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KATTRI-LIIS ESKLA

Therapeutic strategies for ischemia reperfusion injury

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UNIVERSITY OF TARTU
Press

Department of Physiology, Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia

Dissertation was accepted for the commencement of the degree of Doctor of Philosophy (Neurosciences) on June 20, 2019, by the Joint Council for the Curriculum of Neurosciences

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Commencement: September 27th, 2019

This research was supported by the European Regional Development Fund (Project No. 2014-2020.4.01.15-0012) and Estonian Research Council (PUT120, PUT1077, IUT 20-42). It was also funded by grants from the American Heart Association (15POST25610016, 17GRNT33670975, 16GRNT31190016) and the National Institutes of Health (NIH) (R01DK115213, R01HL136915, 5R01HL098481-05, 1R01HL092141, 1R01HL093579, 1U24HL094373, 1P20HL113452). This work was also supported by funding from the Carlyle Fraser Heart Center of Emory University Hospital Midtown and an operation grant from the Canadian Institutes of Health Research (MOP 119565).

ISSN 1736-2792
ISBN 978-9949-03-146-7 (print)
ISBN 978-9949-03-147-4 (pdf)

University of Tartu Press
www.tyk.ee

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LIST OF ORIGINAL PUBLICATIONS

I. **Eskla KL**, Porosk R, Reimets R, Visnapuu T, Hundahl CA, Vasar E, Luuk H. (2018). Hypothermia augments stress response in mammalian cells. Free Radical Biology and Medicine. 121:157–168

II. Shimizu Y, Polavarapu R, **Eskla KL**, Nicholson CK, Koczor CA, Wang R, Lewis W, Shiva S, Lefer DJ, Calvert JW. (2018). Hydrogen sulfide regulates cardiac mitochondrial biogenesis via the activation of AMPK. Journal of Molecular and Cellular Cardiology. 116:29–40

III. Shimizu Y, Polavarapu R, **Eskla KL**, Pantner Y, Nicholson CK, Ishii M, Brunnhoelzl D, Mauria R, Husain A, Naqvi N, Murohara T, Calvert JW. (2018). Impact of Lymphangiogenesis on Cardiac Remodeling After Ischemia and Reperfusion Injury. Journal of the American Heart Association. 7(19):e009565

Contribution of the author:
I – The author participated in designing of the study, performed cell culture work, luciferase-reporter assay, gene and protein expression experiments, carried out the statistical analysis, wrote most parts of the manuscript and handled correspondence.
II – The author participated in designing of the study, performed gene and protein expression experiments (jointly with Yuuki Shimizu and Rohini Polovarapu), mitochondria respiration and ATP measurements (jointly with John W Calvert).
III – The author participated in designing of the study, performed protein expression experiments (jointly with Yuuki Shimizu), and echocardiograph analysis (jointly with Yuuki Shimizu and John W Calvert).

ABBREVIATIONS

3-MST	3-mercaptopyruvate sulfur transferase
ADP	Adenosine diphosphate
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AP1	Activator protein 1
ARE	Antioxidant response element
ATF	Activating transcription factor
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
Atp5b	ATP synthase F1 subunit beta
BCA	Bicinchoninic acid
BNIP3	BCL2 interacting protein 3
BNIP3L	BCL2 interacting protein 3 like
BrdU	Bromodeoxyuridine
CAT	Cysteine aminotransferase
CBS	Cystathionine β-synthase
CEL	Cell-intensity
CGL/CSE	Cystathionine γ-lyase
Cirbp	Cold-inducible RNA-binding protein
CO	Carbon monoxide
COX1	Cytochrome c oxidase subunit I
COX2	Cyclooxygenase 2
COX4i1	Cytochrome c oxidase subunit 4 isoform 1
CREB	cAMP responsive element-binding
CSE	Cystathionase-γ-lyase
DAPI	H-1500-4′,6-Diamidino-2- phenylindole
DC	Detergent compatible
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
dpc	Days post coitum
Drp1	Dynamin related protein 1
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK1/2/MAPK	Mitogen-activated protein kinase
ERRα	Estrogen-related receptor alpha
ERSE	Endoplasmic reticulum stress response element
ETS	E26 transformation-specific or E-twenty-six
EYFP	Enhanced yellow fluorescent protein
FBS	Fetal bovine serum

FDR	False discovery rate
Fis1	Fission, mitochondrial 1
Gclc	Glutamate-cysteine ligase catalytic subunit
GSH	Reduced glutathione
Gss	Glutathione synthetase
GSSG	Glutathione disulfide
H_2S	Hydrogen sulfide
HIF1A	Hypoxia inducible factor 1 subunit alpha
HPRT	Hypoxanthine guanine phosphoribosyl transferase
I/R	Ischemia and reperfusion
IL-1 beta	Interleukin-1 beta
IL-6	Interleukin 6
JAK	Janus kinase
KO	Knock out
LCA	Left coronary artery
LV	Left ventricular
LVEDD	Left ventricular end-diastolic diameter
LVESD	Left ventricular end-systolic diameter
LYVE1	Lymphatic vessel endothelial hyaluronan receptor 1
MEF	Mouse embryonic fibroblast
MEM	Minimum Essential Media
Mfn1	Mitofusin 1
Mfn2	Mitofusin 2
mPTP	Mitochondrial permeability transition pore
Mt-Co1	Mitochondrially encoded cytochrome C oxidase I
NAb	Neutralizing antibody
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NO	Nitric oxide
NRF1	Nuclear respiratory factor 1
Nrf2	Nuclear factor erythroid 2–related factor 2
Opa-1	OPA1 mitochondrial dynamin like GTPase
p38 MAPK	p38 mitogen-activated protein kinases
p5xATF6-GL3	5x activating transcription factor 6 site luciferase reporter gene
PBS	Phosphate buffered saline
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphoinositide 3-kinase
PINK1	PTEN-induced kinase 1
PPARα	Peroxisome proliferator-activated receptor alpha
pRL-CMV	CMV immediate early enhancer/promoter region
pRL-TK	HSV-thymidine kinase promoter
PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR

Rbm3	RNA Binding Motif Protein 3
ROS	Reactive oxygen species
RT	Room temperature
SAMS	AMPK synthetic substrate peptide
SAPE	Phycoerythrin conjugated streptavidin sape
SEM	Standard error of the mean
Sirt1	Sirtuin 1
Sp1	Sp1 transcription factor
Srxn1	Sulfiredoxin 1
STAT	Signal transducer and activator of transcription
Tert-BHQ	tert-Butylhydroquinone
Tfam	Mitochondrial transcription factor 1
Tg	Transgenic
TGX	Tris-Glycine eXtended
TNF-α	Tumor necrosis factor alpha
Trxr1	Thioredoxin reductase 1
UPR	Unfolded protein response
VEGF-A	Vascular endothelial growth factor A
VEGF-C	Vascular endothelial growth factor C
VEGFR2	VEGF receptor 2
VEGFR3	VEGF receptor 3
WST-1	Water soluble tetrazolium salt 1
WT	Wild-Type
XBP1	X-box binding protein 1
Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
ZF5	Zinc finger and BTB domain containing 14
αMHC	α-myosin heavy chain
αMHC-Cre^{+} x AMPK $^{f/f}$	cardiac specific AMPKα2 deficient mice

INTRODUCTION

Ischemic-hypoxic injury is a life-threatening condition that can be triggered by a heart attack, cardiac arrest, stroke, and neonatal hypoxia. In all of these conditions, the patient's survival depends on the duration of ischemic-hypoxic insult until blood circulation is re-established. Modern pharmacological intervention and diagnostic increase chances of survival. Despite these advances, however, the chance of a patient making a full recovery following ischemia reperfusion injury is still very low. There is a need to help these patients not only survive, but also have a normal quality of life. Ischemic-hypoxic injury is a complex condition where molecular events range from metabolic shift to the activation of various stress responses such as inflammation, endoplasmic reticulum (ER) stress and oxidative stress (Burwell et al., 2009; Eltzschig and Eckle, 2011; Murphy and Steenbergen, 2008; Nakka et al., 2010; Tajiri et al., 2004; Yellon and Hausenloy, 2007). Thus, a coordinated approach between different therapeutic approaches may increase the odds of survival and recovery. In this dissertation, the focus is on hypothermia, hydrogen sulfide (H_2S) and lymphangiogenesis. The aim was to study the therapeutic mechanisms that might be protective against ischemia reperfusion injury in order to enhance both our knowledge of ischemia reperfusion injury pathophysiology and mechanisms of protection. First, we demonstrate that mild hypothermia (32°C) activates major stress-inducible transcription factors nuclear factor erythroid 2–related factor 2 (Nrf2) and hypoxia inducible factor 1 subunit alpha (HIF1A), affecting the antioxidant system and hypoxia response pathways, respectively (Eskla et al., 2018). It is widely accepted that the therapeutic effects of hypothermia are due to metabolic depression caused by thermodynamic effects (Van't Hoff's rule) (Luscombe and Andrzejowski, 2006). However, our results suggest that hypothermia activates specific signaling pathways leading to increased stress tolerance during oxygen restriction (Eskla et al, 2018). Next, we studied the molecular mechanisms mediating the actions of hydrogen sulfide (H_2S) in regulating mitochondrial biogenesis. We show here that not only does H_2S regulate mitochondrial biogenesis via AMP-activated protein kinase – peroxisome proliferator-activated receptor gamma coactivator 1-alpha (AMPK-PGC1α) but also that correcting H_2S levels with SG-1002 could protect against heart failure by increasing cardiac mitochondrial content, improving mitochondrial respiration, adenosine triphosphate (ATP) production efficiency, and cardiac function (Shimizu et al., 2018). Finally, we demonstrated that chronic myocardial ischemia and myocardial ischemia reperfusion both stimulate an endogenous lymphangiogenesis response. This study is one of the first to demonstrate that the endogenous lymphangiogenesis response is initiated during the first week after the onset of myocardial ischemia reperfusion injury. Furthermore, inhibition of the endogenous lymphangiogenesis response exacerbates ischemic heart failure (Shimizu et al., 2018).

In long term view, this dissertation aims to provide clues to additional opportunities that could be translated into more efficient therapeutic approaches against ischemia reperfusion injury. There is a surprisingly large gap between the broad application of clinical hypothermia and the understanding of its therapeutic mechanisms. Current results highlight the importance of the cellular stress response systems as potential targets of clinical hypothermia. It offers a novel framework for future investigations seeking to explore the therapeutic mechanisms of hypothermia in animal models and patients. Therapeutic strategies to increase H_2S have shown to be cardioprotective. The merger of H_2S therapy and pharmacological agents targeting AMPK-PGC1α signaling pathway expand insight into strategies for treatment of heart failure. More importantly, this highlights H_2S among the potential new therapies for treatment of heart failure. The lymphangiogenesis study imposes the necessity for further exploration of the use of exogenous vascular endothelial growth factor C (VEGF-C) and its local delivery strategy over systemic delivery. In addition, combination of pro-lymphangiogenic factors with known modulators of inflammation and angiogenesis would extend therapeutic avenues to ischemia reperfusion injury treatment.

REVIEW OF LITERATURE

Pathophysiology of ischemia reperfusion injury

Normal tissue function requires continuous supply of oxygen and nutrients. Lack of blood flow causes an imbalance between the demand and supply of oxygen and nutrients (ischemia) resulting in profound tissue hypoxia and microvascular dysfunction (Visovatti et al., 2011). Surprisingly, restoring blood flow to the ischemic tissue, reperfusion, can trigger an additional cascade of injurious events (Yellon and Hausenloy, 2007). For example, the histopathological signs of injury after 3 h of liver or intestinal ischemia followed by 1 h of reperfusion are more profound than after 4 h of ischemia alone (Varadarajan et al., 2004; Parks et al., 1986). When a coronary vessel is occluded, intracellular respiration switches from aerobic to anaerobic (Figure 1). ATP production by mitochondrial oxidative phosphorylation depends on oxygen, and hypoxia leads to a rapid decrease in ATP production (Braasch et al., 1968), causing ADP, AMP, and adenosine to accumulate in the tissue. As a consequence of anaerobic glycolysis, hydrogen ions (Frank et al., 2012) and lactate accumulate (Braasch et al., 1968; Herdson et al., 1969; Visovatti et al., 2011). Lactate lowers intracellular pH below 7.0. Intracellular acidification activates Na^+-H^+ ion exchanger to extrude H^+ and results in intracellular Na^+ overload. Consequent increase in intracellular Na^+ activates the $2Na^+/Ca^{2+}$ exchanger, which extrudes Na+ from the cell in exchange for Ca^{2+} entry, resulting Ca^{2+} overload. ATP depletion during ischemia also leads to inhibition of Na^+K^+ATPase and an efflux of K^+ and an influx of Na^+, Ca^{2+}, Cl^-, and H_2O (Avkiran and Marber, 2002; Frank et al., 2012; Hausenloy and Yellon, 2013; Visovatti et al., 2011).

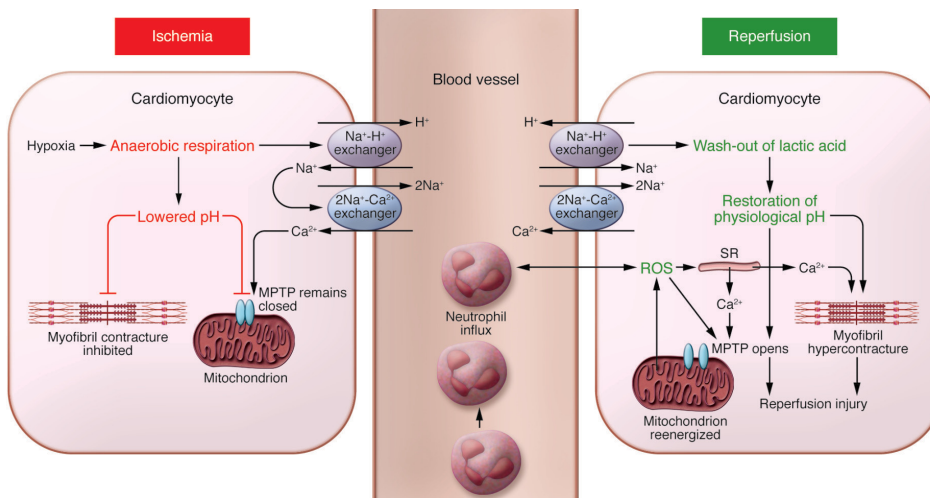

Figure 1. The main proponents of myocardial ischemia reperfusion injury. mPTP – mitochondrial permeability transition pore, SR – sarcoplasmic reticulum, ROS – reactive oxygen species (Hausenloy and Yellon, 2013).

Mitochondria are crucial in cardio-protective mechanisms. The inner mitochondrial membrane is normally impermeable to ions and proteins and maintains its transmembrane potential. Acidic conditions during ischemia prevent the opening of mitochondrial permeability transition pore (mPTP), a non-specific pore in the inner mitochondrial membrane. If mitochondrial permeabilization is minimal, the cell may recover; if moderate the cell may die from apoptosis; if severe, the cell may undergo necrosis. Reperfusion can result in the opening of mPTP in response to oxidative stress and rapid change in pH. Reperfusion and reactivation of Na^+-H^+ exchanger results in the washout of lactate and H^+ is transported into the extracellular space to normalize the pH in exchange for Na^+. The restoration of the mitochondrial membrane potential drives Ca^{2+} into the mitochondria, which can also induce mPTP opening (Frank et al., 2012; Hausenloy and Yellon, 2013).

Furthermore, ischemia triggers the activation of innate and adaptive immune responses and cell death programs (Eltzschig and Eckle, 2011). Thrombolytic therapy and primary percutaneous coronary interventions are treatments of choice for reducing acute myocardial ischemic injury, limiting myocardial ischemia size and improving clinical outcome. During reperfusion electron transport chain is reactivated, but instead of producing ATP it generates reactive oxygen species (ROS), such as superoxide anion (Roberts et al., 1990; Kim et al., 1994; Visovatti et al., 2011). ROS are crucial mediators of ischemia reperfusion injury – accumulation of free radicals overwhelms the tissues' antioxidant capacity, leading to the oxidation and loss of function of various biomolecules (cellular injury) followed by organelle (e.g. mitochondrial) disruption, activation of pro-inflammatory pathways and, ultimately, cell death by necrosis or apoptosis (Frank et al., 2012; Lin et al., 2016; Visovatti et al., 2011). Accordingly, strategies for reducing oxidative stress by increasing antioxidative potential or reducing ROS production would be promising therapeutic approaches to mitigating the effects of reperfusion injury.

Cardiac myocytes consume a large quantities of energy. Thus, it is not surprising that these cells have high density of mitochondria. To maintain mitochondria and aerobic energy reserve, the cell has developed multiple mechanisms that regulate mitochondrial homeostasis. The mitochondrial quality control system, consisting of mitophagy, fission and fusion, and biogenesis, is critically important in maintaining the fidelity of the heart under physiological and pathological conditions (Andres et al., 2015; Murphy et al., 2016; Dorn II et al., 2015). The cell has to identify damaged mitochondria, target them for selective mitochondrial autophagy and finally replace through mitochondrial biogenesis. When mitochondria are depolarized in response to various insults, PTEN-induced kinase 1 (PINK1) is stabilized on depolarized mitochondria. This leads to Parkin translocation to the outer membrane of mitochondria. Once Parkin translocates to the mitochondria, it facilitates the formation of autophagosomes (Ding and Yin, 2012; Youle and Narendra, 2011). Autophagy receptors like BCL2 interacting protein 3 (BNIP3) and BCL2 interacting protein 3 like (BNIP3L/NIX) localize on mitochondria and recruit autophagosomes to

damaged mitochondria by Parkin-independent manner (Zhang et al., 2008; Ding and Yin, 2012). Mitochondrial fission and fusion are important to repair damaged components of mitochondria for preserving mitochondria. Mitochondrial fusion is mediated by Mitofusin 1 (Mfn1), Mitofusin (Mfn2) and OPA1 mitochondrial dynamin like GTPase (OPA1). The exchange of material between healthy mitochondria via fusion allows to preserve the better mitochondria. Mitochondria fission allows for elimination of the bad quality mitochondria and requires the recruitment of dynamin related protein 1 (Drp1) from cytosol to mitochondria (Twig et al., 2008; Vander Bliek et al., 2013). PGC-1α is a master regulator of mitochondrial biogenesis and energy expenditure (Kubli et al., 2012; Fernandez-Marcos et al., 2011). PGC-1α regulates numerous transcription factors including peroxisome proliferator-activated receptor- α (PPARα), estrogen receptor–related α (ERRα) and nuclear respiratory factor 1 (NRF1) (Fernandez-Marcos et al., 2011). By regulating the transcriptional activities of these proteins, PGC-1α modulates genes involved in mitochondrial biogenesis and metabolic pathways. Mitochondrial content is significantly reduced in the failing hearts of both rodents and humans (Karamanlidis et al., 2010; Bayeva et al., 2013). Furthermore, downregulation of PGC-1α signaling has also been observed in experimental heart failure (Faerber et al., 2011). As such, understanding the mechanisms by which PGC-1α signaling is regulated in the heart could lead to the development of therapies aimed at inducing mitochondrial biogenesis and augmenting energy production in the setting of increased contractile demand (Bayeva et al., 2013).

Protection against ischemia reperfusion injury

Timely reperfusion is the only way to salvage ischemic injury. Although many treatments have been identified, the complexity of ischemia reperfusion injury has made difficult to develop effective therapeutics against it. The most studied modalities against ischemia reperfusion injury include non-pharmacological strategies sush as ischemic pre-conditioning, ischemic post-conditioning, and remote ischemic conditioning, or pharmacological strategies (Davidson et al., 2019). So far there have been no widely accepted therapies specifically targeting reperfusion injury. This is, at least in part, due to our limited understanding of the mechanisms of ischemia reperfusion injury that could be exploited therapeutically. Therapeutic approaches can also be divided according to the time they are applied – before, during, or after ischemia. In this regard, it is important that a potential treatment involves combination of two or more mechanisms that are also appropriately timed. An ideal therapeutic strategy would restore oxygen supply to affected tissues while minimizing cellular stress during reperfusion. In this dissertation potentially therapeutic approaches such as therapeutic hypothermia, hydrogen sulfide, and lymphangiogenesis are studied.

Therapeutic effects of hypothermia

Lowering of body temperature to preserve tissues is not a new concept. Numerous studies have shown that even small increases in body temperature (1–2°C) can significantly increase ischemic damage in rodent models (Wass et al., 1995; Dietrich et al., 1996; Kim et al., 1996). Conversely, mild therapeutic hypothermia of 32–35°C has shown consistent benefits against brain injuries in animal models (Barone et al., 1997; Colbourne et al., 1997; Ginsberg et al., 1991; Maher and Hachinski, 1992). Furthermore, it also improves the neurologic outcome of outside hospital cardiac arrest survivors (Bernard et al., 2002 HACA, 2002) and neonatal hypoxia ischemia (Azzopardi et al., 2014; Wagner et al., 1999). A number of studies in both experimental animal models and patients have demonstrated the efficacy of moderate hypothermia initiated a few hours after severe ischemia or circulatory arrest in reducing the subsequent neuronal death and improving behavioral recovery (Bernard et al., 2002; Kawai et al., 2000; Miyazawa et al., 2003; Yanamoto et al., 2001). However, not all patients may benefit from therapeutic hypothermia. While it has been shown to provide protection in cardiac arrest and neonatal encephalopathy, clinical trials for cardioprotection in myocardial infarction showed beneficial effects only in limited subgroups (Chenoune et al., 2010; O'Neill and Dixon, 2004; O'Neill et al., 2005; Villablanca et al., 2016).

Experimentally, the efficacy of hypothermia is highly dependent on the timing of initiation of cooling, its duration and depth. In most instances, the time of initiation should be less than 4–6 hours after the onset of ischemia. The body's core temperature is cooled to 32–34°C for 12–24 hours. Methods of cooling include placing ice packs around the head, torso and neck, use of a cold air mattress, or placing an intravenous catheter and infusing cold saline. After about 24 hours slow re-warming begins. The re-warming process should be very gradual over approximately an eight-hour period to reduce possible side effects (Gupta et al., 2005; Yenari and Hemmen, 2010). Lowered body temperature decreases brain oxygen consumption, glucose metabolism, lactate production, and accumulation of ROS. In addition, it preserves ATP, prevents apoptotic death, and inhibits inflammation. Hypothermia has also been shown to enhance angiogenesis and increase synaptogenesis (Yenari and Han, 2012).

Therapeutic hypothermia has been extensively studied in the laboratory, but most of the studies focus on its applications and efficacy in animal models. Consequently, little evidence is provided to advance our understanding of the underlying mechanisms. Ischemia and reperfusion are time-sensitive processes (Lee et al., 1999; White et al., 2000) and a major problem with therapeutic hypothermia is instituting it within a narrow window of opportunity. Ongoing clinical trials are focused on figuring out the depth (Chenoune et al., 2010; Duncker et al., 1996; Hamamoto et al., 2009) and duration (Colbourne et al., 1999; Maier et al., 2001; Yenari et al., 2008) of hypothermia including how to standardize re-warming (De Georgia et al., 2004; Georgiadis et al., 2002; Polderman et al., 2002; Schwab et al., 1998), prevent and reduce side effects

(Kawai et al., 2000; Meloni et al., 2008; Schubert, 1995), and how to identify patients most likely to benefit from therapeutic hypothermia. Clinical trials and animal studies help to improve hypothermia treatment, but relatively little knowledge has accumulated on why hypothermia provides any protection at all apart from inducing metabolic depression (Luscombe and Andrzejowski, 2006). From a research standpoint, finding molecular mechanisms behind the therapeutic effect of cooling is of paramount importance, as it will pave the way for designing drugs targeting therapeutically relevant pathways without the confounding effects associated with body cooling. While there are many animal studies available to show benefits of applying hypothermia to mitigate ischemic/hypoxic damage, on the molecular level, most have focused on effector mechanisms related to innate immune response and apoptosis. In our study, we took a step back and asked whether hypothermia could modulate the responsiveness of mechanisms coping with cellular stressors.

