Exercise Training Improves Cerebrovascular Oxidative Stress Regulation and Insulin Stimulated Vasodilation in Juvenile and Mature Pigs

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Abstract

Background: Selective insulin resistance in the cerebrovasculature, characterized by augmented vasoconstriction in response to insulin, may relate to enhanced sensitivity to endothelin-1 (ET1) or increased oxidative stress, culminating in attenuated nitric oxide (NO) signalling. Regular exercise has been shown to enhance vascular responses to insulin, but the mechanisms remain unclear. This study tested the hypothesis that exercise training improves oxidative stress regulation and cerebrovascular insulin-stimulated vasodilation in juvenile and mature pigs. *Methods:* Twenty juvenile (n=10F/10M; 3±1 months; mass=11±3 kg) and 17 mature (n=9F/7M; 14±1 months; mass=83±9 kg) Ossabaw miniature-pigs were divided into sedentary or exercise training groups. Pigs in the exercise training groups completed high intensity interval training three times per week for eight weeks. All animals were group housed with access to 1 kg of feed per pig per day, as well as sugar water (~5 L per pig of 10% solution). At euthanasia, cerebral arteries were dissected for pressure myography experiments. Vascular diameter was tracked continuously and vasomotor responses to insulin (1e-9-1e-6 M) and ET1 (1e-12-1e-7 M) were studied under three conditions: 1) untreated (vehicle); 2) superoxide dismutase (SOD) mimetic (TEMPOL; 1e-4 M) and 3) NAD(P)H Oxidase (NOX) inhibition (Apocynin; 1e-4 M). Physiologic maximum and cumulative change in diameter (AUC) for all groups were compared using a two-way ANOVA (independent variables: age and exercise training). *Results*: Whereas sedentary pigs displayed insulin-stimulated vasoconstriction, exercise trained pigs exhibited insulin-stimulated vasodilation. Indices of insulin-stimulated vasodilation were significantly greater in exercise trained vs. sedentary controls (main effect: $P<0.001$). Pretreatment with the SOD mimetic or NOX inhibitor abolished between group differences ($P\geq0.85$). Indices of ET1-induced vasoconstriction were not significantly different between groups under any experimental condition ($P\geq0.11$). *Conclusion:* That insulin-stimulated vasoconstriction was reversible with a SOD mimetic or NOX inhibition in sedentary pigs implicates impaired oxidative stress regulation in the manifestation of selective insulin resistance. Exercise training coincided with improved oxidative stress regulation conjunctional with augmented insulin-stimulated cerebral vasodilation. Given vasoreactivity to ET1 was similar between groups, greater insulin-stimulated vasodilation in exercise trained pigs was likely the result of enhanced oxidative stress regulation yielding improvements in NO signalling.

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In the name of God, the most gracious, the most merciful. It is seldom a unit of one can accomplish what is noteworthy, so this is the most important section of my document. *The happiness of a drop is to die in the river*—Al-Ghazali. My mother and sister carried me to this point, as it is of their strength, I have learned that the only way out is through. The extended arms of my brother and father were essential in pulling me through my endeavours. Collectively, Omaima El Badry, Zeina, Yehia and Ashraf El Karsh were there first. Complaints about the thesis should be directed to Dylan Olver, for it is his insistence that brought me into science. Pertinent words that encapsulate my mentor, and as quoted in Dylan's doctoral dissertation: *If destruction be our lot, we must ourselves be its author and finisher*—Abraham Lincoln. What I received from Dylan extends far beyond scholarly training, for it is to him that I credit the solidification of my convictions. I now understand above all, my defining characteristic is my contentment with discomfort: *Get used to a rough life, for luxury does not last*—Umar Ibn Al-Khattab. Patience in challenges on this path was inspired by Justin, Rory, Adam and Cameron standing alongside, shoulder-to-shoulder. *Desires make slaves of kings and patience makes kings of slaves*—Al-Ghazali. The structure around me in Dr. Farthing, Dr. Unniappan and Cindy Pollard fortified my steadiness, and for their contributions, I express my gratitude.

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Glossary of Terms

Area under the curve	A measure of the overall vascular response derived from dose-response curves.
Autophosphorylation	A biochemical process in which a phosphate is added to a protein kinase by itself.
Catalyzation	The process by which a substance speeds up a chemical reaction without being consumed or altered in the process.
Glucotoxicity	Beta cell failure induced by chronically elevated glucose levels through decreased insulin secretion.
Hyperglycemia	A condition in which an excessive amount of glucose circulates in the blood.
Hyperinsulinemia	A condition in which an excessive amount of insulin circulates in the blood.
Oxidization	The transfer of a negatively charged electron from one organic compound to another organic compound or to oxygen.
Phosphorylation	A biochemical process that involves the addition of phosphate to an organic compound.
Ras	A family of GTPase proteins that are expressed in all animal cell lineages and organs.
Reduction	A half-reaction in which a chemical species decreases its oxidation number, usually by gaining electrons.
Redox reaction	A process in which one molecule is reduced and another is oxidized.
Vascular conductance	The ease of blood flow through a blood vessel, or vascular bed at a given pressure.
Vascular resistance	The impedance of blood flow through a blood vessel, or vascular bed at a given pressure.
Vasoconstriction	The narrowing of the lumen of a vein, artery, or arteriole as a result of smooth muscle cell constriction in the blood vessel wall.
Vasodilation	The widening of the lumen of a vein, artery, or arteriole as a result of smooth muscle cell relaxation in the blood vessel wall.
Vasoreactivity	The responsiveness of blood vessels to stimuli through vasoconstriction or vasodilation.
Vasomotor	Relating to the constriction of dilation of a blood vessel.

Abbreviations

Akt	Protein kinase B
AUC	Area under the curve
BH_4	Tetrahydrobiopterin
Ca^{2+}	Calcium ion
cGK	Cyclic guanosine 3':5'-monophosphate-dependent protein kinase
cGMP	Cyclic guanosine monophosphate
eNOS	Endothelial nitric oxide synthase
ET	Endothelin
Fe^{2+}	Ferritin
GDP	Guanosine diphosphate
GRB	Growth factor receptor-bound protein
GTP	Guanosine-5'-triphosphate
H_2O_2	Hydrogen peroxide
HIIT	High-intensity interval training
IP_3	Inositol 1,4,5-trisphosphate
IR	Insulin receptor
IRS	Insulin receptor substrate
KPH	Kilometers per hour
MAPK	Mitogen-activated protein kinase
$MbFe^{III}$	Ferrous myoglobin
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NO	Nitric oxide
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
$^{*}OH$	Hydroxyl group
O_2	Oxygen
O^{2-}	Superoxide
$ONOO^{-}$	Peroxynitrite
PDK	Pyruvate dehydrogenase kinase
PI3K	Phosphotidylinositol-4,5-bisphosphate 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PLC	Phospholipase C
PP2A	Protein phosphatase 2A

PSS	Physiological saline solution
PTP1B	Protein-tyrosine phosphatase 1B
Raf	Rapidly-accelerated fibrosarcoma
ROS	Reactive oxygen species
Ser^{1177}	Serine group
SOD	Superoxide dismutase
SOS	Son of Sevenless
T2DM	Type-2 diabetes mellitus
TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl

1 Introduction

Insulin is a hormone released from the pancreas in response to an increase in blood glucose concentration. For over 70 years it has been established that insulin interacts with the vasculature and influences blood flow distribution within the cardiovascular system (77). Circulating insulin binds to IRs located on endothelial cells lining the luminal surface of arteries throughout the cardiovascular system. Operating through distinct signalling cascades; this interaction stimulates production of the vasodilator NO as well as production and secretion of the vasoconstrictor ET1 (3). Whereas vasodilation increases vascular conductance and leads to an increase in blood flow, vasoconstriction increases vascular resistance and leads to a decrease in blood flow. The net hemodynamic effect of circulating insulin is dependent on the relative activation of the aforementioned signalling cascades and subsequent changes in local conductance or resistance to blood flow. With respect to the cerebrovasculature, under normal conditions, circulating insulin elicits either no change or causes vasodilation and a resultant increase in blood flow (7, 22, 90). In instances where total blood flow does not change, because the effect of insulin may be site-specific within the cerebrovasculature, insulin may stimulate changes in the regional distribution of brain blood flow without affecting gross perfusion.

The purposes for cerebrovascular actions of insulin remain to be elucidated fully. In skeletal muscle, insulin-stimulated vasodilation serves to increase insulin and glucose delivery, then subsequent insulin-stimulated glucose uptake (7). However, in the brain, insulin-stimulated glucose uptake is minimal (11). Nevertheless, insulin alone can be transported from the vasculature into the brain, through the blood-brain barrier, where it is involved in neuronal cell differentiation and growth (5). Insulin-stimulated vasodilation and the redistribution of blood flow within the brain may serve to enhance regional insulin delivery, uptake and subsequent neurotrophic signalling (11). Accordingly, in pathological conditions, where normal insulin signalling is disrupted, blood flow regulation and end-organ function may also be disturbed. The term selective vascular insulin resistance describes abnormal vascular responses to insulin, characterized by reduced insulin-stimulated NO production and increased insulin-stimulated ET1 production and secretion (32). More recently, this phenomenon has been observed in the cerebrovasculature (53).

Physical inactivity and overnutrition are independent risk factors for the development of cerebrovascular dysfunction (38). Accordingly, these risk factors present with a similar cerebrovascular selective insulin resistance pathophysiology, namely, attenuated NO signalling (78, 105, 106). A primary cause of impaired NO signalling is an accumulation of oxidative stress: an imbalance between pro-oxidants and antioxidants (26). Whereas pro-oxidants elicit ROS production, antioxidants act to remove excess ROS. An aberrant surplus of ROS can lessen NO production and bioavailability. Therefore, an imbalance favouring pro-oxidants can lead to impaired NO signalling (Figure 1). More specifically, an admixture of inactivity and overnutrition lead to hyperglycemic states that could augment oxidative stress and suppress NO-induced vasodilation (50). Given the central role of NO signalling in insulin-stimulated vasodilation, this raises the possibility that the accumulation of oxidative stress serves a critical role in the etiology of selective insulin resistance. Identifying viable interventions to improve oxidative stress regulation could enhance NO signalling and normalize cerebrovascular function in pathological states related to impaired vascular insulin signalling.

