ABSTRACT

Title of Thesis:	RACIAL DIFFERENCES IN ROS PRODUCTION AND SOD ACTIVITY FOLLOWING INDUCED INFLAMMATION
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Black individuals are predisposed to an earlier onset and higher prevalence of cardiovascular disease, such as hypertension. Hypertension may be caused by inflammation and heightened levels of reactive oxygen species (ROS). At rest, Black and White individuals exhibit divergent *in vivo* and *in vitro* inflammation, ROS production, and ROS clearance. This study investigated racial differences in ROS production and ROS clearance following induced inflammation in human plasma and HUVECs from Black (B HUVECs) and White (W HUVECs) individuals.

W HUVECs, but not B HUVECs, exhibited significantly greater ROS production with increased exposure to TNF-α. Further, W HUVECs alone experienced a significant increase in SOD activity with increased time that was abolished with TNF-α. The HUVEC data were also analyzed for sex differences. HUVECs from females exhibited significantly lower ROS production than HUVECS from males basally and following TNF-α treatment. Female HUVECs alone

exhibited significantly greater SOD activity with increased exposure to TNF-α. The findings suggest a ‘priming’ for lower ROS production via greater total antioxidant status (from non-SOD antioxidants) in B HUVECs. Further, male HUVECs may be predisposed to a pro-inflammatory state due to higher androgen exposure in fetal umbilical cord blood.

RACIAL DIFFERENCES IN ROS PRODUCTION AND SOD ACTIVITY FOLLOWING INDUCED INFLAMMATION

by

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Chapter 1: Introduction

A. Overview

Cardiovascular disease (CVD), the leading cause of death in America, demonstrates race-based disparities. Black individuals (B) are predisposed to an earlier onset and higher prevalence of stroke, chronic kidney disease, and hypertension [1-3]. Specifically, increased blood pressure in Black individuals may be caused by subclinical vascular dysfunction from inflammation and elevated systemic reactive oxygen species (ROS) [4]. However, racial differences in the mechanisms linking inflammation, ROS, and vascular dysfunction are not fully elucidated. Thus, studying *in vivo* and *in vitro* inflammatory responses and ROS clearance capacity in Black and White individuals following induced inflammation may elucidate specific targets responsible for racial differences in hypertension (**Figure 1**).

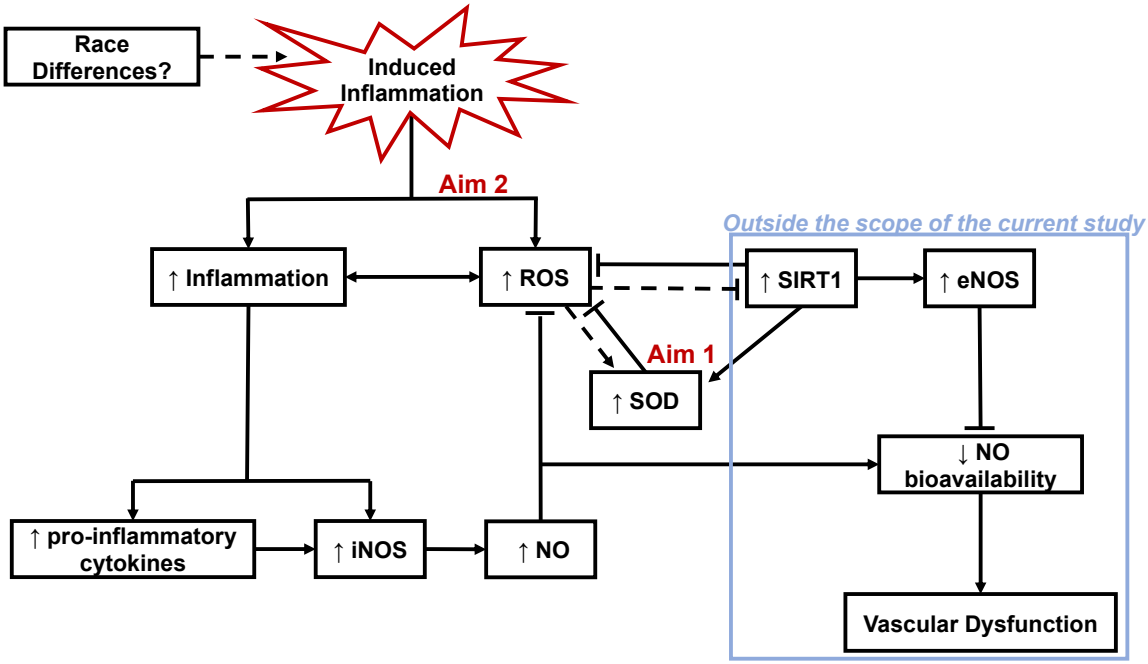

Figure 1. Experimental model of proposed mechanisms of racial differences in inflammation and ROS *in vivo* and *in vitro*. **Aim 1** will investigate racial differences in ROS clearance capacity (SOD activity) in B and W human plasma following induced inflammation. **Aim 2** will investigate racial differences in ROS Production (CellROX/ Hoechst Ratio) and ROS clearance capacity (SOD activity) in B and W HUVECs following induced inflammation. ROS, Reactive Oxygen Species; SIRT1, Sirtuin 1; eNOS, endothelial nitric oxide synthase; SOD, superoxide dismutase; NO, Nitric Oxide; iNOS, inducible nitric oxide synthase.

B. Inflammation and ROS

ROS are free radicals, such as superoxide ions or peroxynitrite. ROS are important cellular signaling molecules that are produced from oxidative metabolism and various proteins, such as NADPH Oxidases and uncoupled endothelial nitric oxide synthase (eNOS) [4, 15]. There is a delicate balance between ROS production and ROS clearance that prevents aberrant increases in ROS levels. During inflammation, ROS levels can increase via heightened ROS production and/or impaired ROS clearance [4, 8]. Superoxide (O_2^-) is a predominant ROS implicated in cellular metabolism and the production of other ROS; superoxide levels have exhibited race-related differences [4, 6, 8-11]. ROS

clearance is predominantly mediated by antioxidants, one of which is superoxide dismutase (SOD), a chief ROS scavenger responsible for the dismutation of superoxide (O_2^-) into hydrogen peroxide (H_2O_2) [12]. When ROS levels are heightened, NO will also scavenge ROS.

There are three forms of nitric oxide synthase (NOS): endothelial (eNOS), neuronal (nNOS), and inducible NOS (iNOS). eNOS and nNOS are generally localized to specific tissues. However, iNOS is present systemically, which partially explains its implication in the inflammatory response. In response to inflammation or increased ROS production, iNOS produces NO [16, 17]. During inflammation, iNOS increases NO production for ROS scavenging, not for vasodilatory purposes (**Figure 1**). Thus, NO bioavailability may decrease even with elevated NO concentrations, an “NO paradox” [4, 9, 11]. Therefore, heightened NO concentrations, but not NO bioavailability, may indicate heightened inflammation, elevated ROS production, and/or inadequate ROS clearance [4-6, 8, 9]. In states of chronic low-grade systemic inflammation, heightened basal ROS levels may impair responses to further perturbation, with impaired ROS clearance resulting in further ROS overproduction following induced inflammation [15].

C. SIRT1, NO, and SOD

At rest, eNOS, the NOS isoform found within the endothelium, produces NO that primarily acts as a vasodilator. In inflammatory states, nuclear factor-kB (NF-kB) activity and heightened interleukin-6 (IL-6) levels stimulate the iNOS production pathway, resulting in an overproduction of NO that then scavenges ROS and prevents cell death [8, 16-20]. When ROS levels are aberrantly elevated,

antioxidants such as SOD may not be able to effectively clear excessive ROS, leading to a reliance on NO to scavenge excess superoxide and form peroxynitrite [8]. Black individuals have reduced bioavailability of nitric oxide (NO), a major vasodilatory signal [7-9]. Importantly, the enzyme Sirtuin1 (SIRT1) is involved in regulating inflammation and ROS via antagonism of NF-kB signaling (**Figure 2**) [6, 21, 22]. SIRT1 directly downregulates NF-kB via deacetylation and indirectly downregulates NF-kB signaling via deacetylation of AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC1α), and peroxisome proliferator activated receptor α (PPARα) [22]. SIRT1's influence on oxidative metabolism and inflammatory pathway is partially mediated via NO production, NADPH oxidase inhibition, SOD promotion, and NF-kB inhibition (**Figure 2**) [6, 21-26].

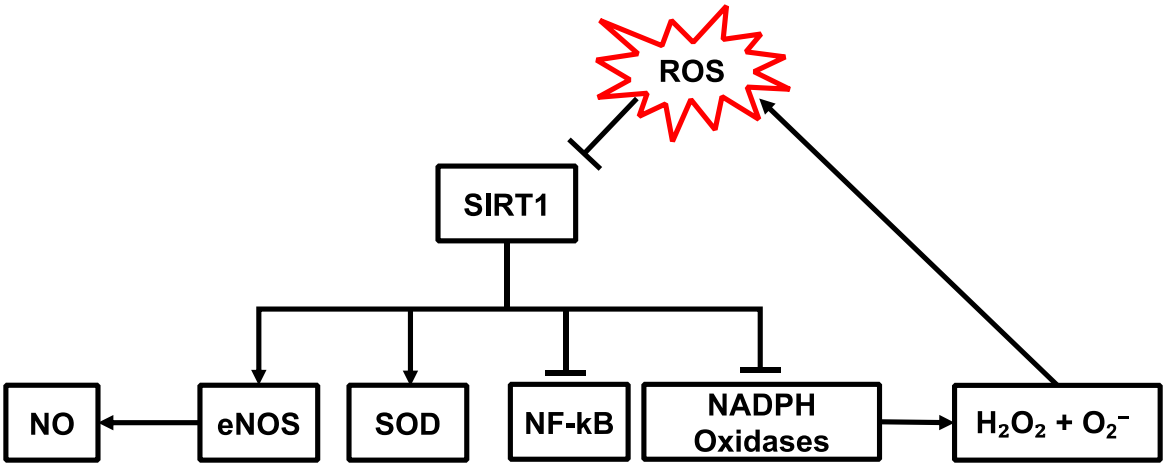

Figure 2. SIRT1 actions on eNOS, SOD, NF-kB, and NADPH oxidases. SIRT1 inhibits inflammation via direct and indirect antagonism of NF-kB and promotion of eNOS-related NO production. SIRT1 influences ROS clearance via promoting SOD activity and hinders ROS production via inhibition of NADPH oxidases ROS, reactive oxygen species; SIRT1, Sirtuin 1; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; SOD, superoxide dismutase; NF-kB, nuclear factor kappa B.

Chapter 2: Review of Literature

A. The Vascular Health Triad

The vascular health triad is the positive feedback loop of inflammation, ROS, and vascular dysfunction (**Figure 3**) [4]. Inflammation is a natural defense mechanism against pathogen infiltration, tissue injury, or tissue infection [20]. Inflammation is initiated by pro-inflammatory cytokines and the movement of immune cells to the site of invasion or injury [4]. Acute inflammation, while protective in nature, may result in chronic inflammation with prolonged overproduction of inflammatory mediators. In the context of aging, prolonged systemic inflammation and heightened ROS both contribute to vascular dysfunction, resulting in eventual clinical vascular impairment (i.e. atherosclerosis or hypertension) [4, 6, 15, 27].

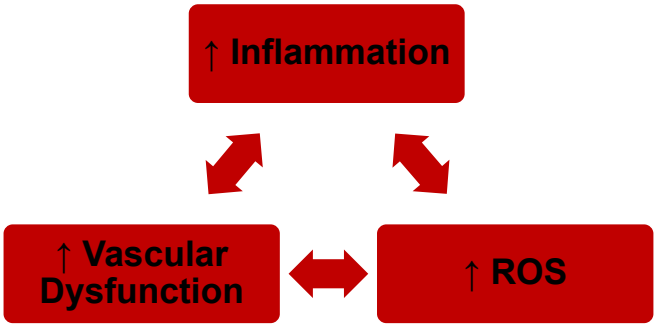

Figure 3. Vascular Health Triad adapted from Wadley, van Zanten, and Aldred [4]. Increased inflammation is caused by and produces elevated ROS. Chronically heightened inflammation and ROS produce vascular dysfunction and eventual arterial remodeling.

In aging and other disease states, there is an overproduction of ROS and a concurrent impairment in ROS clearance capacity [4, 15]. NADPH oxidases are key superoxide producers, so while NADPH oxidases were not a target of the present study, much of the inflammatory-oxidative stress mechanism literature includes measurement of NADPH oxidase protein expression [15, 16, 28-32]. Interestingly, eNOS uncoupling is also thought to be a key producer of superoxide

during inflammation [9, 15, 28, 29]. Antioxidants, such as SOD, glutathione peroxidase, and ascorbic acid, are responsible for ROS clearance capacity [4, 11, 15].

Increased inflammation produces heightened ROS levels, and heightened ROS levels cause increased inflammation [4, 15]. Over time, increased ROS levels and inflammation result in vascular dysfunction, which is characterized by impaired vasodilatory capacity of blood vessels. Vasodilatory capacity may become impaired by imbalanced vasoconstrictive and vasodilatory substances, increased basal sympathetic nervous system activity, and aberrant structural remodeling of blood vessels [4, 15]. Investigating mechanisms linking inflammation and ROS in young, healthy Black and White individuals and in HUVECs from Black and White individuals will elucidate potential mechanisms to target in future mechanistic and clinically applied research. In this context, inducing acute inflammation to investigate inflammatory responses and ROS levels in young, healthy individuals is key to understanding disparities in CVD prevalence, especially hypertension.