Cardioprotective effects of H_2S

H_2S is now recognized as a novel gaseous signaling molecule along with nitric oxide (NO) and carbon monoxide (CO), although it was considered to be a toxic gas for hundreds of years. H_2S is endogenously produced by three key enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CGL or CSE), and 3-mercaptopyruvate sulfur transferase (3-MST) together with cysteine aminotransferase (CAT) (Wang, 2002). CSE is the critical enzyme for H_2S production in the cardiovascular system (Yang et al., 2011).

A number of studies have demonstrated that endogenous and exogenous H_2S have cardioprotective effects in myocardial ischemia reperfusion injury (Bian et al., 2006; Elrod et al., 2007; Sivarajah et al., 2006; Bliksoen et al., 2008; Johansen et al., 2006; Calvert et al., 2010b; Sodha et al., 2008; Osipov et al, 2009; Ji et al., 2008), and cardiac disease may impair the endogenous synthesis of H_2S further exacerbating injury (Jiang et al., 2005; Polhemus et al., 2014). In the cardiovascular system, H_2S is involved in a wide range of protective mechanisms, such as the activation of anti-apoptotic (PI3K/Akt, ERK1/2/MAPK, JAK-STAT) (Hausenloy et al., 2006; Hu et al., 2008; Luan et al., 2012) and anti-inflammatory pathways (eNOS and p38 MAPK) (Kondo et al., 2013; Kaiser et al., 2004), increased antioxidative capacity (Jha et al., 2008; Calvert et al., 2009), and preservation of mitochondrial function (Elrod et al., 2007; Wang et al., 2011). As mentioned previously, ROS production is increased during ischemia reperfusion injury. H_2S is an inhibitor of cytochrome c oxidase and therefore inhibits respiration (Hill et al., 1984), which leads to decreased ROS production and preservation of mitochondrial structure and function (Calvert et al., 2010a; Aon et al., 2004; Chen et al., 2006). H_2S also influences the levels/activation of a number of proteins related to mitochondrial biogenesis (PGC1α (Pan et al., 2014; Untereiner et al., 2016); AMPK (Minamishima et al., 2009; Barr et al., 2015); endothelial nitric oxide synthase (eNOS) (Kondo et al.,

2013; Polhemus et al., 2013; Kind et al., 2014) and there is evidence that mitochondrial content is higher in brains (Pan et al., 2014) and hearts (Calvert et al., 2010b) treated with exogenous H_2S. These studies provided evidence for elevated mitochondrial levels in response to H_2S treatment. It is not clear if the observed increase was due to a direct effect of H_2S or was simply an indirect consequence of H_2S altering injury. Therefore, the main goal of the current study was to address this issue by determining if H_2S levels directly influence cardiac mitochondrial content under non-stressed conditions. Additionally, we sought to gain insights into the mechanisms by which H_2S induces mitochondrial biogenesis in the setting of myocardial ischemia-reperfusion.

Role of lymphangiogenesis in ischemia reperfusion injury

Lymphatic vessels exist throughout the body in the same manner as blood vessels (Kerjaschki et al., 2014). They interconnect with blood vessels to form an elaborate system that functions in interstitial fluid drainage, lipid absorption, and immune cell responses (Zheng et al., 2014). Lymphangiogenesis, or the growth of lymphatic vessels from preexisting vessels, is the major if not the exclusive mode of lymphatic growth. Signaling via VEGF-C and VEGF receptor 3 (VEGFR3) is perhaps the most central pathway for lymphangiogenesis. Disruption in lymphatic vessel formation during development (VEGF-C or VEGFR3 deficient mice) leads to death (Karkkainen et al., 2004; Veikkola et al., 2001). Additionally, the loss of lymphatic function in humans (hereditary disease, lymphatic damage or surgical removal of lymph nodes) leads to lymph edema (Adams et al., 2007; Alitalo et al., 2005). This and other, more recent evidence have led to the understanding that lymphatic vessels are not simply passive conduits for interstitial fluid, but rather are essential for multiple physiological activities (Kerjaschki et al., 2014).

The heart contains an elaborate network of lymphatic vessels, which serve to collect and return macromolecules, proteins, electrolytes, and fluid from the interstitial space to the circulation (Levick et al., 2010; Aspelund et al., 2016; Jones et al., 2011). As a result, the cardiac lymphatic system aids in the control of tissue pressure and edema formation (Aspelund et al., 2016). Interference with cardiac lymphatic flow (i.e. through obstruction) predisposes the heart to edema, inflammation, fibrosis, and infection (Kline et al., 1963). Additionally, impairments in cardiac lymphatic flow have deleterious affects on cardiac dysfunction in the setting of myocardial ischemia (Kline et al., 1964). Despite these well-documented actions, very little is known about the role the cardiac lymphatic system plays in the development of heart failure. Recent studies have begun to focus on this issue with the demonstration that myocardial ischemia initiates an endogenous lymphangiogenesis response (Klotz et al., 2015; Henri et al., 2016). Moreover, treatment with VEGF-C augments endogenous lymphangiogenesis and leads to improvements in cardiac function (Klotz et al., 2015; Henri et al., 2016). Thus, it appears that therapeutic lymphangiogenesis serves

as a promising new treatment of heart failure. While these findings have laid the foundation for the concept of therapeutic lymphangiogenesis, there are a number of issues related to the kinetics and functional significance of the response that need to be addressed. Additionally, the consequences of inhibiting the endogenous lymphangiogenesis response have not been explored. Herein, we addressed these issues in well-defined murine models of ischemia-induced heart failure.

AIMS OF THE STUDY

The general aim of this study was to gain additional insight into the molecular events triggered by ischemia and reperfusion. Ultimately a fused approach of different therapeutic modalities could help to achive optimal outcomes and lower fatality and morbidity of ischemia reperfusion injury. Based on the above-presented analysis of literature the specific aims of present dissertation were:

1. To survey the effect of hypothermia on selected cellular stress pathways with the potential to extend tolerance to ischemia reperfusion injury.
2. To determine if H_2S levels directly influence cardiac mitochondrial content. To gain insights into the mechanisms by which H_2S induces mitochondrial biogenesis in the setting of myocardial ischemia reperfusion.
3. To explore the role of cardiac lymphatic system in the development of heart failure and study the consequences of lymphangiogenesis response inhibition.

MATERIALS AND METHODS

1. In vitro cell culture (I)

1.1. Cell lines (I)

In paper I, HeLa cells (CCL-2) were purchased from ATCC and used for gene expression, glutathione, cell viability, western blot and luciferase reporter activity analysis. HIF luciferase reporter HeLa stable cell line (SL-0023, Signosis) and nuclear factor kappa light chain enhancer of activated B cells (NF-κB) luciferase reporter HepG2 stable cell line (SL-0017, Signosis) were used for luciferase reporter activity assay and gene expression analysis. Antioxidant response element (ARE) luciferase reporter HepG2 stable cell line (60513) was purchased from BPS Bioscience and used for gene expression, cell viability, western blot and luciferase reporter activity analysis. Mouse embryonic fibroblasts (MEFs) (Millipore) were used for gene expression, glutathione, and cell viability analysis.

All cell lines, except MEFs were cultured in low glucose Minimum Essential Media (MEM) (MEM-STA, Capricorn Scientific) supplemented with 10% fetal bovine serum (FBS) (2050-1, PAN Biotech), 1x penicillin/streptomycin (2007, Smart Media) at 37°C with a 5% CO_2 atmosphere and then switched to 32°C incubator with 5% CO_2 atmosphere during hypothermia experiment.

1.2. Isolation of Mouse Embryonic Fibroblasts (I)

To isolate Nrf2-KO (B6.129X1-Nfe2l2^{tm1Ywk}/J, 0017009, The Jackson Laboratory) MEFs, pregnant mice were sacrificed at 15.5 days post coitum (dpc) by cervical dislocation. Embryos were dissected into a 100-mm dish with phosphate buffered saline (PBS) (2002, Smart Media), head and red organs were removed, and washed with PBS three times. Embryos were placed in a clean Petri dish and the tissue was minced using a sterile razor blade. 1 mL of 0.25% trypsin/ ethylenediaminetetraacetic acid (EDTA) (25200056, Gibco, Thermo Fisher Scientific) per embryo was added and Petri dish was incubated for 10 min at 37°C. After 10 min of incubation, cells were dissociated by pipetting up and down. Again, 1 mL of 0.25% trypsin/EDTA per embryo was added followed by 10 min incubation at 37°C. This step was repeated 3 times. Trypsin was inactivated by adding 1 volume of MEF medium. MEFs were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) (E15-883, PAA) supplemented with 10% FBS, 1 mM sodium pyruvate (S11-003, PAA), 1x penicillin/streptomycin (1377475, Gibco, Thermo Fisher Scientific), and 2 mM Glutamax. Cells from 2 embryos were pooled in a 100-mm Petri dish coated with 0.1% gelatin and incubated overnight. Next day, media was changed and cells were grown to confluency. Passage 0 cells were frozen or continued to grow.

1.3. Treatments (I)

In paper I, all treatments were performed for 8 hours at 37°C or 32°C. The following concentrations were used for treatments: tert-Butylhydroquinone (tert-BHQ) (150–500 μM), DL-Sulforaphane (3 μM), N-acetylcysteine (8 mM), brefeldin A (5 μg/mL), tunicamycin (5 μg/mL), cobalt (II) chloride hexahydrate ($CoCl_2$) (100 μM), and recombinant human tumor necrosis factor alpha (TNF-α) (20 ng/mL).

2. Materials (I, II, III)

In paper I, tert-BHQ (112941), DL-Sulforaphane (S4441), brefeldin A (B7651) and tunicamycin (T7765) were dissolved in dimethyl sulfoxide (DMSO) (A3672; PanReac AppliChem) with final concentrations ranging from 0.01 – 0.1%. N-acetylcysteine (A9165) and recombinant human TNF-α (570104) were dissolved in PBS. Cobalt (II) chloride hexahydrate (255599) was dissolved in milli-Q water. All compounds, except for recombinant human TNF-α (BioLegend) were purchased from Sigma Aldrich.

In paper II, the orally active H_2S-releasing prodrug, SG-1002, was provided by Sulfagenix (Cleveland, OH). SG-1002 was administered to mice in the diet (Purina 5001; Research Diets Inc., New Brunswick, NJ) to achieve a dose of 20 mg/kg/day (Kondo et al., 2013). Mice received the diet for 4 weeks. Control mice received standard chow (Chow; Purina 5001) for the same duration.

In paper III, recombinant human VEGF-$C_{Cys156Ser}$ (752-VC-025/CF) was purchased from R&D Systems (Minneapolis,MN). The VEGFR3 inhibitor, MAZ51 (#676492) was purchased from Millipore Sigma (Burlington, MA). VEGF-C NAb (52393) was purchased from GeneTex, Inc. (Irvine, CA). Gelatin-based hydrogels were obtained from MedGEL (Kodaira, Tokyo, Japan) (Tabata et al., 1999; Yamamoto et al., 2003). 5-Bromo-2'-deoxyuridine (BrdU) (#B5002) was purchased from Sigma-Aldrich.

3. Animals (II, III)

In paper II, the following strains of mice on a C57BL/6J background were utilized: (1) C57BL/6J (Jackson Labs, Bar Harbor, ME), (2) Cardiac specific cystathionase-γ-lyase transgenic (CSE Tg^+), (3) Cystathionase-γ-lyase deficient (CSE KO), (4) AMPKα2 floxed (Stock#: 014142, Jackson Labs, Bar Harbor, ME), (5) αMHC-Cre transgenic (Stock#: 011038, Jackson Labs, Bar Harbor, ME). CSE Tg^+ were generated by ligating the full-length *Mus musculus* cystathionine γ-lyase cDNA to the murine α-myosin heavy chain (αMHC) promoter, followed by injection of the DNA into newly fertilized mouse embryos (FVB/n background) (Elrod et al., 2007). The mice were then backcrossed to C57BL/6J for 9 generations. Global CSE KO knockout mice were generated by replacing exon 1 (including the ATG start codon), exon 2, and exon 3 with a neomycin selection cassette (Kondo et al., 2013). The mice were then back-

crossed to C57BL/6J for 9 generations. Cardiac specific AMPKα2 deficient mice (αMHC-Cre^{+} x AMPK$^{fl/fl}$) were generated by breeding AMPKf/f mice with αMHC-Cre^{+} mice. In all experiments, Wild-Type (WT) littermates were used as controls. Male mice between the ages of 8–10 weeks were utilized.

In paper III, C57BL/6J mice (Male; 8–12 weeks of age) were used in all experiments.

All experimental protocols were approved by the Institute for Animal Care and Use Committee at T3 Laboratories and conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996), and with federal and state regulations.

4. Patient samples (II)

Left ventricular (LV) samples were procured from patients with advanced ischemic heart failure undergoing a heart transplant at Emory University in accordance with Institution Review Board protocols. Additional non-failing heart failure samples were obtained from LifeLink. All patient identifiers were removed to strictly maintain donor confidentiality and anonymity. Both sample sets included male and female patients (Table 1).

Table 1. Patient Characteristics

Group	Number	Age	% Female
Non-Failing	6	53.5±3.8	66% (4/6)
Heart Failure	10	56.0±2.6	40% (4/10)

5. Protein extraction and Western blot analysis (I, II, III)

In paper I, after washing with PBS, cells were lysed in 1x passive lysis buffer (E1941, Promega) with gentle shaking at room temperature (RT) for 15 minutes. The extracts were centrifuged at 12,000 x g for 30 sec at 4°C and stored at -80°C. Protein concentration was determined with bicinchoninic acid (BCA) method (Pierce BCA Protein Assay Kit, Thermo Scientific) according to manufacturer's protocol. Proteins extracted from HeLa-HIF stable cells were used to study HIF1A protein expression in response to hypothermia (32 °C) or $CoCl_2$. Proteins extracted from HepG2-ARE stable cells were used to study Nrf2 protein expression in response to hypothermia (32 °C) or tert-BHQ. Protease inhibitors (78430, ThermoFisher Scientific) were added to the lysis buffer after removing an aliquot for the luciferase activity assay. Equal amounts of protein (8 µg) were electrophoresed and transferred to a nitrocellulose membrane using the NuPAGE Electrophoresis System (Life Technologies). Membranes were incubated overnight at 4°C with primary antibodies: rabbit anti-HIF1A antibody (1:2,000) (NB100-479, Novus) or rabbit anti-Nrf2 antibody (1:1,000) (ab62352,

Abcam) and mouse-anti beta-actin (1:10,000) (sc-47778, Santa Cruz) as a loading control. Next day, membranes were probed for 1 h at RT with fluorescent conjugated secondary antibodies goat anti-rabbit antibody (1:40,000 for rabbit anti-HIF1A antibody and 1:5,000 for rabbit anti-Nrf2 antibody) (35569, Jackson ImmunoResearch) and goat anti-mouse antibody (1:15,000) (A21057, Invitrogen). Western blotting signals were detected by using the LI-COR Odyssey CLx system (LI-COR Biotechnologies). Images were converted to grayscale and band intensities were quantified in Image Studio Lite v 3.1.4 (LI-COR Biotechnologies).

In paper II and III, whole cell, cytosolic, or nuclear fractions were obtained from heart homogenates as previously described (Calvert et al., 2009). Protein concentrations were measured with the detergent compatible (DC) protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were loaded into lanes of Criterion™ TGX (Tris-Glycine eXtended) Stain-Free PAGE gels (BioRad). The gels were electrophoresed and activated using a ChemiDoc MP Visualization System (BioRad). The protein was then transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were then imaged using a ChemiDoc MP Visualization System to obtain an assessment of proper transfer and to obtain total protein loads. The membranes were then blocked and probed with primary antibodies (Supplemental Table 2, paper II) overnight at 4 °C. Immunoblots were next processed with secondary antibodies (Cell Signaling) for 1 h at RT. Immunoblots were then probed with a Super Signal West Dura kit (Thermo Fisher Scientific) to visualize signal, followed by visualization using a ChemiDoc MP Visualization System (BioRad). Data was analyzed using Image Lab (BioRad). The total protein images were used as loading controls. For each protein of interest, the portion of the protein load image corresponding to the molecular weight of the protein of interest was used as the loading control (Barr et al., 2015).

6. Quantitative Real-time Reverse Transcription PCR (I, II)

In paper I, cells were plated at a density of 1 million cells in 100-mm culture dishes or 400,000 cells in 6-well plate. On the next day, cells were incubated at 22°C, 27°C, and 32°C to initiate hypothermia or kept at 37°C for various durations followed by extraction of RNA with TRIzol® reagent (15596026, Thermo Fisher Scientific). One microgram of total RNA was used for first strand cDNA synthesis using Random Hexamers (LGC Biosearch Technologies or N8080127, Invitrogen) and SuperScript III Reverse Transcriptase (18080044, Thermo Fisher Scientific). Every reaction was made in four parallel samples to minimize possible errors. All reactions were performed in a final volume of 10 µl, using 5 ng of cDNA. Primers and probes for qPCR assay were purchased from Applied Biosystems or designed for the detection of specific transcripts (LGC Biosearch Technologies) (Supplementary Table S1, paper I). Expression level of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (Ywhaz) or hypoxanthine guanine phosphoribosyl transferase (HPRT) was

used as internal reference for MEFs or HeLa and HepG2 cells, respectively. Real-time qPCR was performed using TaqMan Gene Expression Master Mix (4369016, Thermo Fisher Scientific) or HOT FIREPol EvaGreen qPCR Supermix (08-36-00001, Solis BioDyne). qPCR reactions were run on (1) ABI PRISM 7900HT Fast Real-Time PCR System equipment (PE Applied Biosystems, USA) and quantified with the ABI PRISM 7900 SDS 2.2.2 software, (2) QuantStudio 12K Flex Software v.1.2.2 Real-Time PCR System equipment (Applied Biosystems, USA) and quantified with the QuantStudio 12K Flex Software v.1.2.2.

In paper II, RNA was isolated using the RiboPure kit according to the manufacturer's instructions (Ambion). Reverse transcription was performed in a standard fashion with QuantiTect Reverse Transcription Kit (QIAGEN) supplemented with DNase treatment. Taqman qPCR was carried out according to the manufacturer's instructions using probe sets obtained from Thermo Fisher Scientific.

7. Luciferase-reporter assay (I)

HeLa cells and HepG2-NF-κB stable cells were seeded at a density of 50,000 cells/well onto 12-well plates, grown overnight, and co-transfected with 100 ng renilla and 100 ng firefly luciferase vectors by Effectene Transfection Reagent (301425, Qiagen). The following luciferase vectors were used: HSV-thymidine kinase promoter (pRL-TK) (E2241, Promega), CMV immediate early enhancer/promoter region (pRL-CMV) (E2261, Promega), 5x activating transcription factor 6 (ATF6) site luciferase reporter gene (p5xATF6-GL3) (11976, Addgene) or pd2-EYFP-N1 (EYFP-Enhanced yellow fluorescent protein) (Clontech). The activities of firefly and renilla luciferase were measured using the Dual Luciferase Reporter Assay System (E1980, Promega) according to manufacturer's protocols.

Stable cell lines expressing luciferase reporter were plated at a density of 80,000 cells/well in 12-well plates and grown overnight. After experimental treatment the cells were harvested and analyzed for luciferase activity using Firefly Luciferase Assay (30003-2, Biotium).

The luminescence was quantified using the VICTOR Multilabel Plate Reader (PerkinElmer). Luminometer was programmed to provide a 10-second pre-read delay, followed by a 5-second measurement period.

8. Glutathione levels (I)

The levels of total glutathione and glutathione disulfide (GSSG) were evaluated in HeLa, wild type and Nrf2 KO MEFs using the GSH/GSSG-Glo™ Assay (V6612, Promega). Cells were plated at 10,000 cells/well in 100 µL culture media in 96-well luminometer-compatible tissue culture plates, grown overnight at 37°C in a 5% CO_2 culture incubator, and subjected to normothermia (37°C)

or hypothermia (32°C) for 24 h. Luminescence was measured using the Tecan Sunrise machine.

9. Cell viability assay (I)

Cell viability of MEFs, HeLa and HepG2-ARE cells was determined using of cell proliferation reagent Water Soluble Tetrazolium salt 1 (WST-1) (05015944001, Sigma Aldrich). Briefly, cells were plated at a density of 10,000/well in 100 μL culture media in 96-well plate. Next day, the cells were exposed to normothermia (37°C) or hypothermia (32°C) for 24 hours in a humidified tissue culture incubator. After 24h, culture media was changed to treat the cells with tert-BHQ for 3 hours. Finally, WST-1 reagent was added to wells according to the manufacturer's instructions and incubated for additional 2 hours. Absorbance was measured at 450nm. Reference wavelength was 690nm. To qualify independent experiments for subsequent statistical analysis it was required that the effect of tert-BHQ on the viability of cells pre-incubated at 37°C lie between 0.25-0.75 of the vehicle control viability.

10. Microarray experiments (I)

One million primary MEFs (Millipore) were seeded onto 100-mm culture dishes and grown (atmospheric oxygen, 5% CO_2 at 37°C) in DMEM (high glucose 4.5 g/l, supplemented with 10% FBS and L-glutamine, PAA) until 60–70% confluent. Hypoxia was initiated by lowering oxygen concentration to 1% in a multi-gas incubator (Sanyo). Hypothermia was initiated by lowering temperature in the incubator to 32 ºC. The experiment was carried out in five biological replicates per experimental condition. After 24 h, RNA was extracted from the cells by Trizol® (Life) followed by expression profiling according to manufacturer's protocols. Briefly, 50 ng of total RNA from each sample was amplified using the OvationPico WTA system V2 (Nugene). Fragmentation and biotin labeling was done using the Encore-Ovation cDNA Biotin Module (Nugene). The labeled samples were hybridized to the Mouse Exon 1.0 ST array (Affymetrix). The arrays were washed and stained with phycoerythrin conjugated streptavidin (SAPE) using the Affymetrix Fluidics Station® 450, and the arrays were scanned in the Affymetrix Gene Array® 3000 scanner to generate fluorescent images, as described in the Affymetrix Gene Chip® protocol. Cell-intensity (CEL) files were generated in the Gene Chip® Command Console® Software (AGCC) (Affymetrix).

10.1. Microarray data analysis

Differential gene expression was estimated directly from CEL files using DEMI (Ilmjärv et al., 2014) as implemented in R package version 1.0 (https://CRAN.R-project.org/package=demi). False discovery rate (FDR) ad-

justed differential expression p-values were provided by the DEMI package using a variant of FDR method, which accounts for statistical dependence between the observed variables (Benjamini and Yekutieli, 2001). Differential expression estimates with FDR values below 0.001 were considered statistically significant. Ensemble gene ID-s of significantly differentially expressed genes were submitted to g:Profiler (Reimand et al., 2016) for functional interpretation. Lists of up- and down-regulated genes were submitted separately. KEGG pathways (Kanehisa et al., 2014) and TRANSFAC transcription factor binding sites (Wingender, 2008) with corrected p-values below 0.001 were considered as significantly over-represented in the gene list.