Exercise could be the poly-pill necessary for preserving and rescuing cerebrovascular function and brain health. As far back as 1914, repeated skeletal muscle contractions, constituting exercise, were identified as a viable intervention for improving blood flow and vascular health in the setting of selective insulin resistance (18). Consensus is yet to be established for the magnitude of physical activity, defined as skeletal muscle contractions eliciting any bodily movement, or exercise, a subset of physical activity comprised of deliberate and repeated skeletal muscle contractions in a single bout, that are needed to observe improvements in vascular insulin signalling (20). However, exercise training, that is routine exercise undertaken on a regular basis, is the only well-established method to simultaneously improve NO signalling and prevent or treat selective insulin resistance (63). Of note, the capacity for exercise to confer such benefits appears to be restricted to vascular beds that undergo increases in blood flow during the exercise. Briefly, organ blood flow is coupled directly to metabolic activity (i.e., exercise-induced hyperemia); as metabolic requirements change during exercise, local organ blood flow is adjusted in proportion to changes in local metabolic activity. Both acute and chronic exercise improve NO signalling and responsiveness of arteries to insulin, and the latter effect is appears to be restricted to vascular beds exposed to exercise-induced hyperemia (61, 76, 108). Therefore, exercise-induced hyperemia could underlie enhanced vascular insulin sensitivity. Regarding the brain, moderate-intensity

exercise has been shown to increase cerebral blood flow by as much as 30% during a single bout (97). Furthermore, exercise training improves oxidative stress regulation by normalizing imbalances between pro-oxidants and antioxidants, correspondingly augmenting NO signalling (41). Thus, it is conceivable exercise training could simultaneously prevent the accumulation of excess ROS and improve NO signalling to rescue cerebrovascular selective insulin resistance. However, presently there is insufficient evidence to conclude this prospect.

The following discussion will outline essential components of cerebrovascular insulin signalling in normal and dysfunctional states. In turn, the aim is to deliberate the concomitant roles of oxidative stress and ROS signalling as it pertains to insulin-stimulated vasomotor responses. Furthermore, the subsequent sections will survey mechanisms by which exercise could improve vascular insulin sensitivity. Relevant literature will be reviewed in establishing the thesis of this work. Specifically, how oxidative stress modulates cerebrovascular selective insulin resistance arising with inactivity and overnutrition, which can be rescued at a functional level with exercise training. Fundamentally, distinguishing the mechanisms that underpin differential vasomotor responses to insulin in the brain of sedentary or exercise trained animals is the overarching purpose of this work. Given insulin resistance is increasingly affecting children (84), characterizing the aforementioned phenomena in juvenility is a secondary purpose of the work.

2 Review of the Literature

2.1 Cerebrovascular Insulin Signalling

Insulin is a vasoactive hormone that stimulates endothelium-dependent smooth muscle relaxation and subsequent vasodilation in normal, healthy settings (110). Insulin-stimulated vasodilation is biphasic; initially increasing the diameters of terminal arterioles thus augmenting capillary recruitment (i.e., increasing the number of perfused capillaries), followed by vasodilation of larger resistance vessels leading to greater blood flow (67). Over the last three decades, findings from endothelial cell culture work, isolated vascular function analyses and *in vivo* models have allowed researchers to identify the cellular events underpinning the vascular actions of insulin. Hsueh and Law discuss vascular insulin signalling in great detail in their review (42). The following sections will highlight pertinent molecular pathways outlined in their review.

2.1.1 NO Signalling

Vascular insulin signalling cascades are preceded by the activation of the IR on the endothelial layer of a blood vessel (Figure 1). Circulating insulin binds the α subunit of the receptor, counteracting β subunit inhibition of tyrosine autophosphorylation. Successive activation of the receptor directly phosphorylates IRS-1 on multiple tyrosine residues. Tyrosine-phosphorylated IRS-1 forms the binding site for PI3K; whereby the p85 subunit of PI3K binds IRS-1, constituting a signalling complex that activates PI3K by enhancing the catalytic functions of the p110 subunit (110). Activated PI3K phosphorylates PIP_2, yielding PIP_3, which stimulates PDK-1 autophosphorylation. This leads to Akt-dependent phosphorylation of eNOS at Ser^{1177}, augmenting eNOS activity. Subsequently, eNOS utilizes L-arginine as substrate to increase NO production. Notably, other necessary co-substrates for NO production include NAD(P)H, O_2 and BH_4 (9). NO diffuses into the adjacent vascular smooth muscle layer and activates guanylate cyclase to increase cGMP levels, successively activating cGK. These are enzymes that stimulate vasorelaxation through multiple mechanisms including the reduction of intracellular calcium concentrations and altered contractile protein calcium sensitivity (98). In addition to the aforementioned molecular pathway, stimulating vasodilation, insulin also elicits a vasoconstrictor influence.

2.1.2 ET1 Signalling

Another important signalling cascade downstream from IR activation, which promotes vasoconstriction, is initiated when GRB2 binds phosphorylated IRS-1 to activate SOS (Figure 1). Activated SOS initiates the removal of GDP from Ras, ushering a kinase phosphorylation cascade: Raf, MAPK kinase, and MAPK. Insulin-stimulated MAPK activation regulates growth, mitogenesis as well as ET1 production and secretion from endothelial cells (3). ET1 binds Gq protein-coupled receptors expressed in endothelial (ET_B) and smooth muscle (ET_A) layers of a blood vessel, with a higher binding affinity to the latter. Stimulation of vascular smooth muscle ET_A activates GTP-induced PLCβ, which then hydrolyzes PIP_2 to IP_3. IP_3 activates sarcoplasmic reticulum calcium channels, eliciting intracellular calcium release, thus leading to smooth muscle cell contraction and vasoconstriction. Acting as contradictory arms of insulin signalling, NO (vasodilator) and ET1 (vasoconstrictor) serve paracrine regulatory roles in the production of their counterparts. Notably, it has been shown that increases in endogenous NO attenuates ET1 on a transcriptional level (52). Concurrently, the presence of endothelial ET_B receptors, albeit at relatively lower concentrations, serves as a negative regulatory mechanism for ET1-induced vasoconstriction, by enhancing ET1 clearance and stimulating NO production (88). Vascular actions of insulin are an amalgam of these signalling cascades, which tend to favour NO production and vasodilation in most vascular beds and under normal, healthy conditions. Despite the abundance of literature on vascular insulin signalling, the vast majority of investigations in the past 70 years have focused on studying blood vessels supplying skeletal muscles (7, 54, 77). Recently, the role of insulin in cerebrovascular regulation has been interrogated.

2.1.3 Balance Between Signalling Cascades

The brain was considered classically to be an insulin insensitive organ as the blood-brain barrier was believed to be impermeable to the hormone. Dispelling of this notion in the late 1970s, Havrankova et al. reported on the presence of IRs in the brain (39). Insulin delivery to brain tissue contributes to neuronal development, glucoregulation, and cognition (5). Researchers hypothesize that increased blood flow resulting from insulin-stimulated vasodilation facilitates delivery of the hormone to target tissue (8, 67). In a study examining insulin signalling kinetics in the brains of sexually mature mice lacking cerebrovascular endothelial IRs compared with controls, investigators reported 60%, 85%, and 99% reductions to insulin-stimulated tyrosine phosphorylation of prefrontal cortex, hippocampal, and hypothalamic IR, respectively (51).

Potentially, impaired insulin-stimulated increases in regional blood flow precipitated reductions in the delivery of insulin to target brain tissue.

Although the physiological purpose of endothelial IRs in the cerebrovasculature is not entirely clear, evidence indicates they do serve a role in cerebral blood flow regulation. Using ultrasound imaging, Chaudhuri et al. observed a 13% increase in internal carotid artery diameter 1-hour following an exogenous insulin infusion at physiologic levels in 15 healthy adult subjects (22). In a seminal study, the Katakam group reported a 30% increase in cortical blood flow concurrent with a 32% vasodilation of isolated cerebral arteries in response to exogenous insulin in sexually mature rats (48). Denudation of isolated cerebral vessels abolished insulin-stimulated vasodilation, while pretreatment with non-selective NOS and neuronal NOS inhibitors as well as ET_B blockade attenuated insulin-stimulated vasodilation. Conversely, pretreatment with ET_A blockade augmented insulin-stimulated vasodilation. These data indicate that endothelium-dependent NO production mediates insulin-stimulated vasodilation with competing ET_A/ET_B receptor contributions to insulin-mediated vasomotor responses. Given both insulin-stimulated NO and ET1 are produced in the endothelium, dysregulation of endothelial function could underlie aberrant blood flow control in the brain accompanying cerebrovascular selective insulin resistance.

2.2 Dysregulation of Insulin Signalling

Physical inactivity and overnutrition are leading contributors to the contemporary, worldwide obesity pandemic (38). The loss of tissue responsiveness to the actions of insulin (i.e., insulin resistance) is a key event in obesity. Selective insulin resistance refers to impaired vasomotor responsiveness to circulating or exogenous insulin. This is characterized by an imbalance between PI3K/Akt/NO production and MAPK/ET1 production and secretion, shifting the paradigm towards an attenuation of the former and a potential enhancement of the latter (73). The net result is a decrease in insulin-stimulated vasodilation as well as possible insulin-stimulated vasoconstriction. An investigation in isolated arteries from obese adult human subjects and sexually mature pigs revealed that insulin-stimulated vasoconstriction arises from prolonged insulin stimulation in the setting of PI3K suppression, but this effect can be abolished with MAPK antagonism or ET receptor blockade (73). Importantly, in contrast to combined insulin signalling and PI3K suppression, persistent insulin stimulation alone did not alter subsequent vasomotor responses to the hormone. This observation points to a preservation of the normal balance between signalling cascades with hyperinsulinemia alone and suggests reduced PI3K/Akt/NO signalling

precipitates insulin-stimulated vasoconstriction. There is limited insight on upstream modulatory mechanisms that could alter the balance between PI3K/Akt/NO and MAPK/ET1 signalling cascades. To better understand the manifestation of selective insulin resistance, researchers commonly examine vascular responses to endogenous or exogenous insulin in pathological states of metabolic dysfunction that are often accompanied by systemic insulin resistance (i.e., obesity and T2DM where systemic indicators of insulin resistance are increased).

2.2.1 Etiology of Selective Insulin Resistance

There is a growing body of evidence implicating selective insulin resistance in the skeletal muscle vasculature to the development of cardiovascular disease in obesity, prediabetes and diabetic states (4). Pioneering studies from Alain Baron demonstrate obese, insulin resistant adult subjects exhibit attenuated skeletal muscle blood flow in response to both endogenous and exogenous insulin (6, 58). A subsequent study by Olver and colleagues demonstrated obesity-induced impairments in endogenous insulin-stimulated vasodilation are observed prior to the development of clinical insulin resistance or T2DM (74). Extending on these findings, in experiments on isolated arteries supplying skeletal muscle of obese sexually mature Zucker rats, a 15% vasoconstriction was observed in response to insulin in a dose-dependent manner (32). While ET_A receptor blockade abolished the vasoconstrictor response to insulin, it did not restore vasodilation to similar levels observed in arteries from healthy control rats in this experiment. These data suggest both augmented ET1 and depressed NO signalling in response to insulin contribute to impaired vasoreactivity in pathogenic states.