B. Acute Inflammatory Cascade

Acute inflammation is thought to be a bi-modal process. Initially, acute inflammation is marked by an upsurge in immune cells and pro-inflammatory cytokines which is followed by an increase in anti-inflammatory cytokines [4]. Inflammation is initiated by a localized infiltration of leukocytes and neutrophils at the site of injury or infection. Leukocytes adhere to the vessel wall via cellular adhesion molecules (CAMs) while neutrophils localize through chemotaxis of interleukins IL-1β and IL-8. IL-6, a third pro-inflammatory cytokine, is a chemokine

for monocytes that then differentiate into macrophages at the site of tissue damage or pathogen infiltration. Other notable inflammatory molecules upstream of interleukins include TNF-α and NF-kB [4]. Following the initial pro-inflammatory response, there is an anti-inflammatory response marked by anti-inflammatory cytokine release (IL-10, IL-1ra, IL-4). Importantly, pro-inflammatory cytokine measurement (IL-6 or TNF-α) is common practice for detecting inflammation basally and following induced inflammation [4, 8, 14].

C. TNF-α and Influenza Vaccine

Both TNF-α and the influenza vaccine stimulate an innate immune response, and the influenza vaccine also stimulates a secondary adaptive immune response for long-term immunity [20, 33]. TNF-α is a form of tumor necrosis factor (TNF), and TNFs are predominantly released by monocytes/ macrophages and T-cells as part of the initial innate immune response to a pathogen or tissue injury [33]. TNFs are potent pro-inflammatory cytokines implicated in a wide variety of responses to inflammation, including cellular activation, proliferation, and cell death [33]. Specifically, TNF-α binds to TNF receptor 1 or 2 (TNFR1 or TNFR2), which can be membrane bound or soluble; TNF binding to TNFRs results in a multitude of cascades, including downstream activation of NF-kB [33, 34]. During an inflammatory response, TNF-α contributes to vasodilation at the site of injury via increased prostanoid (PGE2, PGF2a, and PGI2) production, increased endothelial permeability via disruption of the endothelial cell wall, increased leukocyte adhesion via increased E- and P-selectin expression, blood coagulation via increased tissue factor expression, and increased ROS production via NADPH

oxidase activation [34]. Similarly, influenza vaccine virus binding to cell surfaces results in downstream NF-kB activation as part of host cell defenses [35, 36]. Both TNF-α and influenza vaccine activate NF-kB, making NF-kB the crossroads of the two inflammatory stimuli. NF-kB activation causes downstream release of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-8, IL-1β, and expression of inflammatory proteins, such as iNOS [20, 33].

D. IL-6, iNOS, NADPH Oxidases, and SOD Definitions

Mechanisms linking IL-6, iNOS, NADPH oxidases, and SOD are presented in **Figure 4**. IL-6 concentrations increase during the initial pro-inflammatory cascade following inflammation [4]. Importantly, increased IL-6 production and NF-kB activation trigger iNOS expression; iNOS is one form of nitric oxide synthase responsible for NO production during inflammation [17, 19]. NO is a primary vasodilatory molecule that also has anti-inflammatory and anti-atherogenic properties. NO will scavenge superoxide to form peroxynitrite in the presence of ROS overproduction, specifically superoxide (O_2^-) overproduction. Superoxide production is associated with NADPH oxidase activity [30-32]. Further, NADPH oxidases are associated with increased ROS production in the endothelium and in circulating macrophages, reduced NO bioavailability, and atherogenesis. ROS clearance is mediated by antioxidants, and SOD is a major antioxidant that clears superoxide. There are three forms of SOD. SOD1 is cytoplasmic, SOD2 is mitochondrial, and SOD3 is circulating. However, other antioxidants, such as glutathione, vitamins, and other proteins, also clear ROS.

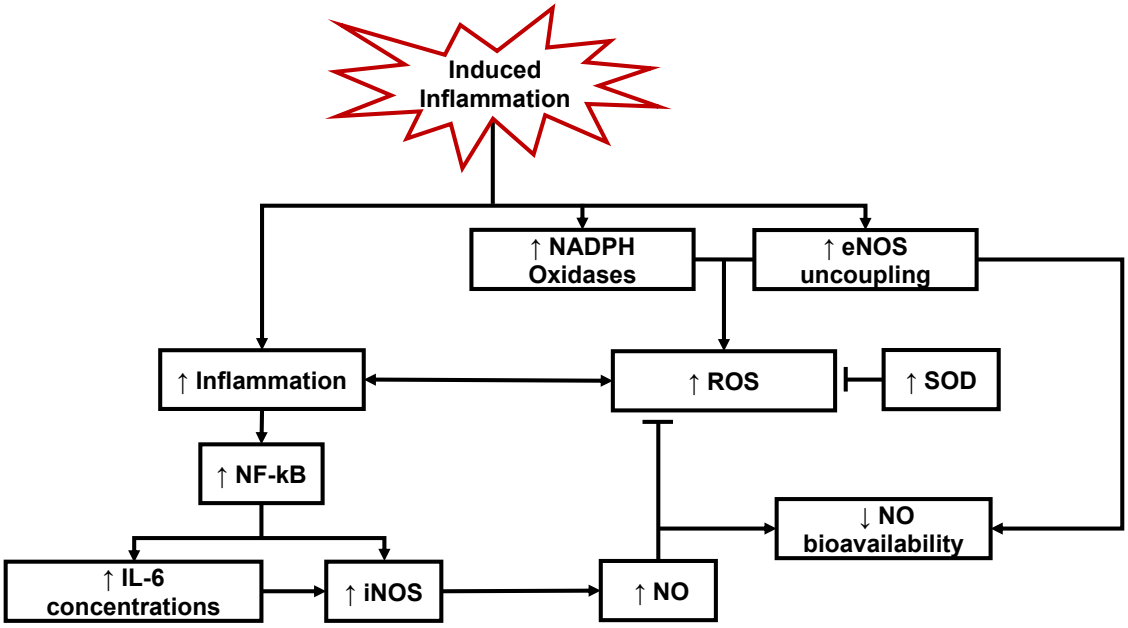

Figure 4. Mechanisms of induced inflammation and heightened ROS during acutely induced inflammation. Induced inflammation triggers NF-kB activation, which causes increased IL-6 concentration and iNOS expression. Induced inflammation also causes heightened NADPH Oxidase activity and eNOS uncoupling, resulting in heightened ROS production. ROS clearance is represented by SOD. NF-kB, nuclear factor kB; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; NO, nitric oxide, eNOS, endothelial nitric oxide synthase; ROS, reactive oxygen species; SOD, superoxide dismutase.

E. Inflammatory Response, ROS levels, and Antioxidant Literature

Clapp and colleagues studied microvascular function and oxidative stress following induced inflammation and local antioxidant treatment [5]. Eight hours after induced inflammation, there was a significant increase in IL-6 and IL-1Ra concentration, a significant decrease in total antioxidant capacity (TAC), a systemic measurement antioxidant capability, and a significant impairment in bradykinin-stimulated microvascular function. Increases in IL-6 and IL-1Ra indicate successfully induced inflammation. Impaired TAC, a marker of the total ROS clearing capability of a sample, and microvascular function suggest

concomitant inhibition of ROS clearance capacity and vascular function. Interestingly, infusion of the antioxidant ascorbic acid resulted in restoration of microvascular function 8 hours following induced inflammation, suggesting a primary role of heightened ROS levels in the impaired vascular function following induced inflammation [5]. Taken together, these findings emphasize the vascular health triad: induced inflammation impairs vascular function likely through a concomitant increase in ROS, and antioxidant infusion reduces ROS levels via bolstering ROS clearance capacity, resulting in restored vascular function.

Wu, Tyml, and Wilson studied iNOS expression following induced inflammation in mouse microvascular endothelial cells [16]. Lipopolysaccharide (LPS) and interferon-γ (IFN-γ) treatment were used to induce inflammation; LPS and IFN-γ treatment significantly increased superoxide production basally, indicating successfully induced inflammation. In p47phox (a NADPH oxidase subunit) knockout cells with and without LPS and IFN-γ treatment, superoxide production was significantly lower than wild-type LPS and IFN-γ treated cells, indicating a primary role of the p47phox NADPH oxidase subunit in superoxide production. Further, iNOS protein expression significantly increased following LPS and IFN-γ treatment, further supporting the protective role of iNOS-related NO production after induced inflammation. iNOS expression was significantly lower in p47phox knockout cells when compared to wild-type cells following LPS and IFN-γ treatment. Because iNOS expression was dampened when a primary superoxide producer was removed from the cells, the findings support the intimate link between ROS production and inflammatory cascades following induced

inflammation. Interestingly, NF-kB inhibition, apocynin (NADPH oxidase inhibitor) treatment, and ascorbate (antioxidant) treatment significantly reduced iNOS protein expression. Taken together, the main findings suggest a key role of inflammation (NF- kB) and superoxide production (p47phox knockout, NADPH oxidase inhibition) in the activation of iNOS and the subsequent protective NO production during heightened inflammation.

Ding and colleagues investigated protein expression of SOD, eNOS, and NADPH oxidase in mouse microvessel endothelial cells following glucose-induced inflammation [28]. Superoxide production was indexed via fluorescence. Following the high glucose load, superoxide fluorescence was significantly increased, indicating increased ROS levels following induced inflammation. Interestingly, treatment with sepiapterin, a tetrahydrobiopterin (BH_4) precursor, significantly reduced superoxide fluorescence in the presence of the high glucose load. Sepiapterin facilitates eNOS coupling; coupled eNOS produces NO while uncoupled eNOS produces superoxide. Thus, induced inflammation likely results in eNOS uncoupling, and increased superoxide following induced inflammation is likely from both eNOS uncoupling and NADPH oxidases. Further, the high glucose load significantly reduced SOD1 and SOD3 protein expression and increased p22phox, an NADPH oxidase subunit, protein expression. Sepiapterin treatment during the high glucose load did not impact SOD1 protein expression. However, sepiapterin treatment did significantly increase SOD3 and significantly reduced p22phox protein expression, indicating the role of sepiapterin in increased ROS clearance capacity and reduced ROS production even during induced

inflammation. High glucose load and sepiapterin treatment did not impact the protein expression of NOX4 or SOD2 [28]. eNOS mRNA was significantly increased 72 hours following high glucose load, yet NO generation was significantly decreased, suggesting a reduction in NO bioavailability with NO scavenging superoxide to form peroxynitrite. These findings suggest that induced inflammation impairs ROS clearance capacity by directly reducing the protein expression of SOD1 and SOD3 while increasing the protein expression of NADPH oxidase subunit p22phox and mRNA expression of eNOS.

Further, Li and colleagues studied the role of NADPH oxidase subunit p47phox in superoxide production in coronary microvascular endothelial cells following induced inflammation [30]. Endothelial cells from p47phox knockout mice and wild type controls were treated with phorbol ester and TNF-α. Basally, there was no difference in ROS production between p47phox knockout and wild-type mice, which highlights the role of other NADPH oxidase subunits (p22phox) and isoforms (NOX2, NOX4) in basal ROS production. Treatment with phorbol ester and TNF-α resulted in a significant increase in ROS production in wildtype mice but had no effect on p47phox knockout mice, and transfecting p47phox cDNA restored the superoxide response to phorbol ester and TNF-α in p47phox knockout mice, suggesting a primary role of p47phox in superoxide production following induced inflammation. Many NADPH oxidase subunits and isoforms likely contribute to basal ROS production, however, in endothelial microvascular cells from mice, p47phox is a main producer of superoxide in response to induced inflammation.

Jacobi and colleagues investigated the impact of superoxide and hydrogen peroxide production on inflammation (via IL-8 mRNA, eNOS and iNOS mRNA, and eNOS protein expression) and oxidative stress (via NADPH oxidase chemiluminescence) in HUVECs [32]. In HUVECS treated with xanthine and xanthine oxidase, superoxide was identified as the primary ROS influencing inflammation and oxidative stress. Specifically, pro-inflammatory cytokine IL-8 concentrations significantly increased 6 hours after exposure, indicating successfully induced inflammation. Additionally, eNOS mRNA and protein expression significantly decreased and iNOS mRNA significantly increased 6 hours after exposure, suggesting eNOS uncoupling and concomitant activation of iNOS via inflammation and ROS production. Taken together, the findings suggest a significant role of superoxide production, likely from NADPH oxidases and eNOS uncoupling, in inflammation and resultant eNOS uncoupling.

Interestingly, NO has been shown to directly suppress NADPH-oxidase superoxide production via S-nitrosylation [29]. Selemidis and colleagues studied the impact of NO donors on human microvascular endothelial cells. The experimental targets were superoxide, NADPH oxidase subunit protein expression, and S-nitrosylation (via assay and antibody immunoblotting). Endothelial cells were treated with NO donors for 6 hours and then NO was washed from the cells prior to any assay. NO donor treatment significantly reduced superoxide chemiluminescence, supporting a direct role of NO in superoxide clearance. Interestingly, a 60-70% reduction in superoxide chemiluminescence was sustained for 6 hours following NO donor washout, suggesting a sustained

suppression of superoxide production by NO that is not the result of direct NO scavenging [29]. Importantly, NO donor treatment did not impact NADPH oxidase (NOX2, NOX4, p47phoc, or g-protein Rac) expression, indicating that superoxide reduction was not due to NADPH oxidase downregulation. To investigate the potential impact of S-nitrosylation, UV light can be used to break S-nitrosylation S-NO bonds. Following UV light treatment, NO donor-induced attenuation in superoxide production was abolished, suggesting that NO reduced superoxide production indirectly through S-nitrosylation [29]. P47phox is S-nitrosylated by NO. Thus, NO is implicated in vascular function (vasodilation), oxidative stress (S-nitrosylating p47phox to reduce superoxide production), and inflammation (NO is overproduced as a result of iNOS activation following inflammation).