11. Electron microscopy (II)

Heart tissue was dissected along the muscle fiber while immersed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2). Samples were stored in the fixative overnight at 4 °C. Samples were then washed with the same buffer and post-fixed in 1% buffered osmium tetroxide, dehydrated through a graded ethanol series to 100%, and embedded in Eponate 12 resin. Ultrathin sections were cut on a Leica UC6rt ultra-microtome at 70–80 nm and counter-stained with 4% aqueous uranyl acetate and 2% lead citrate. Sections were examined using a Hitachi H-7500 transmission electron microscope equipped with a Gatan BioScan CCD camera.

12. Citrate synthase activity (II)

Cardiac citrate synthase activity was measured spectrophotometrically in homogenates (Mo et al., 2012).

13. Sulfide measurements (II)

Hydrogen sulfide and sulfane sulfur levels were measured in heart tissue as previously described (Nicholson et al., 2013). Fresh tissue was homogenized in 5 volumes of PBS (pH 7.4). For measurement of H_2S, 0.2 mL of the sample homogenate was placed in a small glass vial (5182-0553, Agilent Technologies, Santa Clara, CA, USA) along with 0.4 mL of 1 M sodium citrate buffer, pH 6.0, and sealed. The mixture was incubated at 37 °C for 10 minutes with shaking at 125 rpm on a rotary shaker (Fisher Scientific) to facilitate the release of H_2S gas from the aqueous phase. After shaking, 0.1 mL of head-space gas was applied to a gas chromatograph (7890A GC System, Agilent) equipped with a dual plasma controller and chemiluminescence sulfur detector (355, Agilent) and a data processor. The carrier gas was helium with a flow rate of 2.4 mL/min. For the measurement of H_2S released from bound sulfane sulfur, 0.1 mL of the sample

homogenates and 0.1 mL of 15 mM DTT in 0.1 mM Tris/HCl, pH 9.0, were placed in a in a small glass vial, sealed, and incubated at 37 °C for 50 minutes. After the incubation, 0.4 mL of 1 M sodium 4 citrate buffer was injected through the rubber stopper and the mixture was incubated at 37 °C for 10 minutes with shaking at 125 rpm on a rotary shaker to facilitate the release of H_2S gas from the aqueous phase. After shaking, 0.1 mL of head-space gas was applied to a gas chromatograph as detailed above. The amount of H_2S is reported as nmol/mg wet weight.

14. Immunoprecipitation (II)

Heart homogenates were immunoprecipitated with an antibody to PGC-1α using the Dynabeads® Protein G Immunoprecipitation Kit according to manufacturer's instructions. The samples were then subjected to standard Western blot techniques and the membranes probed with antibodies to phosphoserine and acetyllysine.

15. AMPK activity (II)

The activity of AMPK was measured in homogenates prepared from heart tissue. The samples were first immunoprecipitated with a specific anti-AMPKα2 antibody (abcam). An aliquot of the immunoprecipitated samples were incubated in a reaction buffer containing 12.5 mM Tris-hydrochloride (Tris-HCl) (pH 7.5), 2.5 mM β-glycerophosphate, 1 mM dithiothreitol, 0.05 mM sodium orthovanadate (Na_3VO_4), 5 mM magnesium chloride ($MgCl_2$), 0.050 mM ATP, and 0.2 mM of SAMS (AMPK synthetic substrate peptide). The rate of adenosine diphosphate (ADP) formed from the incorporation of ATP in the synthetic peptide was then measured with the ADP-Glo Kinase Assay kit (Promega) according to the manufacturer's instructions. Activity was expressed as ADP generated (in picomoles) per minute per milligram of protein.

16. Sirt1 activity (II)

The activity of cardiac Sirtuin 1 (Sirt1) was evaluated using the SIRT1 Activity Assay (catalog# ab156065, abcam).

17. Mitochondria respiration and ATP (II)

Cardiac fibers were isolated and permeabilized wtih saponin as previously described (Lehman et al., 2008). Respiration was monitored using a Clark-type oxygen electrode (Hansatech Instruments, Amesbury, MA) in the presence of pyruvate or palmitoyl-l-carnitine. To evaluate ATP synthesis, aliquots were taken from the respiration chamber over a 1-minute period after the addition of

ADP. ATP was then quantified with a bioluminescence assay using an ATP determination kit (A-22066; Molecular Probes, Eugene, OR). The rate of ATP synthesis was then normalized to the oxygen consumption rates measured over the time the aliquots were collected to obtain a measure of ATP synthesis efficiency (ATP/Oxygen ratio). This measurement reflects the ratio of state 3 (ADP stimulated respiration) ATP synthesis rates to state 3 oxygen consumption. A higher value indicates better efficiency.

18. Myocardial ischemia reperfusion protocol and echocardiograph analysis (II, III)

In paper II, mice were subjected to surgical ligation of the left coronary artery (LCA) followed by reperfusion for 2 weeks. Echocardiography was performed as previously described (Calvert et al., 2010b).

In paper III, heart failure was induced either by permanent ligation of the LCA or by subjecting mice to 60 minutes of LCA occlusion followed by reperfusion for up to 4 weeks. Surgical ligation of the LCA was performed under anesthesia (ketamine, 100 mg/kg; sodium pentobarbital, 20 mg/kg) as previously described (Calvert et al., 2010b; Shimizu et al., 2016). All animals received prophylactic antibiotic therapy with cefazolin (20 mg/kg) and buprenorphine (0.05 mg/kg) for pain. A total of 185 mice were included in the present study after accounting for animal deaths. All mice were randomly assigned to the treatment groups. For the experiments examining the proliferation of lymph endothelial cells, BrdU (30 mg/mL) (Naqvi et al., 2014) was injected intraperitoneally once daily following the onset of myocardial ischemia until the time of sacrifice.

Transthoracic echocardiography was performed at baseline and 4 weeks after reperfusion using the Vevo 2100 with a 38-MHz linear array scanhead (Shimizu et al., 2016).

19. Gelatin-Based Hydrogel Preparation (III)

Gelatin-based hydrogels were prepared according to the manufacturer's instruction. In brief, to prepare gelatin hydrogels, 10µl of an aqueous solution containing VEGF-$C_{Cys156Ser}$(125 µg/ml) (Shimizu et al., 2012), MAZ51 (25 mg/ml) (Benedito et al., 2012), VEGF-C NAb (0.5 mg/mL) or vehicle was dropped onto the freeze-dried hydrogels. The hydrogels were incubated at RT for at least 30 minutes. The hydrogels are designed to release their content as they degrade with roughly 90% being released by 14 days. This results in the following doses: VEGF-$C_{Cys156Ser}$, 3.214 µg/kg/day; MAZ-51, 0.643 mg/kg/day, and VEGF-C Nab, 0.0218 mg/kg/day. After the onset of reperfusion, the gelatin hydrogels were placed on the surface of myocardium prior to the closing of the chest.

20. Histological Analysis (III)

Hearts were harvested and fixed in 10% formalin and embedded in paraffin. Slices were cut at 7 μm and stained with Masson trichrome (Millipore Sigma (Burlington, MA) (Shimizu et al., 2012). Fibrosis area was quantitatively analyzed with NIH Image software.

21. Immunofluorescence (III)

Frozen sections (7 μm in thickness) were prepared and stained for anti-LYVE1 (lymphatic vessel endothelial hyaluronan receptor 1) to detect lymph vessels (Shimizu et al., 2013). These were followed by incubation with secondary antibodies. Coverslips were mounted using Vectashield H-1500-4′,6-Diamidino-2-phenylindole (DAPI)-containing medium (Vector Laboratories) (Shimizu et al., 2013). Images were acquired on a Leica DM6000. B lymphocytes were detected by staining sections with antibodies against CD45R (B220) and immunoglobulin M (IgM). Proliferating lymph endothelial cells were evaluated by staining sections with antibodies against BrdU and LYVE1.

22. Inflammatory cytokines (III)

The levels of TNF-α, interleukin 1 beta (IL-1 beta), and interleukin 6 (IL-6) were evaluated in heart homogenates using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, #88-7324-22, #88-7013-22, and #88-7064-22, respectively) according to the manufacturer's instructions.

23. Statistics (I, II, III)

In paper I, data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with (1) Student's t-test or (2) paired t-test for comparsion of biological replicates treated with tert-BHQ after normothermic and hypothermic pre-incubation.

In paper II and III, all data are expressed as mean ± SEM. The data was first evaluated for normal distribution using the D'Agostino and Pearson omnibus normality test. Subsequent, statistical significance was evaluated as follows: (1) unpaired Student t-test for comparison between 2 means; (2) a 1-way analysis of variance (ANOVA) with a Tukey test or Dunnett's Multiple Comparison Test as the posthoc analysis for comparison among 3 or more groups; and (3) in paper II, a 2-way ANOVA with a Bonferroni test as the posthoc analysis for comparison among the means from groups of WT and AMPK KO mice. For the echocardiography data, a 2-way repeated measures ANOVA with a Bonferroni test as the posthoc analysis was used. The following comparisons were made separately: (1) baseline vs. post-baseline measurements for each group,

(2) differences between each group's baseline measurements, and (3) differences between each groups post-baseline measurements. The p-value for these evaluations was adjusted by applying the Bonferroni correction for multiple comparisons. A value of $p < 0.05$ denoted statistical significance and p-values were two-sided. All statistical analysis was performed using Prism 5 (GraphPad Software Inc).

RESULTS AND DISCUSSION

1. Paper I

1.1. Hypothermia extensively remodels gene expression

Large-scale gene expression profiling of MEFs revealed 3240 up-regulated and 962 down-regulated genes in response to mild hypothermia (Supplementary file 1 – Microarray analysis, paper I). Bioinformatic analysis of regulatory sequence motifs identified 85 unique transcription factors with significantly over-represented binding sites in promoter regions of hypothermia-induced genes (Supplementary file 1 – Microarray analysis, paper I). RNA-transport was the only enriched KEGG pathway among hypothermia-induced genes concordant with the notion that the two best-studied hypothermia-responsive genes (cold-inducible RNA-binding protein and RNA binding motif protein 3 (Cirbp and Rbm3)) encode RNA-binding proteins (Danno et al., 1997; Fujita, 2000; Nishiyama et al., 1997). In comparison, using the same methodology on cells treated 24h with 1% O_2 hypoxia, which is known to extensively remodel gene expression (Elvidge et al., 2006; Manalo et al., 2005), resulted in the up-regulation of 335 and down-regulation of 477 genes (Supplementary file 1 – Microarray analysis, paper I). Bioinformatic analysis identified three transcription factors (Hif1a, zinc finger and BTB domain containing 14 (ZF5) and Sp1 transcription factor (Sp1)) with significantly over-represented binding sites in promoter regions of hypoxia-induced genes (Supplementary file 1 – Microarray analysis, paper I). As expected, significant enrichment of genes related to HIF1 signaling pathway, glycolysis and pentose phosphate pathway was identified by functional annotation analysis of hypoxia-induced genes (Supplementary file 1 – Microarray analysis, paper I). These results demonstrate more widespread effects of hypothermia on gene expression than initially anticipated and present an extensive list of transcription factors, which are predicted to be hypothermia-responsive (Supplementary file 1 – Microarray analysis, paper I).

In order to provide an independent validation of the hypothesis that hypothermia is able to activate gene expression, we performed reporter experiments in human cell lines transfected with CMV promoter driven luciferase. CMV promoter is a strong activator of gene expression in mammalian cells and contains binding sites of several activating transcription factors including cAMP responsive element-binding (CREB) and activating transcription factor (ATF) (CREB/ATF), NF-κB, activator protein 1 (AP1), E26 transformation-specific or E-twenty-six (ETS) and SP1 (Meier and Stinski, 1996; Thrower et al., 1996). Based on the TRANSFAC database of transcription factor DNA binding motifs, we found 173 and 75 high confidence sites on CMV and HSV-TK promoters, respectively (Supplementary file 2 – TF binding sites, paper I). Bioinformatic analysis suggested that some transcription factors binding to the CMV promoter (most notably CREB/ATF1) had over-represented motifs in the promoters of hypothermia-susceptible genes as identified in the genome-wide expression

screen (Supplementary file 2 – TF binding sites, paper I). Luciferase driven by HSV-TK, a considerably weaker viral promoter, was used as an additional comparison. Mild hypothermia significantly induced the activity of both CMV and TK reporters up to 2.5 fold and 1.8 fold, respectively, above the normothermic level (Figure 2). These results provide further evidence to suggest that mild hypothermia can act as an activator of gene expression.

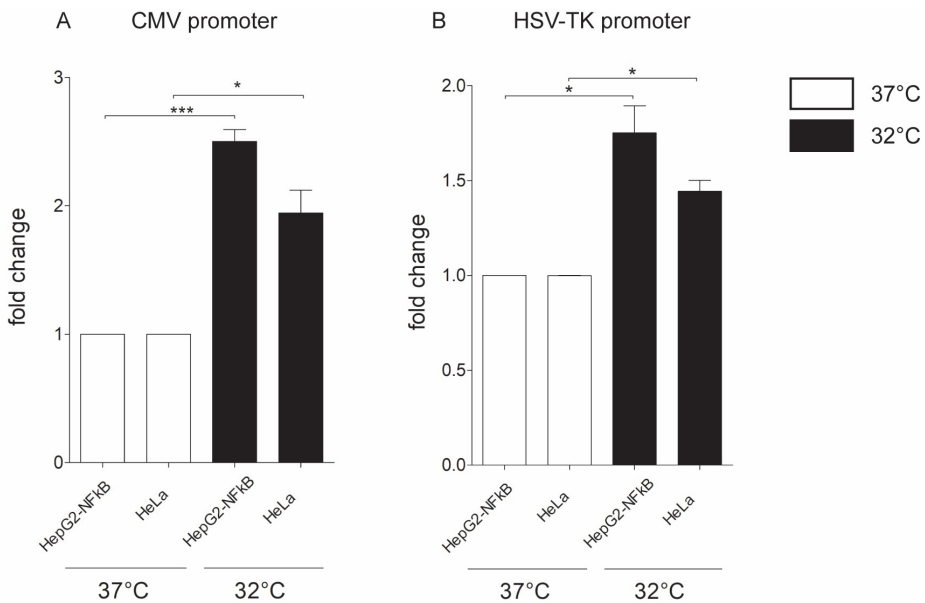

Figure 2. Effect of hypothermia (8h) on the activity of CMV (A) and HSV-TK (B) promoters as reported by renilla-luciferase in HeLa and HepG2 cells. Reporter activity in each cell line is normalized to the corresponding normothermic control group. Figure represents at least three independent experiments (n = 3-4) per cell line. Mean values (+SEM) are shown. *, $p< 0.05$; ***, $p < 0.001$.

1.2. Mild hypothermia activates Nrf2 and HIF1 transcription factors

As the discovery of therapeutically relevant effects of hypothermia is of primary interest, we proceeded to test whether hypothermia can induce responses, which are known to mitigate cellular stress. Production of ROS during hypoxia-reperfusion has been suggested as a possible mechanism of hypoxic injury (Burwell et al., 2009; Eltzschig and Eckle, 2011; Murphy and Steenbergen, 2008; Yellon and Hausenloy, 2007). We investigated whether hypothermia could activate the antioxidant system. Microarray analysis indicated that several genes of the glutathione, thioredoxin and sulfiredoxin systems were upregulated after 24h of hypothermia in wild-type MEFs (Supplementary file 1 – Microarray analysis, paper I). qRT-PCR revealed the upregulation of glutamate-cysteine ligase catalytic subunit (Gclc) and thioredoxin reductase 1 (Trxr1) at 8h after the onset of

hypothermia (Figure 3A, C). Upregulation of Gclc, sulfiredoxin 1 (Srxn1) and glutathione synthetase (Gss) was evident at 24h of hypothermia (Figure 3B, E, F). The fact that Nrf2 is the master regulator of antioxidant gene expression prompted us to study hypothermia response in Nrf2-deficient MEFs. As expected, the expression levels of the studied antioxidant genes were significantly reduced in Nrf2-deficient cells. Gclc and Trxr1 did not respond significantly to hypothermia in Nrf2-deficient cells (Figure 3B, D). Srxn1 and Gss were significantly induced by hypothermia even in the absence of Nrf2 (Figure 3E, F) suggesting that, in selected cases, hypothermia can induce antioxidant gene expression by Nrf2-independent mechanisms.

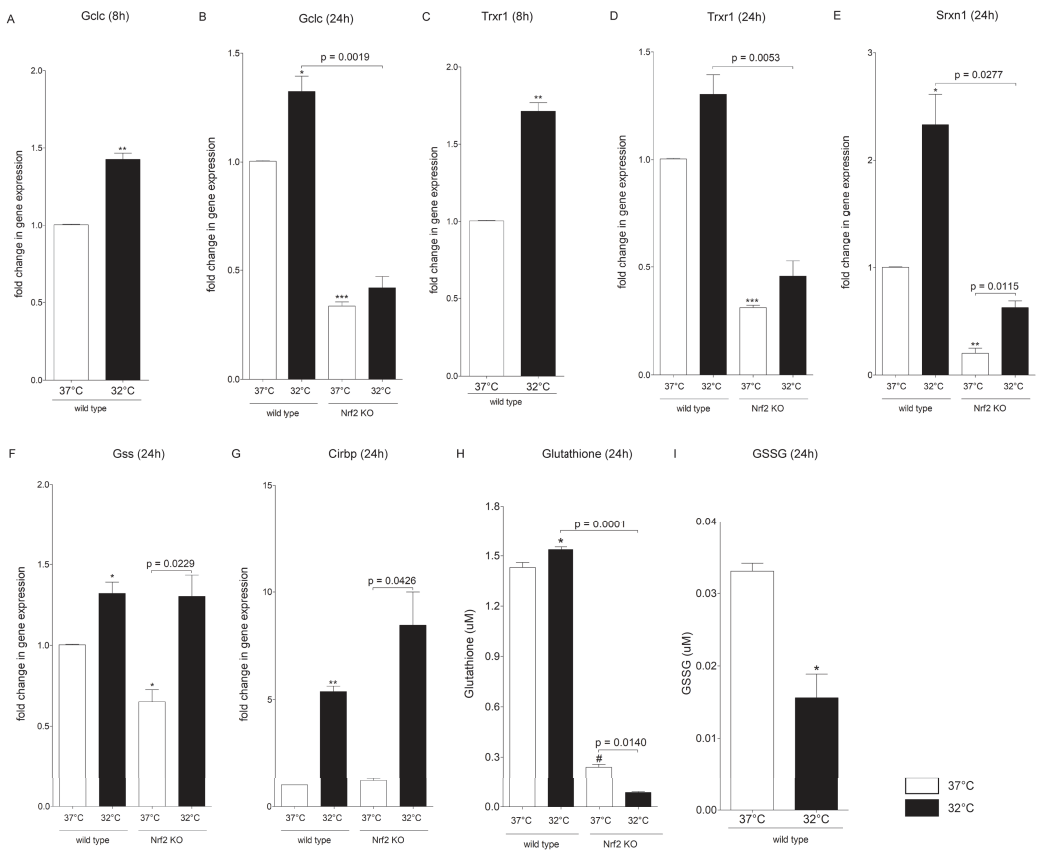

Figure 3. Effect of hypothermia on the expression of antioxidant system genes (A-F). Cold-inducible gene Cirbp was used as a positive control for hypothermic effect (G). Effect of hypothermia on total cellular glutathione (H) and glutathione disulfide (GSSG) (I) in wild-type and Nrf2-deficient mouse embryonic fibroblasts. Figure represents three independent experiments (n = 3) per cell line. Mean values (+SEM) are shown. *, p< 0.05; **, p < 0.01; ***, p < 0.001; #, p < 0.0001 when compared with controls at 37°C.

In order to ascertain whether the apparent induction of glutathione system genes could translate into relevant biochemical changes, cellular glutathione, the major intracellular antioxidant, was measured by the glutathione-S-transferase based enzymatic assay (Promega). Total glutathione, comprised of reduced (GSH) and oxidized forms (GSSG), was significantly higher in wild-type MEFs after 24h of hypothermia when compared to normothermic controls. The opposite pattern was observed in Nrf2-deficient MEFs where total glutathione level was lower in hypothermic cells (Figure 3H). These observations suggest that Nrf2 is required for hypothermia-induced increase of cellular glutathione in MEFs. GSSG level was lower in hypothermic wild-type MEFs when compared to normothermic controls suggesting that hypothermia increases antioxidant potential while lowering oxidative stress (Figure 3I). GSSG was undetectable in Nrf2-deficient cells presumably due to the low level of total glutathione. Hypothermic treatment of HeLa cells was associated with increased total glutathione (Figure 4A) and increase in the expression of GCLC and TRXR1 (Figure 4C–D). Hypothermia had no effect on GSSG (Figure 4B) suggesting, once again, that hypothermia does not lead to elevated oxidative stress. To establish that hypothermic pretreatment can be protective, three different cell lines were subjected to 24 hours of hypothermia followed by oxidative stress and quantification of cell viability (Figure 5A–C). We found that in MEF and HeLa cells, hypothermic pretreatment mitigated the effects of oxidative stress resulting in higher viability when compared to normothermic pretreatment. In sum, we found evidence for increased levels of glutathione and cell-type dependent increase in the resistance to pharmacologically induced oxidative stress after hypothermic pre-incubation.

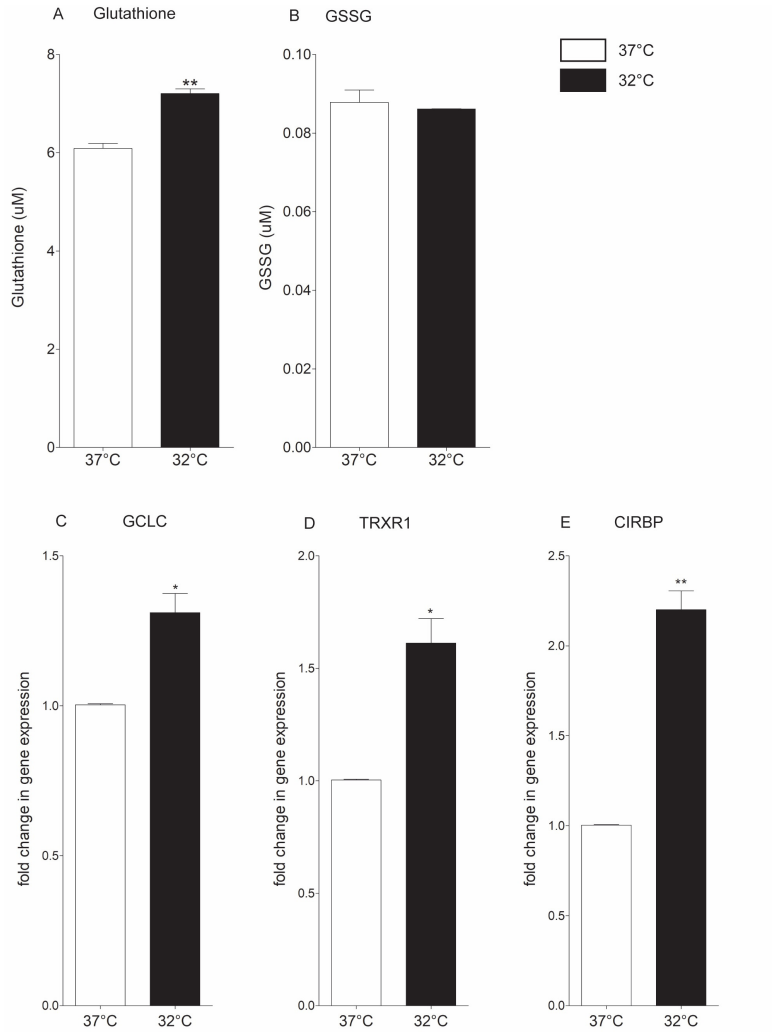

Figure 4. Effect of hypothermia (24h) on total cellular glutathione (A) and glutathione disulfide (GSSG) levels (B), and expression of key genes of glutathione (GCLC) (C), and thioredoxin systems (TRXR1) (D) in HeLa cells. Expression of cold-inducible gene CIRBP was used as positive control for hypothermic effect (E). Mean values (+SEM) from three independent experiments are shown (n = 3). *, $p< 0.05$; **, $p < 0.01$ when compared with controls at 37°C.