Selective vascular insulin resistance has been studied most extensively in the skeletal muscle vasculature, but less is known regarding insulin signalling in other vascular beds. However, it is important to note, several studies have observed evidence of selective insulin resistance in arteries supplying the brain as well as the cerebrovasculature. For example, in another study from Chaudhuri and colleagues, an insulin infusion stimulated a 10% increase of internal carotid artery diameters in the control group, but the dilatory effect was otherwise absent in adult subjects with T2DM (21). Indeed, recent work from Olver and colleagues reported that selective insulin resistance develops simultaneously in brain and skeletal muscle arteries in juvenile obese vs. control pigs and prior to the overt manifestation of more classical indicators of vascular dysfunction (74). Furthermore, reduced insulin-stimulated vasodilation of cerebral arteries coincided with a decreased index of cerebral blood flow and attenuated insulin signalling in the

brain. Accordingly, there may be a relationship between cerebrovascular selective insulin resistance and impaired insulin signalling in the brain itself. Collectively, the data indicate selective insulin resistance, evidenced by impaired insulin-stimulated vasodilation, occurs early in the development of vascular disease, and is reflected by an imbalance in NO vs. ET1 signalling, favouring the latter. However, although selective insulin resistance is observed commonly in obese conditions, modulatory mechanisms contributing the imbalance in NO vs. ET1 signalling, such as oxidative stress, are incompletely understood. Furthermore, few investigations to date have attempted to examine these effects in the cerebral circulation. Given there are currently no pharmacological treatments for selective insulin resistance, investigations into the mechanisms underpinning the dysregulation of vascular insulin signalling are emerging areas of interest.

2.2.2 A Relationship Between Insulin and ROS

Aberrant oxidative stress regulation is characteristic of vascular dysfunction (19). In regard to the cerebrovasculature, impaired oxidative stress defences and ROS overproduction may contribute to pathogenic brain blood flow control and precipitate selective insulin resistance (Figure 1). The direct relationship between vascular insulin signalling and ROS production remains equivocal, but mounting evidence indicates that ROS generated in response to insulin are required to observe the full hormonal effect (59). In contrast, the overproduction of ROS can impair the vascular actions of insulin (49). Briefly, mitochondrial respiration and the actions of NOX modulate cellular production of ROS such as H_2O_2 and O^{2-}, under aerobic conditions, through the transfer of electrons to oxygen molecules (36). Basal levels of ROS are involved in regulation of vascular tone through H_2O_2-stimulated NO production (19). ROS overproduction is regulated by coactivation of antioxidant enzymes such as SOD and O^{2-} catalase, which scavenge O^{2-} to form H_2O_2 and reduce H_2O_2 to form H_2O, respectively (36). IR activation stimulates a burst of intracellular ROS, which at physiological levels act as positive modulators of insulin signalling. Specifically, PTP1B, which dephosphorylates the IR, and PP2A, which prevents Akt phosphorylation, are inactivated by H_2O_2 (23, 59). ROS-mediated facilitation of insulin-stimulated Akt activation could augment downstream NO production. Beyond a role in facilitating augmented NO signalling, ROS accumulation is implicated causally in decreased NO production and bioavailability. Therefore, the balance between pro-oxidants and antioxidants is a major determining factor for the effectiveness of insulin-stimulated NO signalling.

The normal relationship between insulin and ROS is disrupted in pathogenesis, whereby ROS overproduction could lead to impaired NO signalling in the setting of selective insulin resistance (Figure 1). Substrate concentrations for NO production are insufficient in selective insulin resistance, leading eNOS uncoupling, which could enhance O^{2-} generation (9). A requisite molecule for eNOS function is the cofactor BH_4, which plays a direct role in the oxidation of L-arginine and subsequent NO production (96). A dearth of BH_4 fundamentally alters the enzymatic actions of eNOS and its biochemistry (9). The ensuing destabilization in eNOS dimer, resulting in attenuated cellular monomer to dimer ratios, leads to eNOS uncoupling (60). This entails the loss of L-arginine oxidation, while electron transfer from NAD(P)H to molecular oxygen remains intact and results in the formation of O^{2-} from eNOS. Accumulation of O^{2-} results in the scavenging of NO; the reaction between O^{2-} and NO generating $ONOO^-$ occurs three-fold more rapidly than SOD scavenging of O^{2-} (60). Additionally, hyperglycemia, exhibited in states coinciding with selective insulin resistance, elicits the overproduction of mitochondrial NAD(P)H (94). Build up of NAD(P)H stimulates mitochondrial production of O^{2-} which can further scavenge NO. Therefore, insulin-stimulated NO production may be rendered innate owing to diminished production and bioavailability when basal or insulin-stimulated ROS concentration are augmented to a pathological level. This highlights a paradoxical relationship between insulin and ROS signalling, whereby ROS, when synthesis and degradation are regulated, play a role in insulin-stimulated NO production, but when accumulation cannot be regulated sufficiently, can scavenge NO. An illustration of the interplay between vascular insulin signalling and oxidative stress regulatory factors is presented in Figure 1.

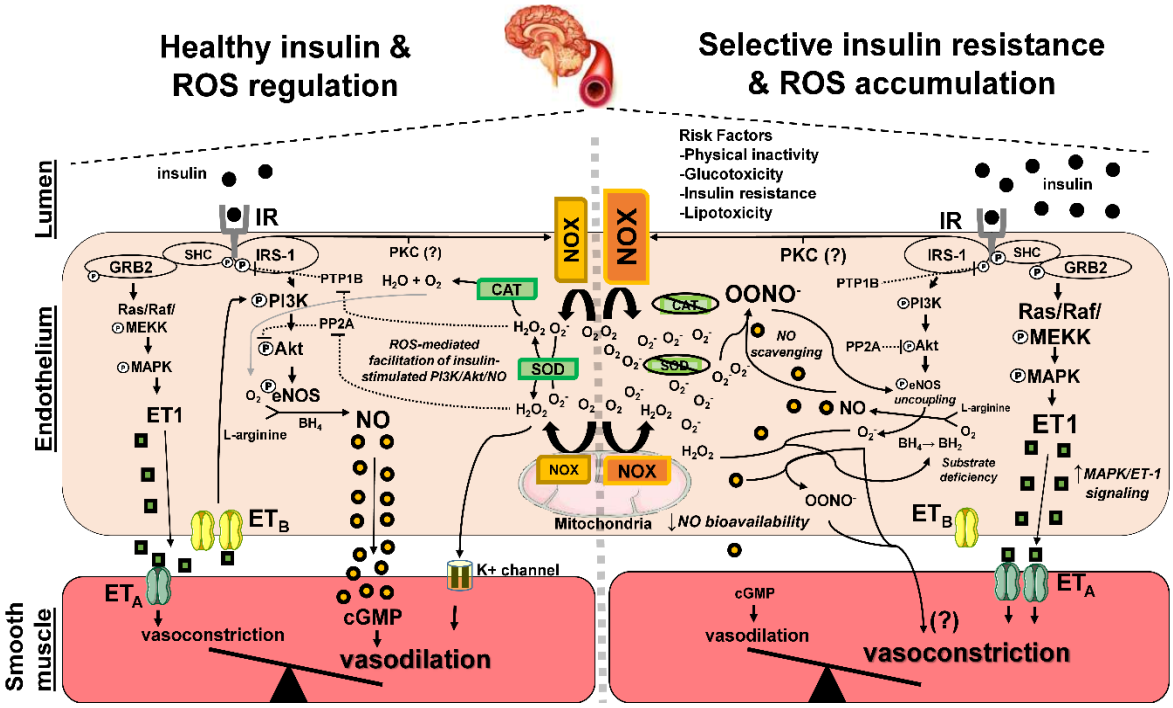

Figure 1. Proposed molecular pathways for vascular insulin and ROS signalling. The normal, healthy state of vascular insulin signalling as well as selective insulin resistance are illustrated in the left and right panels, respectively. In the normal setting of vascular insulin signalling, circulating insulin binds and activates endothelial bound insulin receptors (IR) which directly phosphorylates the insulin receptor substrate (IRS-1). Phosphorylated IRS-1 forms the binding site for phosphotidylinositol-4,5-bisphosphate 3-kinase (PI3K). Subsequently, activated PI3K leads to protein kinase B (Akt)-dependent phosphorylation and activation of endothelial nitric oxide synthase (eNOS), culminating in NO production. Notably, eNOS utilizes L-arginine as substrate and tetrahydrobiopterin (BH_4) as a cofactor to increase NO production. NO diffuses into the adjacent vascular smooth muscle layer to increase cyclic guanosine monophosphate (cGMP) levels, eliciting smooth muscle relaxation and vasodilation. Moreover, activation of the IR stimulates the intracellular mediator SHC, which binds growth factor receptor-bound protein 2 (GRB2). In turn, this initiates a kinase phosphorylation cascade: Ras/Raf/extracellular signal-regulated kinase kinase (MEKK)/mitogen-activated protein kinase (MAPK). As a result, MAPK-induced endothelin-1 (ET1) production and secretion are augmented. ET1 favourably acts on ET_A receptors in the smooth muscle layer of a blood vessel which stimulate vasoconstriction, but it can also activate, to a lesser extent, endothelial ET_B receptors which increase NO production. The balance between the two insulin-stimulated cascades favours vasodilation in normal, healthy states, under which, basal levels of reactive oxygen species (ROS) can facilitate the vasodilatory effect. Activation of IR stimulates nicotinamide adenine dinucleotide phosphate oxidase (NOX), potentially mediated by protein kinase C (PKC). Insulin-stimulated NOX, in conjunction with mitochondrial NOX, produce superoxide (O^{2-}) and hydrogen peroxide (H_2O_2). In turn, H_2O_2 inactivates protein-tyrosine phosphatase 1B (PTP1B) and protein phosphatase 2A (PP2A), which inhibit IR and Akt, respectively. Normal, synergistic superoxide dismutase (SOD) and catalase (CAT) activity respectively yield H_2O_2 from O^{2-}, and subsequently H_2O; thus, preventing excess ROS. However, there is an attenuation of insulin-stimulated NO in selective insulin resistance, which can be precipitated by several risk factors such as physical inactivity and glucotoxicity, among other factors. ROS overproduction is observed in this setting owing to increased NOX and mitochondrial O^{2-} generation, while depressed SOD activity incapacitates the scavenging of O^{2-}. Peroxynitrite ($OONO^-$), which could stimulate vasoconstriction, is produced from the rapid interaction between O^{2-} and NO; thus, decreasing NO bioavailability and insulin stimulated vasodilation. Furthermore, in elevated oxidative stress states, reductions in BH_4 levels, adversely converted to dihydrobiopterin (BH_2), result in eNOS uncoupling; this yields O^{2-}, rather than NO, from eNOS. Ultimately, the ensuing reductions in insulin-stimulated NO, coalescing with intact ET1 activity, result in augmented vasoconstrictor responses to insulin in the setting of selective insulin resistance.