Further, Harrison and colleagues conducted a similar study comparing the impact of eNOS inhibition on superoxide production in p47phox knockout and wild-type mice [37]. Following L-NAME treatment, wild-type mice exhibited increased superoxide production, p47phox protein expression, and vascular cell adhesion molecule-1 protein expression, further underlying the role of NO in superoxide clearance, p47phox inhibition, and anti-atherogenesis. In further support, p47phox knockout mice did not exhibit an increase in superoxide production, p47phox protein expression, or vascular cell adhesion molecule-1 protein expression following L-NAME treatment. Taken together, the findings from the Selemidis et al. and Harrison et al. studies suggest that NO is both a crucial superoxide production inhibitor and a direct superoxide scavenger [29, 37].

Induced inflammation and ROS clearance capacity are intimately linked, as sepiapterin treatment restores NO production and SOD3 protein expression and reduces NADPH oxidase p22phox subunit expression [28]. While SOD2 was not impacted by the high glucose load or sepiapterin treatment in the study by Ding and colleagues, SOD2 protein expression is the target of choice in the present study [28]. Studies of racial differences in SOD expression have hinted at a potential differential impact of inflammation on SOD2 in B and W HUVECs [13]. In this context, quantifying inflammatory response markers (IL-6 concentration, iNOS protein expression, and NO concentration [via nitrite]) alongside ROS clearance capacity markers (SOD activity, TAC, and SOD2 protein expression) provides a unique insight into the inflammatory response to TNF-α proposed in the current study.

F. Racial Differences in Inflammation and ROS Clearance Capacity

There are well established differences in inflammation and ROS clearance capacity between Black and White individuals [7-9, 11-14]. Feairheller, Brown, and colleagues conducted a series of studies investigating racial differences in inflammation and ROS clearance capacity [8, 12-14]. In human plasma (*in vivo*) and HUVECs (*in vitro*) from Black and White individuals, Feairheller et al. measured NO concentration, IL-6 concentration, SOD activity, TAC, and protein carbonylation (an index of elevated ROS levels) [8]. SOD 1 activity and protein expression of eNOS, iNOS, IL-6, SOD2, and NADPH oxidases (isoforms NOX2 and NOX4 and subunit p47phox) were also measured *in vitro* [8]. There were no differences in *in vivo* NO concentration, suggesting systemic maintenance of NO

concentrations in young, healthy Black and White individuals. However, B HUVECs had significantly greater *in vitro* NO concentration, eNOS protein expression, and iNOS protein expression than W HUVECs, suggesting increased inflammation in B HUVECs basally and in isolation of circulating factors. There were no *in vivo* racial differences in IL-6 concentration, suggesting similar inflammation in young, healthy Black and White individuals. However, there were *in vitro* racial differences in IL-6 concentration, with Black HUVECs exhibiting significantly greater IL-6 concentration and IL-6 protein expression. Higher pro-inflammatory cytokine IL-6 concentrations and protein expression in B HUVECs further suggest heightened inflammation basally.

In regard to ROS clearance, *in vivo* SOD activity was significantly greater in plasma from Black individuals (B plasma) as compared with plasma from White individuals (W plasma), suggesting a higher ROS clearance capacity in Black individuals. Though, *in vitro* SOD activity was significantly lower in B HUVECs compared with W HUVECs. Importantly, SOD3 is circulating, and, thus, racial differences in *in vitro* SOD activity may be due to the missing influence of SOD3. There were no differences in SOD1 activity or SOD2 protein expression *in vitro*, further supporting a potential critical role of SOD3 in differential *in vivo* and *in vitro* SOD activity. Further, B plasma and HUVECs exhibited significantly greater TAC and protein carbonylation than W plasma and HUVECs, respectively, suggesting greater ROS clearance capacity in B plasma and B HUVECs. In regard to ROS production, B HUVECs exhibited significantly greater expression of NADPH oxidases (subunit p47phox and isoforms NOX2 and NOX4) [8]. Heightened eNOS

protein expression, iNOS protein expression, and NO levels in B HUVECs suggests an inflammation-related increase in NO production to scavenge overproduced superoxide via heightened NADPH oxidase protein expression and protein carbonylation.

Deo and colleagues studied superoxide production and NADPH oxidase expression in peripheral blood mononuclear cells (PBMCs) from Black and White individuals [10]. PBMCs are circulating immune cells that play a crucial role in superoxide production (and, thus, circulating ROS levels) and eventual atherogenesis [10]. Superoxide production was directly quantified in isolated PBMCs via fluorescence. B PBMCs exhibited significantly greater superoxide fluorescence than W PBMCs, indicating greater superoxide levels in B PBMCs. The greater superoxide levels may be due to heightened ROS production or impaired ROS clearance. Because B PBMCs exhibited a significantly higher protein expression of NADPH oxidase subunits gp91phox and p47phox and angiotensin II type I receptors, increased ROS production (from NADPH oxidases) likely contributed to the increased superoxide levels in B PBMCs [10]. Taken together, these findings further support a racial difference in ROS production, with Black individuals exhibiting significantly greater superoxide production via NADPH oxidases.

The time course of NO, superoxide, and peroxynitrite release gives insight into potential racial differences in basal ROS levels and ROS clearance. Kalinowski and colleagues studied oxidation kinetics of NO, superoxide, and peroxynitrite and protein expression of NADPH oxidases and eNOS in B and W

HUVECs [9]. Basally, B HUVECs had significantly greater superoxide and peroxynitrite release and significantly lower NO release as compared with W HUVECs [9]. Thus, reduced NO bioavailability in Black individuals is likely due to NO scavenging superoxide and heightened superoxide production.

Following the study of basal cell responses, various groups investigated the impacts of inflammatory stimuli and various inhibitors on cell inflammatory and ROS responses. Inhibitors used include: NADPH oxidase inhibitors apocynin and S17834, xanthine oxidase inhibitor oxypurinol, mitochondrial complex I inhibitor rotenone, cyclooxygenase inhibitor meclofenamate, and eNOS inhibitor L-NAME. Kalinowski and colleagues reported that racial differences in oxidation kinetics were sustained with oxypurinol, rotenone, and meclofenamate, suggesting a minimal role of xanthine oxidase, mitochondrial complex I, and cyclooxygenase in the racial differences in oxidation kinetics. With both NADPH oxidase inhibitors and the cyclooxygenase inhibitor, B HUVECs saw a significant decrease in superoxide and peroxynitrite release and a significant increase in NO release. Importantly, only the NADPH oxidase inhibitors abolished the racial differences in oxidative kinetics, suggesting a primary role of NADPH oxidase-produced superoxide in reduced NO bioavailability [9]. Further, B HUVECs exhibited a significantly greater protein expression of eNOS and NADPH oxidase subunits p47phox, p22phox, and p67phox. Heightened eNOS expression suggests either increased NO production or, if eNOS is uncoupled by the heightened ROS, increased superoxide production. Further, there were racial differences in SOD-inhibitable ferricytochrome c reduction (superoxide production calculated by the amount of

ferricytochrome c reduction inhibited by SOD) with the control and with oxypurinol, rotenone, meclofenamate, and L-NAME, indicating a significantly greater superoxide production in B HUVECs at rest and with xanthine oxidase inhibition, mitochondrial complex I inhibition, cyclooxygenase inhibition, and eNOS inhibition. There were no racial differences in SOD-inhibitable ferricytochrome c reduction with apocynin and S17834, suggesting a primary role of NADPH oxidases in racial differences in superoxide production [9]. As compared to W HUVECs, B HUVECs exhibit differential oxidation kinetics of NO, superoxide, and peroxynitrite and these differences are abolished with NADPH oxidase inhibition. B HUVECs likely experience heightened superoxide levels that likely result in NO scavenging, eNOS uncoupling, and reduced NO bioavailability.

Then, Mason and colleagues studied oxidative kinetics of NO, superoxide, and peroxynitrite release basally and following nebivolol and apocynin (NADPH oxidase inhibitor) treatment in B and W HUVECS and iliac artery endothelial cells [11]. Nebivolol is a β_1-receptor blocker and is implicated in vasodilation, antioxidant activity, and inhibition of eNOS uncoupling. B HUVECs exhibited significantly greater release rates and concentrations of superoxide and peroxynitrite and a significantly reduced release rate and concentration of NO as compared to W cells, further supporting the previous findings by Kalinowski and colleagues [9]. However, with sustained nebivolol or apocynin treatment, racial differences in NO, superoxide, and peroxynitrite levels were abolished [11]. These findings suggest that beta-blockers and NADPH oxidase inhibitors can successfully reduce ROS levels and, thus oxidative stress, and B endothelial cells.

Brown and colleagues studied racial differences in TNF-α and SOD treatment in endothelial cell microparticle release, SOD activity, and IL-6 production [14]. Elevated endothelial microparticle release is indicative of increased inflammation and endothelial cell activation. There were no basal racial differences in endothelial microparticle levels. However, following TNF-α treatment, B HUVECS exhibited a significant increase in endothelial microparticle release as compared with W HUVECS and the basal B HUVECs condition, suggesting a heightened inflammatory response and greater endothelial cell activation following induced inflammation. Compared with TNF-α treatment, both W and B HUVECS exhibited a significant decrease in endothelial microparticle release with SOD treatment and SOD + TNF-α treatment [14]. Decreased endothelial microparticle release with SOD treatment indicates a role of increased superoxide production and overall ROS levels in endothelial microparticle release. In terms of ROS clearance capacity, B HUVECS had significantly lower SOD activity basally and following TNF-α treatment, which is in agreement with findings from Feiarheller et al. [8]. Basal IL-6 protein expression, but not IL-6 concentration, was significantly greater in B HUVECs as compared to W HUVECs [14]. Taken together, the findings suggest that B HUVECs are more susceptible to an inflammatory insult.

Following the study of basal racial differences in inflammation and oxidative stress in HUVECS, Feairheller and colleagues studied potential racial differences in inflammatory responses and oxidative stress in B and W HUVECs following low and moderate laminar sheer stress [8, 13]. Laminar shear stress, the frictional

force of blood flow along the arterial wall, increases with aerobic exercise onset. Thus, laminar shear stress on HUVECs is thought to be an *in vitro* aerobic exercise mimetic. Basally, B HUVECS exhibited significantly greater NADPH oxidase subunit (p47phox) and isoform (NOX2 and NOX4) protein expression as compared to W HUVECs, which is in agreement with the previous Feairheller et al. study [8, 13]. Following low shear stress, B HUVECS exhibited a significant decrease in NOX4 expression; with moderate shear stress, B HUVECs exhibited a significant decrease in p47phox and NOX4 protein expression as compared to basal p47phox and NOX4 protein expression [13]. The decrease in NADPH oxidase protein expression indicates an efficacious effect of the exercise mimetic on ROS production in B HUVECs. Consistent with the previous Feairheller study [8], B HUVECs exhibited significantly greater basal eNOS protein expression than W HUVECs. In response to low and moderate shear stress, both racial groups experienced progressive, significant increases in eNOS protein expression to similar levels. There were no differences in basal NO levels between racial groups, and both groups exhibited progressive, significant increases in NO with low and moderate laminar shear stress, suggesting similar exercise-induced changes in eNOS expression and NO production in B and W HUVECs [13].

In terms of ROS clearance capacity, B HUVECs exhibited significantly lower basal SOD2 protein expression and SOD activity level as compared to W HUVECs, suggesting impaired ROS clearance capacity in B HUVECs. There were no basal racial differences in catalase protein expression or TAC. B and W HUVECs both exhibited a significant increase in catalase protein expression at low

laminar shear stress; however, B HUVECs exhibited a significant decline in catalase protein expression from low laminar shear stress to moderate laminar shear stress, suggesting potential differential impacts of low and moderate exercise shear stress in B HUVECs. For SOD2 protein expression and total SOD activity, B HUVECs exhibited a significant increase from basal in SOD2 protein expression and SOD activity at low laminar shear stress that significantly declined, but was still significantly increased from basal, with moderate laminar shear stress. For TAC, B HUVECs exhibited a significant increase from basal in TAC with low and moderate laminar shear stress; B HUVECs exhibited a significantly greater TAC than W HUVECs at low laminar shear stress [13]. The findings further support racial differences in ROS production and ROS clearance capacity, with B HUVECs exhibiting significantly greater ROS production and significantly lower ROS clearance capacity basally. However, laminar shear stress, an aerobic exercise mimetic, was able to abolish racial differences in NADPH oxidase protein expression, eNOS protein expression, SOD2 protein expression, and SOD activity. Thus, aerobic exercise may be an efficacious intervention for lowering oxidative stress in Black individuals.

