the SMC and cardiomyocyte responses to hypoxia. One point of caution in moving forward in this avenue of investigation is that both H_2S and HIF-1 are widely influenced, and influential, and careful reductionist control would therefore need to be exercised.

I-R injury is a major complicating feature of clinical disease entities including MI and stroke [2, 13]. A cardioprotective role for endogenous H_2S/CSE pathway has been demonstrated [85], but few *in vitro* studies exist, leaving much yet to be explained. Given the present observation of simulated hypoxia-induced CSE protein expression, we endeavoured to investigate whether H-R stress would have a similar effect. Thus, following consideration of the available literature, and in an effort to produce conditions that would approximate the *in vivo* situation for cardiomyocytes experiencing I-R injury, HL-1 cardiomyocytes were subjected to an H-R regimen as follows: 12 hours of hypoxia (1 % O_2) followed by 4 hours of normoxia (~20 % O_2). Cell viability was assessed via the colourimetric MTT assay, wherein MTT reagent was added to cultures at t = 12 hours (upon completion of the hypoxia phase) and incubated for the remaining duration of the H-R regimen (4 hours normoxia), before analysis at t = 16 hours. Cell viability was significantly decreased in H-R-challenged HL-1 cardiomyocytes (**Figure 22**). These data indicate that HL-1 cardiomyocytes were significantly susceptible to H-R stress; thus was the present H-R protocol retained for subsequent experiments. CSE protein expression was assessed via immunoblot in H-R-challenged HL-1 cardiomyocytes, with analysis via densitometry. H-R induced significantly increased CSE expression (**Figure 23**) in the HL-1 cardiomyocytes, on the order of that observed under $CoCl_2$ stress (**Figure 19**). Indeed, taken together with the observed increase in CSE expression in response to $CoCl_2$-mediated HIF-1 activation, these data

collectively indicate that simulated and true hypoxia alike induce H_2S/CSE pathway activation, possibly downstream of HIF-1-mediated signaling, and are thus consistent with the concept of a cardioprotective role for H_2S/CSE pathway.

There are a number of limitations to this study that must be acknowledged. First, H_2S proper was not measured directly. Instead, the H_2S-synthesizing enzyme, CSE, was used as a proxy for H_2S/CSE pathway. Indeed, CSE was measured and its endogenous activity altered (via chemical inhibition and genetic knockdown), but no specific reconciliation of CSE and cellular H_2S levels are provided here in support of these findings. That said, in the case of the genetic CSE knockout model, we had already shown that the CSE-deficient SMCs featured no detectable CSE protein or mRNA expression, and that H_2S levels were very low (~7 % versus WT) [94]. It was due to these recent data, as well as the project time constraints, that these experiments were not repeated. However, as a result it is not possible to say for certain that a given change in CSE expression correlated with a similar change in H_2S level. Here we use the term "H_2S/CSE pathway" in the context of WT versus KO SMCs, chemically inhibited CSE (via PPG) and CSE protein expression data.

Second, cellular metabolism was measured via mitochondrial dehydrogenase activity (MTT assay). This assay is typically used to measure decreases in cell viability upon some intervention. However, although atypical, the test principle is suitable for the type of interpretation presented here. That said, the strength of these interpretations would be greatly enhanced by another, sufficiently different cellular metabolism readout, such as ATP measurement. Finally, the use of exogenous H_2S, administered via an H_2S donor compound such as sodium hydrosulfide [212], would provide some proof-of-concept support for the present data.

Conclusions

A rapidly growing body of evidence links H_2S with NO and CO as the third endogenously-produced gaseous signaling transmitter, or gasotransmitter. Reports of its physiopharmacological effects indicate its ubiquitous presence throughout mammalian tissues, where it has been shown to exert many and varied effects. H_2S, which is generated enzymatically by the enzyme, CSE, is a critical regulator of cardiovascular homeostasis, with essential roles in maintenance of vascular tone, pre- and post-conditioning and cardioprotection against ischemia-reperfusion injury, as well as inhibition of pathological processes underlying hypertension, atherosclerosis and diabetes. Hypoxia is a common feature of normal and disease processes alike, and the relevance of H_2S to O_2 homeostasis is increasingly relevant by virtue of the recent evidence for its role in O_2-sensing and interaction with the master transcriptional regulator of the hypoxia stress repsonse, HIF-1. The results of these *in vitro* studies demonstrated that CSE-deficient smooth muscle cells were significantly more susceptible to hypoxic stress than their normal counterparts, as evidenced by redox and inflammatory imbalance, and heightened mitochondrial activity and cell metabolism. As per their blunted HIF-1 expression upon hypoxic insult, deficient hypoxia stress response may have been the underlying cause of the observed susceptibility. Moreover, in models of cobalt-induced, simulated hypoxia, as well as hypoxia-reoxygenation, adult cardiomyocytes exhibited increased CSE expression; inhibition of which caused decreased cell viability. Taken together, these findings suggest an essential role for H2S/CSE pathway in the hypoxia stress reponse, and indicate the potential involvement of HIF-1 in their hypoxic regulation. Further study is warranted, and, if validated, these findings may represent novel, highly relevant knowledge of H_2S's function in hypoxia and HIF-1-mediated processes in cardiovascular tissues.

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