2.2.3 Oxidative Stress Modulates Vascular Insulin Signalling

At the functional level, ROS may be requisite for insulin-stimulated vasodilation. However, dysregulation of oxidative stress defences and ROS overproduction exhibited in the setting of selective insulin resistance, yielding an accumulation of oxidative stress, result in impaired vascular function. In investigating endothelial signalling cascades, Shinozaki et al. observed an attenuation of maximal endothelium-dependent vasorelaxation coupled with augmented O^{2-} generation in aortic segments from hyperglycemic sexually mature rats compared to control rats (96). eNOS inhibition attenuated O^{2-} production in hyperglycemic rats, implicating eNOS uncoupling in hyperglycemic rats. Notably, exogenous administration of BH_4 elicited a threefold increase in NO production in hyperglycemic but not control rats; potentially owing to 10% lower BH_4 concentrations observed in hyperglycemic rats. Katakam and colleagues extended on these findings, reporting on attenuated insulin-stimulated vasodilation in isolated cerebral arteries of obese rats vs. lean sexually mature rats (49). Pretreatment with NOX inhibitor and SOD mimetic restored insulin-stimulated vasodilation in obese rats but had no effect in lean rats. Further, pretreatment with non-selective NOS and nNOS inhibitors, as well as a BH_4 precursor, enhanced insulin-stimulated vasodilation in obese but not lean rats. Elevated ROS levels in obese rodents could have contributed to eNOS uncoupling stemming from a BH_4 deficiency. Thus, in the previous experiments BH_4 levels were insufficient for NO production, leading to attenuated eNOS monomer to dimer ratios, and subsequent ROS overproduction.

Improving oxidative stress regulation and normalizing ROS signalling to basal levels may enhance, as well as rescue, endothelial function, and insulin-stimulated vasodilation. The Frisbee group provides evidence for this postulate, reporting that endothelium-dependent vasodilation of isolated cerebral arteries from obese sexually mature rats was attenuated when compared with their lean counterparts; while treatment with a SOD mimetic enhanced NO signalling in the obese group only (14). Furthermore, Olver et al. reported lower insulin-stimulated vasodilation in isolated cerebral arteries of obese compared with lean sexually mature rats (78). An effect size analysis revealed that obesity had a large, negative effect on cerebral artery vasodilation to insulin at physiologic doses. eNOS blockade enhanced insulin-stimulated vasodilation in obese but not lean rats, pointing to eNOS uncoupling and ROS overproduction exhibited by obese rodents. Additionally, 20 weeks of spontaneous wheel running (i.e., a model for physical activity), after the onset of selective insulin resistance in obese rats, improved eNOS contributions to, and enhanced,

insulin-stimulated vasodilation. This affirms the prospect that alleviation of oxidative stress and normalized ROS signalling are necessary for reversing and improving endothelial NO signalling and cerebrovascular insulin sensitivity. Consequently, physical activity and exercise were presented as a method for improving cerebrovascular function in the aforementioned investigation by the Olver group, warranting an inquisition of the prospect. The subsequent sections will discuss exercise-induced adaptations in the brain briefly and expound the relationship between normalized ROS regulation and insulin signalling in the cerebrovasculature.

2.3 Exercise

Physical activity can improve vascular function in healthy and pathogenic states. In a longitudinal investigation following 27,000 participants over 11 years, Mora et al. reported on the relationships between trends in adverse cardiovascular events, cardiovascular disease risk factors and physical activity levels (64). Investigators ascribe 65% of the inverse relationship between physical activity levels and adverse cardiovascular events to modulatory factors outside of biomarkers traditionally measured in clinical settings (e.g., HbA1c, LDL and HDL cholesterol). To better understand how exercise training confers a therapeutic benefit, it is necessary to explicate mechanisms that underlie cardiovascular adaptations occurring in conjunction with physical activity beyond improvements in traditional risk factors. Particularly germane to the present work are cerebrovascular adaptations to exercise training that include improved insulin-stimulated NO production and oxidative stress regulation. The following discussion will consider exercise training adaptations, and potential mechanisms that modulate these adaptations, in the cerebral circulation and brain.

The brain remains active under all living conditions (72). Therefore, to sustain function, neuronal activity is matched by a constant supply of oxygenated, nutrient-rich blood flow (~50-60 mL 100 g^{-1} min^{-1}) (83). Owing to the low substrate storage capacity of the brain, metabolic demands can only be met for ~1-second if blood flow to the organ is discontinued (17). For the better part of the twentieth century, it was thought that cerebral blood flow during exercise remained unchanged from resting states (89, 111). However, advancements in measurement techniques (e.g., microspheres, transcranial Doppler ultrasound and functional magnetic resonance imaging) have allowed investigators to detect increases in global and regional brain blood flow during exercise intensities ranging from ~20-100% of VO_2max (15, 33, 65). Increases in brain blood flow during exercise to match the metabolic requirements of elevated neuronal activity are

the result of neurovascular coupling (80). Accordingly, repeated bouts of exercise (i.e., exercise training) result in vascular adaptations that optimize neurovascular coupling. These adaptations include structural changes, such as outward remodeling (i.e., larger arteries), arteriogenesis (i.e., new arteries) and angiogenesis (i.e., new capillaries), as well as functional changes, such as improved vasodilatory capacity (i.e., improved endothelial function) (72, 103, 107). The underlying mechanisms responsible for such adaptations remain incompletely understood, but likely involve both systemic and local stimuli. The following discussion will focus on mediators of improved vascular function with exercise training.

2.3.1 Exercise Mediators

Investigators have interrogated the relationship between changes in brain blood flow and neuronal activation responsible for generating movement through skeletal muscle contractions for over a century (87). In this time, research has likewise elucidated mediators of exercise-induced adaptations in the brain that include blood-borne circulating factors such as brain-derived neurotrophic factor and vascular endothelial growth factor, as well as localized, mechanical stimuli such as vascular shear stress (25, 40, 77). Of note, improvements in vascular insulin sensitivity do not occur uniformly throughout the cardiovascular system with exercise training, but instead appear to be restricted to vascular beds that experience increases in blood flow during the exercise bout (77). This suggests, rather than blood-borne circulating factors, improvements in insulin-stimulated NO signalling are mediated by localized, mechanical stimuli.

Increases in cerebral blood flow during sub-maximal exercise may be a primary stimulus for improving vascular insulin sensitivity. Indeed, the hyperemic/neurovascular coupling response to exercise may impose a mechanical stress on the cerebrovasculature that parallels insulin-stimulation in some respects (77). That is, when blood flow through an artery increases, it causes friction between blood and the endothelium, which generates shear stress in the artery. Briefly, shear stress frictional forces activate a series of endothelial mechanoreceptors, referred to as glycocalyx receptors, which induce the PI3K/Akt/NO pathway, subsequently leading to vasodilation. Human endothelial cell culture experiments demonstrate that *in vitro* shear stress stimulated PI3K-mediated Akt phosphorylation is augmented at greater magnitudes of the stimulus (28). These data indicate that shear stress stimulated NO production is intensity-dependent. Further investigations in sexually mature pig aortic endothelial cells show that shear stress augmented levels of insulin-stimulated tyrosine phosphorylation of eNOS (34). Additionally, Walsh and

colleagues documented augmented insulin-stimulated vasodilation of isolated skeletal muscle arterioles of sexually mature pigs following shear stress stimulation (108). This raises the possibility that improved vascular insulin sensitivity in response to exercise or exercise training is highly specific and related to a priming effect of exercise-induced functional hyperemia or vascular shear stress (76, 79). Nonetheless, whether findings on the improvements to vascular insulin sensitivity can be extended to the cerebral circulation or work in conjunction with other mechanisms has never been tested. Furthermore, in pathological states characterized by excessive ROS accumulation, increasing NO production alone may not be beneficial, as NO reacts with O^{2-}, forming $ONOO^{-}$. As outlined previously, this interaction decreases NO bioavailability and subsequently decreases NO production through eNOS uncoupling. Thus, exercise training related improvements in NO production must occur in concert with improved ROS regulation to realize the full functional benefit.

2.3.2 Exercise Improves ROS Regulation and Endothelial NO

Exercise enhances vascular insulin sensitivity, evidenced by increases in insulin-stimulated vasodilation following acute exercise and chronic exercise training in humans and rodents (68, 78). Furthermore, it has been demonstrated in sexually mature rodents that indicators of cerebrovascular selective insulin resistance can be reversed with daily physical activity through improvements in NO signalling (78). In addition to the vascular shear stress hypothesis outlined above, evidence indicates improved ROS regulation represents an additional mechanism through which exercise training enhances endothelial function. Although this mechanism of improved insulin-stimulated NO signalling has not been studied in the context of exercise training, given evidence reveals ROS dysregulation underlies selective insulin resistance in the cerebrovascular, this prospect warrants further discussion.

Evidence indicates enhanced endothelial function is underpinned by exercise training induced adaptations including reductions in oxidative stress yielding greater NO bioavailability (93). For example, NOX concentrations are decreased and endothelium-dependant vasodilation is increased in trained vs. sedentary adult subjects (1). Extending on these observational reports, Durrant and colleagues set out to examine the effects of 14 weeks of wheel running in overfed sexually mature mice (31). *In vivo* measurements of endothelium-dependent carotid artery vasodilation demonstrated overnutrition-induced deficits were reversed by wheel running. Moreover, experiments on isolated carotid arteries of overfed sexually mature mice showed that

wheel running improved maximal endothelium-dependent vasodilation and restored NO bioavailability, corresponding with enhanced carotid artery eNOS and SOD, but attenuated NOX concentrations. These results indicate that exercise can restore healthy vascular function partly by improving protective mechanisms against excessive oxidative stress. Notably, it is apparent that a single bout of moderate or high intensity exercise (60-100% of VO_{2max}) can enhance insulin signalling (40, 77). However, work from Gibala suggests between six and 12 weeks of HIIT, thrice weekly for 25 minutes per bout, as an ideal exercise training modality for improving cardiometabolic health in various settings accompanying selective insulin resistance (37). Although promising, there is limited direct evidence for the effectiveness and underlying protective mechanisms of routine exercise or HIIT on cerebrovascular insulin signalling.

