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Review article

An emerging perspective on sex differences: Intersecting *S*-nitrosothiol and aldehyde signaling in the heart

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ABSTRACT

Cardiovascular disease is the leading cause of the death for both men and women. Although baseline heart physiology and the response to disease are known to differ by sex, little is known about sex differences in baseline molecular signaling, especially with regard to redox biology. In this review, we describe current research on sex differences in cardiac redox biology with a focus on the regulation of nitric oxide and aldehyde signaling. Furthermore, we argue for a new perspective on cardiovascular sex differences research, one that focuses on baseline redox biology without the elimination or disruption of sex hormones.

1. Introduction

At the center of cardiovascular physiology is the heart, which drives blood flow to all organ systems, delivering oxygen and collecting waste to maintain homeostasis. The basis for this blood flow results from contraction of the heart, which is ostensibly similar between males and females. New investigations are revealing important sex differences in the cardiovascular system during health and disease. These findings are beginning to garner more attention as the field begins to appreciate that cardiovascular disease often manifests differently in males and females. Traditional experimental strategies to study sex differences involve the disruption or removal of hormones by pharmacologic agents, genetic ablation of key endocrine signaling pathways, or surgical excision of reproductive organs (i.e. ovaries or testes). While these techniques have been instrumental in determining that female hearts are different in several important ways from male hearts, there are few studies that have investigated the baseline biological differences between male and female hearts absent the purposeful disruptions to the endocrine system. It is undeniable that sex hormones, specifically estrogens, progestins, and androgens, play vital roles in regulating cardiac physiology and signaling at a molecular level, however these molecules also set the stage for the cardiac response to injury and disease. As such, it is essential to understand the downstream and fundamental molecular processes with the intact influence of these hormones on heart physiology. In this review, we argue for a new approach to investigations that examine sex as a biological variable, one that moves beyond endocrine disruption and aims to study the male and female heart in a "natural" state with intact hormonal signaling. Throughout this review, we will highlight studies that have utilized this approach to study redox-dependent signaling mechanisms in the male and female heart during health and disease.

2. Sex differences in coronary heart disease and cardioprotection

Coronary heart disease is a leading cause of death for both men and women in the United States, typically manifesting as a myocardial infarction (MI) [1]. An MI is primarily caused by the occlusion of a coronary artery or arteries, which are the blood vessels responsible for supplying the heart with oxygen and other nutrients [2]. This lack of oxygen leads to an ischemic zone in the heart, which can result in the death of precious cardiomyocytes. To date, the only clinically established treatment for MI that has been shown to reduce infarct size in humans is the restoration of blood flow, or reperfusion of the ischemic heart tissue [3]. Although timely reperfusion is critical for reducing infarct size, it is also responsible for some portion of the total injury and cell death. This total injury is referred to as ischemia-reperfusion (I/R) injury [4]. Diminishing the risk for MI and the ensuing injury with novel and effective strategies is a necessary goal to prevent the development of more severe cardiovascular disease in both men and women.

Insight into the prevention and reduction of injury associated with MI may be garnered from the female heart, as pre-menopausal women have a reduced incidence for coronary heart disease compared to age-

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Non-standard abbreviations

E2	17β − estradiol
Akt	protein kinase B
ALDH	aldehyde dehydrogenase
cGMP	cyclic guanosine monophosphate
eNOS	endothelial NOS
ER	estrogen receptor
GSNO	S-nitrosoglutathione
GSNO-R	S-nitrosoglutathione reductase
I/R	ischemia-reperfusion
iNOS	inducible NOS
MI	myocardial infarction
NO	nitric oxide
NO^+	nitrosonium
NO	nitroxide
NOS	NO synthase
nNOS	neuronal NOS
OVX	ovariectomy
PI3K	phosphoinositide 3-kinase
PK	protein kinase
Redox	reduction-oxidation
RISK	reperfusion injury salvage kinase
sGC	soluble guanylyl cyclase
SHMG	S-hydroxymethyl-glutathione
SNO	S-nitrosation
THF	tetrahydrofolate

matched men [5,6]. This female-specific cardioprotection has also been observed in pre-clinical animal models, with female hearts exhibiting endogenous sex-dependent cardioprotection and reduced susceptibility to ischemic heart injury [7–9]. During the menopausal transition, however, the risk for coronary heart disease rises dramatically [10]. Researchers have largely attributed this increase in risk to the decline of estrogens, namely 17β-estradiol, and progesterone [11–13]. However, in the 1990s, two clinical trials were designed with the intent to determine the efficacy and safety of Hormone Replacement Therapy by administering estrogens and progestin to post-menopausal women. Unfortunately, these trials failed to demonstrate a reduction in coronary heart disease events between the placebo and treatment hormone groups, and even increased the risk cardiovascular disease and certain forms of cancer in some groups [14,15]. While these results and those of subsequent trials were inconclusive, they provided crucial information on the role of estrogens and progestin in post-menopausal cardiovascular disease and further underscored the need for additional studies examining the female heart. Although estrogens, progestins, and androgens are known to play a critical role in the prevention and/or progression of cardiovascular disease, the results of these trials, as well as pre-clinical animal studies, suggest that sex differences in cardiovascular disease must extend beyond hormonal changes and include fundamental sex differences in biochemistry and redox biology.