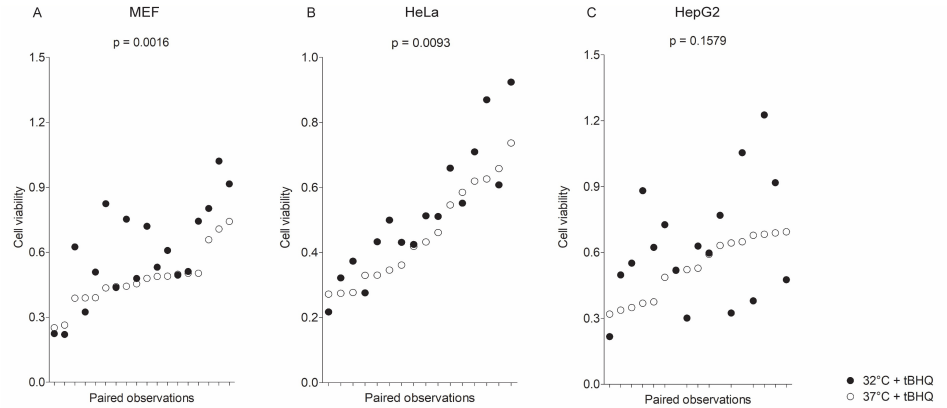

Figure 5. Cell viability in response to tert-BHQ (150-500 μM) after normothermic and hypothermic pre-incubation (24h) in MEF (A), HeLa (B) and HepG2 cells (C). Mean values (+SEM) from at least 16 independent experiments are shown (n = 16–18). Cell viability is normalized relative to the corresponding vehicle control at 37°C. Paired observations represent one biological replicate treated with tert-BHQ after normothermic and hypothermic pre-incubation.

In order to gain insight into the dynamics of Nrf2 induction, HepG2 cells constitutively expressing Nrf2 luciferase reporter were subjected to increasing durations of hypothermia. In parallel, antioxidant gene expression was monitored with real-time qPCR. Nrf2 reporter activity peaked at 8h and declined below the baseline level by 24h (Figure 6A). The expression dynamics of antioxidant system genes GCLC and TRXR1 (Figure 6B-C) correlated positively with Nrf2 reporter activity (average Pearson Product-Moment correlation 0.86). The accuracy of Nrf2 reporter assay was confirmed by oxidative stress-inducing agents tert-BHQ and DL-Sulforaphane, which increased Nrf2 reporter activity and antioxidant N-acetylcysteine, which decreased it (Figure 6E). No change in the expression of Nrf2 protein was observed after hypothermia on Western blot (Figure 6F) suggesting that hypothermia associated increase in Nrf2 activity might be due to a post-translational mechanism.

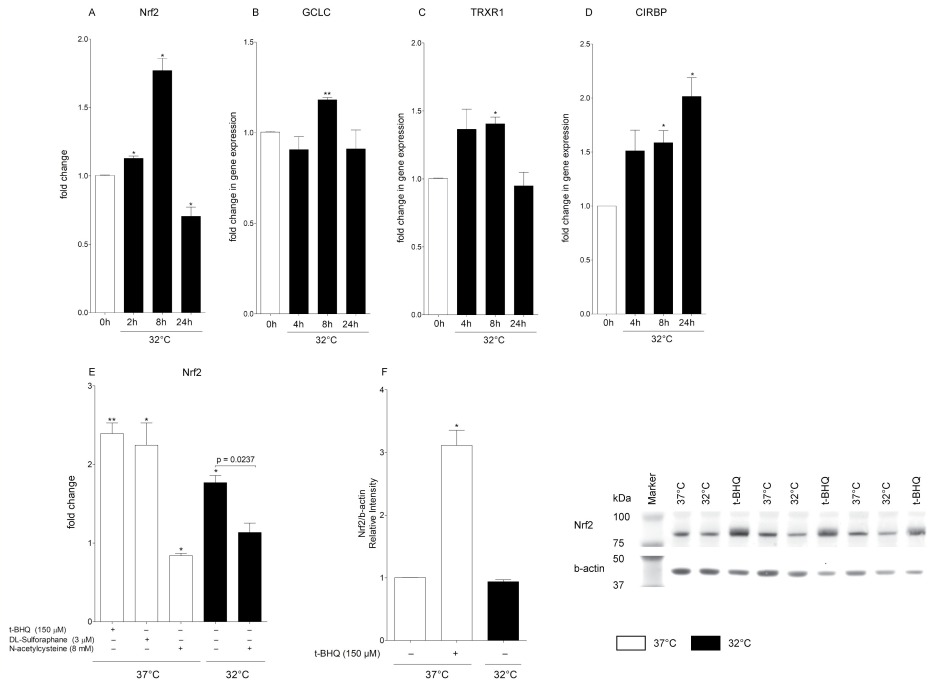

Figure 6. Time-course of Nrf2 luciferase reporter activity in HepG2 cells under hypothermia (A). Time-course of GCLC (B) and TRXR1 (C) gene expression in HepG2 under hypothermia. Cold-inducible gene CIRBP was used as positive control for hypothermic effect (D). Activity of Nrf2 luciferase reporter in HepG2 cells in response to hypothermia and oxidative stress-modulating compounds (E). Activity is normalized relative to the corresponding vehicle control at 37°C. ROS-inducing compounds tert-BHQ (150 µM), DL-Sulforaphane (3 µM) and antioxidant N-acetylcysteine (8 mM) were used as positive controls. All treatments were performed for 8h. Nrf2 protein expression in HepG2 cells under hypothermia and representative immunoblot (F). Tert-BHQ (150 µM) was used as positive control for Nrf2 induction. All treatments were performed for 8 h. Mean values (+SEM) from at least three independent experiments are shown (n = 3–4). *, $p < 0.05$; **, $p < 0.01$ when compared with corresponding vehicle controls at 37°C or 0h time point.

Next, we focused on HIF1A, the major oxygen-dependent transcriptional activator (Elvidge et al., 2006; Forsythe et al., 1996; Manalo et al., 2005; Semenza and Wang, 1992), to explore the interplay between hypoxia-sensitive stress mechanisms and hypothermia. Under normoxic conditions, HIF1A reporter activity increased monotonically in time when exposed to increasing durations of hypothermia (Figure 7A). Marked amplification of HIF1A activity by hypothermia was observed in $CoCl_2$-treated cells and N-acetylcysteine abolished this effect (Figure 7B). Hypothermia in isolation increased HIF1A protein abundance modestly but significantly as measured by Western blot. When hypothermia was applied together with $CoCl_2$, however, HIF1A protein induction was amplified reminiscent of a synergistic effect (Figure 7C). Gene expression

analysis of HIF1A target genes after 24h of normoxic hypothermia revealed no activation of BNIP3 and vascular endothelial growth factor A (VEGFA) (Figure 7D-E), which are commonly induced under oxygen deprivation. These findings suggest that hypothermia can augment the activation of HIF1A in hypoxia-mimicking conditions. Furthermore, in normoxic settings, hypothermia increases HIF1A activity without producing a downstream response typical of oxygen deprivation.

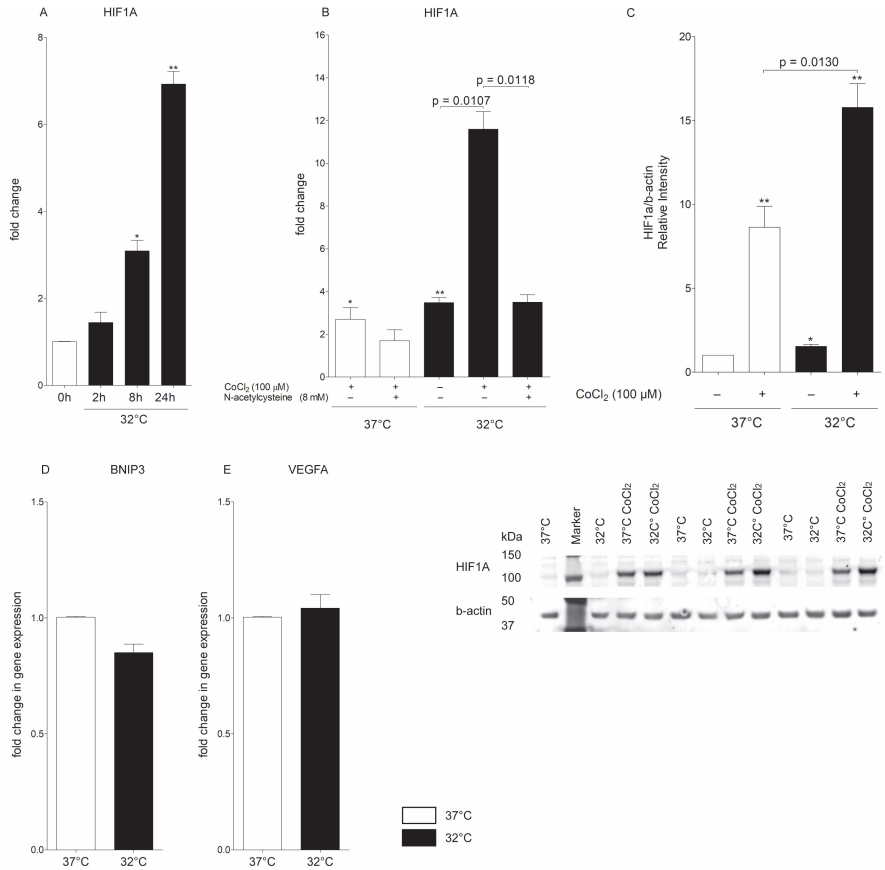

Figure 7. Time-course of HIF1A luciferase reporter activity in HeLa cells under hypothermia (A). Activity of HIF1A luciferase reporter in response to hypothermia with respect to the corresponding vehicle control group at 37°C (B). Hypoxia mimetic $CoCl_2$ (100 µM) and antioxidant N-acetylcysteine (8 mM) were used as positive controls for HIF1A activity. All treatments were performed for 8 h. HIF1A protein expression in HeLa cells under hypothermia and representative immunoblot (C). $CoCl_2$ (100 µM) was used as positive control for HIF1A induction. All treatments were performed for 8 h. Expression of BNIP3 (D) and VEGFA (E), HIF1-inducible genes, in HeLa cells under hypothermia (24 h). Mean values (+SEM) from three independent experiments are shown (n = 3). *, $p < 0.05$; **, $p < 0.01$ when compared with corresponding vehicle controls at 37°C or 0h time point.

1.3. Lower temperatures do not activate Nrf2 and HIF1A pathways as efficiently as 32°C

Previous studies in animal models have suggested that relatively small decreases in body temperature are as protective as lower temperatures while not providing a mechanistic rationale for such observations (Krieger and Yenari, 2004; van der Worp et al., 2007). We subjected cells to a temperature titration curve from 37ºC to 22ºC in 5ºC steps and measured the effect on Nrf2 and HIF1A activities and their downstream targets. Incubation at 27ºC or 22ºC did not increase Nrf2 and HIF1A transcription factor activities above the levels found at 32ºC (Figure 8A). Similarly, GCLC, TRXR1 and BNIP3 gene expression levels did not exhibit consistent temperature-dependent increase below 32ºC (Figure 8B–D). Incubation at 22ºC had no effect on Nrf2 activity and its downstream targets suggesting no additional benefits from lowering the temperature further.

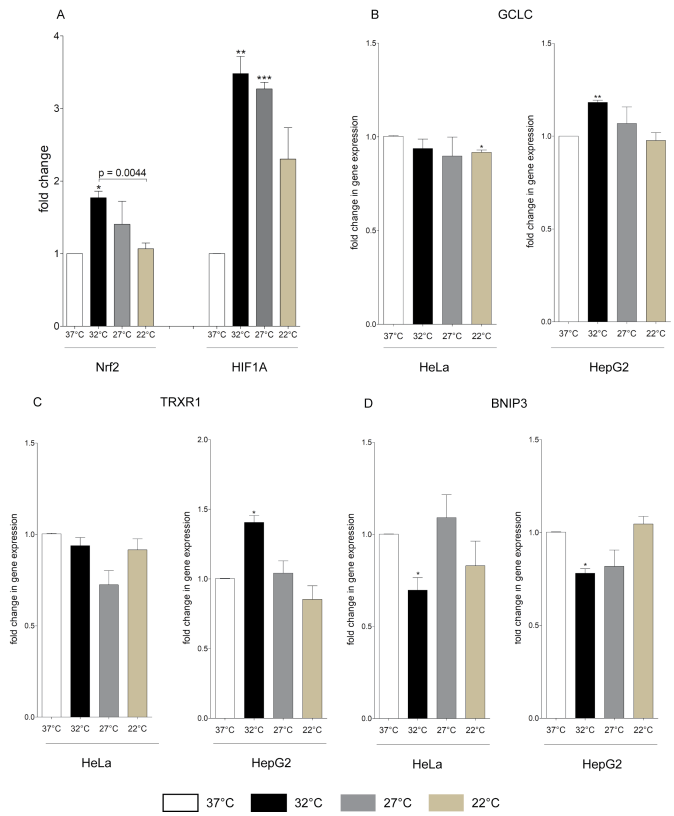

Figure 8. Response of Nrf2 and HIF1A luciferase reporters at various temperatures in HepG2 and HeLa cells, respectively (A). All treatments were performed for 8 h. Gene expression of GCLC (B), TRXR1 (C), and BNIP3 (D) at various temperatures in HeLa and HepG2 cells (8 h). Mean values (+SEM) of at least three independent experiments per cell line are shown (n = 3–4). *, p< 0.05; **, p < 0.01; ***, p < 0.001 when compared with normothermic controls.

1.4. Mild hypothermia does not trigger unfolded protein response and inflammation

Ischemic hypoxic injury perturbs ER homeostasis, leads to ER stress and consequent activation of unfolded protein response (UPR) (Nakka et al., 2010; Tajiri et al., 2004). As hypothermia can decrease protein synthesis (Hofmann et al., 2012; Knight et al., 2015; Radford et al., 2015), possibly ameliorating ER-stress, we studied its effect on transcriptional activators, which bind to the mammalian endoplasmic reticulum stress response element (ERSE). No increase in ERSE reporter activity (Figure 9A) or in the level of X-box binding protein 1 (XBP1) spliced isoform (Figure 9B), which is translated to a potent transcription factor that binds to ERSE (Yoshida et al., 2001), was found in response to hypothermia. ER stress inducers tunicamycin and brefeldin A increased ERSE-driven luciferase activity approximately 1.5 – 1.65-fold (Figure 9A). Furthermore, the induction of ERSE reporter activity by ER stress inducing compounds was attenuated by hypothermia (Figure 9A) suggesting that hypothermia is more likely to alleviate than to facilitate ER stress.

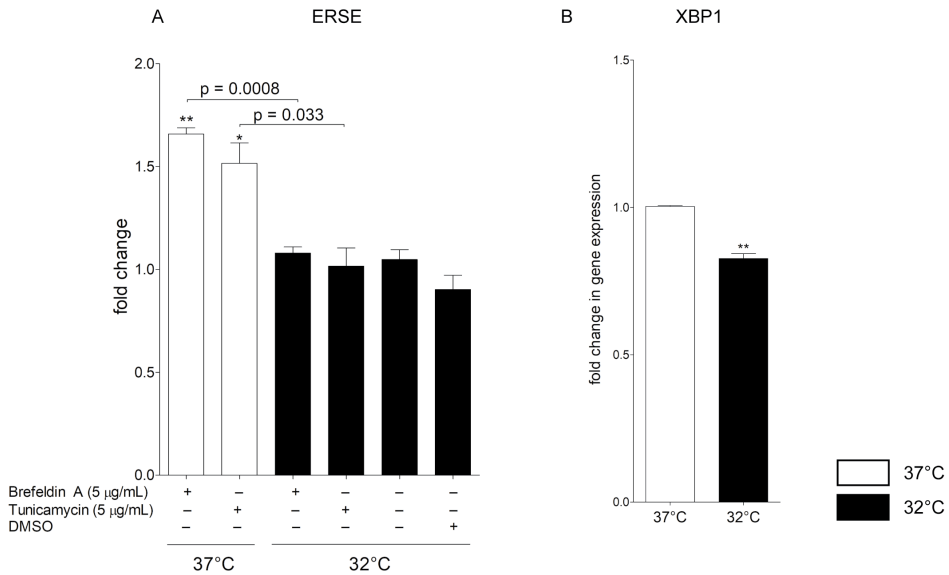

Figure 9. Induction of ERSE-dependent luciferase reporter in HeLa cells in response to hypothermia (A). Activity is reported with respect to the vehicle control group at 37°C. ER-stress inducing compounds brefeldin A (5 μg/mL) and tunicamycin (5 μg/mL) were used as positive controls. All treatments were performed for 8 h. Expression of XBP1, an activating transcription factor 6 (Atf6) inducible gene, in HeLa cells in response to hypothermia (24 h) (B). Mean values (+SEM) of three independent experiments are shown (n = 3). *, $p < 0.05$; **, $p < 0.01$ when compared with corresponding vehicle controls at 37°C

NF-κB transcription factor plays a major role in inflammation and immune response. It is also involved in cell growth and apoptosis (Barkett and Gilmore, 1999; Ghosh et al., 1998; Guttridge et al., 1999; Pahl, 1999). Hypoxia and ROS production both modulate NF-κB response (Koong et al., 1994a; 1994b; Shreck et al., 1992). We found TNFα, a pro-inflammatory cytokine, to activate NF-κB reporter activity 11-fold. In comparison, 1.6-fold induction of NF-κB reporter by hypothermia (Figure 10A) was not significantly different from the baseline (see HSV-TK reporter in Figure 2A). The expression level of cyclooxygenase 2 (COX2) gene, a downstream target of NF-κB, appeared to be decreased by hypothermia (Figure 10B) supporting the notion that hypothermia is not a primary inducer of pro-inflammatory response in cells.

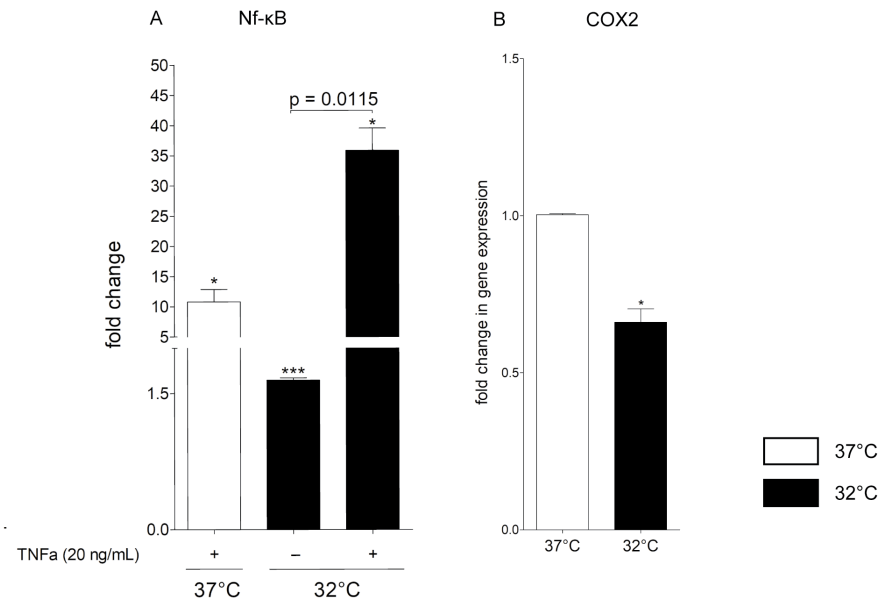

Figure 10. Induction of NF-κB luciferase reporter in HepG2 cells in response to hypothermia with respect to the vehicle control group at 37°C (A). NF-κB -inducing compound TNFα (20 ng/mL) was used as positive control. All treatments were performed for 8h. Expression of COX2, a NF-κB-inducible gene, in HepG2 cells in response to 24h hypothermia (B). Mean values (+SEM) from at least three independent experiments are shown (n = 3-4). *, $p < 0.05$; ***, $p < 0.001$ with respect to vehicle controls at 37°C.

As hypothermia has become a routine clinical therapy for specific hypoxic/ischemic conditions worldwide (Azzopardi et al., 2014; Bernard et al., 2002; HACA, 2002; Wagner et al., 1999), questions have arisen regarding the efficacy of current cooling protocols in providing optimal protection. In the past two years, a number of reviews have addressed this issue while paying little attention to the therapeutic mechanisms (Arrich et al., 2016; Chavez et al., 2016;

Dumitrascu et al., 2016; Gunn et al., 2016). Only a handful of studies have previously studied the role of signaling pathways in the therapeutic effects of hypothermia (Lee et al., 2017; Tahir and Pabaney, 2016; Talma et al., 2016) and the widely accepted mechanistic explanation is lacking in molecular detail. In general, proposed mechanisms rely on the conjecture that the reduction of metabolic demand in response to hypothermia leads to decreased oxidative stress, apoptosis, autophagy and inflammation (Lee et al., 2017; Talma et al., 2016). Let us call this conjecture the thermodynamic hypothesis. First, one should note that apoptosis, autophagy and inflammation are likely to be secondary events triggered by cellular damage and, hence, it is not clear from the hypothesis whether therapeutic hypothermia affects these processes directly or by alleviating the root causes of cellular damage. Oxidative stress can be a potential root cause and hypothermia could mitigate it either by reducing the production of ROS (e.g. by reducing electron leak from the electron transport chain) or by enhancing the antioxidant response (e.g. by up-regulating reduced glutathione).