In summary, normal cerebrovascular insulin signalling is necessary for the maintenance of brain vascular health. Although physiological ROS regulation appears requisite for healthy endothelial function, evidence indicates excessive ROS accumulation, culminating in impaired NO production and bioavailability, precipitate impaired insulin-stimulated vasodilation in settings of physical inactivity and overnutrition. As alluded to previously, the vast majority of discussed literature inadequately explores the uniformity of selective insulin resistance etiology throughout stages of development (i.e., juvenility vs. maturity). Given that insulin resistance is increasingly being documented in children (84), examining the manifestation and treatment of selective vascular insulin resistance in the developing brain is necessary. Exercise training has been shown to improve insulin-stimulated vasodilation through improvements in NO signalling, the source of which remains unknown. Potentially, by enhancing NO signalling concurrently with improving ROS regulation in the cerebrovasculature, exercise training has emerged as the ideal intervention to prevent or treat selective insulin resistance; yet this prospect has not been validated. Understanding the pathophysiology of selective insulin resistance and the mechanisms through which exercise training confers therapeutic benefits at different developmental stages holds extraordinary promise in the advancement of gene therapies, pharmacological treatments, and targeted exercise prescriptions for brain vascular health.

2.4 Objectives

Our group set out to explore the modulatory roles of ROS producing enzymes (NOX) and oxidative stress defences (SOD) in insulin-stimulated and ET1-induced vasomotor responses in the cerebrovasculature at different stages of development (juvenility vs. maturity) in pigs. Further,

we examined the effects of exercise training on oxidative stress regulation and cerebrovascular insulin signalling through the application of a translationally relevant exercise training intervention.

2.5 Purpose

Using pigs as an experimental model, the primary purpose of this study was to provide insight into pathways relevant to the vasomotor actions of insulin in the cerebrovasculature. We endeavour to understand better the etiology of selective insulin resistance in the cerebrovasculature, and beneficial adaptations to exercise training. A secondary purpose of the study was to explore the pathophysiological state at different stage of development. Collectively, we aim to advance current knowledge on mechanisms of cerebrovascular regulation in pathological and exercise-trained states.

2.6 Hypotheses

We hypothesized that inactive juvenile and mature pigs will display cerebrovascular insulin resistance, evidenced by insulin-stimulated vasoconstriction (and not vasodilation). The vasoconstrictor response to insulin will be ameliorated with acute SOD mimetic (augments oxidative stress defences) or NOX inhibitor (attenuates ROS overproduction) pretreatments, implicating excess ROS accumulation as an underlying mechanism of cerebrovascular selective insulin resistance. We also hypothesized that juvenile and mature pigs that underwent an 8-week exercise training (HIIT) intervention will exhibit enhanced cerebrovascular insulin sensitivity, evidenced by insulin-stimulated vasodilation (and not vasoconstriction). Moreover, neither acute SOD mimetic nor NOX inhibitor pretreatments will further improve insulin-stimulated vasodilatory responses, suggesting the exercise training improved vascular insulin sensitivity through enhanced ROS regulation. Likewise, owing to enhanced ROS regulation, relative to sedentary pigs, exercise-trained pigs would display decreased vasocontractile responses to ET1. Lastly, we hypothesized the magnitude of insulin-stimulated vasodilation would be decreased and the magnitude of ET1-induced vasoconstriction would be increased in mature vs. juvenile groups.

3 Materials and Methods

This study was conducted at the University of Saskatchewan, in the Western College of Veterinary Medicine. All study procedures were approved by the Animal Research Ethics Board of the University of Saskatchewan (Animal Use Protocol #20190036).

3.1 Study Sample and Design

A primary prerequisite for any animal model of disease is the translational potential to the human clinical context. Pig models have been used to study the etiology of several cerebrovascular disorders, on account of similarities between humans and pigs in both cerebrovascular and central nervous system structure and function (i.e., human and pig brains are gyrencephalic, contain >60% white matter, and rely on the internal carotid, circle of Willis, and middle cerebral arteries for perfusion) (44, 100). The correspondence between species extends to cardiovascular responses to sedentary behaviour as well as adaptations to acute exercise and chronic training. Although pigs are naturally sedentary, they can learn to exercise at speeds and intensities similar to humans (82). Regarding pig breed, the Ossabaw miniature-pig may be an ideal breed of pig to serve as a model for studying the pathophysiology of selective insulin resistance, owing to their unique propensity to develop diet-induced obesity and cardiovascular disease. Ossabaw miniature-pigs were subject to repeated feed-famine phases throughout their evolution on Ossabaw island; hence, researchers speculate that this breed developed a thrifty genotype, facilitating survival through famine phases by promoting excess body fat storage (102). Accordingly, when provided with the same access to feed as other commercially-available breeds, the Ossabaw miniature-pig can develop obesity and corresponding cardiovascular pathologies more rapidly (71). Of note, our group has established expertise in studying cerebrovascular dysfunction in pig models of human disease, including selective insulin resistance in Ossabaw miniature-pigs (71, 73–75). Therefore, the work described herein utilized the Ossabaw miniature-pig as a model for studying the effects of exercise training on cerebrovascular actions of insulin and ROS regulation.

Thirty-seven female and castrated male pigs (19F/18M) from the same line were obtained from a commercial supplier for this study. Seventeen sexually mature (14±2 months) and 20 juvenile (3±1 months) pigs were allocated to exercise or sedentary groups. Within each age group,

sex and weight matching were established between exercise training and sedentary groups prior to the onset of exercise training. Of the matched pigs, where necessary, selection of the exercisers was based on treadmill running compliance. Briefly, pigs that consistently required frequent electric shocks during familiarization and did not respond to positive reinforcement were allocated to the sedentary group to ensure animal welfare and researcher safety. Following a two-week acclimatization period, 10 juvenile (5F/5M; 11±3 kg) and eight mature (5F/3M; 83±9 kg) pigs began the exercise training protocol. All animals were group housed in an outdoor enclosure in the animal care unit (study was conducted in a single cohort and operated from May to September of 2020. Each day pigs were provided with feed that met the dietary reference values for macronutrients (26% fat; 60% carbohydrates; 14% protein; 1 kg per pig per day; ingredients list presented in the Appendix: Table 3) and had unlimited access to sugar water (~5 L of 10% sugar solution per pig per day).

3.2 Exercise Training

Exercise training protocols consisted of three high intensity interval exercise sessions per week, for eight weeks (i.e., HIIT). The durations of the exercise training protocol, individual exercise bouts as well as training frequency, were based on achieving a minimum effective dose for detecting training adaptations as previously reported by Gibala (37). Exercise intensity and duration increased incrementally over four weeks to the following: a three-minute warm up at 2 KPH, followed by five successive three-minute bouts of high-intensity running at 5 KPH (mature pigs) or 8 KPH (juvenile pigs), interspersed by four X one-minute low-intensity bouts at 2 KPH and ending with a three-minute cool-down period at 2 KPH all performed on a motorized treadmill set to an incline grade of 5%. Progression of high-intensity intervals throughout the eight-week period is presented in Table 1. Speeds at high-intensity intervals were chosen to suffice maximal intensity within an allotted 25-minute exercise period; animals were unable to complete an additional high-intensity bout. That is, pigs were unable to complete an additional (a sixth) high-intensity interval at their respective speeds. Accordingly, we concluded that the determined speeds were appropriate for our exercise training protocol.

Table 1. High-intensity intervals.

		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Juvenile	Day 1	4x3 min. at 5 KPH	4x3 min. at 5 KPH	5x3 min. at 5 KPH	5x3 min. at 5 KPH	5x3 min. at 8 KPH	5x3 min. at 8 KPH	5x3 min. at 8 KPH	5x3 min. at 8 KPH
	Day 2	4x3 min. at 5 KPH	4x3 min. at 5 KPH	5x3 min. at 5 KPH	5x3 min. at 5 KPH	5x3 min. at 8 KPH	5x3 min. at 8 KPH	5x3 min. at 8 KPH	5x3 min. at 8 KPH
	Day 3	4x3 min. at 5 KPH	4x3 min. at 5 KPH	5x3 min. at 5 KPH	5x3 min. at 5 KPH	5x3 min. at 8 KPH	5x3 min. at 8 KPH	5x3 min. at 8 KPH	5x3 min. at 8 KPH
Mature	Day 1	4x3 min. at 4 KPH	4x3 min. at 5 KPH	4x3 min. at 5 KPH	4x3 min. at 4 KPH	5x3 min. at 5 KPH	5x3 min. at 5 KPH	5x3 min. at 5 KPH	5x3 min. at 5 KPH
	Day 2	4x3 min. at 4 KPH	4x3 min. at 5 KPH	4x3 min. at 5 KPH	4x3 min. at 4 KPH	5x3 min. at 5 KPH	5x3 min. at 5 KPH	5x3 min. at 5 KPH	5x3 min. at 5 KPH
	Day 3	4x3 min. at 4 KPH	4x3 min. at 5 KPH	4x3 min. at 5 KPH	4x3 min. at 4 KPH	5x3 min. at 5 KPH	5x3 min. at 5 KPH	5x3 min. at 5 KPH	5x3 min. at 5 KPH

Presented as number of bouts by time in minutes; KPH=kilometer per hour

3.3 Primary Outcomes

Small arterioles are considered resistance vessels, owing to their role in regulating upstream vascular tone of larger feed arteries and organ perfusion (56). Pial arterioles are intracranial arterioles that span the surface of the brain and contribute significantly to cerebrovascular resistance, gross cerebral blood flow and blood flow distribution within the brain (83). Functional studies of vascular insulin signalling and pial arteriole vasomotor control are conducted routinely using the pressure myography technique (48, 49, 74, 78). Pressure myography was first developed in 1967 to study blood vessels 50 µm in diameter, and later refined by Dulling and colleagues to study blood vessels as narrow as 12 µm, in 1981 (30). Pressure myography is an *in vitro* technique used to interrogate mechanisms underlying vasomotor responses of isolated small arterioles, under isobaric conditions. In some respects, the technique parallels *in vivo* conditions of the blood vessel, as isolated arterioles are intraluminally cannulated and pressurized. Although *in vivo* and *in vitro* assessments of vascular control may produce differing results, some studies have provided evidence for the resemblance of isolated pressurized vascular segments to *in vivo* conditions of blood vessels (16, 92). A major advantage of pressure myography is that the direct or interactive effects of singular or multiple inputs on vasomotor control can be isolated in the absence of many confounding variables present under *in vivo* conditions. Accordingly, the pressure myography technique is a standard tool in investigations on the vascular actions of insulin in physiological and pathological states. The technique allows the study of the effects of insulin on

vascular diameter, and hence whether it would lead to vasodilation or vasoconstriction. Further, the use of blockers, agonists and mimetics can be implemented to provide insight on modulatory mechanisms involved in vasomotor responses to insulin. Therefore, pressure myography was employed herein to investigate modulatory mechanisms underlying differential vasomotor responses to insulin and ET1 in the cerebral circulation.