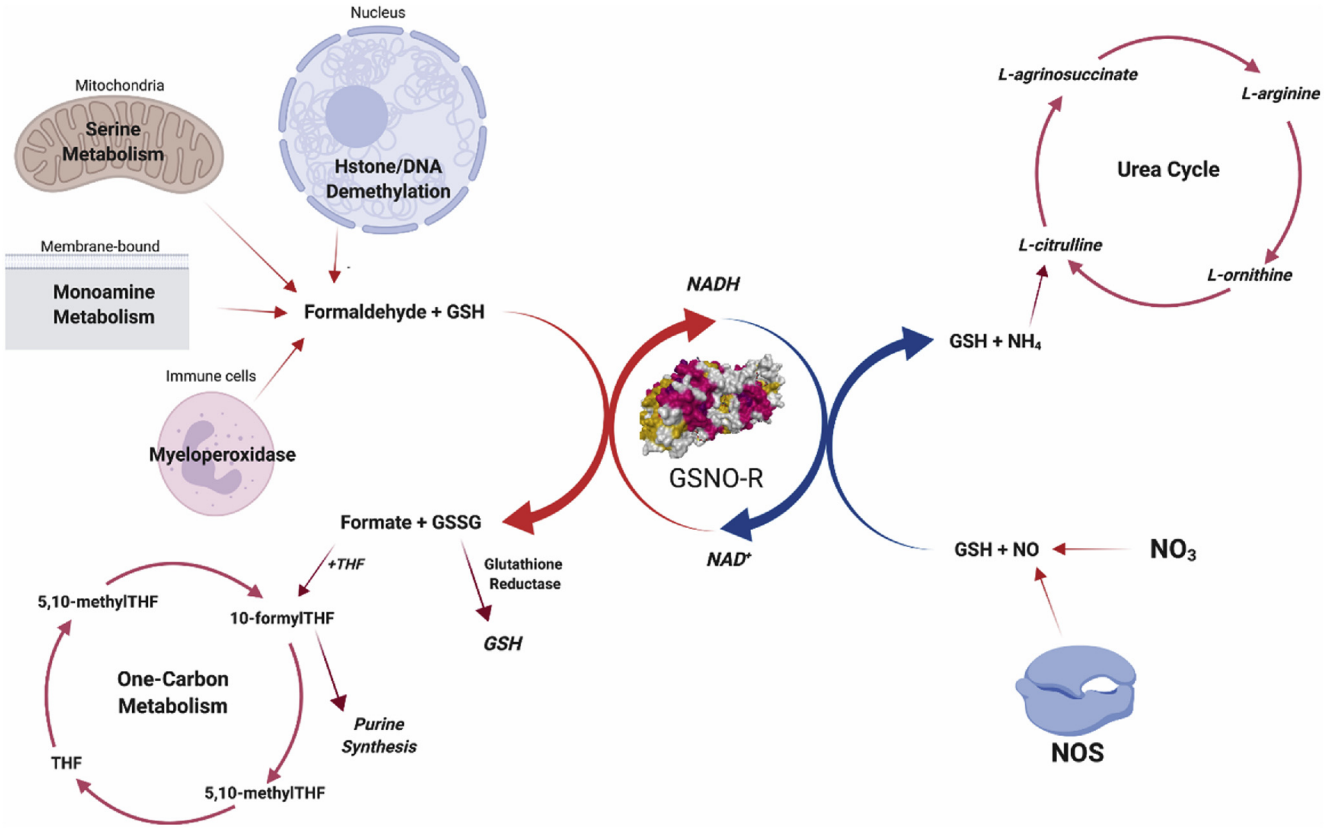

Fig. 1. *GSNO-R sits at the intersection of endogenous formaldehyde and nitric oxide production.* Nitric oxide (NO) and formaldehyde are produced in a variety of reactions that occur in various cellular compartments. NOS isoforms and the nitrate (NO_3)-nitrite axis are two ways to produce NO. GSH captures NO to form S-nitrosoglutathione (GSNO), which is the substrate for S-nitrosoglutathione reductase (GSNO-R), the breakdown products of which can be shunted into the urea cycle. Formaldehyde is produced by histone/DNA demethylation (nucleus), serine metabolism (mitochondria), monoamine metabolism (membrane-bound), and myeloperoxidase oxidation (immune cells). Formaldehyde from these reactions binds to GSH, to form S-hydroxymethyl-glutathione, and is oxidized by GSNO-R into formate, which can participate in one-carbon metabolism and purine synthesis. Abbreviations in figure: THF, tetrahydrofolate; NOS, NO synthase; NH_4, Ammonium.