There is, however, a more fundamental problem, which casts doubt on the thermodynamic hypothesis as a whole. If the thermodynamic hypothesis was to hold, one would expect larger therapeutic effects at lower temperatures (i.e. a positive correlation between therapeutic efficacy and temperature reduction) since it is well known that the metabolic rate of tissue is positively correlated with body temperature (not considering hyperthermia) (Chaui-Berlinck et al., 2002; Geiser, 2004). Substantial body of evidence stands in conflict with this prediction, however, as the effect of therapeutic hypothermia peaks at around 32ºC in mammals and no clear benefits arise from decreasing the temperature further (Bernard et al., 2002; Bona et al., 1998; Dalen et al., 2012; HACA, 2002; Holzer, 2010; Wood et al., 2016). In line with these observations, we found no additional gain in HIF1A/Nrf2 activity and corresponding target gene expression at 27 and 22ºC when compared to 32ºC. These observations suggest a more complicated model where the efficacy of hypothermia possibly depends on a mechanism with inhibitory (e.g. the slowing down of metabolic rate) and activating (e.g. activation of stress mitigation pathways) components with the highest therapeutic effect emerging in the window of mild hypothermia (32–35ºC). Our experiments with mild hypothermia revealed large-scale rearrangements of gene expression in cell culture concomitant with the activation of transcription factors with potentially therapeutic downstream effects. It is conceivable that mild hypothermia is accompanied by metabolic adaptation, which boosts resilience to oxidative stress to deal with the negative effects of hypoxia and decreased energy expenditure (Alva et al., 2010; Camara et al., 2004; Diestel et al., 2011; Huang et al., 2009; Maier et al., 2001). In hibernating species such as ground squirrels and bats, for example, metabolic adaptation to energy restriction has been shown to include upregulation of antioxidant proteins and glutathione (Carey et al., 2003; Storey, 2010; Yin et al., 2016).

Our experiments in cell culture indicate that mild hypothermia triggers a temporary induction of Nrf2, the master regulator of antioxidant response, and

its target genes 8h after the onset followed by an increase in reduced glutathione level which is sustained at least 24h. Since the levels of oxidized glutathione were not affected by hypothermia, the up-regulation of cellular glutathione should be interpreted as an increase in antioxidant potential (i.e. a proactive effect) and not a reaction to elevated oxidative stress. Furthermore, we found that hypothermic pretreatment (24h) increased the viability of MEFs and HeLa cells subjected to oxidative stress. To my knowledge, we are the first to report evidence that hypothermia effect can translate into the activation of the anti-oxidant system on three levels of the hierarchy 1) Nrf2 transcription factor activation 2) up-regulation of Nrf2 target genes 3) up-regulation of reduced glutathione.

Another therapeutically relevant effect of hypothermia was revealed in our experiments with the HIF1 reporter, where hypothermia amplified HIF1 induction when applied together with the hypoxia-mimetic $CoCl_2$. Hypothermic facilitation of HIF1 response to hypoxic stress is likely to speed up the cascade of metabolic adaptations such as switching to anaerobic ATP production by diverting glucose degradation to lactate and reduction of mitochondrial mass (Semenza, 2011; Zhang and Bosch, 2008).

On the other hand, hypothermia had negligible impact on the activity ER stress and NF-kB reporters suggesting that immune signaling (Han et al., 2002; Webster et al., 2009; Yenari and Han, 2006) and ER stress pathways (Aoki et al., 2001; Poone et al., 2015) are unlikely to be among the primary targets of therapeutic hypothermia in cells.

2. Paper II

2.1. Endogenous H2S influences cardiac mitochondrial content

We sought to determine if there was a direct relationship between the cardiac H_2S levels and mitochondrial content. First, we used a genetic model to address this question. We collected heart samples from mice with significantly elevated levels of H_2S (CSE Tg^+) and from mice with attenuated levels of H_2S (CSE KO) (Supplemental Figure 1, paper II). Analysis revealed that the hearts of CSE Tg^+ mice displayed an increase in mitochondrial DNA levels and citrate synthase activity (Figure 11A–B). In contrast, both were decreased in the hearts of CSE KO mice. Together these findings suggest that H_2S levels influence cardiac mitochondrial content. This was further confirmed by analysis of electron microscopy images of cardiac ventricles from CSE Tg^+ and CSE KO mice demonstrating an increase and decrease in the number of mitochondria per field, respectively (Figure 11C–D).

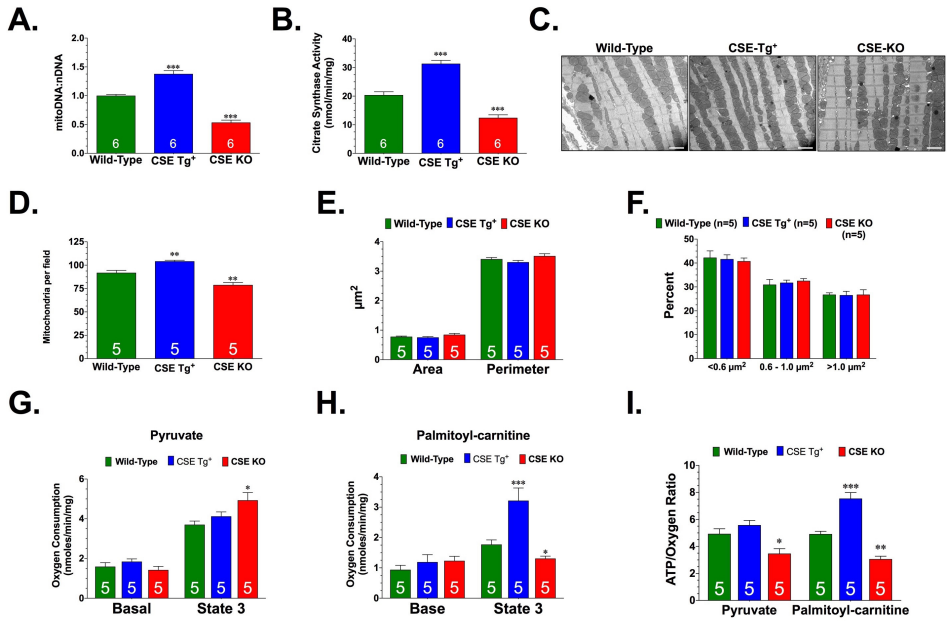

Figure 11. (A) Ratio of mitochondrial DNA to nuclear DNA. (B) Citrate synthase activity. (C) Representative electron microscopy images of mitochondria. Scale bar equals 3 µm. (D) Number of mitochondria per field of view. (E) Summary of mitochondria area and perimeter measurements (µm^2) and (F) percentage of mitochondria in a given field that fell into three size categories based on area: < 0.6 µm^2, 0.6 µm^2–1.0 µm^2, and > 1.0 µm^2. (G) Basal and maximum (state 3) oxygen consumption rates for permeabilized myocardial fibers in the presence of pyruvate and (H) palmitoyl-l-carnitine. (I) Efficiency of ATP synthesis [ATP produced per oxygen consumed (ATP/O)] in permeabilized myocardial fibers. All samples were collected from hearts of Wild-Type, cystathionase-γ-lyase transgenic (CSE Tg$^+$), and CSE deficient (CSE KO) mice. Values are means ± SEM. Numbers in bars indicate sample size. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. Wild-type.

Alterations in mitochondrial content can arise from biogenesis or from fusion and fission. A series of experiments were, therefore, undertaken to determine the contribution of fusion-fission to the altered mitochondrial content observed in the hearts of CSE Tg$^+$ and CSE KO mice (Supplemental Figure 2, paper II). First, the expression of proteins involved in mitochondrial fusion and fission were evaluated. The expression of the fusion proteins, Mfn1, Mfn2, and Opa-1, as well as the fission protein, fission-1 (Fis1) were not altered in the hearts of CSE Tg$^+$ or CSE KO mice. Second by electron microscopy, we observed no differences in the area or perimeter of the mitochondria in the hearts of each strain (Figure 11E). Finally, further analysis of mitochondrial fusion-fission was achieved by calculating the percentage of mitochondria in a given field that fell into three size categories based on area: < 0.6 µm2, 0.6 µm2–1.0 µm2, and

> 1.0 μm^2 (Wang et al., 2011). In support of the overall area and perimeter calculations, this analysis also revealed no differences in the hearts of each strain (Figure 11F). Overall these results suggest that H_2S levels influence cardiac mitochondrial content via biogenesis.

To characterize the mitochondrial functional phenotype of hearts from WT, CSE Tg^+ and CSE KO mice in a manner that would detect potential differences in mitochondrial volume density or function, respiration (oxygen consumption) experiments were performed on saponin-permeabilized myocardial fibers. This technique can measure the maximal respiratory capacity of specific mitochondrial oxidative pathways via selective use of different metabolic substrates (Lehman et al., 2008). Experiments were performed with LV fibers using pyruvate and palmitoyl-carnitine. For these experiments, basal respiration was assessed followed by maximal ADP-stimulated state 3 respiration. LV fibers from CSE Tg^+ mice did not display any changes in state 3 respiration rates in the presence of pyruvate (Figure 11G). They did however display increased state 3 respiration rates in the presence of palmitoyl-carnitine (Figure 11H). In contrast, LV fibers from CSE KO mice displayed increased state 3 respiration rates in the presence of pyruvate and decreased rates in the presence of palmitoyl-carnitine. Additionally, maximal rates of ATP synthesis from ADP were normalized to state 3 respiration rates to determine the efficacy of ATP synthesis in the presence of each substrate (Figure 11I). LV fibers from CSE Tg^+ mice displayed enhanced ATP/oxygen ratios in the presence of palmitoyl-carnitine. In contrast, fibers from CSE KO mice displayed lower ATP/oxygen ratios in the presence of both pyruvate and palmitoyl-carnitine.

2.2. Exogenous H_2S influences cardiac mitochondrial biogenesis

Next, we sought to determine if exogenous H_2S could influence cardiac mitochondrial content. For these studies, we administered SG-1002 (20 mg/kg/day) in the chow for 4 weeks. SG-1002 is an H_2S prodrug that we have previously shown to exert cardioprotective effects (Kondo et al 2013; Barr et al., 2015). Initial studies confirmed that the administration of SG-1002 significantly increased cardiac H_2S levels (Supplemental Figure 1, paper II). We then examined if the increase in H_2S influenced cardiac mitochondrial content. Analysis revealed that SG-1002 increased mitochondrial DNA levels, citrate synthase activity, and the number of mitochondria per field of electron microscopy images (Figure 12A–D). Further analysis confirmed that SG-1002 did not influence mitochondrial fusion-fission (Supplemental Figure 2, paper II and Figure 12E–F). Finally, SG-1002 did not alter the state 3 respiration rates of LV fibers in the presence of pyruvate (Figure 12G). It did, however, improve the state 3 respiration rates and ATP/oxygen ratios of LV fibers in the presence of palmitoyl-carnitine (Figure 12H–I).

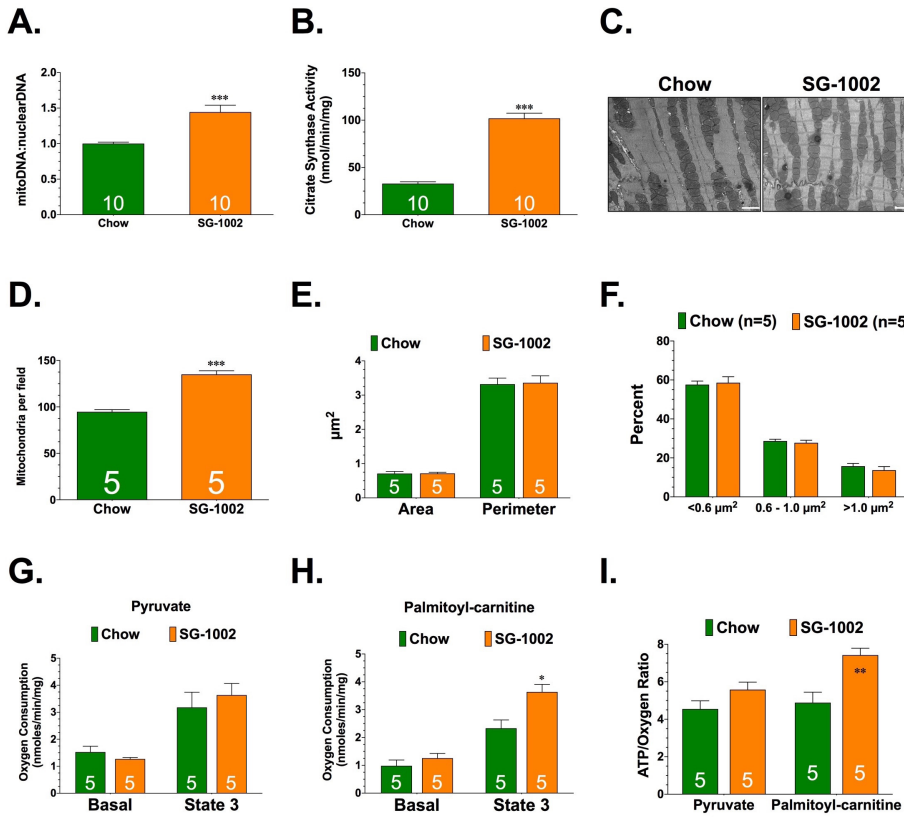

Figure 12. (A) Ratio of mitochondrial DNA to nuclear DNA. (B) Citrate synthase activity. (C) Representative electron microscopy images of mitochondria. Scale bar equals 3 μm. (D) Number of mitochondria per field of view. (E) Summary of mitochondria area and perimeter measurements (μm^2) and (F) percentage of mitochondria in a given field that fell into three size categories based on area: < 0.6 μm^2, 0.6 μm^2–1.0 μm^2, and > 1.0 μm^2. (G) Basal and maximum (state 3) oxygen consumption rates for permeabilized myocardial fibers in the presence of pyruvate and (H) palmitoyl-l-carnitine. (I) Efficiency of ATP synthesis [ATP produced per oxygen consumed (ATP/O)] in permeabilized myocardial fibers. All samples were collected from hearts of mice administered standard diet (Chow) or diet supplemented with SG-1002 (SG-1002; 20 mg/kg/day) for 4 weeks. Values are means ± SEM. Numbers in bars indicate sample size. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. Chow.

2.3. H_2S levels influence PGC1α

Given that PGC-1α is a master regulator of mitochondrial biogenesis (Kubli et al., 2012; Fernandez-Marcos et al., 2011), we sought to determine if H_2S influenced the expression or activation of PGC-1α. Initial analysis revealed that the whole cell expression of PGC-1α was not altered in the hearts of CSE Tg^+ or CSE KO mice (Figure 13A–B). However, the cytosolic levels of PGC-1α were significantly lower in the hearts of CSE Tg^+ mice (Figure 13A–B). Correspon-

dingly, the nuclear levels of PGC-1α were significantly higher in the hearts of CSE Tg^+ mice. In contrast, the cytosolic levels of PGC-1α were unaltered in the hearts of CSE KO mice. However, the nuclear levels of PGC-1α were significantly lower in the hearts of CSE KO mice (Figure 13A–B). In agreement with these changes, the hearts of CSE Tg^+ mice displayed elevated expression of a number of PGC-1α target genes, whereas the expression of the same genes were lower in the hearts of CSE KO mice (Figure 13D). Similarly, SG-1002 did not alter the whole cell expression of PGC-1α, but it did decrease the cytosolic levels of PGC-1α, increase the nuclear expression of PGC-1α, and increase the expression of PGC-1α target genes (Figure 13D–F). Together, this data suggests that cardiac H_2S levels influence the nuclear localization and transcriptional activity of PGC-1α.

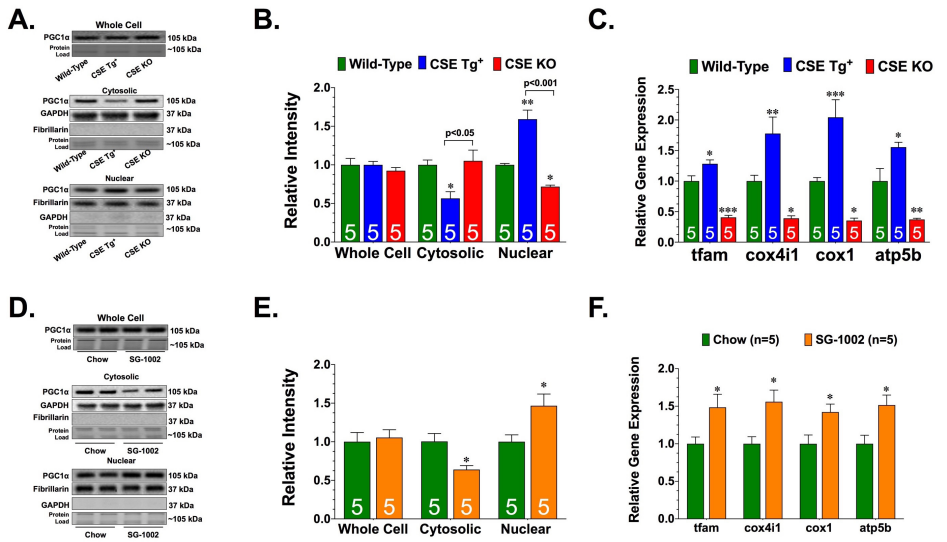

Figure 13. (A) Representative immunoblots and (B) analysis of the whole cell, cytosolic and nuclear expression of PGC1α. (C) Relative gene expression of PGC1α target genes associated with mitochondrial biogenesis (tfam, cox4i1, cox1, atp5b). Samples were collected from Wild-Type, CSE Tg+, and CSE KO mice. Values are means ± SEM. *p < 0.05 and **p < 0.01 vs. Wild-type. (D) Representative immunoblots and (E) analysis of the whole cell, cytosolic and nuclear expression of PGC1α. (F) Relative gene expression of PGC1α target genes associated with mitochondrial biogenesis (tfam, cox4i1, cox1, atp5b). Samples were collected from mice administered Chow or diet supplemented with SG-1002. Values are means ± SEM. Numbers in bars indicate sample size. *p < 0.05 vs. Chow.

2.4. H_2S induces mitochondrial biogenesis via AMPK

The transcriptional activity of PGC-1α is regulated by post-translational modifications. For instance, AMPK regulates the transcriptional activity of PGC-1α via phosphorylation (Jager et al., 2007), whereas Sirt1 regulates its transcriptional activity via deacetylation (Fernandez-Marcos et al., 2011). Evidence suggests that AMPK both activates Sirt1 and cooperates with it in enhancing the ability of PGC-1α to stimulate mitochondrial biogenesis (Yan et al., 2013). So, the next series of experiments sought to determine if H_2S-induced mitochondrial biogenesis was channeled through AMPK and Sirt1. SG-1002 increased the phosphorylation and activity of AMPK (Figure 14A–C). No changes were observed in the expression of Sirt1, but SG-1002 did increase its activity (Figure 14D–F). In agreement with these changes, SG-1002 increased the serine phosphorylation of PGC-1α and decreased its acetylation (Figure 14G–I). To determine if AMPK was directly responsible for H_2S induced PGC-1α-signaling and mitochondrial biogenesis, αMHC-Cre^{+} x $AMPK^{fl/fl}$ mice were given SG-1002 for 4 weeks. The deficiency of AMPK did not affect the expression of PGC-1α or Sirt1 (Figure 15A–C). However, SG-1002 failed to increase the serine phosphorylation of PGC-1α, failed to increase Sirt1 activity, and failed to decrease the acetylation of PGC-1α (Figure 15D–H). Importantly, SG-1002 was unable to increase the expression of PGC-1α target genes and mitochondrial DNA levels in the hearts of αMHC-Cre^{+} x $AMPK^{fl/fl}$ mice (Figure 15I). Together this data suggests that H_2S induced mitochondrial biogenesis via an AMPK-Sirt1-PGC-1α signaling cascade.

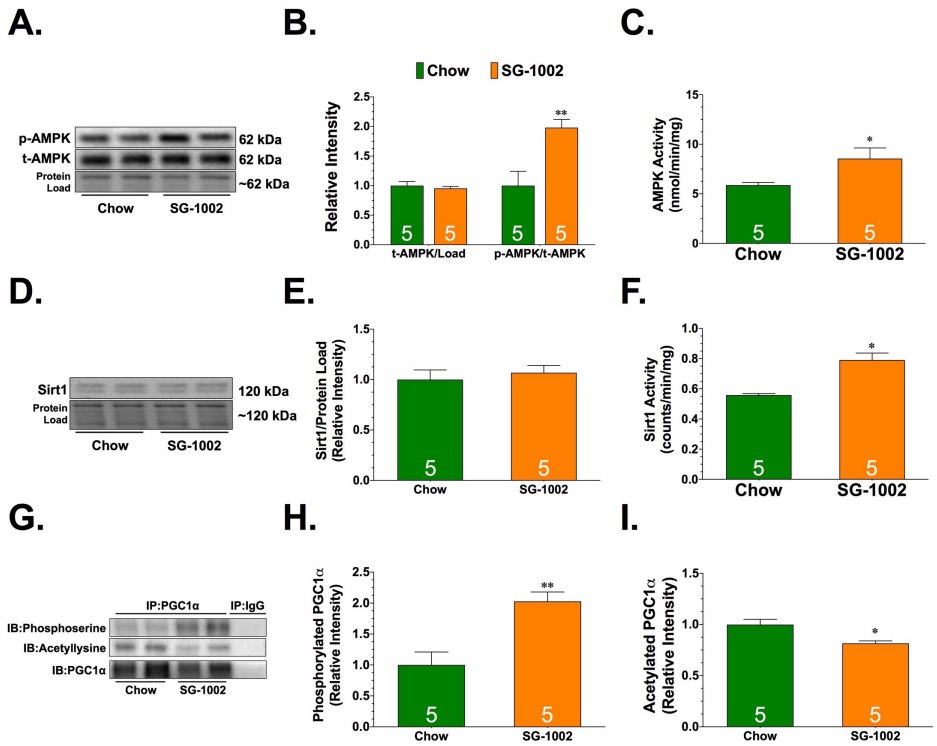

Figure 14. (A–B) Representative immunoblots and analysis of phosphorylated AMPK and total AMPK. (C) AMPK activity. (D–E) Representative immunoblots and analysis of Sirt1. (F) Sirt1 activity. (G) Representative immunoblots and analysis from immunoprecipitation experiments examining the (H) serine phosphorylation and (I) acetylation status of PGC1α. All samples were collected from hearts of mice administered standard diet (Chow) or diet supplemented with SG-1002 (SG-1002; 20 mg/kg/day) for 4 weeks. Values are means ± SEM. Numbers in bars indicate sample size. *$p < 0.05$ and **$p < 0.01$ vs. Chow.

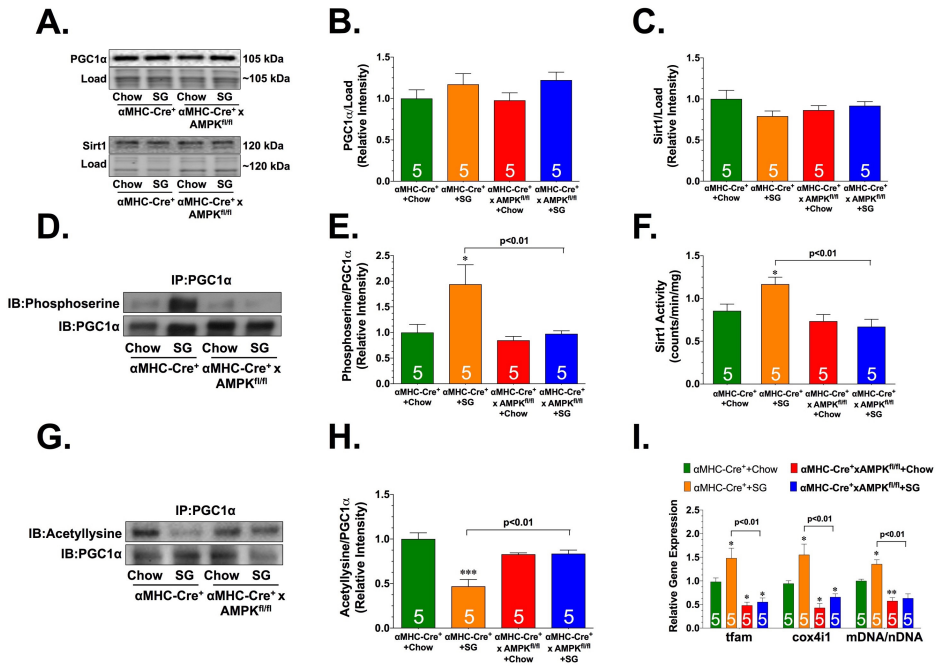

Figure 15. (A) Representative immunoblots and analysis of (B) PGC1α and (C) Sirt1. (D) Representative immunoblots and analysis from immunoprecipitation experiments examining the serine phosphorylation status of PGC1α. (E) Sirt1 activity. (F) Representative immunoblots and analysis from immunoprecipitation experiments examining the acetylation status of PGC1α. (G) Relative gene expression of tfam, cox4i1, and mitochondria DNA. All samples were collected from hearts of Wild-type (αMHC-Cre+) and αMHC-Cre^{+} x AMPK floxed (αMHC-Cre^{+} x AMPK$^{fl/fl}$) mice administered standard diet (Chow) or diet supplemented with SG-1002 (SG; 20 mg/kg/day) for 4 weeks. Values are means ± SEM. Numbers in bars indicate sample size. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. WT Chow.