Feairheller and colleagues then examined oxidative stress responses to acute exercise in Black and White adults [12]. Black individuals exhibited a significant progressive increase in SOD activity up to 120 minutes post-exercise, suggesting a protective, acute increase in ROS clearance capacity in Black individuals. White individuals exhibited a significant increase in SOD activity immediately, 30 minutes, and 60 minutes after exercise; however, at 60 minutes,

and 120 minutes post-exercise, W exhibited a significant decrease in SOD activity as compared to SOD activity at 30 minutes post-exercise [12]. While there were no impacts of exercise on any of their other targets, Black individuals exhibited significantly greater protein carbonylation than White individuals pre- exercise and immediately, 30 minutes, and 60-minutes post-exercise, suggesting higher systemic ROS levels in Black individuals independent of exercise. Black individuals also exhibited significantly greater TAC pre-exercise, immediately, and 30-minutes post exercise, suggesting higher systemic antioxidant levels in Black individuals independent of exercise [12]. Consistent with the previous Feairheller study [8], the basal differences in SOD activity, PC, and TAC in this study further underpin racial differences in oxidative stress, with Black individuals exhibiting greater ROS clearance capacity and markers of elevated ROS levels *in vivo* [8, 12].

In a separate acute aerobic exercise study, McKenzie and colleagues assessed inflammatory response and oxidative stress markers before and after an acute bout of aerobic exercise in Black and White women [38]. Lipid hydroperoxides, protein carbonyls, and malondialdehyde are markers of increased oxidative stress, as they all form in the presence of elevated ROS. Xanthine oxidase is a ROS producer, and glutathione is an antioxidant implicated in total antioxidant capacity. Following exercise, both Black and White subjects exhibited significant increases in lipid hydroperoxide concentration, protein carbonyl concentration, malondialdehyde concentration, IL-6 concentration, and TNF-α concentration with no pre- or post-exercise differences between racial groups.

Xanthine oxidase concentrations and reduced glutathione concentrations were not altered by exercise in either racial group, and there were no between group pre- or post-exercise differences. However, there was a significant increase in oxidized glutathione following exercise in both groups with no between group differences [38]. These findings suggest a similar response to aerobic exercise in Black and White women. The differential findings between the previous Feairheller study [8] and McKenzie study [38] may be due to differences in exercise duration (Bruce submaximal exercise test that was stopped at 75-80% of heart rate reserve versus 30 minutes of treadmill exercise at 70% of VO2max) and sex differences (women and men versus all women subjects).

With resistance exercise training, Cook et al. [39] studied the impact of 6 weeks of resistance training in young Black and White men. Following the 6 weeks of training Black men exhibited reduced levels of matrix remodeling protein matrix metalloproteinase-9 and oxidative stress biomarker 8-isoprotane while W men matrix metalloproteinase-9 and 8-isoprotane levels were similar to pre-training levels [39]. Taken together, the findings of aerobic exercise and resistance training studies of racial differences further support differential inflammatory oxidative responses in Black and White individuals, which may be modulated by sex and circulating factors (differential *in vitro* and *in vivo* responses). Sex differences are outside of the scope of the presently proposed study. However, the proposed study aims to assess potential factors influencing racial differences in inflammatory responses and ROS clearance capacity by studying *in vivo* plasma blood samples and *in vitro* HUVECS basally and following induced inflammation.

Aims and Hypotheses

Aim 1: To evaluate *in vivo* SOD activity (a major contributor to ROS clearance) in plasma from Black (B plasma) and White (W plasma) individuals following induced inflammation.

Aim 1 Hypothesis: Following induced inflammation, we hypothesized that B plasma would have greater SOD activity as compared with W plasma.

Aim 2: To evaluate *in vitro* ROS Production (CellROX/ Hoechst ratio) and SOD activity (a major contributor to ROS clearance capacity) in HUVECs from Black (B HUVECs) and White (W HUVECs) individuals following induced inflammation.

Aim 2 Hypotheses: Following induced inflammation, we hypothesized that B HUVECs would have increased ROS production as compared with W HUVECs. We also hypothesized that SOD activity would be lower in B HUVECs as compared with W HUVECs.

Exploratory aim: (a) To evaluate *in vitro* ROS production and SOD activity in female and male HUVECs following induced inflammation. (b) To evaluate *in vivo* SOD activity in plasma from women and men following induced inflammation.

Exploratory aim hypotheses: Following induced inflammation, we hypothesized that female HUVECs would have lower ROS production and higher SOD activity than male HUVECs. We also hypothesized that plasma from women would have higher SOD activity than plasma from men following induced inflammation.

Chapter 3: Methods

IRB Approval and Human Participant Eligibility

This study was approved by the Institutional Review Board at the University of Maryland, College Park. For human blood sampling, a trained researcher explained study details, including the research protocol, study measurements, and potential risks, to all human participants prior to obtaining written informed consent. All human participants were not taking any medications (antibiotics, NSAIDS, or blood pressure medication) and were free from any diseases that may have altered inflammatory profile (metabolic diseases, inflammatory diseases, or bleeding disorders). Participants had not been sick with the common cold, influenza, other bacterial or viral infections, or an upper respiratory tract infection in the previous two months. Participants could not have received the current seasonal influenza vaccine, were not currently pregnant, weighed greater than 110 pounds, and were eligible to receive the influenza vaccine according to Center for Disease Control guidelines. All women were tested in the early follicular phase of the menstrual cycle during menses or during the low hormone phase (placebo pill or ring phase) if taking hormonal contraceptives. HUVECs for the *in vitro* experiments were obtained from a commercial company, Lonza, from young, healthy donors. 8 total cell lines were obtained: two cell lines of each race and each sex (n=4 for each racial group).

Human Subjects Experimental Overview

For the human induced inflammation portion of the study, participants had blood drawn at baseline, 24 hours after, and 48 hours after influenza vaccine

administration. Blood was collected from the antecubital vein via sterile venipuncture techniques by a researcher certified through the University of Maryland Health Center. Blood was collected into acid citrate dextrose (ACD) tubes and serum separator tubes (SST). The plasma ACD tube was immediately spun at room temperature at 2000xg for 20 minutes. The SST sat on the bench top for 45 minutes to allow clotting of blood clotting factors prior to being spun at 4°C at 1500xg for 15 minutes. Immediately following centrifugation, plasma and serum samples were pipetted as 500μL aliquots and stored at -80°C until sample analysis. **Figure 5** shows the experimental targets for Aim 1.

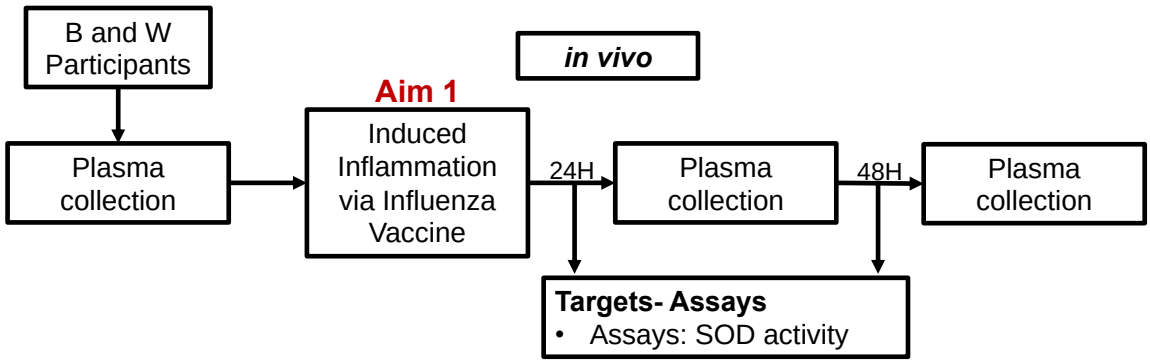

Figure 5. The experimental target for Aim 1 is SOD activity in human plasma taken at baseline 24 hours, and 48 hours following induced inflammation.

In vitro HUVECs Experimental Overview

TNF-α, which is a naturally produced pro-inflammatory cytokine, was used to induce inflammation in the HUVECs. First, optimization was performed to determine the TNF-α concentration needed to elicit inflammation but not cause unnecessary cell death. Pooled HUVECs in endothelial basal media and 2% fetal bovine serum were exposed to increasing concentrations of TNF-α for 24 hours.

At 24 hours, cells were visually analyzed for cell death and detachment. ROS production was measured via a CellROX assay and cell count was assessed via Hoechst assay. Based on the results, the experimental TNF-α concentration was determined and shown below (**Figure 6**).

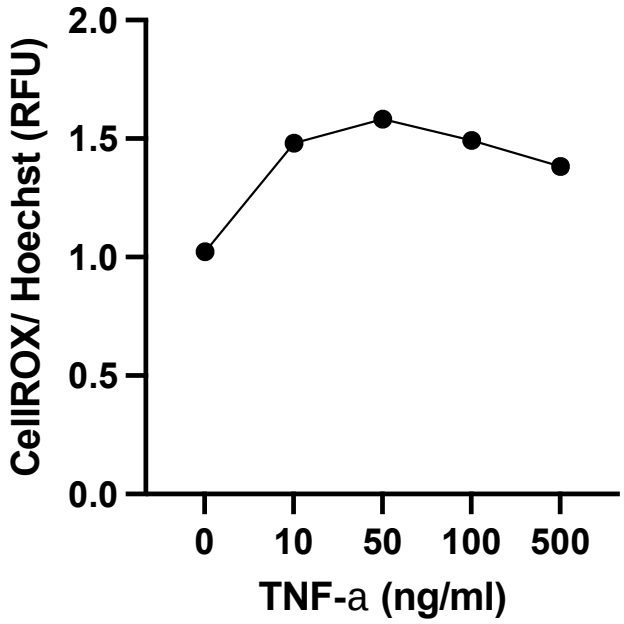

Figure 6. Optimization data in pooled HUVECS following 24 hours of TNF-α exposure or time-matched control (0 ng/ml).

To determine the optimal protein concentration for the iNOS and SOD2 antibodies, varying amounts of protein from 1 ug/well to 5 ug/well were added to pre-cast gels from Bio-Rad. After electrophoresis, the protein was transferred onto PVDF membranes and incubated for 1 hour in blocking buffer. The membranes were cut in two places to probe for iNOS, SOD2, and beta-actin on the same membrane. Following an overnight incubation with either iNOS, SOD2, or beta-actin antibody, the membranes were incubated with a compatible secondary antibody. Band intensity was measured using a ChemiDoc Imaging System

(BioRad; Hercules, California) with iNOS and SOD2 concentration being normalized to beta-actin concentration. After plotting intensity against protein, it was determined that a protein concentration of 4 ug/well is needed to attain bands for both iNOS and SOD2 protein expression.

Figure 7 shows the experimental targets and protocol for Aim 2, which utilized HUVECs from Black and White individuals following induced inflammation. Following the purchase of 4 cell lines per race (2 female cell lines and 2 male cell lines per race, cell sex determined from sex of the baby), all experiments were performed in triplicate, with B and W HUVECS treated identically and cultured in parallel. Previous studies have used similar sample sizes as those proposed for this study, suggesting that these sample sizes are adequately powered to detect statistical significance [8, 13, 14]. HUVECs were received at passage 1 (P1) and stored in liquid nitrogen. HUVECs were thawed, grown out, and stored in P2 aliquots. All experiments were performed in P3-5 HUVECs. HUVECs were grown to 80% confluency before being cultured in either (1) endothelial growth media and 2% fetal bovine serum (time-matched control) or (2) endothelial growth media, 2% fetal bovine serum, and the experimental TNF-α concentration. At 4 hours and 24 hours, cell media was collected, centrifuged at 2500xg for 20 minutes to remove cell debris, and stored at -80°C until analysis. For cell lysate collection for protein concentration determination, the 6-well cell culture plate were placed on ice. Cells were lysed via RIPA buffer with 1% protease inhibitor. Cells were then scraped with a rubber scraper or bent sterile pipette tip and collected intro microcentrifuge

tubes. Cells were rotated for 20 minutes at 4°C then centrifuged at 16000xg for 20 minutes at 4°C. The supernatant was collected and stored at -20°C until analysis.

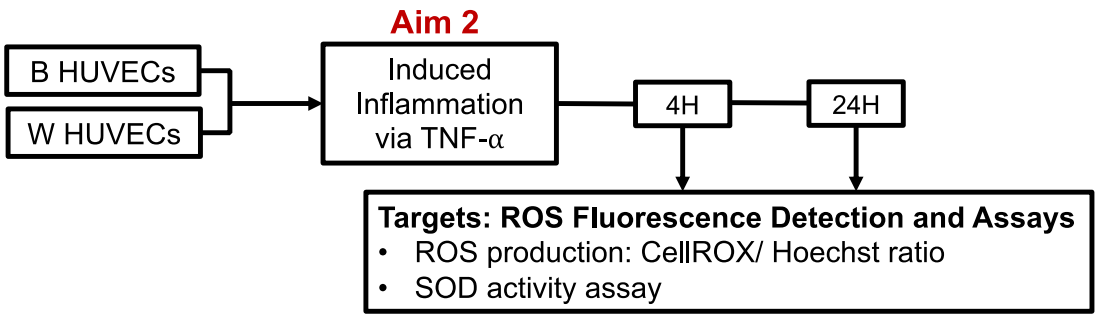

Figure 7. Experimental targets for Aim 2 include quantification of ROS production and SOD activity in HUVECS 4 hours and 24 hours following induced inflammation.