3. Sex differences in redox biology and ischemia-reperfusion injury

Sex differences in cardiac biology can be fundamentally linked to the fluidic, and often rapid, exchange of subatomic particles (i.e. electrons and protons) that participate in balanced reduction-oxidation (redox) reactions. A hallmark component of I/R injury is the sudden burst of reactive oxygen species that occurs at the onset of reperfusion [16,17]. This burst of reactive oxygen induces oxidative stress and injury by promoting the excessive contraction and death of cardiomyocytes [18–20]. While oxidants play a key role in I/R-induced damage to both male and female hearts, when in balance, these reactive molecules play an important role in maintaining cardiac homeostasis in a sex-dependent manner [21–25]. Hormones can influence redox reactions in the heart through several important pathways, but we are still actively working to define the mechanistic basis of these pathways and we have much to learn [26–28]. As such, it is critical for future studies to examine the fundamental differences in redox signaling that exist between males and females. Recently, we demonstrated that disruption of a key enzyme that regulates nitric oxide signaling also resulted in the sex-dependent dysregulation of reactive aldehyde signaling in the context of myocardial I/R injury [29]. Importantly, these experiments were performed using intact animals, and thus estrogen and androgen signaling was preserved. Beyond the study of hormonal changes, investigations into sex-dependent differences in redox signaling and imbalance can help inform new avenues of research and provide mechanistic insight into the pathogenesis of I/R injury in the heart. Because sex-dependent differences in reactive oxygen species have been well-studied (please see Kander et al. 2017), for this review, we will focus on the following two areas: 1) nitric oxide and 2) aldehyde signaling and toxicity [30]. These axes are linked by an enzyme, *S*-nitrosoglutathione reductase (GSNO-R), that is an essential component in the maintenance of cardiac redox homeostasis as we recently demonstrated [29].

3.1. Nitric oxide: Production and source regulation

Nitric oxide (NO), originally discovered as endothelium-derived relaxation factor, is an endogenous, gaseous signaling molecule that is produced in two ways: (1) nitric oxide synthase (NOS) and (2) nitrite reduction (Fig. 1) [31–33]. This free radical can be produced by three NOS isoforms: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) NOS. These enzymes convert L-arginine into L-citrulline in a NA(P)DH-dependent reaction that requires oxygen, Ca^{2+}/calmodulin, and 5,6,7,8-tetrahydrobiopterin to produce NO [34]. Sex differences have been noted with regard to the expression and activation of the various NOS isoforms in the heart [9,35–38]. In addition to NOS, under specific conditions, it was proposed that nitric oxide can be generated within the mitochondria [39–41], but whether the production mechanism is either an alternative NOS isoform or a Complex III-mediated nitrite reduction remains in dispute [42]. The nitrite/nitrate (NO_3)/NO axis can also be used to produce this important gaseous molecule under certain conditions.

Nitrate can enter the body in two ways: 1) the diet and 2) the oxidation of NOS-derived nitric oxide. Once inside the body, nitrate is reduced to nitrite by nitrate reductase in the oral cavity by the oral microbiome. Nitrate and nitrite circulate to various tissues, and NO is produced either by enzyme-mediated reactions or under acidic conditions, such as hypoxia [43]. In the presence of oxygen and water, NO can undergo a reversible reaction to produce dinitrogen trioxide and nitrite, which under anaerobic or non-aqueous conditions can become NO donors [44,45]. These varying chemistries help to dictate the biological interactions between NO and macromolecules, and along with concentration, determine its cytoprotective or cytotoxic properties [46].

NO signaling can be controlled in part by regulating the source, and each of the NOS isoforms has its own form of regulation. Phosphorylation of the NOS isoforms is an important mechanism for enhancing or suppressing NO production. Modification of these sites facilitates electron flow through the oxidase and reductase domains that yields an increase in L-arginine reduction, thus altering NO output [47]. Activation of eNOS to enhance NO synthesis is mediated through phosphorylation of serines 615, 633, and 1177 by phosphoinositide 3-kinase (PI3K)/Protein kinase B (Akt), protein kinase (PK) G, PKA, adenosine monophosphate-activated protein kinase, and Ca^{2+}/calmodulin kinase II [48–52]. Phosphorylation of eNOS at threonine 495 by PKC in the absence of calmodulin, is known to be inhibitory [53]. nNOS is also regulated by phosphorylation and can be inhibited when phosphorylated by Ca^{2+}/calmodulin kinase II at serine 847. It is also important to note that these phosphorylation sites vary by species, for instance the analogue of eNOS serine 1177 in humans, is serine 1176 in murine and serine 1179 in bovine (sites reviewed elsewhere) [54,55]. Sex differences, which will be discussed later, have been noted regarding the post-translational modification of the NOS isoforms in the heart [9,38]. In contrast to the other isoforms, iNOS is not constitutively expressed and is transcriptionally regulated by factors such as hypoxia-inducible factor [56,57]. The NO output from these NOS isoforms can induce or modulate signaling cascades that are critical for cardiac function and cardioprotection.