2.5. H_2S levels and mitochondria content are reduced in response to heart failure

There is evidence for impaired mitochondrial biogenesis from animal models of heart failure and from studies utilizing human heart failure samples (Karamanlidis et al., 2010; Arany et al., 2006). Additionally, studies report lower circulating H_2S levels in human heart failure patients (Polhemus et al., 2014). However, an association between cardiac H_2S levels and cardiac mitochondrial content has not been explored. Here, we were able to obtain LV samples from end-stage heart failure patients at the time of transplant. Analysis revealed diminished H_2S levels (Figure 16A–B), as well as lower levels of PGC-1α target genes (Figure 16C) and mitochondrial DNA levels (Figure 16D). Similar results in regards to H_2S levels and mitochondrial content were also found in samples from mice collected at 2 weeks following myocardial ischemia and reperfusion

(Figure 17A–C). We, therefore, sought to determine if restoring H_2S levels could influence the mitochondrial content of the ischemic heart via the proposed AMPK-PGC1α signaling cascade. Mice subjected to myocardial ischemia and reperfusion were administered normal diet or diet supplemented with SG-1002 beginning 24 h after reperfusion. The mice were then followed for 2 weeks. Our analysis revealed that restoring H_2S levels via the dietary supplementation of SG-1002 was associated with an increase in the phosphorylation of AMPK, an increase in the gene expression of PGC-1α target genes, an increase in cardiac mitochondrial content, improved mitochondrial respiration, improved ATP production efficiency, and improved cardiac dilatation and function (Figure 17C–H and Supplemental Figure 6F–H, paper II).

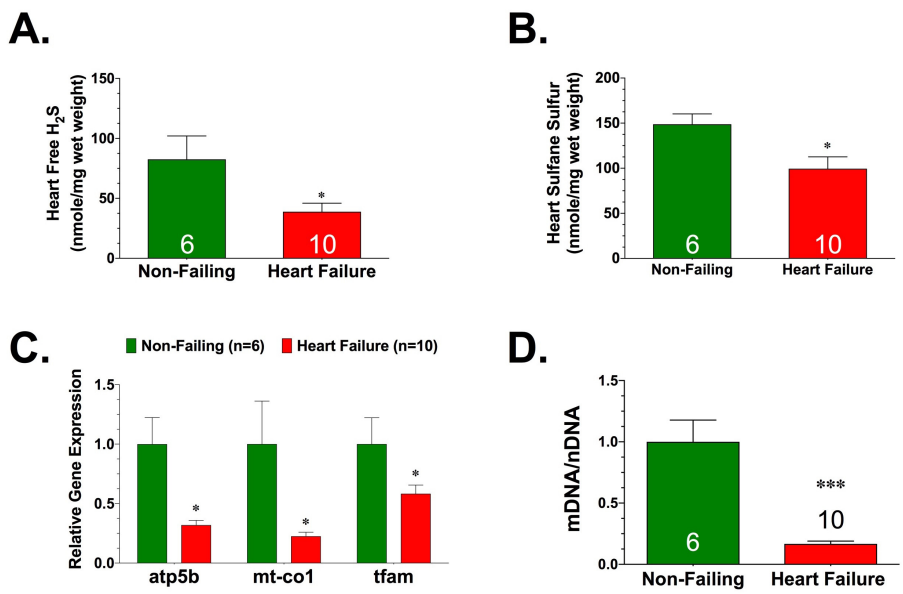

Figure 16. Cardiac levels of (A) free H_2S and (B) sulfane sulfur. (C) Relative gene expression of atp5b, mt-co1, and tfam. (D) Ratio of mitochondrial DNA to nuclear DNA. LV samples were collected from non-failing and end stage heart failure patients. Number in bars represent sample sizes. Values are means ± SEM. Numbers in bars indicate sample size. *$p < 0.05$ and ***$p < 0.001$ vs. Non-Failing.

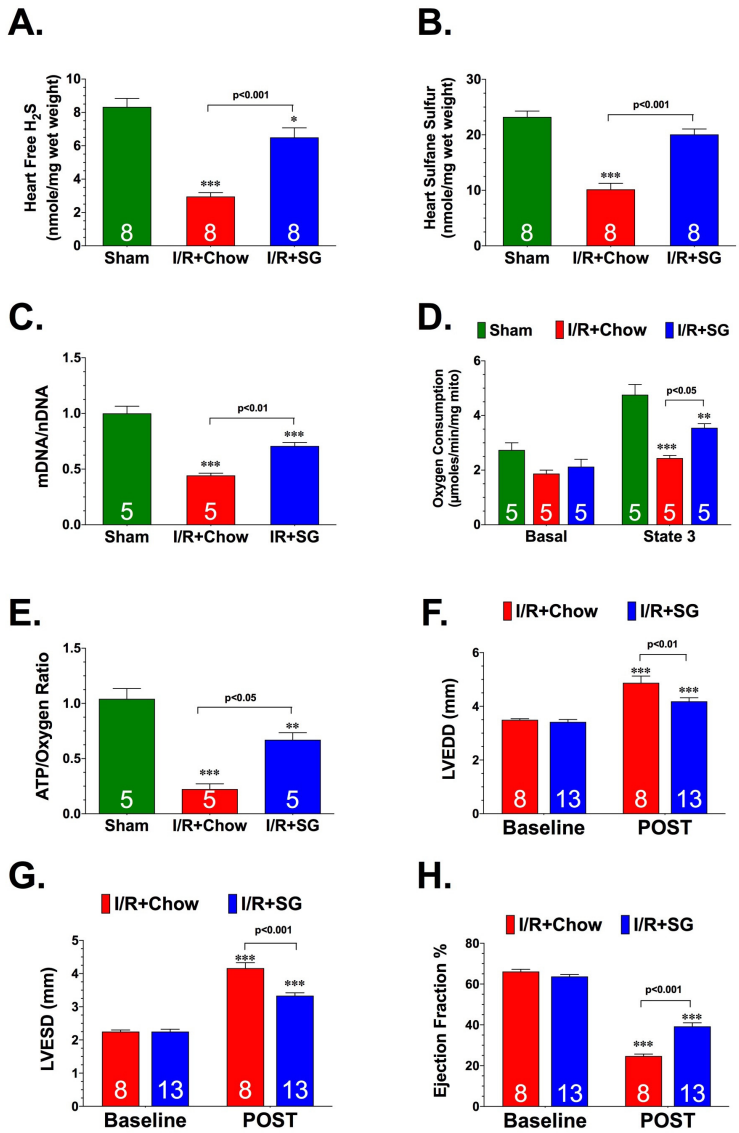

Figure 17. Cardiac levels of (A) free H_2S and (B) sulfane sulfur. (C) Ratio of mitochondrial DNA to nuclear DNA. (D) Basal and maximum (state 3) oxygen consumption rates for permeabilized myocardial fibers in the presence of palmitoyl-l-carnitine. (E) Efficiency of ATP synthesis [ATP produced per oxygen consumed (ATP/O)] in permeabilized myocardial fibers. Samples were collected from hearts of mice subjected to 60 min of ischemia and 2 weeks of reperfusion. Values are means ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. Sham. (F) LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and LV Ejection Fraction measured in groups of mice using echocardiography images 2 weeks following myocardial ischemia and reperfusion (Post). Values are means ± SEM. Numbers in bars indicate sample size. ***$p < 0.001$ vs. Baseline.

The transcriptional activity of PGC-1α is regulated by a number of stimuli, exemplifying the range of settings in which mitochondrial biogenesis is induced. The mechanisms governing this regulation have been extensively studied and have been determined to vary between tissues and different settings (Fernandez-Marcos et al., 2011). AMPK, a member of the metabolite-sensing protein kinase family, is activated in response to alterations in cellular energy levels (Zaha et al., 2012). Activation of AMPK acts to maintain cellular energy stores, switching on catabolic pathways that produce ATP, while switching off anabolic pathways that consume ATP (Canto et al., 2009). AMPK also induces mitochondrial biogenesis via the activation of PGC-1α. Specifically, AMPK regulates PGC-1α directly through serine phosphorylation, as well as indirectly via the activation of Sirt1, which leads to the deacetylation of PGC-1α (Fernandez-Marcos et al., 2011). This coordinated signaling cascade, whereby the phosphorylation of PGC-1α by AMPK is required for the subsequent Sirt1-mediated deacetylation, demonstrates the complex and specific nature by which PGC-1α is regulated by AMPK (Nisoli et al., 2004). Here, we found that increasing cardiac H_2S levels through the dietary supplementation of SG-1002 led to the activation of AMPK and Sirt1 resulting in the phosphorylation and deacetylation of PGC-1α. More importantly, we found that SG-1002 failed to alter PGC-1α, induce PGC-1α target genes, and induce mitochondrial biogenesis in the hearts of AMPKα2 deficient mice – indicating that H_2S induces mitochondrial biogenesis via AMPK.

As noted, H_2S is a known regulator of cellular bioenergetics via its actions on mitochondrial function. For instance, H_2S acts as a stimulator of mitochondrial bioenergetics through its ability to donate electrons to the mitochondrial electron transport chain (Modis et al., 2013a; Fu et al., 2012). This action serves a physiological role in the maintenance of mitochondrial electron transport, as well as complementing and balancing the bioenergetic role of Krebs cycle-derived electron donors (Modis et al., 2013a). In contrast, H_2S is a potent and reversible inhibitor of mitochondrial function via its regulation of cytochrome c oxidase (complex IV of the mitochondrial electron transport chain) (Hill et al., 1984). Paradoxically, this action contributes to the cardioprotective effects of exogenous H_2S, as inhibition of mitochondrial respiration during the early stages of reperfusion injury limits the generation of ROS, which ultimately preserves mitochondrial function (King et al., 2014; Calvert et al., 2010b; Elrod et al., 2007). In addition, H_2S targets several cellular pathways that influence mitochondrial function (Modis et al., 2013b). PGC-1α not only regulates mitochondrial biogenesis, but also regulates energy expenditure in the heart. Specifically, PGC-1α is essential for the maintenance of maximal, efficient cardiac mitochondrial fatty acid oxidation and ATP synthesis (Lehman et al., 2008). Consistent with our findings regarding the induction of PGC-1α signaling, we observed that augmenting cardiac H_2S levels increased the maximal capacity for mitochondrial fatty acid β-oxidation and ATP synthesis, whereas lower H_2S levels had the opposite effect.

The AMPK-PGC-1α signaling cascade has been extensively studied in the context of cardiac metabolism (Zaha et al., 2012). Current study provides novel evidence that endogenous and exogenous H_2S modulates mitochondrial biogenesis via AMPK-PGC-1α signaling. Furthermore, the finding that a decrease in endogenous H_2S levels led to an impairment in AMPK-PGC-1α signaling becomes important when considering the evidence that H_2S levels are decreased in pathological conditions – i.e. heart failure (Fig. 15 and (Polhemus et al., 2014)) – that also present with reduced mitochondrial content (Karamanlidis et al., 2010; Arany et al., 2006). Based on this evidence it can be suggested that endogenous H_2S levels not only play an important role in maintaining the mitochondrial content of the heart, but that a reduction in endogenous H_2S levels contributes to the pathophysiology of heart failure through a disruption in mitochondrial biogenesis. This idea is supported by our findings that restoring H_2S levels with SG-1002 increased mitochondrial content, improved ATP production, and attenuated LV dysfunction in a murine model of ischemia reperfusion injury.

3. Paper III

3.1. Kinetics of lymphangiogenesis early after the onset of myocardial ischemia

Previous studies report an endogenous lymphangiogenesis in response to myocardial ischemia (Klotz et al., 2015; Henri et al., 2016). However, the kinetics of the response have not been evaluated during the early period following the onset of ischemia. We, therefore, addressed this here. For these experiments, mice were subjected to permanent myocardia ischemia and followed for up to 7 days. First, we assessed the protein expression of VEGF-C and VEGFR3 in heart homogenates obtained from mice subjected to various periods of ischemia (Figure 18). The expression of VEGF-C was significantly increased 1 day after the onset of ischemia. This increase persisted for up to 7 days of ischemia. The expression of VEGFR3 was increased from 3 days to 7 days of ischemia. Next, we evaluated the remodeling of the cardiac lymphatics by focusing on the lymph density in the subendocardium (an area that experiences a robust increase in lymphatic density in response to myocardial ischemia (Henri et al., 2016). Our analysis revealed a significant increase in the number of LYVE1 positive cells starting at 3 days of ischemia that persisted for up to 7 days of ischemia (Figure 19A–B). To investigate if the observed increase in LYVE1 positive cells was indicative of lymphatic cell proliferation, we treated a subset of mice with BrdU starting after the onset of myocardial ischemia. Analysis revealed a significant increase in the number of LYVE1 positive cells labeled with BrdU starting at 3 days of ischemia with a gradual increase noted at 7 days of ischemia (Figure 19C). Finally, we also evaluated the diameter of lymphatic pre-collectors in the epicardium as an evaluation of lymphatic drainage capacity (Henri et al., 2016). Our analysis revealed a significant increase in lumen area starting at 1 day of ischemia that persisted for up to 7 days of ischemia (Figure 19A and D).

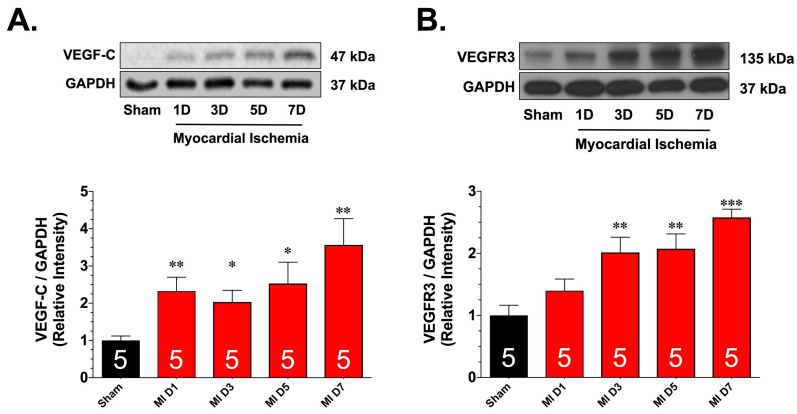

Figure 18. Representative immunoblots and analysis of (A) VEGF-C and (B) VEGFR3 protein expression levels in samples collected from hearts subjected to different periods of ischemia. Values are means ±SEM. Numbers in bars indicates sample size. *p<0.05, **p<0.01, and ***p<0.001 vs. Sham. MI, myocardial ischemia.

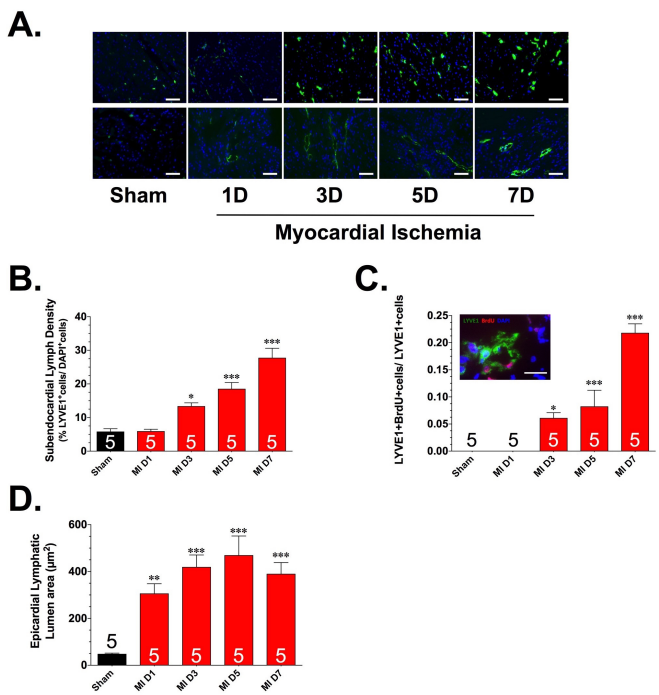

Figure 19. Representative images of LV sections stained with an anti-LYVE1 antibody to denote LYVE1 positive cells (top row) and lymphatic collecting vessel luminal area. (B) Summary of LYVE1 positive cells. (C) Summary of LYVE1 positive cells labeled with BrdU. (D) lymphatic lumen area. Samples were collected from hearts subjected to different periods of ischemia. Scale bar denotes 50 μm(A) or 20 μm (C). Values are means ±SEM. Numbers in bars indicate sample size. *p<0.05, **p<0.01 and ***p<0.001 vs. Sham.

3.2. Kinetics of lymphangiogenesis in the setting of myocardial ischemia reperfusion injury

The next series of experiments evaluated the kinetics of the lymphangiogenesis response in a more clinically relevant model of myocardial ischemia reperfusion injury. For these experiments, mice were subjected to 60 minutes of myocardial ischemia followed by up to 7 days of reperfusion. First, we assessed the protein expression of VEGF-C and VEGFR3 in heart homogenates obtained from mice subjected to ischemia and to various periods of reperfusion (Figure 20). The expression of VEGF-C was significantly increased 1 day after reperfusion. This increase persisted for up to 7 days of reperfusion with a peak elevation observed at 3 days of reperfusion. The expression of VEGFR3 was likewise increased from 1 day to 7 days of reperfusion. However, the increase was similar at all times evaluated. Additional analysis revealed a significant increase in the number of LYVE1 positive cells starting at 3 days of reperfusion that persisted for up to 7 days of reperfusion (Figure 21A–B). Finally, we noted a significant increase in epicardial lymphatic lumen area starting at 3 days of reperfusion that persisted for up to 7 days of reperfusion (Figure 21A and C).

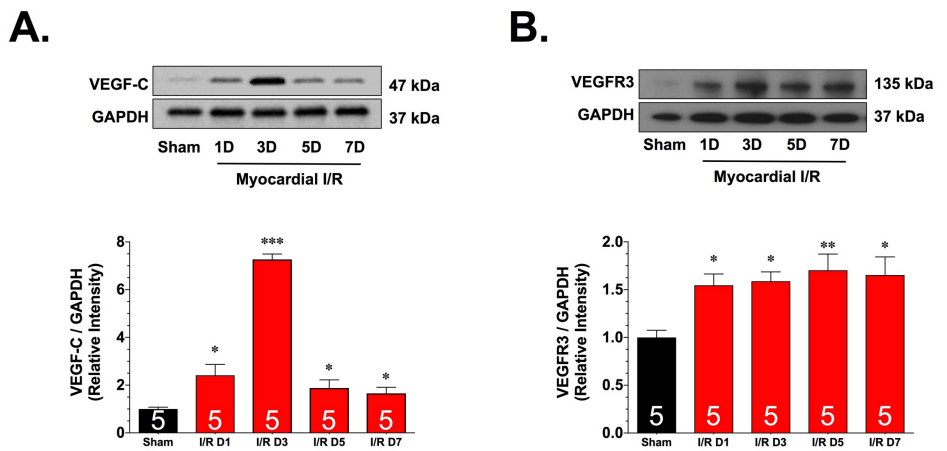

Figure 20. Representative immunoblots and analysis of (A) VEGF-C and (B) VEGFR3 protein expression levels in samples collected from hearts subjected to 60 minutes of myocardial ischemia and different periods of reperfusion. Values are means ±SEM. Numbers in bars indicate sample size. *p<0.05, **p<0.01, and ***p<0.001 vs. Sham. I/R, ischemia and reperfusion.

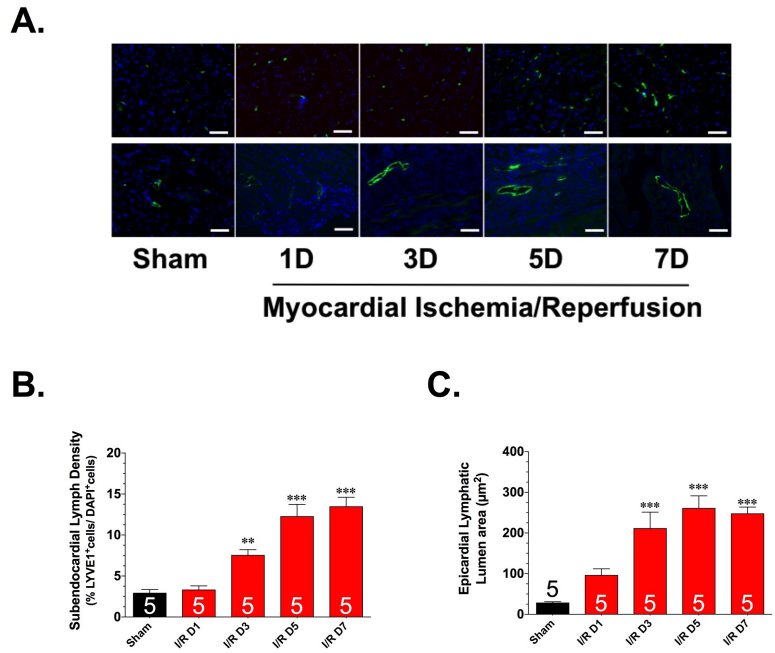

Figure 21. (A) Representative images of LV sections stained with an anti-Lyve1 antibody to denote Lyve1 positive cells (top row) and lymphatic collecting vessel luminal area. Summary of (B) Lyve1 positive cells and (C) lymphatic lumen area. Samples were collected from hearts subjected to 60 minutes of myocardial ischemia and different periods of reperfusion. Scale bar denotes 50 μm. Values are means ±SEM. Numbers in bars indicate sample size. **p<0.01 and ***p<0.001 vs. Sham.