SOD activity

SOD activity was determined in human plasma and cell lysate via a commercially available SOD activity assay kit (Cayman Chemical; Ann Arbor, Michigan). One unit of SOD is the amount of SOD needed to elicit 50% dismutation of superoxide. Through xanthine oxidase dependent superoxide production, this assay indexes activity of all three SODs: SOD1 (cytoplasmic), SOD2 (mitochondrial), and SOD3 (extracellular). Human plasma samples were thawed and diluted 1:5 with sample buffer for the SOD activity assay. For the HUVECs, cell lysate samples were utilized for the SOD activity assay. Six well plates were placed on ice, where 20mM HEPES buffer containing 1mM EGTA, 210mM mannitol, and 70mM sucrose was pipetted onto the cells. After scraping, cell lysate samples were collected in Eppendorf tubes and rotated for 20 minutes at 4°C. The samples were then centrifuged at 1500xg for 20 minutes at 4°C. The supernatant was removed and stored at -80°C for future assay.

On the day of assay, 200μL of the diluted radical detector was added to each well in the 96 well plate. Then, 10μL of serially diluted standard or diluted sample was added to each well. 20μL of xanthine oxidase was added to each well as quickly as possible. Following a 30 minute incubation on a plate shaker, absorbance was read between 440 and 460 nm using a plate reader. The absorbance of the known standards was used to create the absorbance-concentration equation. The determined equation was then used to calculate the concentration of each sample based on absorbance. Standards and samples were analyzed in duplicate and averaged across duplicates. SOD activity is reported in U/ml and normalized to protein content (U/mg).

Statistical Analysis

This proposed study evaluated racial differences in inflammatory responses and ROS clearance capacity. All data were assessed for normality and outliers via Shapiro-Wilk test for normality and the ROUT 1% method, respectively. For HUVECs, 2-way ANOVAs with factors of race and time (4 hours and 24 hours) were performed separately for the time-matched Control and TNF-α conditions. For human plasma, the analyses were performed using repeated measures analysis of variance (ANOVA) with race as the between-subject condition and time points (baseline, 24 hours, and 48 hours post-stimulus for human plasma) as the repeated measures, within subject variable. Post-hoc t-tests were performed on any significant model effects. Data are presented as mean (SD) and effect sizes of significant findings were calculated using a supplemental effect size spreadsheet [40]. Aim by aim statistical analyses include:

Aim 1: To evaluate *in vivo* SOD activity (a major contributor to ROS clearance) in plasma from Black and White individuals following induced inflammation.

Repeated measures analysis of variance (ANOVA) were used with race as the between-subject condition and time points (baseline, 24 hours, and 48 hours for human plasma and baseline, 4 hours, and 24 hours for HUVECs) as the repeated measures, within subject variable The primary outcome variable for Aim 1 was SOD activity in human plasma from Black and White individuals before and following induced inflammation. Post-hoc analyses were performed on any significant within model effects.

Aim 2: To evaluate *in vitro* ROS Production (CellROX/ Hoechst ratio) and SOD activity (a major contributor to ROS clearance capacity) in HUVECs from Black (B HUVECs) and White (W HUVECs) individuals following induced inflammation.

Repeated measures analysis of variance (ANOVA) were used with race as the between-subject condition and time points (baseline, 24 hours, and 48 hours for human plasma and baseline, 4 hours, and 24 hours for HUVECs) as the repeated measures, within subject variable The primary outcome variables for Aim 2 were ROS production, SOD activity (U/ml), and SOD activity normalized to protein content (U/mg) in B and W HUVECs before and following induced inflammation. Post-hoc analyses were performed on any significant within model effects.

Exploratory aim: (a) To evaluate *in vitro* ROS production and SOD activity in female and male HUVECs following induced inflammation. (b) To evaluate *in vivo* SOD activity in plasma from Women and Men following induced inflammation.

Repeated measures analysis of variance (ANOVA) were used with sex as the between-subject condition and time points (baseline, 24 hours, and 48 hours for human plasma and baseline, 4 hours, and 24 hours for HUVECs) as the repeated measures, within subject variable The primary outcome variables for the exploratory aim were ROS production, SOD activity (U/ml), and SOD activity normalized to protein content (U/mg) in female and male HUVECs before and following induced inflammation. Post-hoc analyses were performed on any significant within model effects.

Sample size and statistical power considerations

For the *in vivo* portion of the study, minimal detectable effect/ sensitivity power analyses for t-tests and f-tests (ANOVA) were performed to determine if sample sizes previously collected are adequate for the proposed study. With n=12 for B plasma and n=15 for W plasma, α=0.05, and 1-β=0.80 or 0.85, there is a detectable effect size of 1.13 and 1.21, respectively, for t-tests. An effect size greater than 0.80 is considered large for t-tests. For ANOVA f-test minimal detectable effect/ sensitivity power analyses, effect sizes were 0.64 (1-β=0.80) and 0.68 (1-β=0.85). For ANOVAs, an effect size greater than 0.40 is considered a large effect size.

Three previous studies conducted by Feairheller, Brown, and colleagues have utilized a similar study design and study methodology [8, 13, 14]. The three studies utilized sample sizes of n=3 per group and either performed experiments in triplicate (n=9) or duplicate (n=6). Using estimated means and standard deviations of SOD activity, a prior power calculations for t-tests with calculated effect sizes (0.48 [8], 0.84 [13], and 0.89 [14]), α=0.05, and 1-β=0.80 and 0.85 were performed to determine necessary sample size. Sample sizes for 1-β=0.80 and 0.85 were between 15-81 (n=71 and 81 for [8], n=24 and 27 for [13], and n=15 and 17 for between group TNF-α [14]).

Chapter 4: Results

Induced inflammation

While there was similar ROS production in the 4H and 24H time-matched condition, the TNF-α treatment significantly increased ROS production (CellROX/ Hoechst ratio) in all HUVECs at 24H as compared to 4H (CellROX/Hoechst Control 4H vs 24H: $p>0.05$; TNF-α 4H vs 24H: $p=0.0015$, Hedges's $g_s=0.97$; Hoechst Control 4H vs 24H: $p=0.028$, Hedges's $g_s=0.65$; TNF-α 4H vs 24H: $p=0.0001$, Hedges's $g_s=1.21$; **Table 1; Figure 8**). The significant increase in ROS production in the TNF-α condition alone suggests the model successfully induced inflammation. For the subsequent by race and by sex comparisons, *in vivo* (SOD activity) *and in vitro* (ROS production and SOD activity) findings will be presented by aim.

Table 1. ROS production and cell viability for all HUVECs following induced inflammation.

	All HUVECs			
	C 4H (n=23)	**TNF-α 4H** (n=23)	**C 24H** (n=24)	**TNF-α 24H** (n=24)
CellROX/Hoechst	1.27 (0.20)	1.26 (0.17)*****	1.37 (0.21)	1.44 (0.20)
Hoechst	39901 (5929)*****	39290 (5973)*****	36006 (5849)#	32896 (4395)

Data presented as Mean (SD). C, control, TNF-α, tumer necrosis factor-α; 4H, 4 hours post-stimulus; 24H, 24 hours post-stimulus. ***** indicates $p<0.05$ as compared with 24H, within same condition. # indicates $p<0.05$ as compared with TNF-α 24H.

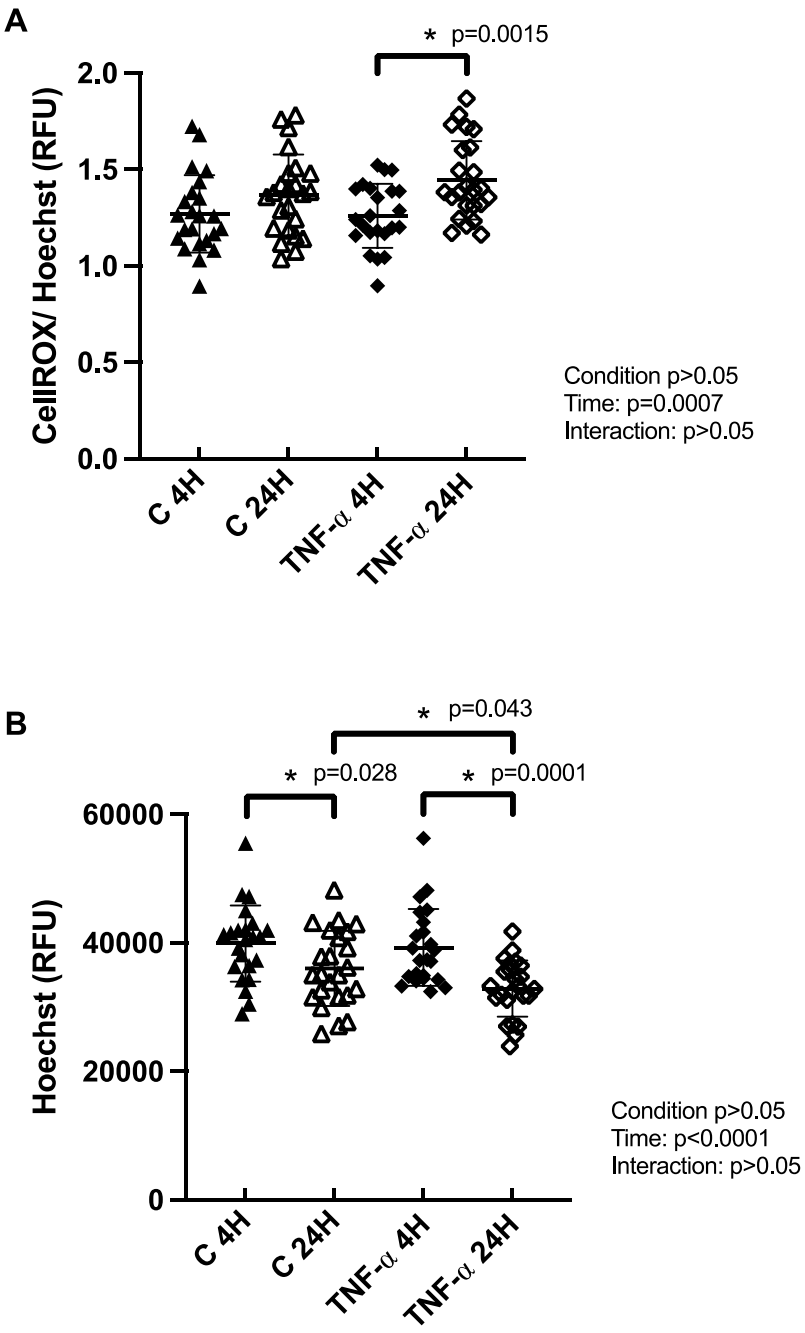

Figure 8. (A) CellROX/ Hoechst (ROS production normalized to living cells) in all HUVECs at 4H and 24H for Control and TNF-α conditions. (B) Hoechst (living cells) in all HUVECs at 4H and 24H for Control and TNF-α conditions.

Aim 1: No race differences in SOD activity in plasma

Participant characteristics are detailed below in **Table 2.** Black participants exhibited a significantly lower VO_2Peak than White participants (p=0.025). For human plasma analysis, there were no between or within group differences in SOD activity, suggesting similar SOD activity across the groups at rest and following influenza vaccine induced inflammation (p>0.05; **Table 2; Figure 9**).

Table 2. B and W human participant characteristics and plasma SOD activity levels at baseline and following induced inflammation.

	B Plasma			W Plasma		
	BL n=12	**24H** n=11	**48H** n=7	**BL** n=13	**24H** n=15	**48H** n=14
Age (y)	21 ± 2			22 ± 3		
Height (m)	1.73 ± 0.10			1.74 ± 0.10		
Weight (kg)	72.3 ± 15.1			70.3 ± 14.1		
BMI (kg/m^2)	24.1 ± 3.5			23.1 ± 2.5		
VO_2Peak	34.3 ± 7.1			41.1 ± 8.0 *		
SOD activity (U/ml)	2.35 (0.90)	2.67 (1.21)	3.14 (1.69)	2.73 (1.21)	2.24 (1.23)	2.53 (1.34)

Data presented as Mean (SD). BL, baseline; 24H, 24 hours following induced inflammation; 48H, 48 hours following induced inflammation. Participant characteristics reported for n=13 for Black participants and n=17 for White participants (n=12 for Black participant VO_2Peak). * indicates p<0.05, between race.

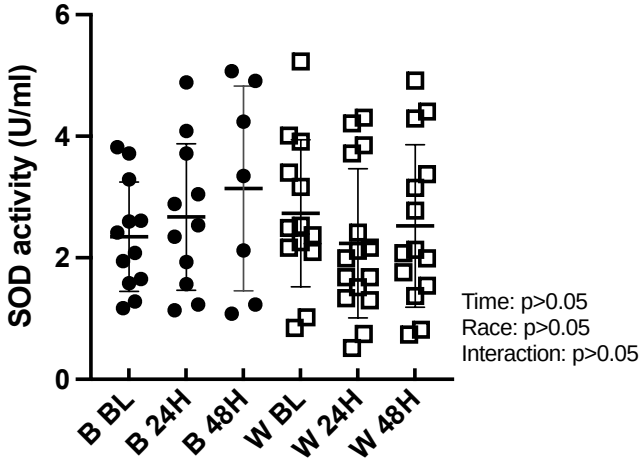

Figure 9. SOD activity (U/ml) in human plasma from Black and White individuals at baseline (BL), 24 (24H), and 48 hours (48H) following induced inflammation.