NO availability is also regulated through its reactivity with other molecules, especially free radicals and heme groups. NO is considered an antioxidant molecule due to its high reactivity with superoxide and other radicals. Superoxide reacts with NO to produce peroxynitrite, which is cytotoxic and can influence cardiac physiology through thiol oxidation and tyrosine nitration [58,59]. Various heme-containing proteins, such as myoglobin and hemoglobin, can scavenge NO and yield a heme-NO and an *S*-nitrosothiol group [60]. This reaction is exploited by the body to scavenge, generate, and transport NO to various organs. Within tissues, NO participates in essential signaling cascades that govern cellular homeostasis [61,62].

3.2. Nitric oxide: The signaling molecule

As an essential regulator of cardiovascular homeostasis, NO can be generated by many reactions in different cellular compartments, and this property can be used for molecular signaling pathways. NO-mediated signaling occurs when the molecule is converted into two ionic forms: cationic nitrosonium (NO^+) or anionic nitroxide (NO^-) [63]. In these states, NO can promote signaling cascades either by binding to metals, such as heme groups, or by reacting with thiols, amines, and aromatic groups on proteins [46]. Physiologically, vasorelaxation can occur via NO binding to the heme group of soluble guanylyl cyclase (sGC). Upon binding, NO induces the synthesis of cyclic guanosine monophosphate (cGMP) that acts as a second messenger to activate PKG, an enzyme that phosphorylates multiple targets [64–66]. Many consider this pathway to be the canonical NO signaling pathway, however there is an additional signaling mechanism that involves the oxidation of cysteines.

Cysteine modifications, specifically oxidation, are an integral part of redox homeostasis by modulating various signaling pathways. NO can post-translationally modify cysteines; this modification is known as *S*-nitrosation (SNO) [63,67–69]. It is thought that the secondary reaction of NO oxidation into nitrogen dioxide, dinitrogen trioxide, or peroxynitrite, is necessary for NO to modify amino acid side chains, however the ability of NO to directly react with cysteines has been reported [67,70,71]. Based on the thiol-binding properties of NO, the NO molecules can be stored and transported in the blood [72]. NO can react with glutathione, which is an abundant low molecular weight thiol, to form *S*-nitrosoglutathione (GSNO). Cardiomyocytes use GSNO as a physiologic reservoir for NO, which is a major participant in *trans-S*-nitrosation reactions because GSNO exists in steady-state with protein SNO [73]. Our group and others have shown that GSNO and other

nitrosating agents are important for reducing I/R injury by increasing SNO of key proteins [74–79]. In addition to SNO, the sGC/cGMP/PKG-pathway is also considered to be protective against myocardial I/R injury, though there is evidence that it may not be involved in all forms of protection [80]. It is important to note that SNO is postulated to be an unstable precursor to *S*-glutathionylation and intramolecular disulfide bonds, however, a significant amount of work has shown that SNO is a bona fide post-translational modification (as reviewed recently by Stomberski et al. (2018)) [81–83]. Nevertheless, it is necessary to understand the interplay between *S*-glutathionylation and SNO, as the former may be involved in long-term protein regulation versus the latter, which is a more transient modification, and may act as an acute "redox switch" [84]. Since intracellular NO is stored as GSNO in cardiomyocytes, the regulation of NO signaling largely occurs through the modulation of intracellular GSNO concentrations.

The major pathway for protein SNO regulation occurs through the metabolism of GSNO by GSNO-R [85–87] (Fig. 1). This enzyme is a member of the alcohol dehydrogenase family (protein: ADH5, gene: *Adh 3*), and functions as a homodimer. To date, GSNO-R has three main substrates: (1) GSNO, (2) *S*-hydroxymethyl-glutathione, and (3) medium- and long-chain (i.e. > 4 carbon-chain) alcohols, which may include retinol [88]. Interestingly, GSNO-R can act as a reductase or an oxidase, depending on the redox state of NAD. In an NADH-dependent reaction, GSNO-R metabolizes GSNO and depending on the redox environment, glutathione will either be oxidized or converted to hydroxysulfenamide and ammonia [89]. Depleting GSNO levels tilts the steady-state and indirectly lowers protein SNO levels. This is evident from the high and low SNO protein levels found in GSNO-R knockout and overexpression models respectively [86,87,90]. On the other hand, in an NAD^+-dependent manner, GSNO-R facilitates the oxidation of *S*-hydroxymethyl-glutathione, which is the spontaneous reaction product of formaldehyde and glutathione. Formaldehyde regulation via GSNO-R will be discussed later in this review. As a potential node to increase SNO, GSNO-R is a target of investigation, especially to protect against ischemic injury.

Studies have shown that genetic deletion of this enzyme can reduce I/R injury, in part, by increasing protein SNO, but our recent study has demonstrated that this protection is sex dependent. Initially, in a study from Lima et al. (2009), the authors demonstrated that hearts from male GSNO-R knockout mice were protected from *in vivo* I/R injury by increasing capillary density, mediated by SNO of hypoxia-inducible factor [91]. A different study in 2015, implicated GSNO-R in the regulation of cardiomyocyte proliferation post-I/R injury [92]. Protection with GSNO-R ablation also extends to the brain, where male GSNO-R knockout mice subjected to cardiac arrest and cardiopulmonary resuscitation were less susceptible to ischemic brain injury via preserved cerebral SNO protein levels [93]. These studies were essential in linking GSNO-R to protection against cardiac I/R injury in male animals. However, we recently reported that while GSNO-R knockout or inhibition protected male hearts from I/R injury, females hearts no longer exhibited sex-dependent protection from I/R, and injury was actually exacerbated [29]. Taken together, these studies implicate GSNO-R as an essential component of NO-mediated, sex-dependent protection from I/R injury and indicate that while a particular intervention may prove beneficial in one sex, the same intervention may be ineffective or debilitating in the other sex.