3.3. Blocking endogenous lymphangiogenesis response exacerbates ischemic-induced heart failure.

As noted, previous studies have reported that treatment with VEGF-C augments endogenous lymphangiogenesis and leads to improvements in cardiac function in rat models of permanent myocardial ischemia and myocardial ischemia reperfusion (Klotz et al., 2015; Henri et al., 2016). However, there is not any information available linking the consequences of inhibiting the endogenous lymphangiogenesis response to the development of heart failure. We, therefore, addressed this issue using two different pharmacological approaches. In the first set of experiments, mice were subjected to 60 minutes of ischemia followed by reperfusion. At the time of reperfusion, a hydrogel containing the VEGFR3 inhibitor, MAZ-51, was placed on the surface of the infarcted myocardium prior to the closing of the chest cavity. Mice treated with MAZ-51 displayed a significant decrease in subendocardial lymph density at 7 days of reperfusion when compared to vehicle treated mice (Figure 22A–B). Given that the lymphatic system functions in the clearance of inflammatory cells and edema (Alitalo, 2011; Bryere and Noel, 2010; Karaman and Detmar, 2014), we next evaluated

the relationship in the development of lymphatic vessels and the inflammatory response following MAZ-51 treatment. First, we observed a significant increase in the diameter of epicardial lymphatic pre-collectors in MAZ-51-treated hearts when compared to vehicle treated mice (Figure 22A and C). This was associated with the accumulation of B lymphocytes (detected as B220 and IgM double positive cells) (Zouggari et al., 2013) and an increase in the levels of TNFα, IL-1b, and IL-6 (Figure 23A–E). In the second set of experiments, different groups of mice had a hydrogel containing a VEGF-C NAb placed on the surface of the infarcted myocardium. In a similar manner to MAZ-51, treatment with VEGF-C NAb led to a decrease in subendocardial lymph density (Figure 22A–B), an increase in the diameter of epicardial lymphatic pre-collectors (Figure 22A and C), and an increase in the inflammatory response at 7 days of reperfusion (Figure 23A-E). Postischemic cardiac remodeling and dysfunction are closely linked to inflammation. (Frangogiannis, 2012) We, therefore, investigated the effects of MAZ-51 and VEGF-C NAb treatment on cardiac remodeling and dysfunction. Analysis at 28 days of reperfusion revealed that both treatments increased infarct scar size, increased ventricular hypertrophy, increased LV dilatation, and decreased LV function (Figure 24).

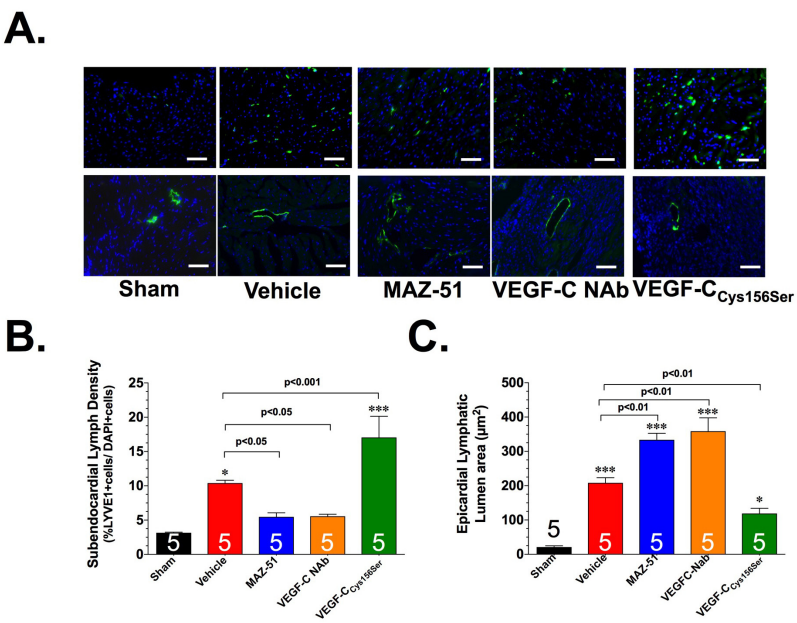

Figure 22. (A) Representative images of LV sections stained with an anti-Lyve1 antibody to denote Lyve1 positive cells (top row) and lymphatic collecting vessel luminal area. Summary of (B) Lyve1 positive cells and (C) lymphatic lumen area. Samples were collected from hearts subjected to 60 minutes of myocardial ischemia and 1 week of reperfusion. Different groups of mice were treated with Vehicle, MAZ-51, VEGF-C neutralizing antibody (NAb), or VEGF-$C_{Cys156Ser}$. Scale bar denotes 50 μm. Values are means ±SEM. Numbers in bars indicate sample size. *p<0.05 and ***p<0.001 vs. Sham.

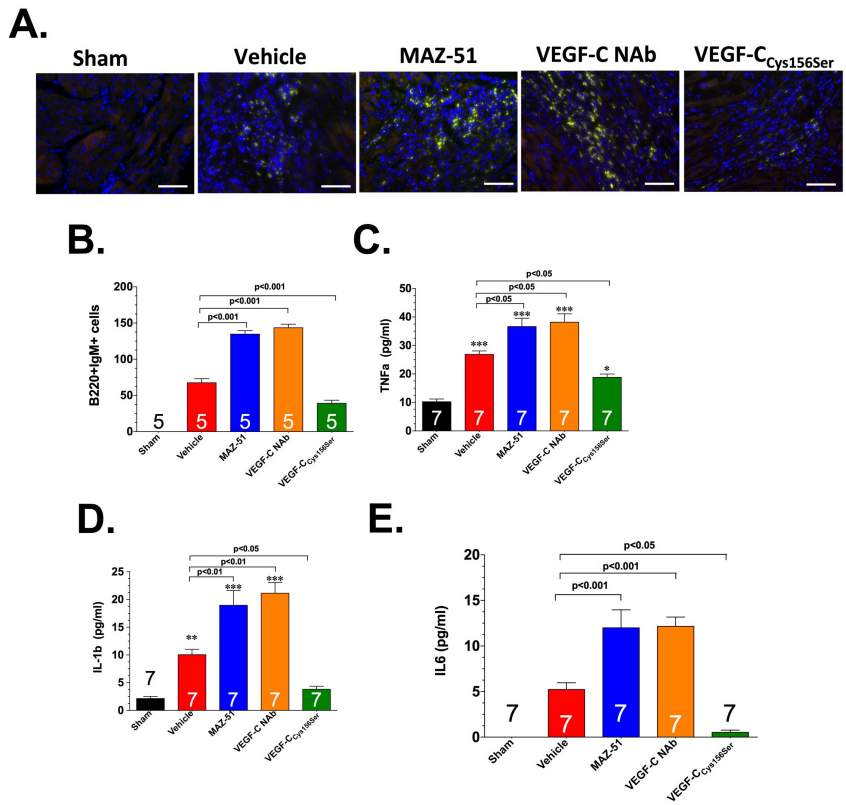

Figure 23. (A) Representative images of LV sections stained with B220 and IgM to denote inflammatory cells. Bar denotes 50 μm. (B) Summary of B220 and IgM positive cells. Levels of (C) TNFα, (D) IL-1b, and (E) IL-6. Samples were collected from hearts subjected to 60 minutes of myocardial ischemia and 1 week of reperfusion. Different groups of mice were treated with Vehicle, MAZ-51, VEGF-C neutralizing antibody (NAb), or VEGF-$C_{Cys156Ser}$. Values are means ±SEM. Numbers in bars indicate sample size. *p<0.05, **p<0.01, and ***p<0.001 vs. Sham.

3.4. Enhancing endogenous lymphangiogenesis response attenuates ischemic-induced heart failure

Next, we sought to determine the therapeutic potential of a VEGF-C releasing hydrogel in the setting of myocardial ischemia reperfusion injury. For these experiments, mice were subjected to 60 minutes of ischemia followed by reperfusion. At the time of reperfusion, a hydrogel containing VEGF-$C_{Cys156Ser}$ (a mutant form that specifically binds to VEGF-R3 (Joukov et al., 1998) was placed on the surface of the infarcted myocardium prior to the closing of the chest cavity. Mice treated with VEGF-$C_{Cys156Ser}$ displayed a significant increase in subendocardial lymph density at 7 days of reperfusion when compared to vehicle treated mice (Figure 22A–B). This was associated with a decrease in the

diameter of epicardial lymphatic pre-collectors (Figure 22A and C), a decrease in the accumulation of B lymphocytes (Figure 23A–B), and a decrease in the levels of TNFα, IL-1b, and IL-6 (Figure 23C-E) when compared to vehicle treated mice. Further analysis at 28 days of reperfusion revealed that VEGF-$C_{Cys156Ser}$ treatment decreased infarct scar size, decreased ventricular hypertrophy, decreased LV dilatation, and increased LV function (Figure 24).

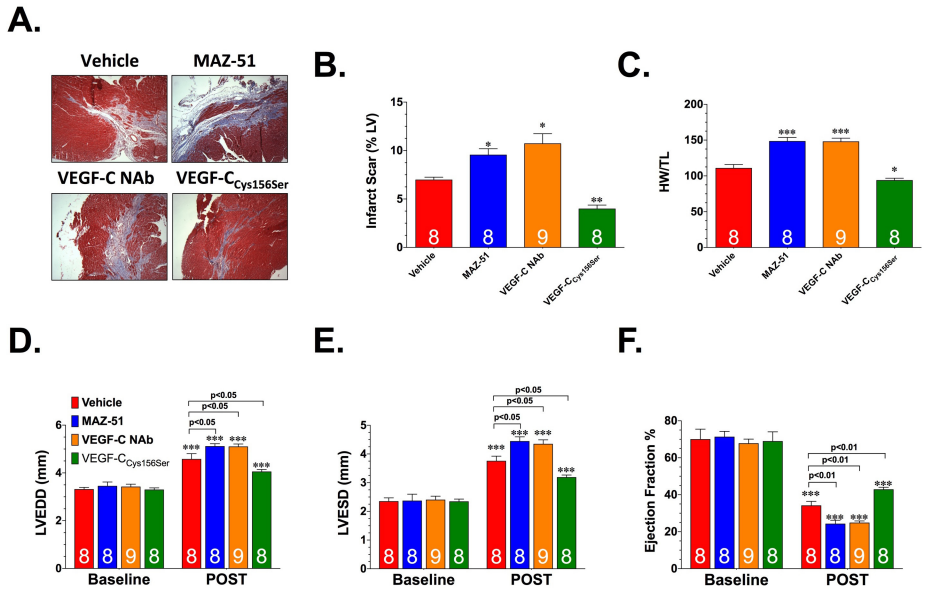

Figure 24. (A) Representative images of heart sections stained with Masson's trichrome to denote infarct scar. (B) Infarct scar area as a percentage of LV area. (C) Heart weight to tibia length ratios (HW:TL). Values are means ±SEM. *p<0.05, **p<0.01, and ***p<0.001 vs. Vehicle. (D) Left ventricular end-diastolic diameter (LVEDD), (E) LV end-systolic diameter (LVESD), and (F) LV ejection fraction were measured in groups of mice using echocardiography images 4 weeks following myocardial ischemia and reperfusion (Post). Values are means ±SEM. Numbers in bars indicate sample size. ***p<0.001 vs. Baseline.

The molecular and cellular events initiated with hours to days following the onset of myocardial infarction dictate the ultimate consequences of the injury. Therefore, it is important to understand early signaling events that contribute to myocardial damage and repair (Rainer et al., 2014). Following the onset of ischemic injury, cardiomyocytes undergo irreversible injury leading to cell death. The initiation of a highly regulated inflammatory response consisting of neutrophils and monocytes/macrophages occurs with hours after the insult to remove dead cells and matrix debris (Jung et al., 2013; Swirski et al., 2013; Frangogiannis, 2006). As a result, the temporal sequence of events occurring

after the onset of ischemic injury must be finely tuned to promote healing while at the same time minimizing adverse remodeling (Rainer et al., 2014).

The functional significance of the cardiac lymphatic system was first demonstrated by the observation that surgical ligation of a lymphatic vessel in the canine heart induced edema, cardiac fibrosis and cardiac dysfunction (Kline et al., 1963). This was further substantiated by the observations that obstructing lymphatic flow following myocardial ischemia exacerbated ischemic-induced edema, fibrosis and cardiac dysfunction (Kline et al., 1964). However, until recently, few advances in our understanding of the physiological role of the cardiac lymphatic system have been made. In the past several years, two elegant studies have independently demonstrated that myocardial ischemia induces an endogenous lymphangiogenesis response (Klotz et al., 2015; Henri et al., 2016). In the current study, we confirm these results in mouse models of myocardial ischemia and ischemia reperfusion injury. In our study, we focused on the kinetics of the endogenous lymphangiogenesis response during the early periods following either the onset of ischemia or reperfusion. Although we found a similar response in regards to an increase in the protein expression of VEGF-C and VEGFR3 preceding an increase in subendocardial lymph density, there was a subtle difference between the two models. Specifically, we observed a difference in the kinetics of VEGF-C expression. In the ischemia model, VEGF-C was elevated to a similar level at all times evaluated. In contrast, the expression of VEGF-C rose dramatically at 3 days of reperfusion before declining to a lower level by 5 days of reperfusion. Currently, the underlying cause for this difference is not known. However, the difference did not seem to influence the endogenous lymphangiogenesis response, as a similar pattern and onset of an increase in subendocardial lymph density was observed in both models. Our findings are in slight contrast with those previously reported (Klotz et al., 2015; Henri et al., 2016). Specifically, the previous studies reported that the endogenous lymphangiogenesis response occurs weeks after the onset of myocardial ischemia. Currently, we do not completely understand the conflicting observations regarding the timing of the response. However, it should be noted that the previous studies used a rat model of myocardial ischemia and focused on later time points. Here, we used a mouse model and focused on the early periods following the onset of ischemia or reperfusion. Despite the differences in timing all 3 findings clearly indicate that the response occurs.

In agreement with the two previous studies (Klotz et al., 2015; Henri et al., 2016), we also found that treatment with a mutated version of VEGF-C augmented the endogenous lymphangiogenesis response and attenuated ischemic-induced cardiac dysfunction. Specifically, we found that VEGF-$C_{Cys156Ser}$ treatment increased ischemic-induced subendocardial lymph density and decreased inflammation at 7 days of reperfusion. This was associated with a reduction in scar formation and improvement in cardiac function at 28 days of reperfusion. More importantly, we provide direct evidence that blocking the endogenous lymphangiogenesis response exacerbates cardiac injury and dysfunction following myocardial ischemia reperfusion. As noted, signaling via VEGF-C

and VEGFR3 are the most central pathway for lymphangiogenesis. Here, we found that inhibiting VEGFR3 with MAZ-51 blunted the ischemic-induced increase in subendocardial lymph density, increased inflammation, increased scar formation, and increased cardiac dysfunction. Likewise, targeting endogenous VEGF-C with a NAb disrupted the endogenous lymphangiogenesis response and exacerbated cardiac injury. Together, this data suggests that the endogenous lymphangiogenesis response plays an adaptive role in response to myocardial ischemia reperfusion injury.

4. Concluding remarks and future directions

The clinical manifestations of ischemia reperfusion injury are diverse. Even more, the process of reperfusion is a 'double-edge sword' and can, in itself, induce cell death. From a treatment standpoint, ischemia reperfusion injury is a critical challenge. We have shown three interventions that are capable of reducing ischemia reperfusion injury such as hypothermia, hydrogen sulfide and lymphangiogenesis (Figure 25). Therapeutic modalities can be categorized according to the time of application. Hypothermia treatment is normally administered during ischemia (Yenari and Hemmen, 2010; Yenari and Han, 2012). In our settings, diet supplemented with SG-1002 began 24 h after reperfusion and gelatin hydrogels containing VEGF-C were placed on the surface of myocardium after the onset of reperfusion. Hypothermia may protect against ongoing ischemic injury and blunt the immediate hyperaemia (Yenari and Han, 2012). Since most cell death occurs during the first minutes of reperfusion, treatments that protect only against reperfusion injury should be administered as early as possible. In fact, hypothermia applied together with interventions against reperfusion injury (e.g. hydrogen sulfide or lymphangiogenesis) may protect the heart from ongoing ischemic injury and reduce damage caused by restoration of blood flow.

Therapeutic strategies can also be classified according to their end target. The first group includes molecular targets involved in cell death, such as necrosis, apoptosis, reactive oxygen species, and autophagy. Hypothermia (Frink et al., 2012; Yenari and Han, 2012) and hydrogen sulfide (Wu et al., 2015) are known to target these pathways. However, there has been some controversy on the role of H_2S in autophagy (Wu et al., 2018). Second group includes activation of signaling pathways related to cytoprotective response, pro-survival, and mitochondrial function. Again, hypothermia and hydrogen sulfide target this group. Third group includes inflammation and all here studied therapeutics reduce inflammation (Szuba et al., 2002; Kataru et al., 2009; Frink et al., 2012; Yenari and Han, 2012; Shimizu et al., 2012; Wu et al., 2015; Henri et al., 2016).

Protective strategies can also be catecorized based on the cellular target. Hypothermia affects virtually all organ systems. In the heart, hydrogen sulfide is produced in the myocardium, blood vessels and fibroblasts (Andreadou et al.,

2015; Calvert et al., 2010b; Polhemus and Lefer, 2014). VEGF-C binds to VEGFR-3 on lymphatic endothelial cells and to either VEGFR-3 or vascular endothelial growth factor receptor 2 (VEGFR-2) on blood endothelial cells (Brakenhielm and Alitalo, 2009; Cao et al., 1998; Joukov et al., 1997; Henri et al., 2016).

Translation of individual therapeutic to clinical settings has controversy results (Doukas et al., 2006; Garcia-Prieto et al., 2017). Combination of 2 or more interventions, each which has different time point of action (during ischemia, at reperfusion, and late into reperfusion), distinct target and end-point may provide additive benefits and maximal protection.

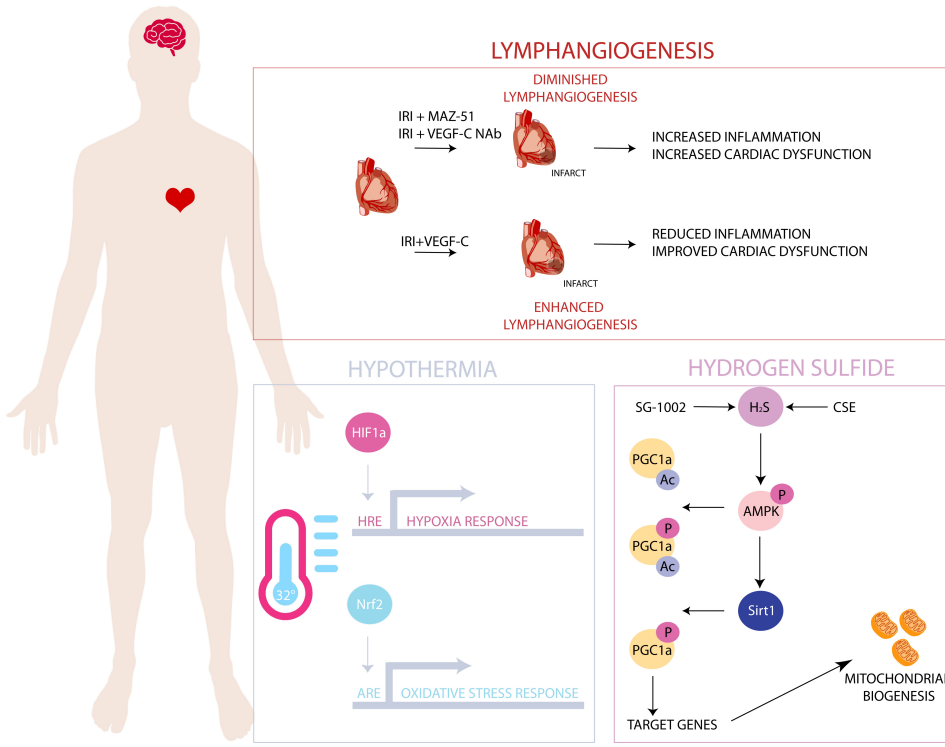

Figure 25. Graphical overview of therapeutic strategies for ischemia reperfusion injury investigated in the present study. IRI – ischemia reperfusion injury, NAb – neutralizing antibody, HRE – hypoxia response element, ARE – antioxidant response element, P – phosphorylation, Ac – acetylation

Therapeutic hypothermia is one of the most promising future interventions for the treatment of ischemic injury. However, the mechanisms of action are diverse and not completely understood. Hypothermia is probably able to modify the cascade of ischemic events on many levels. Firstly, knowing the mechanisms of action would allow to plan more controlled clinical trials and evaluate syner-

gistic effects of therapeutic hypothermia combined with other existing therapies. Secondly, the development of drugs designed to target therapeutically relevant pathways would eliminate the confounding effects associated with body cooling. We argue that systems coping with cellular stressors are plausible targets of therapeutic hypothermia and deserve more attention in clinical hypothermia research. Hypothermia had a major impact on Nrf2 and HIF1a signaling pathways suggesting that antioxidant system and hypoxia response are likely to be among the primary candidates for testing. H_2S is one of the potential options to treat ischemia reperfusion. An accumulating body of evidence shows that exogenous or endogenous H_2S have cardioprotective effects (Bian et al., 2006; Elrod et al., 2007; Sivarajah et al., 2006; Bliksoen et al., 2008; Johansen et al., 2006; Calvert et al., 2010b; Sodha et al., 2008; Osipov et al, 2009; Ji et al., 2008). Numerous proteins and pathways have been identified as cellular targets of H_2S (Calvert et al., 2009; Elrod et al., 2007; Hausenloy et al., 2006; Hu et al., 2008; Jha et al., 2008; Kondo et al., 2013; Kaiser et al., 2004; Luan et al., 2012; Wang et al., 2011). However, a common cellular target for many studies aimed at understanding the biology and therapeutic potential of H_2S has been the mitochondria (Aon et al., 2004; Barr et al., 2015; Calvert et al., 2010a; Calvert et al., 2010b; Chen et al., 2006; Elrod et al., 2007; Kind et al., 2014; Kondo et al., 2013; Minamishima et al., 2009; Pan et al., 2014; Polhemus et al., 2013; Untereiner et al., 2016; Wang et al., 2011). H_2S has a dual effect on mitochondrial bioenergetics with low concentrations serving as electron donors to the electron transport chain and higher concentrations serving as inhibitors of cytochrome c oxidase (Hill et al., 1984; Modis et al., 2013a; Fu et al., 2012). We showed here that H_2S is an important regulator of cardiac mitochondrial content and requires AMPK to induce PGC1α signaling and mitochondrial biogenesis. In addition, we found that restoring H_2S levels with SG-1002 in the setting of heart failure increased cardiac mitochondrial content, improved mitochondrial respiration, improved ATP production efficiency, and improved cardiac function. Our study has, therefore, identified a potentially important regulatory mechanism in the mitochondrial biogenesis pathway that if corrected by restoring H_2S levels could protect the failing heart and ultimately reduce mortality and morbidity associated with heart failure. Future studies aimed at determining if H_2S therapy coupled with other pharmacological agents that target the AMPK-PGC-1α signaling pathway are warranted to fully test this postulate. In recent years, there has been a growing interest in lymphatic research due to several key breakthroughs into the biology of lymphatic endothelial cells. The discovery of VEGF-C, and its receptor, VEGFR3 (Lohela et al., 2009), followed by the identification of proteins that discriminate between endothelial cells of the blood and lymphatic vessel lineages (i.e. LYVE-1) (Tammela et al., 2010), lead to the understanding that lymphatic vessels serve an active role in disease processes (Alitalo, 2011; Bruyere and Noel, 2010; Karaman and Detmar, 2014). Current findings provide evidence that the endogenous lymphangiogenesis response is induced within 3 days following myocardial ischemia. Moreover, we provide evidence that the response plays an

adaptive role in the development of ischemic-induced heart failure. Thus, our findings support the emerging concept that therapeutic lymphangiogenesis is a promising new approach for the treatment of cardiovascular disease (Henri et al., 2016). We anticipate that the clinical use of therapeutic lymphangiogenesis will not be achieved by systemic delivery, but rather local delivery. As such, the use of hydrogels could be a viable delivery strategy. Due to their highly tunable chemical, physical, and mechanical properties, hydrogels have been widely used as a tool in regenerative medicine (Hastings et al., 2015; Slaughter et al., 2009). In regards to the heart, hydrogel-based materials have been used as a structural/ mechanical support for the injured myocardium (Fujimoto et al., 2009) and have also been used as a means to deliver small molecules (Zustiak et al., 2013). The ability to fine-tune the properties of the hydrogel provides the unique opportunity to control the delivery of pharmacological agents (Slaughter et al., 2009). Here, we used a hydrogel to deliver VEGF-$C_{Cys156Ser}$ to the injured myocardium by placing the gel on the surface of the heart. While this strategy might not be clinically feasible, it does show proof of concept that the delivery mechanism is viable. As such, future approaches such as injecting the VEGF-C containing hydrogel directly into the myocardium are warranted.