After approximately eight weeks of sedentary or exercise training conditions, pigs were fasted overnight (full access to water) and weighed. In the morning, pigs were sedated (IM injection of Ketamine at 20-30 mg per kg of body mass) and transported from the outdoor enclosure to the laboratory where sedation was maintained with inhaled isoflurane (1 L/min O_2, 1-5% isoflurane). While under sedation, a 6 mL blood sample was taken from a cardiac puncture (EDTA coated Lavender Top BD Vacutainer). Samples were centrifuged and the supernatant was transferred to an Eppendorf and frozen for subsequent blood content analyses (all blood analyses were performed by the Animal Health Diagnostic Center at Cornell University). Pigs were euthanized with an overdose of inhaled isoflurane (1 L/min O_2, 5% isoflurane) followed by exsanguination. Exercise trained animals completed their final bout of the protocol 48 hours prior to tissue extraction, in order to minimize acute exercise effects.

Following euthanasia, pig brains were carefully removed and a portion of the brain that contained the middle cerebral artery and downstream pial arterioles was then transferred to a dissecting dish containing ice-cold PSS (145 mM NaCl, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.17 mM $MgSO_4$ with 10 g/L albumin added) with a pH of 7.4. Branches of the middle cerebral artery were dissected, transferred to a Plexiglas chamber filled with PSS and cannulated with two glass micropipettes (60–75 μm). The chambers were then transferred to the stage of an inverted microscope (Amscope, Irving, CA) attached to a video camera (ThorLabs, Newton, NJ) and warmed to 37^{o}C using a circulating hot water bath. Fluid-filled reservoirs were used to set intraluminal pressure at 60 mmHg, and luminal diameter was monitored throughout the experiment using VasoTracker open-source pressure myography data acquisition software (57). An experimental setup for pressure myography studies is illustrated in Figure 2. After cannulation, arteries were allowed 45 minutes to stabilize, at which point maximal arterial vasoconstriction in response to 80 mM KCl was determined. Arteries that did not vasoconstrict ≥20% to KCl were discarded. To test the prospect that alleviation of oxidative stress improves vascular function, arteries remained either untreated (vehicle) or were pretreated with a SOD mimetic (TEMPOL;

1e−4 M) or a non-selective NOX inhibitor (Apocynin; 1e-4 M) prior to undergoing dose-response curves for insulin (1e−9 – 1e−6 M) and ET1 (1e−12 – 1e−7 M). TEMPOL and Apocynin were brought to solution with DMSO (0.5% final concentration). This concentration of DMSO does not alter vasomotor reactivity to endothelium-dependent vasodilators in isolated arteries as indicated in previous studies (47, 81, 99). Arteries that did not have spontaneous tone (≥20%) prior to the insulin dose-response curve were preconstricted with U46619 (thromboxane A2 analogue; ranging from 1e−6 − 1e−4 M) to 20-50%. After the final dose of ET1, vessels were washed twice with Ca^{2+}-free PSS to determine maximal diameter. All drugs were obtained from Cayman Chemicals (Cayman Chemicals Company, Ann Arbour, MI), and solutions were administered abluminally. Vasomotor responses were expressed as percent change from baseline diameter (i.e., the Δ diameter from baseline/baseline diameter, multiplied by 100%).

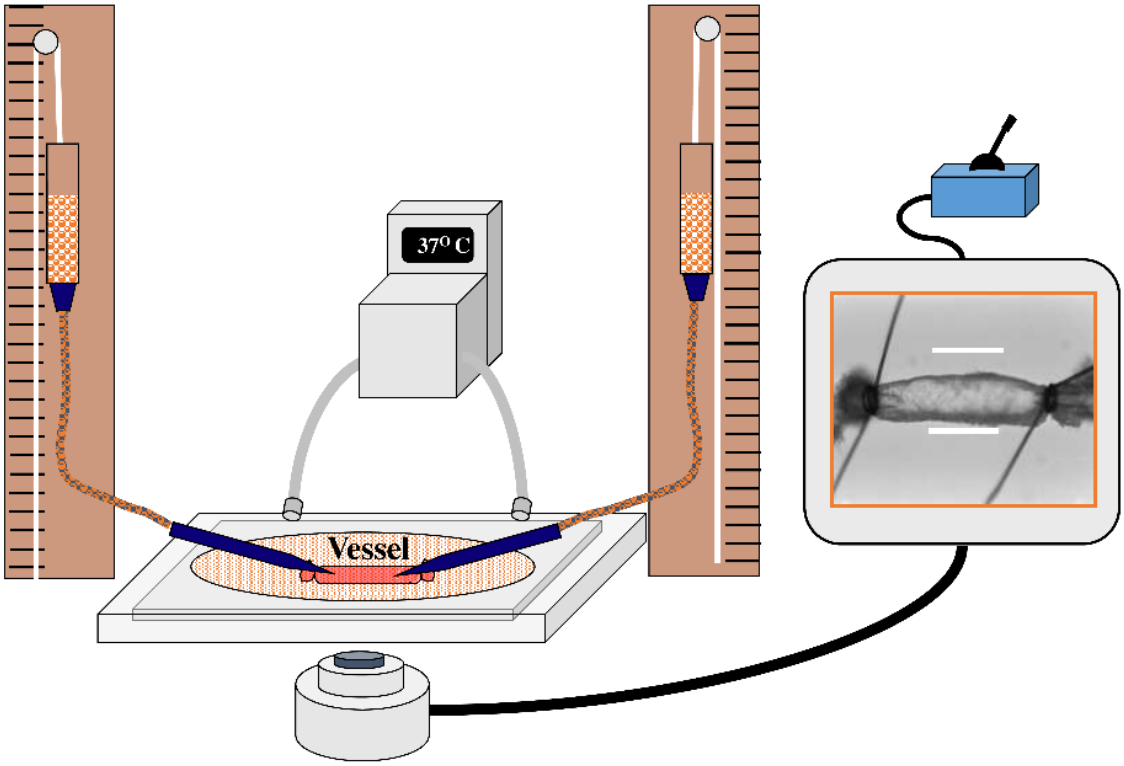

Figure 2. Pressure myography setup. An illustration of the pressure myograph for an artery in a Plexiglas chamber filled with physiological saline solution and double-cannulated with two glass micropipettes. The chamber was warmed to 37° by a circulating hot water bath, while micropipettes were connected to a fluid-filled reservoir to maintain intraluminal pressure at 60 mmHg. Vascular diameter was monitored constantly on a computer.

3.4 Data Analyses

Statistical analyses were conducted using SyStat SigmaPlot version 14.0 and GraphPad Prism version 8.0 was used for graphical presentation of data. Two-way ANOVA were used to

compare between group differences (i.e., independent variables are age and exercise status) in blood markers and vasoreactivity parameters (maximum physiological response, AUC as well as the Δmaximum physiological response and ΔAUC). Additionally, mixed model repeated measures ANOVA were used to assess differences in body mass (at intake and terminal timepoints for all experimental groups), and group by dose differences for insulin (%baseline diameter at insulin concentrations ranging from 1e-9 to 1e-6 M for each experimental group) or ET1 (%baseline diameter at ET1 concentrations ranging from 1e-12 to 1e-8 M for each experimental group). Post-hoc Student Newman-Keul's test were used to determine the location of significance. To determine the contributions of SOD mimetic and NOX inhibition to insulin-stimulated vasodilation and ET1-induced vasoconstriction within each group, delta analyses of max response and AUC were conducted by subtracting responses during untreated condition from responses in the SOD mimetic or NOX inhibitor pretreatment conditions. Fisher's exact analyses were used to compare proportion of pigs from each group that displayed either enhanced or attenuated insulin-stimulated vasodilation or ET1-induced vasoconstriction with SOD mimetic and NOX inhibitor pretreatment. Significance was set at $P \leq 0.05$. Cohen's *d* (24) effect size analyses were conducted to evaluate the magnitude and directional differences in insulin-stimulated vasodilation and ET1-induced vasoconstriction between juvenile and mature pigs as well as sedentary and exercise trained pigs. Results are presented as mean ± standard error of the mean and where possible individual data are presented.

4 Results

Body mass at intake was not significantly different between age-matched groups ($P \geq 0.37$). However, there was a significant group x time interaction effect that revealed terminal body mass was significantly lower for juvenile and mature exercise trained vs. sedentary age-matched pigs ($P < 0.001$; Figure 3). Plasma glucose and insulin were significantly higher, but triglyceride content was significantly lower in juvenile vs. mature pigs (main effect of age: $P \leq 0.03$; Table 2). There was a significant exercise x age interaction effect that revealed total cholesterol and HDL were significantly lower in juvenile exercise trained vs. sedentary age-matched pigs ($P \leq 0.04$; Table 2); but there were no other between group differences in these blood markers ($P \geq 0.07$). The vLDL+LDL was significantly lower in exercise trained vs. sedentary pigs (main effect of exercise training: $P = 0.02$; Table 2) as well as in juvenile vs. mature pigs (main effect of age: $P = 0.01$; Table 2).

Maximal vascular diameter achieved in Ca^{2+}-free PSS was significantly lower in juvenile (N=60: 219±10 µm) vs. mature (N=51: 257±11 µm) pigs (main effect of age: $P = 0.01$). However, there were no significant differences in maximal vascular diameter between sedentary and exercise trained pigs ($P = 0.53$). Moreover, maximal vasoconstriction following KCl was not significantly different between groups under any experimental conditions ($P \geq 0.57$). Absolute diameters achieved during experiments are presented in the Appendix: Table 4.

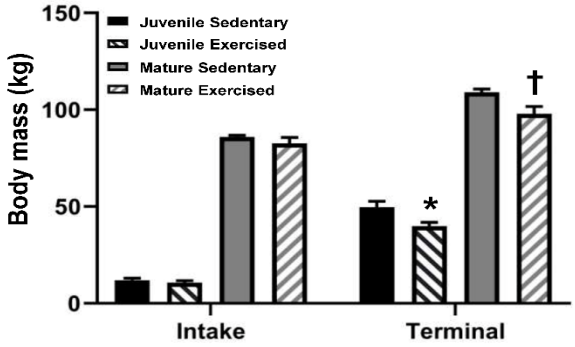

Figure 3. Body mass at intake and terminal time points. Body mass (kilograms) of juvenile sedentary (filled bars; N=10) and exercise trained (black hatched bars; N=10) as well as mature sedentary (grey bars; N=9) and exercise trained (grey hatched bars; N=8) groups. Data analyzed using a mixed model repeated measures ANOVA. Only interaction effects are denoted. *Significantly different from juvenile sedentary ($P < 0.05$). †Significantly different from mature sedentary ($P < 0.05$).