3.3. Nitric oxide: Sex differences in cardioprotection

Sex differences in nitric oxide signaling have been extensively studied over the years and have largely focused on the expression of the NOS isoforms. In humans, it was recently reported that female gender was associated with greater nitric oxide content in heart tissues sampled before surgery [28]. These data, along with epidemiologic and preclinical data indicating that female hearts have lower risk and injury from cardiovascular disease, suggest that baseline NO signaling may be important for preventing cardiovascular disease development. While we argue that future studies should examine baseline redox signaling without disruptions to endocrine signaling, these previous studies have significantly advanced our understanding of sex differences in cardiovascular disease and these studies are reviewed below.

Estrogen, specifically 17β−estradiol (E2), is an inducer of eNOS and nNOS expression. A well-characterized axis of protection from I/R injury is known as the Reperfusion Injury Salvage Kinase (RISK) pathway, defined as the signaling cascade from PI3K/Akt to eNOS or glycogen synthase kinase 3β to ultimately prevent cell death from I/R injury [94–96]. E2 binds to estrogen receptors (ER-alpha/beta and GPR30) and stimulates the RISK pathway to activate eNOS, thus increasing NO output and protein SNO [37,97–100]. Estrogen also regulates nNOS expression, which in turn regulates cardiomyocyte contraction [101–104]. In addition to eNOS, nNOS is cardioprotective largely by preventing ventricular arrhythmias after I/R via SNO [105–108]. Furthermore, in 2006, Sun et al. demonstrated that endogenous female cardioprotection was derived in part from SNO of the L-type Ca^{2+} channel, thereby reducing Ca^{2+} entry and the potential for I/R-induced Ca^{2+} overload. Interestingly, this protection was dependent upon both eNOS and nNOS, as knockout of either isoform ablated sex-dependent cardioprotection [7]. Androgens have also been reported to increase eNOS expression and activation (via ERK1/2), which in turn can induce vasorelaxation likely by the NO-mediated activation of sGC and PKG [109]. Since sex hormones are regulators of NO signaling, studies that involved the removal of these hormones have been an integral part of understanding the importance of these molecules in cardioprotection.

Ovariectomy (OVX)- and castration-based models have been utilized to demonstrate that estrogen is an essential component of female cardioprotection, as these OVX animals have larger infarcts compared to intact females following I/R injury (Table 1) [37,110,111]. Furthermore, various studies have shown that estradiol treatment can reduce heart and brain I/R injury in males and OVX hearts, partly by regulating eNOS and nNOS expression and by stimulating the RISK pathway (Table 1) [112–114]. In addition to NOS, phosphodiesterase 5, which is a critical regulator of sGC/cGMP/PKG-pathway, is also estrogen-dependent, as it has been shown that phosphodiesterase 5 inhibitors do not exert commonly-reported anti-remodeling properties in OVX mice (Table 1) [115]. These studies focused on the effects of estrogens on cardioprotection from I/R injury, but testosterone also has a role to play in cardioprotection.

The role of testosterone in the heart, however, is controversial as some studies have demonstrated that testosterone increases infarct size

Table 1
Summary of sex differences in cardiac redox biology. Column "Male" is compared to the intact female, and column "Female" is compared to male. Arrows indicate the increase (↑) or decrease (↓) in reactive species for each sex. Abbreviations in table: NOS, nitric oxide synthase; cGMP, cyclic guanosine monophosphate; PKG, protein kinase G; GSNO-R, S-nitrosoglutathione reductase; ALDH, aldehyde dehydrogenase.

	Male	Female	References
Nitric oxide			
NOS expression/Activity	↓	↑	88, 191-193
CGMP/PKG	ND	ND	195
S-Nitrosation	↓	↑	88, 205
GSNO-R Activity	↓	↑	88, 240
Aldehyde			
Formaldehyde	↓	↑	90
Lipid peroxidation	↑	↓	242, 243
Carbonylation	↑	↓	241
ALDH2 activity	↓	↑	240
Antioxidants			
Antioxidant expression	↓	↑	250
Glutathione capacity	↓	↑	262, 263

with I/R injury, while others have reported opposing findings [116,117]. Both male and female mice deficient in aromatase, which is the enzyme responsible for converting testosterone into estrogen, have significantly more testosterone compared to wild-types [118]. Hearts from female aromatase knockout mice, relative to wild-type hearts, have improved left ventricular function after I/R injury, indicating that these mice are cardioprotected, likely by improved myocardial Ca^{2+} handling [119]. Conversely, male mice that overexpress aromatase, which should increase estrogen and lower testosterone levels, had depressed left ventricular functional recovery after I/R injury and more reperfusion-induced arrhythmias [120]. These investigations demonstrated that testosterone may be protective to both male and female hearts in certain contexts. Several studies have shown that sex hormones are a crucial component of cardiac physiology, in part by regulating redox homeostasis. Surgical and pharmacologic disruption of hormones can only take investigations into redox biology so far because these reactions take place in short time frames. Few studies have examined baseline sex differences in redox biology.