Aim 2: Race differences in ROS production in HUVECs

B HUVECs exhibited similar ROS levels at 24H as compared with 4H for both conditions (B Control 4H vs 24H: p>0.05; TNF-α 4H vs 24H: p=0.079; **Table 3**; **Figure 10**). However, W HUVECs exhibited significantly greater ROS levels at 24H as compared to 4H for the Control and TNF-α condition (W Control 4H vs 24H: p=0.035, Hedges's g_s=0.88; TNF-α 4H vs 24H: p=0.0015, Hedges's g_s=1.37*;* **Table 3**; **Figure 10**). The higher ROS production in W HUVECs may suggest a greater sensitivity to TNF-α induced inflammation. On the contrary, the similar ROS levels in B HUVECs across time points and conditions may suggest a better ability to clear ROS and/or a lower sensitivity to TNF-α induced inflammation.

Table 3. B and W HUVECs ROS production and cell viability following induced inflammation.

	B HUVECS				**W HUVECs (n=12)**			
	C 4H n=11	**TNF-α 4H** n=11	**C 24H** n=12	**TNF-α 24H** n=12	**C 4H**	**TNF-α 4H**	**C 24H**	**TNF-α 24H**
CellROX /Hoechst	1.27 (0.20)	1.23 (0.20)	1.30 (0.26)	1.40 (0.25)	1.28 (0.21)	1.29 (0.13)*****	1.44 (0.13)	1.49 (0.15)
Hoechst	40179 (6537)	40842 (7670)*****	38455 (7034)**†**	33878 (5583)	39645 (5596)*****	37867 (3640)*****	33558 (2995)	31915 (2669)

Data presented as Mean (SD). C, control, TNF-α, tumer necrosis factor-α; 4H, 4 hours post-stimulus; 24H, 24 hours post-stimulus. ***** indicates p<0.05 as compared with 24H, within same condition. **†** indicates p<0.05 as compared with W HUVECs.

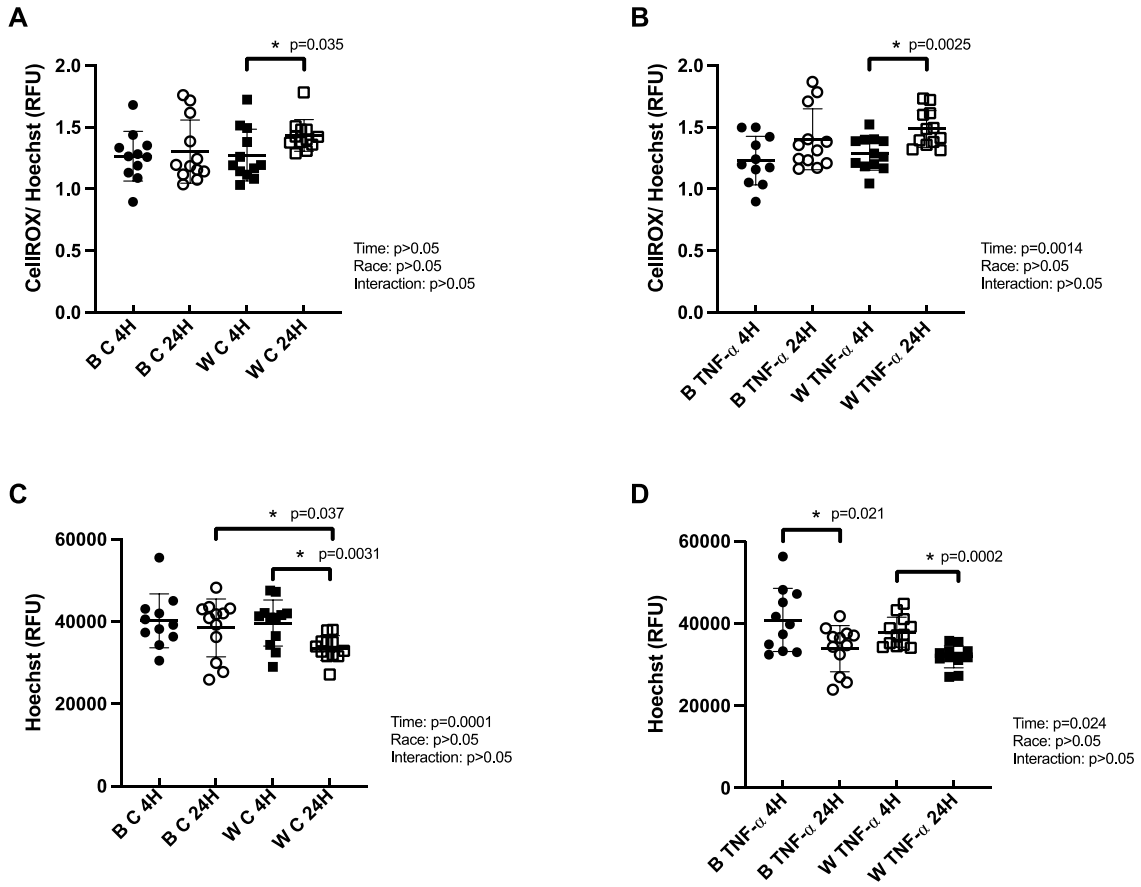

Figure 10. CellROX/ Hoechst (ROS production normalized to living cells) in B and W HUVECs at 4H and 24H for Control (A) and (B) TNF-α treatment. (C) Hoechst (living cells) in B and W HUVECs at 4H and 24H for (C) Control and (D) TNF-α conditions.

Aim 2: Race differences in SOD activity in HUVECs

B and W HUVECs both exhibited significantly higher SOD activity (U/ml) at 24H as compared with 4H for both conditions, within race (SOD activity (U/ml)- B Control 4H vs 24H: p=0.0004, Hedges's g_s=2.04; TNF-α 4H vs 24H: p=0.0007, Hedges's g_s=1.90; W Control 4H: vs 24H: p<0.0001, Hedges's g_s=2.47; TNF-α 4H vs 24H: p=0.0011, Hedges's g_s=1.80; **Table 4; Figure 10**). However, SOD activity normalized to protein content in B HUVECs was similar between conditions and time points (SOD activity (U/mg)- B Control 4H vs 24H: p>0.05; TNF-α 4H vs 24H:

p>0.05; **Table 4; Figure 11**), SOD activity normalized to protein content in W HUVECs was significantly greater at 24H as compared to 4H in the Control condition alone (SOD activity (U/mg)- W Control 4H vs 24H: p=0.0034, Hedges's g_s=1.54; TNF-α 4H vs 24H: p>0.05; **Table 4; Figure 11**). Focusing on the SOD activity normalized to protein content, the increase in SOD activity in the time-matched Control condition that was then abolished in the TNF-α condition further underpins a greater sensitivity to TNF-α induced inflammation in W HUVECs. The lack of perturbation in SOD activity normalized to protein content in the B HUVECs suggests a lower sensitivity to TNF-α induced inflammation and/or a greater ability to clear ROS, likely from non-SOD antioxidant sources.

Table 4. B and W HUVECs SOD activity following induced inflammation.

	B HUVECs (n=9)				**W HUVECs (n=9)**			
	C 4H	**TNF-α 4H**	**C 24H**	**TNF-α 24H**	**C 4H**	**TNF-α 4H**	**C 24H**	**TNF-α 24H**
SOD activity (U/ml)	0.231 (0.054)*****	0.247 (0.075)*****	0.530 (0.19)	0.483 (0.15)	0.240 (0.12)*****	0.220 (0.090)*****	0.622 (0.17)	0.476 (0.17)
SOD activity (U/mg)	3.49 (1.02)	3.30 (1.72)	4.58 (2.25)	4.37 (1.50)	2.86 (1.31)*****	2.92 (1.32)	5.57 (1.97)	4.18 (1.45)

Data presented as Mean (SD). C, control, TNF-α, tumer necrosis factor-α; 4H, 4 hours post-stimulus; 24H, 24 hours post-stimulus. ***** indicates p<0.05 as compared with 24H, within same condition.

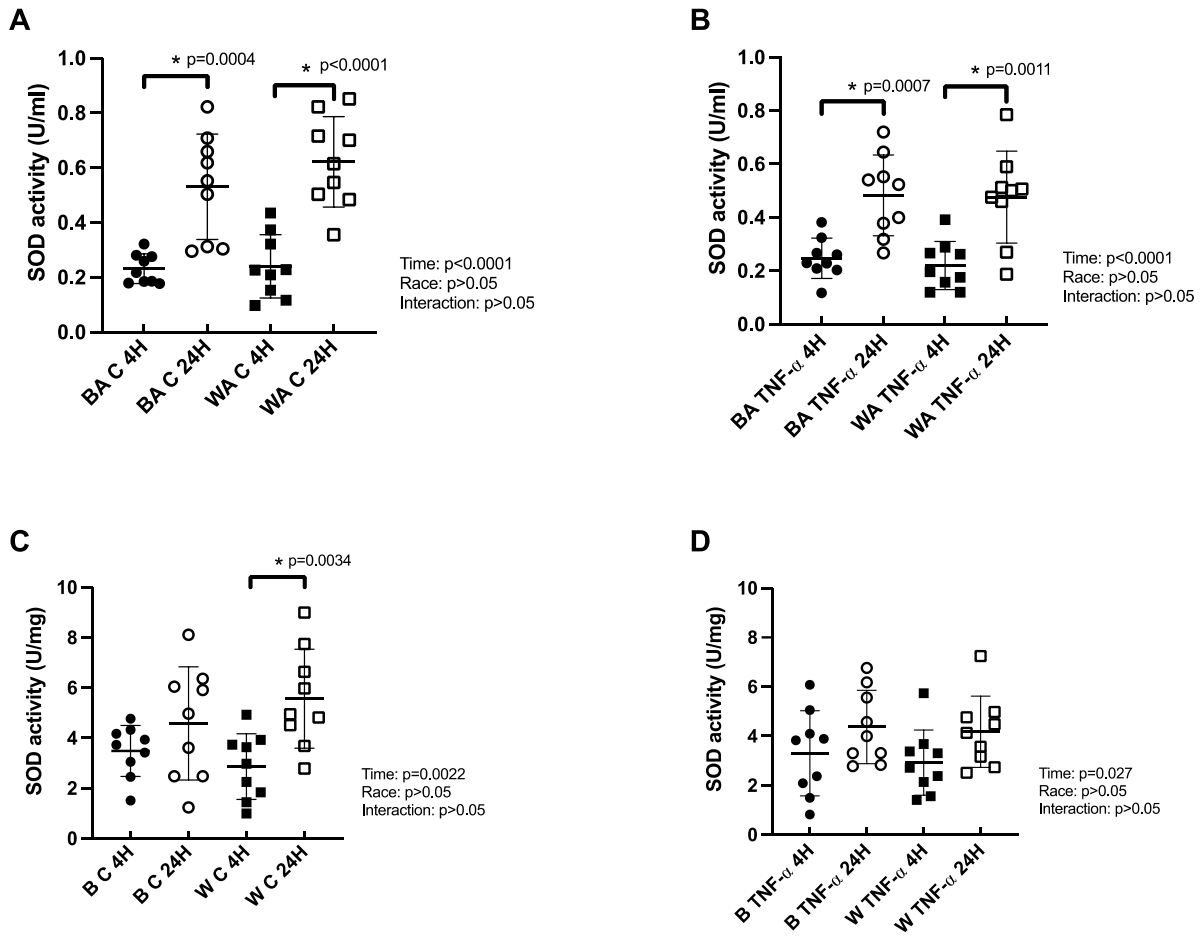

Figure 11. SOD activity (U/ml) in B and W HUVECs at 4H and 24H for Control (A) and (B) TNF-α treatment. SOD activity normalized to protein content (U/mg) in B and W HUVECs at 4H and 24H for Control (C) and (D) TNF-α treatment.

Aim 3: No Sex differences SOD activity in plasma

Participant characteristics are detailed below in **Table 5.** Women exhibited significantly lower height, weight, and VO_2Peak than men (height p<0.001; weight p=0.0006; VO_2Peak p=0.0054)**.** There were no sex differences in human plasma SOD activity (U/ml), suggesting similar between sex SOD activity at rest and following influenza vaccine induced inflammation (p>0.05 for all effects, between sex comparisons, and within sex comparisons; **Table 5; Figure 12**). It is plausible that the time course of change in circulating SOD activity is different than the 24 hour and 48 hour time points sampled, the infleunza vaccine was not strong enough to elicit effects in human plasma SOD activity levels, or SOD activity is not sensitive to influenza vaccine induced inflammation.

Table 5. Human participant characteristics and plasma SOD activity levels at baseline and following induced inflammation, by sex.

	Women			**Men**		
	BL n=13	**24H** n=13	**48H** n=10	**BL** n=12	**24H** n=13	**48H** n=11
Age (y)	21 ± 3			21 ± 2		
Height (m)	1.67 ± 0.08 *****			1.80 ± 0.07		
Weight (kg)	62.9 ± 10.5 *****			79.5 ± 13.0		
BMI (kg/m^2)	22.5 ± 2.2			24.5 ± 3.4		
VO_2Peak	34.4 ± 7.0 *****			42.5 ± 7.5		
SOD activity (U/ml)	2.52 (1.28)	2.19 (1.17)	2.47 (1.51)	2.58 (0.84)	2.65 (1.26)	2.97 (1.42)

Data presented as Mean (SD). BL, baseline; 24H, 24 hours following induced inflammation; 48H, 48 hours following induced inflammation. Participant characteristics reported for n=15 for men and women (n=14 for men's VO_2Peak). ***** indicates p<0.05, between sex.