CONCLUSIONS

1. Traditionally, hypothermia is considered as a passive rather than active agent in shaping cellular physiology. In paper I, it is shown that, contrary to the predictions of the thermodynamic hypothesis, mild hypothermia can activate regulatory factors that govern cellular coping with stressors. It is demonstrated for the first time that mild hypothermia (32°C) activates major transcription factors Nrf2 and HIF1A, which orchestrate adaptive responses to hypoxic stress. Evidence suggesting that hypothermia is able to augment stress response in mammalian cell lines motivates to inspect corresponding mechanisms more closely in animal studies.
2. H_2S is an important regulator of mitochondrial bioenergetics, but its role in regulating mitochondrial biogenesis is not well understood. Mice deficient in the H_2S-producing enzyme, cystathionine γ-lyase (CSE KO) display diminished cardiac mitochondrial content when compared to wild-type hearts. In contrast, mice overexpressing CSE (CSE Tg) and mice supplemented with the orally active H_2S-releasing prodrug, SG-1002, display enhanced cardiac mitochondrial content. Studies aimed at evaluating the underlying mechanisms found that H_2S requires AMPK to induce PGC1α signaling and mitochondrial biogenesis. Finally, restoring H_2S levels with SG-1002 in the setting of heart failure increases cardiac mitochondrial content, improved mitochondrial respiration, improved ATP production efficiency, and improved cardiac function.
3. Although the lymphatic system has been known to exist in a heart, very little is known about the role the cardiac lymphatic system plays in the development of heart failure. Results revealed that both, myocardial ischemia or myocardial ischemia and reperfusion, increase VEGF-C and VEGFR3 protein expression starting at 1 day following the onset of injury, whereas a significant increase in lymphatic vessel density is observed starting at 3 days. Further studies aimed to determine the consequences of inhibiting the endogenous lymphangiogenesis response on the development of heart failure. Using two different pharmacological approaches, it was found that inhibiting VEGFR3 with MAZ-51 and blocking endogenous VEGF-C with a NAb blunts the increase in lymphatic vessel density, increased inflammation, and increased cardiac dysfunction. Subsequent studies revealed that augmentation of the endogenous lymphangiogenesis response with VEGF-C treatment reduces inflammation, and improved cardiac dysfunction. These results suggest that the endogenous lymphangiogenesis response plays an adaptive role in the development of ischemic-induced heart failure.

   Taken together, it is likely that no single intervention can provide extensive protection against such a complex event like ischemia reperfusion injury with many interlinked processes. It is important that a potential course of treatment addresses all major aspects of this pathological condition while being appropriately timed to ensure a complete protection against ischemia reperfusion injury.

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SUMMARY IN ESTONIAN

Isheemia reperfusiooni kahjustuse terapeutilised mehhanismid

Hüpoksilis-isheemilist seisundit põhjustavad ajukahjustus, südameinfarkt, südame seiskumine ja hüpoksilis-isheemiline entsefalopaatia vastsündinutel. Hetkel kasutusel olevad ravimeetodid aitavad küll vähendada kahjustust, kuid pole kaugeltki ideaalsed. Hapniku ja verevoolu taastamine aitavad kõrvaldada tekkinud kahjustuse. See kõrvaldab esmalt tekkinud kahjustuse, kuid põhjustab omakorda reperfusiooni kahjustuse, mille tagajärjed võivad olla tõsisemad kui isheemial üksi. Ideaalne ravimeetod võimaldaks taastada verevoolu võimalikult väikese reperfusiooni kahjustusega. Selleks, et välja töötada tõhusamaid ravimeetodeid on meil vaja paremini mõista molekulaarseid mehhanisme, mis on seotud isheemia reperfusiooni kahjustusega. Käesolev doktoritöö koosneb kolmest artiklist, milles uurisime kolme erinevat terapeutilist mehhanismi (hüpotermia, vesiniksulfiid ja lümfangiogenees). Antud töö aitab paremini mõista, millised tegurid võiks kaitsta kude hapnikupuuduse eest, mis kaasneb erinevate südameveresoonkonna haigustega.

Esimene artikkel annab uut informatsiooni hüpotermia molekulaarsete toimemehhanismide kohta. Kliinikus on hüpotermia (32°C) kasutusel eelkõige kesknärvisüsteemi hüpoksilis-isheemiliste kahjustuste leevendamiseks. Kuna hüpotermial on võime kaitsta rakku isheemilise seisundi korral, siis on selle molekulaarsete mehhanismide tuvastamine äärmiselt oluline. Meie töö näitab esmakordselt, et mõõdukas hüpotermia (32°C) aktiveerib transkriptsioonifaktoreid nagu Nrf2 ja HIF1A, mis vahendavad adaptiivset vastust hüpoksilisele stressile. Hüpotermiat on seni vaadatud kui passiivset termodünaamilist tegurit (vähendab metabolismi ja hapniku tarbimist) raku füsioloogia mõjutamisel. Meie viimased tulemused viitavad aga sellele, et hüpotermia kutsub esile iseloomuliku vastuse raku füsioloogias spetsiifiliste molekulaarsete regulaatorite vahendusel, misläbi suureneb stressile vastupanu olukorras, kus puudub hapnik.

Teises artiklis uurisime, kas vesiniksulfiid võib mõjutada südames mitokondrite hulka. Vesiniksulfiid on mitokondrite bioenergeetikas oluline regulaator, kuid selle roll mitokondrite biogeneesi regulatsioonis on ebaselge. Tsüstationiini γ-lüaasi puudulikel (CSE KO) hiirtel puudub vesiniksulfiidi tootev ensüüm. Nendel hiirtel on südames vähenenud mitokondrite hulk võrreldes metsiktüüpi hiirtega. Samas CSE üleekspressioon ja vesiniksulfiidi vabastava eelravimi, SG-1002, manustamine suurendavad mitokondrite hulka. Leidsime, et vesiniksulfiidi tase südames mõjutab PGC1α tuuma lokalisatsiooni ja transkriptsioonilist aktiivsust. Lisaks vajab vesiniksulfiid AMPK-d, et indutseerida PGC1α signaal ja mitokondrite biogenees. Meie tulemused näitavad ka, et kui taastada SG-1002-ga vesiniksulfiidi tase südamekahjustuse olukorras, siis suureneb südame mitokondrite hulk, paraneb mitokondrite hingamine, ATP tootlikkuse efektiivsus ja südame funktsioon. Koos näitavad need tulemused, et vesiniksulfiid on südame mitokondrite koguse oluline regulaator ja eksogeenne

vesiniksulfiid indutseerib mitokondrite biogeneesi läbi AMPK-PGC1α signaaliraja.

Kolmas artikkel aitab paremini kirjeldada südame lümfaatilise süsteemi rolli südame isheemia-reperfusiooni kahjustuse korral. Selleks tekitati hiirtele müokardi isheemia või isheemia-reperfusiooni kahjustus, mis kestis kuni 28 päeva. Meie tulemused näitavad, et mõlemad isheemiamudelid stimuleerivad endogeenset lümfangiogeneesi suurendades VEGF-C (*vascular endothelial growth factor C*) ja VEGFR3 (*VEGF receptor 3*) valgu ekspressiooni juba päev pärast kahjustust. Lümfisoonte tihedus suureneb alates kolmandast päevast. Järgmiseks inhibeerisime endogeense lümfangiogeneesi südamekahjustuse olukorras. Leidsime, et selle tagajärjel ei suurenenud lümfisoonte tihedus, vaid suurenes põletikuvastus ning südame düsfunktsioon (talitushäire). Samas on äärmiselt oluline leid, kus VEGF-C manustamine vähendas põletikku ja parandas südame talitushäiret. Nende tulemuste põhjal saab järeldada, et endogeensel lümfoangiogeneesil on adaptiivne roll südamepuudulikkuse tekkes ja terapeutiline lümfangiogenees on üks lootustandvamaid ravimeetodeid kardiovaskulaarsete haiguste ravis.

Ilmselgelt on isheemia-reperfusiooni kahjustuse näol tegemist väga kompleksse haigusseisundiga, kus interakteeruvad omavahel erinevad molekulaarsed signaalirajad. Seega ei ole antud olukorras ühte kindlat ravimeetodit, mis aitaks toime tulla tekkinud kahjustusega. Pigem on see kombinatsioon mitmest erinevast terapeutilisest mehhanismist, mille koostoime vähendab isheemia-reperfusiooni kahjustust.

ACKNOWLEDGEMENTS

Writing a dissertation takes a tribe and there is no way I could have done it alone. So, before the curtain comes down, there are few people I am bursting to express my love and gratitude.

I am deeply grateful to my mentor Hendrik Luuk, for your direction and ability to see the whole picture and keep things moving forward. I do not know what I enjoy more – working with you professionally or our dear friendship. Your work has inspired me to be scientist I am today. Thank you for setting me on my path. Thanks, too, for the crispbread and pizzas and wine and desserts and all the untold snacks and group dinners yet to be.

Thank you, Eero Vasar, for your understanding, your patience, your attention to details, your insightful stewardship, and your continues help on building this dream. I am grateful to know you.

To peer-reviewers Allen Kaasik and Kalle Kilk – thank you for taking the time to read my words and offer honest and valued feedback. I am beyond grateful to have had your eyes over the thesis before anyone else.

I am truly lucky to have met Sten Ilmjärv, who has been on this journey with me from the very beginning. You provided the initial spark years ago when you introduced me to Tanel Visnapuu. You two are far more than just colleges, you are my dear friends.

Thank you, thank you, thank you to everyone at Department of Physiology. There is absolutely no way that I could have completed this journey without an incredibly gifted Christian Ansgar Hundahl, Riin Reimeits, Rando Porosk, Hans Vellama, Anton Terasmaa, Mari-Anne Philips, Karina Karis, Kersti Lilleväli, Toomas Jagomäe, Jane Narvik, and Paula Reemann. Thank you for deep, rich, stimulating, intellectual conversations and for holding the space for me to feel supported every step of the way.

Endless thanks to John Calvert and his hardworking team at Emory University. Your hard work and brilliance continue to floor me. You are a dream to work with.

Thank you to all the amazing women in my life who have kept me lifted up. You have been there for me at every turn on the road. You all know who you are and what you mean to me – my longest friends Irmen Noormaa, Marianne Pisukov, Maarja-Liis Elland, Katrin Mark, Marilin Saaroja. There are two special people, earth angels really... They are Liisa Mahlapuu and Maarja Grünbach whom I'm blessed to have on speed dial at any hour of the day!

To my parents, Maiken and Arvi – I am truly grateful for the pieces I carry of both of you that have made me the woman I am today. I am who I am because of who I come from. I have always wanted to be like my mom. Maybe it was a choice, or maybe that is just how all little girls feel. You are the strongest woman I know. Thank you for all you have done, all you were willing to do, and all you continue to do. You are the greatest example of unconditional love. Thank you for being my guiding light.

And to my sister and brother, Kätlin and Kristjan. Where do I begin? I literally do not know how I could do life without you. Kätlin, thank you for being such a beautiful example of pure love, for your endless kindness, support and for being my biggest cheerleader. Kristjan, you are the best brother a sister could ask for, a loving and caring son, a best battle buddy who always has everybody's back.

First and foremost, thank you to my partner and my best friend Raavo for being the most beautiful being to hold hands and walk this path with. You inspire me to be more, to show up more and to always go inward before looking outward for answers. Thank you for believing in me more than I believed in myself.

ORIGINAL PUBLICATIONS

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ACADEMIC QUALIFICATIONS:

2014–...	University of Tartu, Faculty of Medicine, neurosciences, doctoral studies
2012–2014	University of Tartu, Faculty of Science and Technology, molecular and cell biology, MSc
2009–2012	University of Tartu, Faculty of Science and Technology, biology, BSc

PROFESSIONAL EXPERIENCE:

September 2017–...	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine, Department of Physiology, Junior Research Fellow
January–June 2017	Visiting Research Scholar at Emory University School of Medicine, Division of Cardiothoracic Surgery, Carlyle Fraser Heart Center, Atlanta GA USA
2016–2016	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine, Department of Physiology, specialist
2015–2016	Medical Technology Group OÜ, statistical data analyst
2015–2015	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine, Department of Physiology, specialist
July–August 2014	University of Tartu, Faculty of Medicine, Institute of
July–August 2013	Biomedicine and Translational Medicine, Department of
August 2012	Physiology
July–August 2011	Preparation and conducting of scientific experiments to determine the genotype of transgenic animals

RESEARCH ACTIVITY:

Areas of expertise: Molecular biology, cell biology, bioenergetics, biochemistry, neuroscience

Previous scholarships

2018 Archimedes Foundation Dora Plus short study visits, Estonia
2016 Archimedes Foundation Dora Plus PhD student mobility, Estonia
2016 Archimedes Foundation Dora Plus short study visits, Estonia
2015 Cold Spring Harbor Laboratory Bursary, USA
2015 Wellcome Trust Advanced Course Bursary, UK

Research experience

- The balance between tricarboxylic acid cycle and oxidative phosphorylation in health and disease (2016-present), principal investigator Dr. Hendrik Luuk
- Therapeutic mechanisms of mild hypothermia (2014-present), principal investigator Dr. Hendrik Luuk
- Mechanisms of cardioprotection in the setting of acute myocardial ischemia-reperfusion injury and heart failure (January – June 2017), principal investigator Assoc. Prof. John W. Calvert
- IgLON family of cell adhesion molecules as the potential targets for neuropsychiatric disorders (2016), principal investigator Dr. Mari-Anne Philips
- Wfs1 gene involvement in the regulation of limbic system (2010–2014), principal investigator Prof. Eero Vasar

Publications:

Shimizu Y, Polavarapu R, **Eskla KL**, Nicholson CK, Koczor CA, Wang R, Lewis W, Shiva S, Lefer DJ, Calvert JW. (2018). Impact of Lymphangiogenesis on Cardiac Remodeling Following Ischemia and Reperfusion Injury. JAHA 7(19): e009565.

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Karis K, **Eskla KL**, Kaare M, Täht K, Tuusov J, Visnapuu T, Innos J, Jayaram M, Timmusk T, Weickert CS, Väli M, Vasar E, Philips MA. (2018). Altered Expression Profile of IgLON Family of Neural Cell Adhesion Molecules in the Dorsolateral Prefrontal Cortex of Schizophrenic Patients. Front Mol Neurosci 11:8.

Shimizu Y, Polavarapu R, **Eskla KL**, Nicholson CK, Koczor CA, Wang R, Lewis W, Shiva S, Lefer DJ, Calvert JW. (2018). Hydrogen sulfide regulates cardiac mitochondrial biogenesis via the activation of AMPK. J Mol Cell Cardiol 116:29-40.

Vanaveski T, Singh K, Narvik J, **Eskla KL**, Visnapuu T, Heinla I, Jayaram M, Innos J, Lilleväli K, Philips MA, Vasar E. (2017). Promoter-Specific Expression and Genomic Structure of IgLON Family Genes in Mouse. Frontiers in Neuroscience 11:38.

Visnapuu T, Plaas M, Reimets R, Raud S, Terasmaa A, Kõks S, Sütt S, Luuk H, Hundahl CA, **Eskla KL**, Altpere A, Alttoa A, Harro J, Vasar E. (2013). Evidence for impaired function of dopaminergic system in Wfs1-deficient mice. Behavioural Brain Research 244:90–9.

SELF-PERFECTION & SOCIAL ACTIVITIES

2018 MITOEST 2018 oral presentation "Hypothermia augments stress response in cells", Tallinn, Estonia
Conference in the cellular bioenergetics and mitochondrial metabolism

2016–... **Dissertations supervised/under supervision**
Ave Auser, Master of Science, 2017, "The effects of hypothermia on the induction of the antioxidant system", University of Tartu
Hans Vellama, Master of Science, 2017, "Evaluating Sf9 cell line metabolism in anoxia and normoxia through CO2 measurements", University of Tartu
Elisabeth Sainast, Bachelor of Science, under supervision

2016 FEBS Advanced Lecture Course on "Redox Regulation of Metabolic Processes", Spetses, Greece
A vigorous lecture course that covers redox regulation, the interaction between reactive species, metabolic processes and signalling cascades, and novel methods and approaches.

2016 10th European Summer School
EMBO workshop "Advanced Proteomics", Brixen/Bressanone, South Tirol, Italy
An insight into state-of-the-art proteomic technologies and applications in the life sciences.

2016–... Member of Programme Commitee in the programme of Neuroscience, University of Tartu, Estonia

2015 Eukaryotic Gene Expression course, Cold Spring Harbor Laboratory, NY, USA
A comprehensive laboratory-oriented course in cellular and molecular biology with almost 20 hands-on experiments.

2015 Functional Genomics and System Biology course, Wellcome Trust Advanced Course, Cambridge, UK
Intensive laboratory course complemented by computer-based training course

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Juuli–August 2014	Tartu Ülikool, Meditsiiniteaduste valdkond,
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August 2012	preparaator
Juuli–August 2011	

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Stipendiumid:

2018 SA Archimedes, Dora Pluss lühiajaline õpiränne, Eesti
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2015 Cold Spring Harbor Laboratory stipendium, USA
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Teadustöö

Tsitraaditsükli ja oksüdatiivse fosforüleerimise tasakaal normaalsetes ja patoloogilistes tingimustes (2016–...), juhtivteadlane Dr. Hendrik Luuk

Hüpotermia terapeutilised mehhanismid (2014–...), projekti juht Dr. Hendrik Luuk

Kardioprotektiivsed mehhanismid südame isheemia reperfusiooni kahjustuse ja südamepuudulikkuse korral (Jaanuar–Juuni 2017), projekti juht dotsent John W. Calvert

IgLON perekonna valgud kui potentsiaalsed sihtmärgid neuropsühhiaatriliste häirete ravis (2016), projekti juht Dr. Mari-Anne Philips

Wfs1 geeni osaluse uurimine emotsionaalsete närviringete regulatsioonis kasutades Wolframi sündroomi loommudelit Wfs1 gene involvement in the regulation of limbic system (2010–2014), projekti juht Prof. Eero Vasar

Teaduspublikatsioonid

Shimizu Y, Polavarapu R, **Eskla KL**, Nicholson CK, Koczor CA, Wang R, Lewis W, Shiva S, Lefer DJ, Calvert JW. (2018). Impact of Lymphangiogenesis on Cardiac Remodeling Following Ischemia and Reperfusion Injury. JAHA. 7(19):e009565

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Shimizu Y, Polavarapu R, **Eskla KL**, Nicholson CK, Koczor CA, Wang R, Lewis W, Shiva S, Lefer DJ, Calvert JW. (2018). Hydrogen sulfide regulates cardiac mitochondrial biogenesis via the activation of AMPK. J Mol Cell Cardiol. 116:29-40

Vanaveski T, Singh K, Narvik J, **Eskla KL**, Visnapuu T, Heinla I, Jayaram M, Innos J, Lilleväli K, Philips MA, Vasar E. (2017). Promoter-Specific Expression and Genomic Structure of IgLON Family Genes in Mouse. Frontiers in Neuroscience. 11:38

Visnapuu T, Plaas M, Reimets R, Raud S, Terasmaa A, Kõks S, Sütt S, Luuk H, Hundahl CA, **Eskla KL**, Altpere A, Alttoa A, Harro J, Vasar E. (2013). Evidence for impaired function of dopaminergic system in Wfs1-deficient mice. Behavioural Brain Research. 244:90-9

ENESETÄIENDUS JA MUU TEADUSLIK VÕI ÜHISKONDLIK TEGEVUS

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	Hans Vellama, magistrikraad, 2017, Sf9 rakuliini metabolismi hindamine anoksias ja normoksias CO2 järgi, Tartu Ülikool, Loodus- ja täppisteaduste valdkond, molekulaar- ja rakubioloogia instituut Elisabeth Sainast, bakalaureuse astme üliõpilane, juhendamisel
2016	FEBS loengukursus "*Redox Regulation of Metabolic Processes*", Spetses, Kreeka
2016	10. Euroopa suvekool "*Advanced Proteomics*", EMBO, Varna, Itaalia
2016–...	Neuroteaduste doktoriõppe programminõukogu liige, Tartu Ülikool
2015	Kursus "*Eukaryotic Gene Expression*", Cold Spring Harbor Laboratory, USA
2015	Kursus "*Functional Genomics and System Biology*", Wellcome Trust Advanced Course, Cambridge, UK
2013–2013	Biostart loengukursus bioettevõtlus (40 tundi)
2008–2009	MTÜ Võru Noortekeskus juhatuse liige

DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS

1. **Sirli Raud.** Cholecystokinin$_2$ receptor deficient mice: changes in function of GABA-ergic system. Tartu, 2005.
2. **Kati Koido.** Single-nucleotide polymorphism profiling of 22 candidate genes in mood and anxiety disorders. Tartu, 2005.
3. **Dzhamilja Safiulina.** The studies of mitochondria in cultured cerebellar granule neurons: characterization of mitochondrial function, volume homeostasis and interaction with neurosteroids. Tartu, 2006.
4. **Tarmo Areda.** Behavioural and neurogenetic study of mechanisms related to cat odour induced anxiety in rodents. Tartu, 2006.
5. **Aleksei Nelovkov.** Behavioural and neurogenetic study of molecular mechanisms involved in regulation of exploratory behaviour in rodents. Tartu, 2006.
6. **Annika Vaarmann**. The studies on cystatin B deficient mice: neurochemical and behavioural alterations in animal model of progressive myoclonus epilepsy of Unverricht-Lundborg type. Tartu, 2007.
7. **Urho Abramov.** Sex and environmental factors determine the behavioural phenotype of mice lacking CCK_2 receptors: implications for the behavioural studies in transgenic lines. Tartu, 2008.
8. **Hendrik Luuk.** Distribution and behavioral effects of WFS1 protein in the central nervous system. Tartu, 2009.
9. **Anne Must.** Studies on molecular genetics of male completed suicide in Estonian population. Tartu, 2009.
10. **Kaido Kurrikoff**. Involvement of cholecystokinin in chronic pain mechanisms and endogenous antinociception. Tartu, 2009.
11. **Anu Aonurm-Helm.** Depression-like phenotype and altered intracellular signalling in neural cell adhesion molecule (NCAM)-deficient mice. Tartu, 2010.
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28. **Maarja Toots.** Pharmacological challenge in rodent models of Wolfram syndrome with emphasis on diabetic phenotype. Tartu, 2018, 114 p.
29. **Katyayani Singh.** Neuropsychiatric endophenotypes – focusing on IgLON adhesion molecules in the mouse brain. Tartu, 2019, 148 p.