Moving beyond experiments that surgical or pharmacologically disrupt the endocrine system, Chen et al. (2003) reported that adult cardiomyocytes isolated from female rats had more baseline nNOS protein expression with no change in eNOS [121]. Recently, we demonstrated that eNOS, but not nNOS, expression and serine 1176 phosphorylation was greater in whole hearts from intact female mice compared to males (Fig. 2) [9]. These findings are consistent with previous studies [122]. Furthermore, we reported that Akt is more highly phosphorylated in female hearts, which may contribute to our observed increase in eNOS phosphorylation [37,38]. While there is conflicting evidence about the species-specific expression of certain NOS isoforms, these enzymes are undoubtably working in concert to maintain redox homeostasis. While eNOS and nNOS are commonly studied in sex-dependent cardioprotection, iNOS is also known to participate in cardioprotection [123]. Male hearts overexpressing iNOS had smaller infarcts than wild-type and impaired opening of the mitochondrial permeability transition pore [124]. Other mechanisms of protection involve a reduction in nitrosative and oxidative stress, and improved cardiac remodeling [125–128]. Our group has not detected a difference in baseline iNOS expression [9], which is to be expected since iNOS is only expressed with certain stimuli. In 2004, Xu et al. showed that estrogen treatment in aged, female rats increased iNOS expression and induced protection from I/R injury [23]. Therefore, it may be valuable to determine if iNOS is involved in other cardioprotective interventions, but these data show that iNOS can be protective in both male and female hearts.

Cardioprotective SNO proteins have largely been investigated in male tissue, but our group and others are working to rectify this dearth. We reported that hearts from intact female mice had more SNO proteins at baseline, yet more GSNO-R activity compared to males with no difference in GSNO-R expression (Table 1) [9]. Also, under hypercontractile conditions, female hearts have been reported to have more protein SNO than males [7]. Recently, we showed that GSNO-R inhibition or genetic deletion impaired endogenous cardioprotection in female hearts, in part by increasing post-ischemic protein SNO levels [29]. These studies support SNO, GSNO, and GSNO-R as essential mediators of cardioprotection, especially sex-dependent protection. Maintenance of the nitroso-redox equilibrium is important, but the augmentation of the system must be made with care. High doses of NO can be damaging by inducing nitrosative stress. Furthermore, our study has shown that while an intervention that regulates NO signaling may be beneficial to male hearts, the same intervention can be detrimental to females. While NO is a well-studied component of cardioprotection, other redox systems, such as reactive aldehyde species, are emerging as critical players in I/R injury.

4. Sex differences in aldehyde signaling and toxicity

4.1. Aldehydes: Formaldehyde production and regulation

Aldehydes are reactive electrophiles produced by various biochemical processes and are in turn regulated through enzymatic and non-enzymatic reactions [129]. The most commonly referenced aldehydes are those formed from lipid peroxidation reactions (e.g. 4-hydroxy-nonenal and malondialdehyde) and are reviewed elsewhere, however there are others that have not been as well studied. Formaldehyde, for instance, is the simplest aldehyde and is intimately linked to NO signaling through GSNO-R. Therefore, this section will focus on this reactive aldehyde. Formaldehyde is highly reactive with amine groups, especially on lysine, to form imines. These modifications, known as methylol groups, are Schiff bases that bond with other Schiff bases to form crosslinks that can impair enzymes involved in cellular degradation, thus preventing decay [130]. This reactivity makes formaldehyde a popular chemical for preservation and laboratory techniques (e.g. chromatin immunoprecipitation sequencing) [131–133]. Also, biomarker studies take advantage of this chemistry: N [6]-formyllysines are often used to detect formaldehyde exposures [134,135]. It has also been proposed that formaldehyde can react with other amino acids, especially cysteines, however, the reaction with these thiols is much slower than with amines [136–138]. Apart from environmental exposures, formaldehyde is endogenously produced through various biochemical reactions, and due to its reactivity, these sources are tightly regulated.