Table 2. Blood markers.

	Juvenile		Mature		Age	EX	Age x
Blood marker	SED	EX	SED	EX	Effect	Effect	EX
Plasma glucose (mmol/L)	10.4±0.9	10.3±0.6	8.5±0.5	9.1±0.7	*P*=0.03	*P*=0.76	*P*=0.62
Plasma insulin (µIU/mL)	11±1	9±1	13±2	18±4	*P*=0.01	*P*=0.40	*P*=0.07
Triglycerides (mmol/L)	0.9±0.1	0.7±0.1	1.4±0.3	1.3±0.2	*P*=0.02	*P*=0.30	*P*=0.65
Total Cholesterol (mmol/L)	3.3±0.2	2.5±0.1*	3.0±0.2	3.0±0.2	*P*=0.50	*P*=0.01	*P*=0.01
HDL (mmol/L)	1.9±0.1	1.5±0.1*	1.4±0.1	1.5±0.2	*P*=0.12	*P*=0.15	*P*=0.04
vLDL+LDL (mmol/L)	1.5±0.1	1.0±0.1	1.6±0.1	1.5±0.1	*P*=0.01	*P*=0.02	*P*=0.09

Blood markers presented as mean±standard error of the mean; HDL=high density lipoprotein; vLDL=very low density lipoprotein; LDL=low density lipoprotein; vLDL+LDL is calculated as ΔTotal cholesterol-HDL. *Significantly different from juvenile sedentary (*P*<0.05).

4.1 Vasomotor Responses to Insulin

Juvenile and mature sedentary pigs exhibited insulin-stimulated vasoconstriction. Conversly, juvenile and mature pigs that were exercise trained exhibited insulin-stimulated vasodilation (Figure 4A). There was a group x dose interaction effect that revealed insulin-stimulated vasodilation was significantly greater in juvenile exercise trained vs. juvenile sedentary pigs at each dose of insulin (*P*≤0.03; Figure 4A). Furthermore, insulin-stimulated vasodilation was significantly greater in mature exercise trained vs. juvenile sedentary pigs at 1e-7 and 1e-6 M of insulin *(P*≤0.02; Figure 4A). Both juvenile and mature exercise trained pigs displayed significantly greater insulin-stimulated vasodilation compared to mature sedentary pigs from 1e-8-1e-6 M of insulin (*P*≤0.02; Figure 4A). Moreover, there was a main effect of group indicating both the cumulaitve vasodilation (AUC) and maximal vasodilation in response to insulin were significantly greater in exercise trained vs. sedentary pigs (*P*<0.01; Figure 4B & C). However, there were no differences between juvenile and mature groups (*P*≥0.14; Figure 4B & C). The magnitude and directional effect of exercise and age on insulin AUC and the maximal physiological response are presented in the Appendix: Table 5.

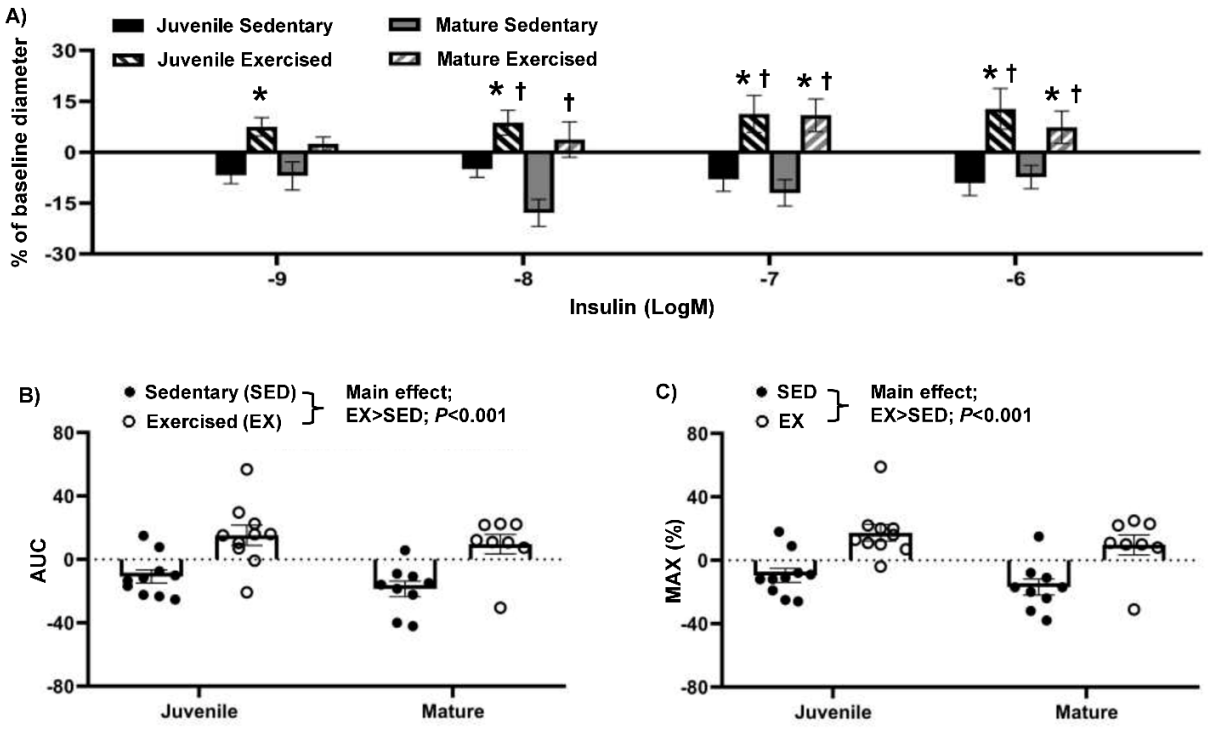

Figure 4. Vasoreactivity to insulin. A) Change in pial arteriolar diameter in response to insulin (1e-9-1e-6 M), relative to baseline, in juvenile sedentary (filled bars; N=10) and exercise trained (black hatched bars; N=10) as well as mature sedentary (grey bars; N=9) and exercise trained (grey hatched bars; N=8) groups. B&C) AUC and maximal response (MAX) derived from the insulin dose-response curve in juvenile and mature sedentary (filled circles) and exercise trained (open circles) groups. Data were analyzed using a mixed model repeated measures ANOVA (A) and a two-way ANOVA (B&C). *Significantly different from juvenile sedentary (P<0.05). †Significantly different from mature sedentary (P<0.05).

4.2 Oxidative Stress Regulation with Insulin Stimulation

In arteries preincubated with the SOD mimetic TEMPOL, the cumulative response to insulin was similar bewteen groups (P≥0.55; Figure 5A). However, the contribution of SOD mimetic pretreatment to the cumulative insulin response was significantly lower in exercise trained vs. sedentary pigs (main effect exercise training: P<0.01; Figure 5B). Probing further, whereas SOD mimetic pretreatment improved insulin-stimulated vasodialtion in 15 of 19 cases in sedentary pigs, it improved insulin-stimulated vasodilation in only 6 of 18 cases in exercise trained pigs (Figure 5C).

Similar to the cumulative responses, maximal responses to insulin were not significantly different between groups with SOD mimetic pretreatment (P≥0.47; Figure 5D). Additionally, the contribution of SOD mimetic pretreatment to the maximal insulin response was significantly lower in exercise trained vs. sedentary pigs (main effect of exercise training: P<0.01; Figure 5E). Indeed, whereas SOD mimetic pretreatment improved maximal insulin-stimulated vasodilation in 14 of 19

cases in sedentary pigs, it only imporved vasodilation in 4 of 18 cases in exercise trained pigs (Figure 5F). Taken together, a statistically significant proportion of exercise trained vs. sedentary pigs exhibited an attenuation in cumulative and maximal insulin-stimulated vasodilation with SOD mimetic pretreatment (P<0.05; Figure 5C & F).

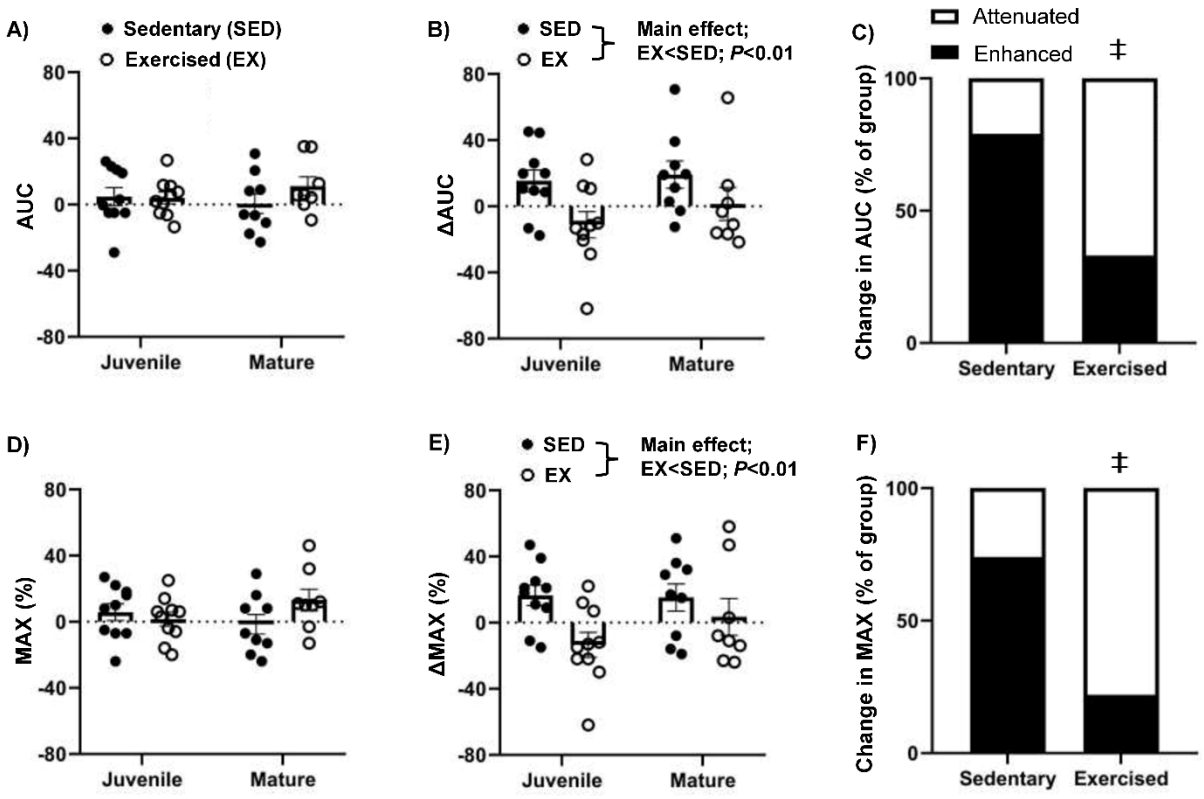

Figure 5. Vasoreactivity to insulin following SOD pretreatment. A&B) AUC and ΔAUC (responses from SOD condition–untreated) derived from the insulin dose-response curve in juvenile and mature sedentary (filled circles; N=9-10) and exercise trained (open circles; N=8-10) groups. C) Proportion of animals that SOD pretreatment either enhanced (filled portion) or attenuated (open portion) cumulative insulin-stimulated vasodilation. D&E) Maximal response (MAX) and ΔMAX (responses from SOD condition–untreated) derived from the insulin dose-response curve in juvenile and mature sedentary and exercise trained groups. F) Proportion of animals that SOD pretreatment either enhanced or attenuated maximal insulin-stimulated vasodilation. Data were analyzed using a two-way ANOVA (A, B, D and E) and Fisher's exact test (C&F). ‡Significantly less enhancement than sedentary animals (P<0.05).