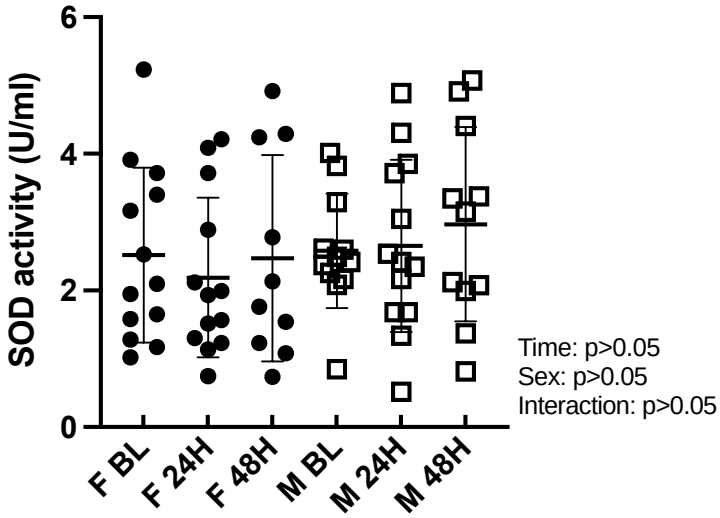

Figure 12. SOD activity (U/ml) in human plasma from Women (F) and Men (M) at baseline (BL) and 24 (24H) and 48 hours (48H) following induced inflammation.

Aim 3: Sex differences in ROS production in HUVECs

Both female and male HUVECs exhibited greater ROS production at 24H TNF-α treatment when compared to 4H TNF-α treatment (female: p=0.050, Hedges's g_s=0.81; male: p=0.0030, Hedges's g_s=1.34). Interestingly, female HUVECs exhibited significantly lower ROS production than male HUVECs at 24H for both the control and TNF-α conditions (Control: p=0.0060, Hedges's g_s=1.20; TNF-α: p=0.0040, Hedges's g_s=1.29; **Table 6; Figure 13**). The higher ROS production in male HUVECs alone suggests a greater sensitivity to TNF-α induced inflammation in male HUVECS as compared with female HUVECs.

Female HUVECs also exhibited significantly higher cell viability than male HUVECs at 24H for both the Control and TNF-α conditions (Control: p=0.014, Hedges's g_s=1.05; TNF-α: p=0.0060, Hedges's g_s=1.19). Female HUVECs maintained similar cell viability at 4H and 24H of Control (p>0.05) but exhibited a significantly lower cell viability at 24H when compared to 4H for TNF-α (p=0.0037,

Hedges's g_s=1.32). Male HUVECs exhibited significantly lower cell viability at 24H as compared to 4H for both conditions (Control 4H vs 24H: p=0.034, Hedges's g_s=0.89; TNF-α 4H vs 24H: p=0.0012, Hedges's g_s=1.46; **Table 6; Figure 12**). The sex differences in ROS production and cell viability suggest a more protective oxidative state in female HUVECs and a more pro-inflammatory oxidative state in male HUVECs.

Table 6. Female and male HUVECs ROS production and cell viability following induced inflammation.

	female HUVECs				**male HUVECs** (n=12)			
	C 4H n=11	**TNF-α 4H** n=11	**C 24H** n=12	**TNF-α 24H** n=12	**C 4H**	**TNF-α 4H**	**C 24H**	**TNF-α 24H**
CellROX/ Hoechst	1.20 (0.21)	1.20 (0.17)*****	1.26 (0.15)**†**	1.33 (0.14)**†**	1.34 (0.18)	1.32 (0.14) *****	1.48 (0.20)	1.56 (0.20)
Hoechst	42337 (6447)	42317 (6695)***** **†**	38835 (5304) **†**	35221 (3296) **†**	37668 (4599)	36514 (3610) *****	33177 (5092)	30572 (4211)

Data presented as Mean (SD). C, control, TNF-α, tumer necrosis factor-α; 4H, 4 hours post-stimulus; 24H, 24 hours post-stimulus. ***** indicates p<0.05 as compared with 24H, within same condition. **†** indicates p<0.05 as compared with male HUVECs.

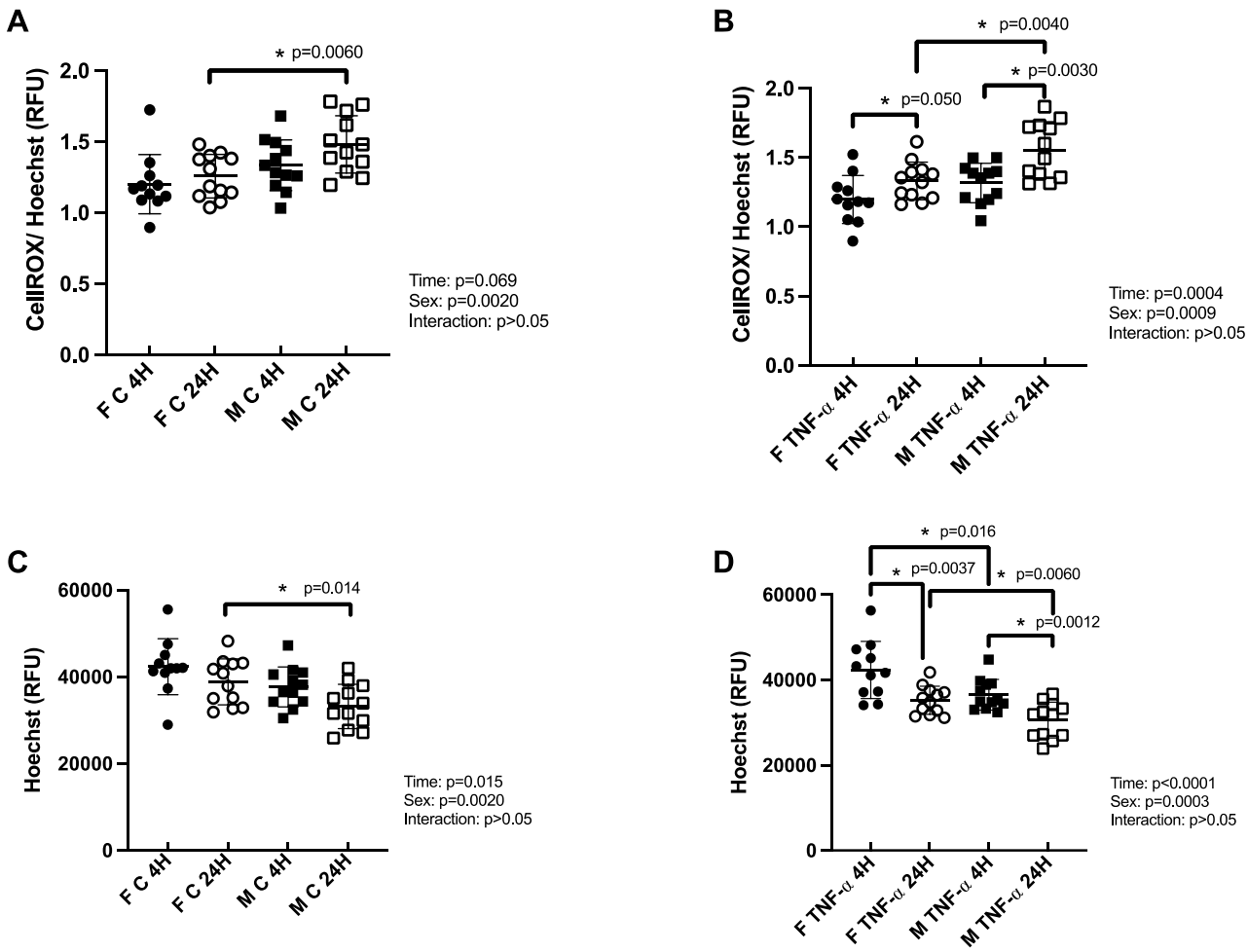

Figure 13. CellROX/ Hoechst (ROS production normalized to living cells) in female (F) and male (M) HUVECs at 4H and 24H for Control (A) and (B) TNF-α treatment.

Aim 3: Sex differences in SOD activity in HUVECs

For ROS clearance, female HUVECs exhibited significantly higher SOD activity than male HUVECs following 24H of TNF-α treatment (p=0.0001, Hedges's g_s=0.86). Within sex comparisons revealed that both female and male HUVECs exhibited greater SOD activity (U/ml) at 24H TNF-α treatment as compared with 4H TNF-α treatment (female: p=0.0001, Hedges's g_s=2.27; male: p=0.0021, Hedges's g_s=1.67; **Table 7; Figure 14**). The sex differences in SOD activity

suggests a greater SOD-related ROS clearance response to TNF-α induced inflammation in female HUVECs as compared with male HUVECs.

There were no between sex differences in SOD activity when normalized to protein content (U/mg) ($p>0.05$). However, within sex, female HUVECs exhibited significantly greater SOD activity normalized to protein content at 24H as compared with 4H for both conditions (Control: $p=0.0035$, Hedges's $g_s=1.52$; TNF-α: $p=0.0098$, Hedges's $g_s=1.31$; **Table 7; Figure 14**), and male HUVECS exhibited similar SOD activity (U/mg) between conditions and time points ($p>0.05$). Focusing on the SOD activity normalized to protein content, female HUVECs alone exhibited significant increases in SOD activity with increased TNF-α exposure, suggesting SOD activity is a major ROS clearer in female HUVECs and likely contributes to the lower ROS production.

Table 7. Female and male HUVECs SOD activity following induced inflammation.

	female HUVECs (n=9)				**male HUVECs** (n=9)			
	C 4H	**TNF-α 4H**	**C 24H**	**TNF-α 24H**	**C 4H**	**TNF-α 4H**	**C 24H**	**TNF-α 24H**
SOD activity (U/ml)	0.200 (0.07)	0.227 (0.08)	0.647 (0.16)	0.544 (0.17)	0.271 (0.09)	0.239 (0.09)	0.505 (0.18)	0.415 (0.11)
SOD activity (U/mg)	2.34 (1.01)	2.93 (1.10)	5.38 (2.50)	4.82 (1.60)	4.01 (0.61)	3.30 (1.87)	4.77 (1.80)	3.72 (1.06)

Data presented as Mean (SD). C, control, TNF-α, tumer necrosis factor-α; 4H, 4 hours post-stimulus; 24H, 24 hours post-stimulus.

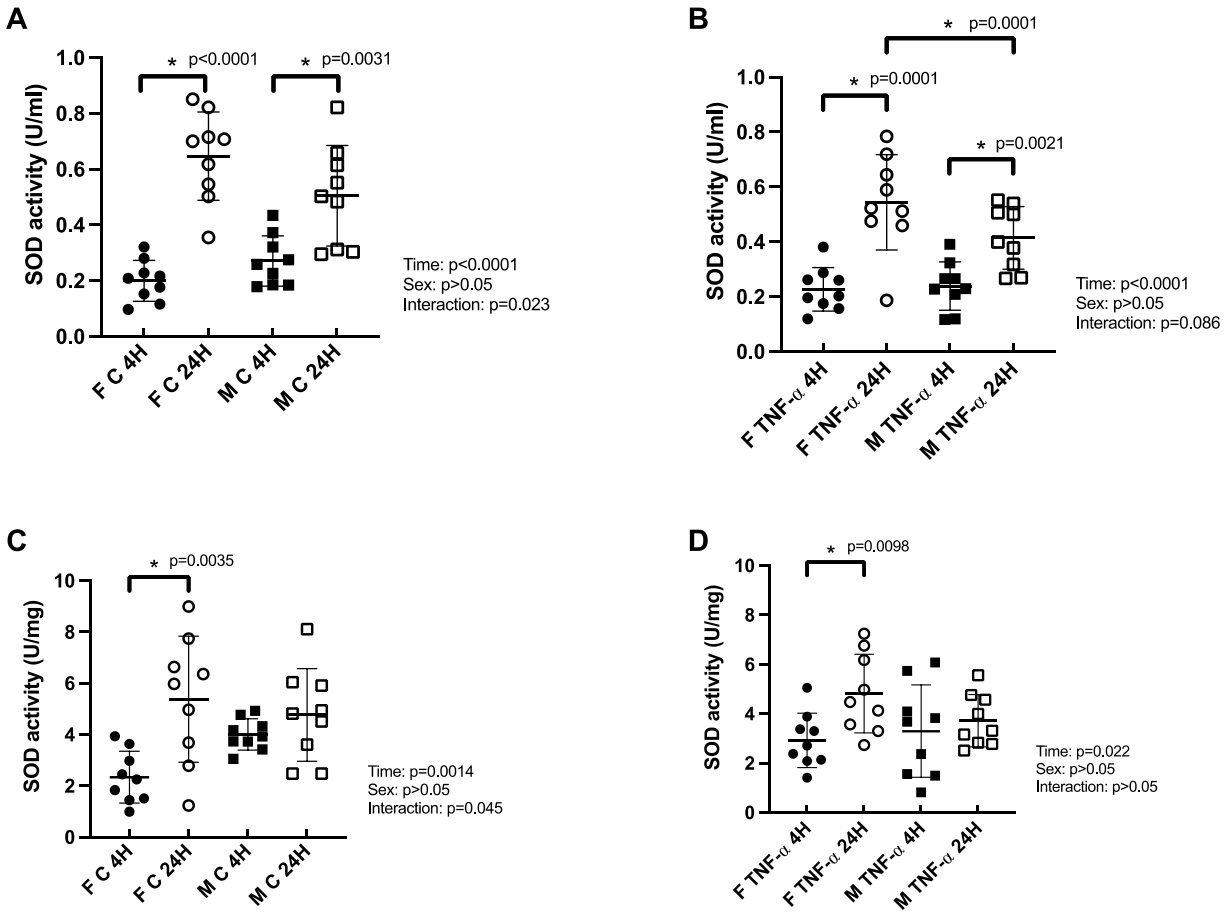

Figure 14. SOD activity (U/ml) in female and male HUVECs at 4H and 24H for Control (A) and (B) TNF-α treatment. SOD activity normalized to protein content (U/mg) in female (F) and male (M) HUVECs at 4H and 24H for Control (C) and (D) TNF-α treatment.