Cells produce formaldehyde in different compartments (Fig. 1). A common mechanism of formaldehyde generation is the reaction between methanol and water; however, this is an unlikely mechanism for *in vivo* formaldehyde production because methanol is not readily produced by cells. In the mitochondria, formaldehyde is produced by

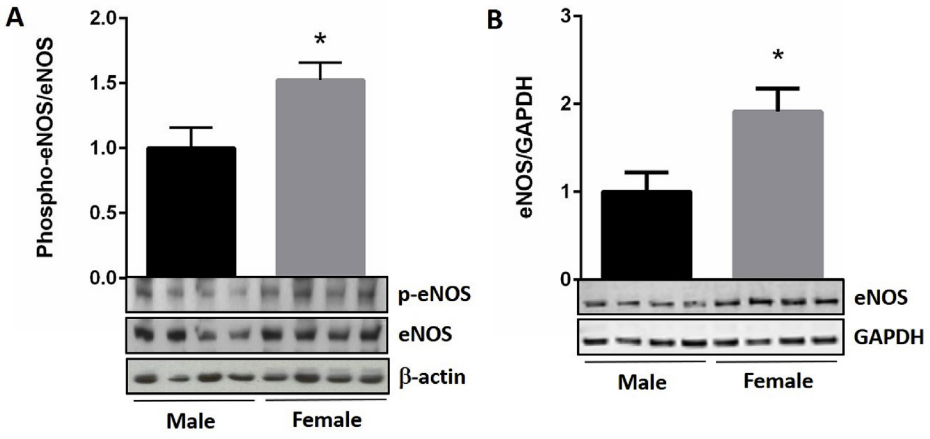

Fig. 2. *Phosphorylated and total endothelial nitric oxide synthase (eNOS) expression in male and female mouse hearts.* (A) Phospho-eNOS at Ser 1176 and (B) total eNOS expression (male: black bar, female: gray bar, *p < 0.05 vs. male) as assessed via western blot using whole heart homogenates from male and female mouse hearts. Figure reproduced from Shao, Q., Fallica, J., Casin, K., Murphy, E., Steenbergen, C., Kohr, M.J. Characterization of the sex-dependent myocardial S-nitrosothiol proteome. *Am J Physiol Heart Circ Physiol.* 310 (4): H505-15; 2016.

serine hydroxymethyltransferase 1 and 2, which is an enzyme that converts serine to glycine [139,140]. Serine oxidation by oxidants produced via myeloperoxidase after cardiac I/R injury is also a potential source of formaldehyde [141]. Formaldehyde is also a byproduct of demethylation reactions in the nucleus. N-methyl demethylation is a reaction that occurs with JmjC histone demethylases and TET DNA demethylases that yield formaldehyde as an intermediate product [142,143]. Finally, semicarbazide-sensitive amine oxidases, such as vascular adhesion protein 1, are also a source of formaldehyde. These copper-containing amine oxidases convert primary amines to their corresponding aldehydes, thus formaldehyde is produced by the conversion of methylamines [144]. Formaldehyde liberated from the aforementioned reactions can bind to tetrahydrofolate (THF) to form 5,10-methylene-THF and is then shunted into the one-carbon metabolism cycle. In 2017, Burgos-Barragan et al. demonstrated that formaldehyde can be released by 5,10-methylene-THF and that this formaldehyde is used for purine synthesis in mammalian cells [145]. Various biochemical processes throughout the cell are sources of this reactive carbonyl, and as a result, there are enzymes to keep formaldehyde in check.

Although formaldehyde does not react easily with protein thiols, it does react with glutathione to yield *S*-hydroxymethyl-glutathione (SHMG), which is a substrate for GSNO-R (Fig. 1) [146,147]. In the past, GSNO-R and aldehyde dehydrogenase (ALDH) 2 were respectively described as a cytosolic GSH-dependent, and a low Km-mitochondrial formaldehyde dehydrogenase. It was discerned that formaldehyde metabolism by the former was impaired in the absence of glutathione [148]. In a series of papers, Saghani et al. elegantly described the coordination involved in the oxidation of SHMG [149–153]. The metabolism of SHMG involves the coordinated oxidation of the hydroxymethyl group via active site residues. The electron is transferred to NAD^+ and SHMG is converted to *S*-formylglutathione, which is cleaved by *S*-formylglutathione reductase to release formate and glutathione [154,155]. Control of formaldehyde levels may be an essential role for GSNO-R, in addition to regulating SNO, because GSNO-$R^{-/-}$ mice have increased susceptibility to formaldehyde-induced toxicity and have exacerbated formaldehyde levels after myocardial I/R injury [29,156]. GSNO-R occupies a unique niche where it can regulate both SNO and formaldehyde, however, GSNO-R is not the only enzyme that protects against formaldehyde toxicity.

Mitochondrial ALDH2 is a GSH-independent formaldehyde dehydrogenase that can also use NAD^+ to oxidize formaldehyde into formate, though formaldehyde is not the preferred substrate [157]. Other substrates include acetaldehyde, 4-hydroxynonenal, and acrolein, which are also reactive species. ALDH2 is demonstrated to be cardioprotective under certain circumstances such as I/R injury, heart failure progression after MI, and lipotoxic cardiomyopathy, but has been reported to exacerbate cardiac hypertrophy [29,158–162]. Compounds have been created to increase the catalytic activity of the enzyme, and among them, Alda-1 is commonly used due to its ability to shield a critical cysteine in the active site from oxidation [158,163]. At baseline, female hearts have higher GSNO-R and ALDH2 activity compared to males, which may be the reason that Alda-1 fails to provide additional protection to female hearts subjected to I/R injury, as it does with male hearts [9,164]. These data suggest that there is an important interaction between GSNO-R and ALDH2 that can protect the female heart from I/R injury. This link is understood with DNA repair, but its role in the heart remains to be understood [145,165]. Since GSNO-R is capable of regulating endogenous formaldehyde concentrations, it demonstrates a potential link between nitric oxide and aldehyde signaling.

4.2. Aldehydes: Sex differences in cardiac biology

Few studies have directly evaluated cardiac sex differences in aldehyde signaling and dysregulation, but we and others have demonstrated a sex-dependent phenotype that is likely mediated through these oxidants. One such aldehyde with a reported sex difference is formaldehyde. We and others have shown that female hearts have more free formaldehyde at baseline compared to male hearts, yet we find no difference in other reactive species, such as 4-hydroxy-nonenal, which is a product of lipid peroxidation. We also found that I/R injury further increased formaldehyde levels in GSNO-R inhibited female hearts (Table 1) [29]. Furthermore, we and others have demonstrated sex differences in the enzymes that regulate formaldehyde. Reports show that both the heart and lungs have increased GSNO-R activity in females, which may be regulated by estrogen [9,166]. Lagranha et al. (2010) also reported higher phosphorylation and activity of ALDH2 in WT female hearts compared to males, and that Alda-1 treatment protected male hearts, but provided no further protective benefit to females [167].

Thus far, impaired formaldehyde metabolism has been discussed, but there are other forms of carbonyl dysregulation, such as lipid peroxidation, that are demonstrated to have important sex differences. Reactive oxidants can overwhelm antioxidant systems and these radicals can oxidize fatty acids to release aldehydes. These species react with nucleophilic sites in the cell, such as nucleic acids and proteins, to damage critical macromolecules. Protein carbonylation is reported to be lower in female rats compared to males, but with no difference in malondialdehyde, a common marker of lipid peroxidation [168,169]. However, it was recently reported that women have significantly less malondialdehyde compared to men, and this compound increases with age [170]. These results indicate that females have lower lipid peroxidation and are consistent with evidence that females have less oxidative stress and greater antioxidant capacity. Redox homeostasis can be altered without the need to disrupt or remove sex hormones by targeting enzymes that are uniquely poised to influence different redox pathways.

4.3. Nitric oxide signaling and reactive aldehyde species

GSNO-R is a prime example of unique enzyme that can influence redox signaling and which yields a striking sex-disparate phenotype without the alteration of sex hormones. As a unique enzyme that can control multiple redox pathways, GSNO-R is poised to influence cardiac redox homeostasis and contribute to biochemical cycles. GSNO-R regulates NO signaling by metabolizing GSNO and along with the loss of GSH, ammonium is produced, which is shunted into the urea cycle (Fig. 1). However, GSNO-R also helps to regulate formaldehyde levels, which detoxifies this aldehyde, by helping to convert formaldehyde into formate. The latter is then sent to the one-carbon metabolism cycle and contributes to nucleotide synthesis [149,171]. There is a unique interaction between NO signaling and formaldehyde detoxification that is mediated by GSNO-R. Further studies into enzymes, like GSNO-R, that can regulate multiple redox nodes may provide novel insight into the mechanism of sex-dependent protection from I/R injury and other forms of cardiovascular disease.

Apart from GSNO-R, SNO of enzymes that regulate aldehydes can influence redox homeostasis. Several studies have demonstrated that SNO of different ALDHs can inhibit their activity [172,173]. Also, SNO can inhibit glutathione *S*-transferases, which conjugate aldehydes to glutathione and detoxify these species [174]. SNO of GSNO-R has been shown to inhibit the enzyme in plants [175,176]. If GSNO-R activity can be impaired by protein SNO in mammalian hearts, then SNO may have a profound impact on aldehyde signaling and toxicity which may confer or block protection from I/R injury in a sex-specific manner. However, estrogen has been shown to induce a SNO-dependent increase in GSNOR activity in the female mouse lung, so additional research is warranted [166]. Further investigations of NO-aldehyde interactions are also essential for furthering our understanding of the pathology of cardiovascular disease.

5. Conclusion

Cardiovascular disease is the leading cause of death amongst both men and women. Therefore, it is paramount that we understand disease development and progression in both sexes, and the role of redox signaling in this process. Many studies have demonstrated fundamental sex differences in cardiovascular and redox biology. While these findings made significant impacts in both areas of research, there is still more to understand. In order to learn more about sex differences in cardiovascular disease, we must develop a new experimental perspective, one that does not depend on the disruption or removal of the sex hormones that are foundational to the physiology of both sexes. Instead, we should interrogate the impact of altered redox signaling pathways, in part, by modulating the enzymes that can influence these pathways. Furthermore, it is important to note that female hearts are typically smaller than male hearts for most species. Several studies have also demonstrated that female hearts express higher levels of glutathione, catalase, and superoxide dismutase, in addition to nitric oxide synthases. As such, nitric oxide signaling, and antioxidant capacity may be maximized due to smaller diffusion distances. With these factors in mind, the study of the heart in its natural state will provide valuable insight into the distinct molecular mechanisms that govern cardiovascular physiology, redox homeostasis, and disease in both men and women.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

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