Chapter 5: Discussion

The novel findings of the study are: 1) W HUVECs, but not B HUVECs, exhibited significantly greater ROS production with increased exposure time to TNF-α; 2) W HUVECs alone experienced an increase in SOD activity with increased time that was abolished with TNF-α treatment; 3) female HUVECs exhibited significantly lower ROS production than male HUVECs both basally and following TNF-α treatment; and 4) female HUVECs exhibited significantly greater SOD activity with increased exposure time to TNF-α, as compared with male HUVECs.

The higher ROS production in all HUVECs after 24H of TNF-α treatment, but not 24H of time-matched Control exposure, suggests the TNF-α (50ng/ml) induced inflammation in the HUVECs. Further, the different ROS production and SOD activity responses within each race following induced inflammation may be due to two reasons; namely 1) W HUVECs being more sensitive to acute inflammation and/or 2) B HUVECs being better equipped to respond to acute inflammation. In regard to the third, exploratory aim, the sex differences in ROS production and SOD activity may be due to 1) female HUVECs experiencing augmented protective effects of estrogen and/or 2) male HUVECs being primed to exhibit a pro-inflammatory state due to higher androgen exposure [41].

Racial Differences in ROS Production

Previously, B HUVECs have exhibited significantly higher basal protein expression of various superoxide-producing NADPH oxidases, suggesting higher basal ROS production [8, 9, 13]. Interestingly, as compared to W HUVECs, B

HUVECs also exhibit greater eNOS expression (to increase NO-related clearance of superoxide) and greater NO concentration, suggesting a greater contribution of NO to ROS clearance in B HUVECs [8, 9, 13]. However, the findings from the current study are not in agreement with previous literature. In the present study, B HUVECs exhibited statistically similar ROS production at 24H and 4H of TNF-α treatment. The similar ROS production and SOD activity (U/mg) in B HUVECs with increased exposure time to induced inflammation in the present study may be explained by higher eNOS expression and NO production, as noted in previous literature [9]. Elevated NO production can combat heightened ROS production directly and indirectly: NO indirectly inhibits ROS production and directly scavenges superoxide when ROS is overproduced [29].

Racial Differences in SOD activity

SOD is an enzyme that primarily attenuates ROS levels by clearing superoxide. Previously, B HUVECs have exhibited significantly lower SOD activity normalized to protein content basally and following 4 hours of TNF-α exposure when compared with W HUVECs [8, 13, 14]. Interestingly, B HUVECs have also exhibited significantly greater total antioxidant capacity than W HUVECs basally [8]. In the present study, when comparing 4H to 24H Control, W HUVECs, but not B HUVECs, exhibited greater SOD activity normalized to protein content that was then blunted with TNF-α exposure, suggesting a greater impact of TNF-α on reducing the increase in SOD activity in W HUVECs. The current findings support similar SOD activity between races basally and following 4 and 24 hours of TNF-α exposure, with divergent within race responses to 24 hours of TNF-α exposure.

Thus, it is plausible that B HUVECs may be primed to respond to inflammatory stimuli due to a higher capacity to clear ROS.

The lack of perturbation in B HUVECs ROS production and SOD activity in the current study is plausibly explained by previously observed heightened basal total antioxidant capacity in B HUVECs, supporting a greater ROS clearance capacity via various antioxidants. Interestingly, in the present study, W HUVECs also exhibited a significant increase in ROS production at 24H as compared with 4H for the time-matched control, while B HUVECs exhibited similar ROS production across the time-matched control. The similar ROS production across time points and conditions in B HUVECs further supports an efficient ROS clearing capability, potentially via other antioxidant systems in B HUVECs and could explain the lack of response in ROS production and SOD activity following acute inflammation.

Taken together, the *in vivo* and *in vitro* previous literature suggests that HUVECs from Black individuals exhibit a greater total antioxidant capacity, which explains the lack of change in ROS production with increased exposure time to acute inflammation in the current study [8]. However, young, healthy Black individuals exhibit greater plasma oxidative stress *and* total antioxidant capacity and SOD activity [8, 12]. Taken together, previous literature and the current results suggest that B HUVECs have higher expression of ROS producing enzymes (NADPH oxidases), yet do not overproduce ROS (similar CellROX/ Hoechst ratio in B HUVECs with increased TNF-α exposure), likely due to higher total antioxidant capacity from non-SOD antioxidants. Further, young, healthy Black individuals

exhibit greater oxidative stress and concurrently higher total antioxidant capacity as compared with W individuals, suggesting higher ROS production that the heightened total antioxidant capacity is unable to completely buffer in Black individuals.

Considering the vascular health triad, aging humans experience increases in ROS production with an initially concurrent increase in ROS clearance via antioxidants [6, 15]. However, with increased exposure time to aging, ROS clearance machinery becomes exhausted and inefficient, contributing to overall greater oxidative stress and downstream inflammation and vascular dysfunction [15]. Thus, it is plausible that B HUVECs and young, healthy Black individuals are primed to respond to acute inflammation-related increases in ROS production via heightened antioxidant capacity (from non-SOD antioxidants). However, with increased exposure time to heightened ROS levels, as indicated by higher protein carbonylation in young, healthy Black individuals, there may be an impairment to ROS clearance machinery, and, thus, a greater predisposition to oxidative stress, inflammation, and vascular dysfunction that may partially explain greater hypertension prevalence and earlier onset in Black individuals [8, 12].

B and W Human Plasma SOD activity

Two previous studies note significantly higher SOD activity (U/ml) at baseline and following intense exercise in B plasma when compared with W plasma [8, 12]. B plasma SOD activity (U/ml) was significantly greater than W plasma SOD activity at baseline and 60 and 120 minutes post-exercise. B plasma exhibited a stepwise increase in SOD activity when comparing baseline to the

various post-exercise time points while W plasma exhibited an initial increase followed by a stark decline in SOD activity post-exercise. The findings at baseline and following exercise suggest a higher contribution of SOD to ROS clearance in young, healthy B individuals [8, 12]. However, in the present study, B and W human plasma exhibited similar SOD activity (U/ml) at baseline, 24 hours, and 48 hours following induced inflammation. The disparity in previous findings and the current study may be due to the inflammatory stimulus used (intense exercise versus influenza vaccine) or the time course of sampling (30, 60, and 120 minutes post-exercises versus 24 and 48 hours post-influenza vaccine). The influenza vaccine may not be strong enough to influence SOD activity or the time course of change in SOD activity may be shorter than 24 hours.

At the onset of exercise, there is an increase in ROS production from NADPH oxidases, xanthine oxidases, and other non-mitochondrial sources of ROS [42]. The resultant cellular damage activates protective pathways, such as the production of IL-6 as a myokine [43]. Myokine IL-6 may increase SOD activity, specifically SOD2 (mitochondrial SOD; in hepatocytes), which could plausibly explain the lack of heightened ROS production from mitochondrial sources during exercise [42-44]. The activation of downstream antioxidant and cellular protective pathways following increased acute exercise-related cellular damage results in the protective effects of exercise training. Thus, in Black individuals, the continual increase in SOD activity, yet similar total antioxidant capacity and protein carbonylation (oxidative stress) concentrations, suggest either (1) greater cellular damage from the acute exercise eliciting a greater antioxidant response or (2) a

greater magnitude of antioxidant response to acute exercise, likely through IL-6 myokine and other myokine signaling.

Sex Differences in ROS Production and SOD activity

The sex differences in ROS production and SOD activity may be due to female HUVECs being better equipped to increase ROS clearance with inflammation-related increases in ROS production. In the present study, male HUVECs exhibited significantly greater ROS production than female HUVECs basally and following induced inflammation. Further, both female and male HUVECs exhibited greater ROS production with greater exposure time to TNF-α.

Considering SOD activity, female HUVECs exhibited significantly greater SOD activity (U/ml) than male HUVECs at 24H of TNF-α exposure. Interestingly, both groups' SOD activity (U/ml) was significantly greater (within sex) at 24 hours of exposure time to TNF-α when compared with 4 hours of exposure time, suggesting either more potent SOD activity or greater overall antioxidant capacity in female HUVECs. With SOD activity normalized to protein content, only female HUVECs exhibited significantly greater SOD activity (U/mg) at 24H when compared with 4H for both conditions, further supporting better ROS clearance capabilities via increasing SOD activity in female, but not male, HUVECs.

The sex differences in ROS production and SOD activity may be due to differences in estrogen or androgen actions across the different sexed cells. It is suggested that umbilical cord blood estrogen concentrations do not differ between fetal sex, and, thus, it is plausible that male and female HUVECs experience similar estrogen concentrations [41]. Considering the anti-inflammatory and anti-

oxidative effects of estrogen, it is plausible that female HUVECs may have higher estrogen receptor density or a greater magnitude of post-estrogen binding actions. Alternatively, lower action of testosterone/ androgens in female HUVECs as compared to male HUVECs could explain the lower ROS production and higher SOD activity in female HUVECs. Previously, androgens have been shown to synergize the inflammatory impacts of TNF-α exposure in female and male HUVECs, and umbilical cord blood has shown sex differences in testosterone levels, with male cords exhibiting higher testosterone concentrations [41, 45]. Thus, it is plausible that the greater androgen concentrations in umbilical cord blood of male HUVECs 'primes' a more pro-inflammatory state in male HUVECS as compared with female HUVECs. Whereby, exposure to a subsequent inflammatory stimulus, such as TNF-α, results in greater oxidative stress (heightened ROS production) and impaired SOD activity in male HUVECs as compared with female HUVECS that experience lower concentrations of androgens in umbilical cord blood. Indeed, premenopausal women exhibit a lower cardiovascular disease risk than age matched men, and this cardio protection may be due to a combination of protective effects of estrogen and lower exposure to androgens.

Chapter 6: Conclusions, Considerations, Limitations, and Future Directions

Conclusions

B and W HUVECs exhibit divergent responses to TNF-α-induced inflammation, with W HUVECs, but not B HUVECs, exhibiting greater ROS production and a blunted increase in SOD activity with increased exposure time to TNF-α. The findings suggest, for the first time, a greater sensitivity to TNF-α treatment in W HUVECs and further support a greater ROS buffering capacity in B HUVECs following TNF-α exposure. Interestingly, female and male HUVECS exhibited sex differences in ROS production and SOD activity; female HUVECs exhibiting significantly lower ROS production and significantly higher SOD activity than male HUVECs following TNF-α exposure, suggesting sex differences in susceptibility to induced inflammation in HUVECs. The current findings underly the importance of noting the race and sex (or indicating that race and sex are pooled if using pooled HUVECs) of HUVECs used in *in vitro* research.

Considerations

These disparate physiological responses, especially in the HUVECs, could "*stem from disparities in socio-economic status, educational status, as well as other social determinants of health which a growing body of research has shown to be* ***linked to*** *structural racism*" faced by the mothers (beyond the scope of the current study) (p. H2372) [46]. The purpose of this study (thesis) is not to suggest one race has inherently lower physiological function. On the contrary, it is recognized that 'race' is a social construct- one with real, material consequences-

and the purpose of the study (thesis) is to present the disparities between races, which may be a result of social determinants of health [46]. Therefore, one major limitation is that the data does not take into consideration how various social determinants of health, including systemic racism faced by the mothers, could impact the HUVEC responses in the present study [46].

Limitations

The current study includes some limitations. First, only SOD activity was measured, and, thus, a complete picture of ROS clearance pathways were not fully elucidated. Second, due to slow growth rates in some cell lines, SOD activity data is only reported for n=9 per race and sex. However, based on literature utilizing a sample size of n=9, the study is still powered to detect differences between groups. Third, NO could not be measured in any samples due to detectability issues and low nitrate yields with the assay kit. Lastly, because of the observed sex differences and within race divergences, sex and race likely confounded one another and require further research.

Future Directions

To further investigate the divergent within race HUVEC responses to induced inflammation, future research should aim to evaluate total antioxidant capacity and the protein expression of NADPH oxidases (ROS production), SOD2 (mitochondrial SOD activity), iNOS (inflammation-related increases in NO production) in B and W HUVECs following induced inflammation. To further investigate HUVECs sex differences following induced inflammation, future research should also evaluate levels of total antioxidant capacity, protein

expression of NADPH oxidases, concentrations of androgens and estrogen, receptor density of androgens and estrogens, and the time course/ persistence of androgen pro-inflammatory effects in female and male HUVECs following induced inflammation.

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