In arteries preincubated with the NOX inhibitor Apocynin, the cumulative response to insulin was similar bewteen groups (P≥0.37; Figure 6A). However, the contribution of NOX inhibition pretreatment to the cumulative insulin response was significantly lower in exercise trained vs. sedentary pigs (main effect of exercise training: P<0.01; Figure 6B). Whereas NOX

inhibition pretreatment improved insulin-stimulated vasodilation in 15 of 19 cases in sedentary pigs, it only improved vasodilation in 4 of 18 cases in exercise trained pigs (Figure 6C).

Similar to the cumulative responses, maximal responses to insulin were not significantly different between groups with NOX inhibition ($P \geq 0.29$; Figure 6D). The contribution of NOX inhibiton pretreatment to maximal insulin-stimulated vasodilation was significantly lower in exercise trained vs. sedentary pigs (main effect of exercise training: $P<0.01$; Figure 6E). Although NOX inhibition pretreatment improved insulin-stimulated vasodilation in 15 of 19 cases in sedentary pigs, it only improved vasodilation in 4 of 18 cases in exercise trained pigs (Figure 6F). Accordngly, a statistically significant proportion of exercise trained vs. sedentary pigs exhibited an attenuation in cumulative and maximal insulin-stimulated vasodilation with NOX inhibitor pretreatment ($P<0.05$; Figure 6C & F).

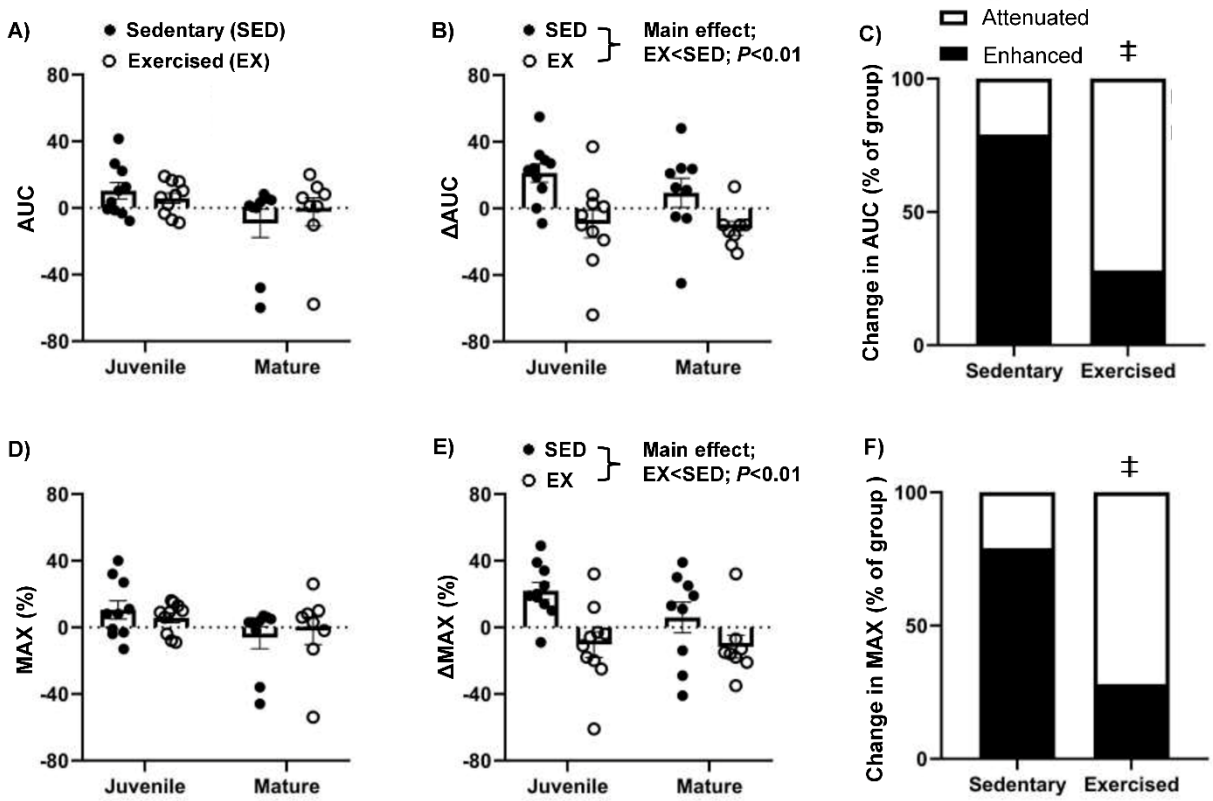

Figure 6. Vasoreactivity to insulin following NOX inhibition. A&B) AUC and ΔAUC (responses from NOX condition–untreated) derived from the insulin dose-response curve in juvenile and mature sedentary (filled circles; N=9-10) and exercise trained (open circles; N=8-10) groups. C) Proportion of animals that NOX pretreatment either enhanced (filled portion) or attenuated (open portion) cumulative insulin-stimulated vasodilation. D&E) Maximal response (MAX) and ΔMAX (responses from NOX condition–untreated) derived from the insulin dose-response curve in juvenile and mature sedentary and exercise trained groups. F) Proportion of animals that NOX pretreatment either enhanced or attenuated maximal insulin-stimulated vasodilation. Data were analyzed using a two-way ANOVA (A, B, D and E) and Fisher's exact test (C&F). ‡Significantly less enhancement than sedentary animals ($P<0.05$).

4.3 Vasomotor Responses to ET1

ET1-induced vasoconstriction was similar between groups ($P \geq 0.35$; Figure 7A). Likewise, cumulative and maximal responses to ET1 were not statistically different between groups ($P \geq 0.11$; Figure 7B & C). Neither SOD mimetic nor NOX inhibition pretreatment influenced vasomotor responses to ET1 differentially between groups ($P \geq 0.18$; Figures 8 & 9). The magnitude and directional effect of exercise and age on ET1 AUC and the maximal physiological response are presented in the Appendix: Table 5.

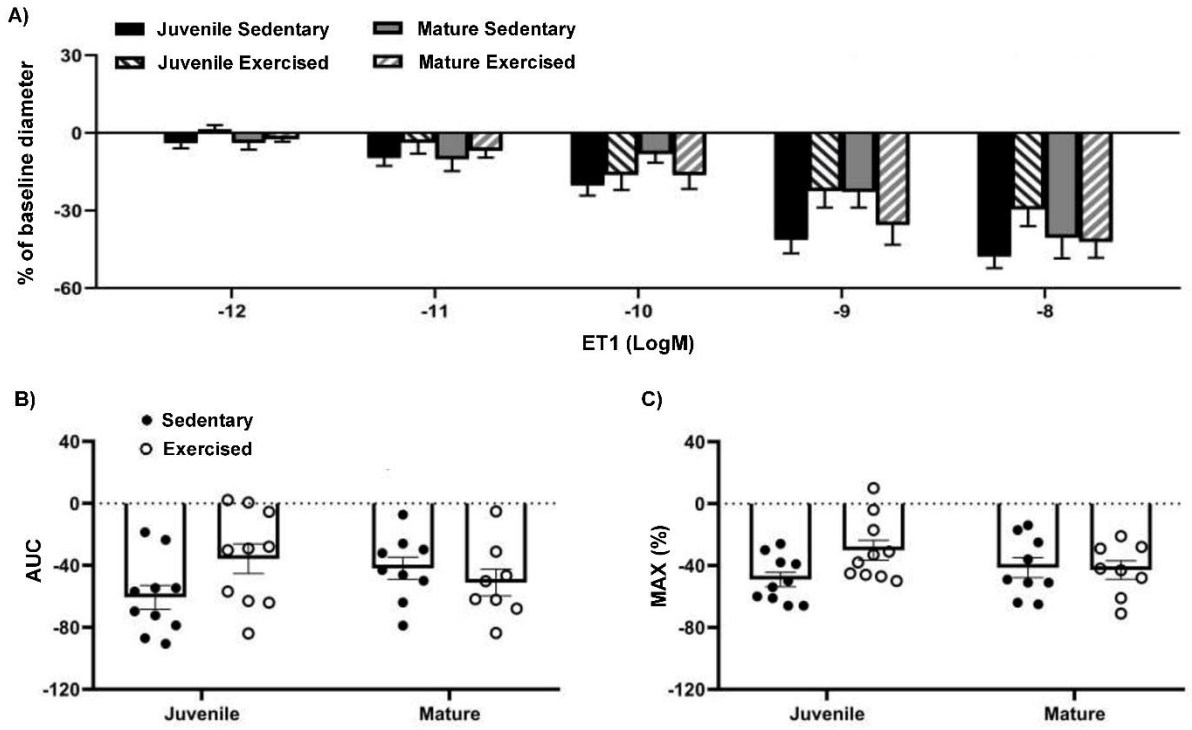

Figure 7. Vasoreactivity to ET1. A) Change in pial arteriolar diameter in response to ET1 (1e-12-1e-7 M), relative to baseline, in juvenile sedentary (filled bars; N=10) and exercise trained (black hatched bars; N=10) as well as mature sedentary (grey bars; N=9) and exercise trained (grey hatched bars; N=8) groups. B&C) AUC and maximal response (MAX) derived from the ET1 dose-response curve in juvenile and mature sedentary (filled circles) and exercise trained (open circles) groups. Data were analyzed using a mixed model repeated measures ANOVA (A) and a two-way ANOVA (B&C).

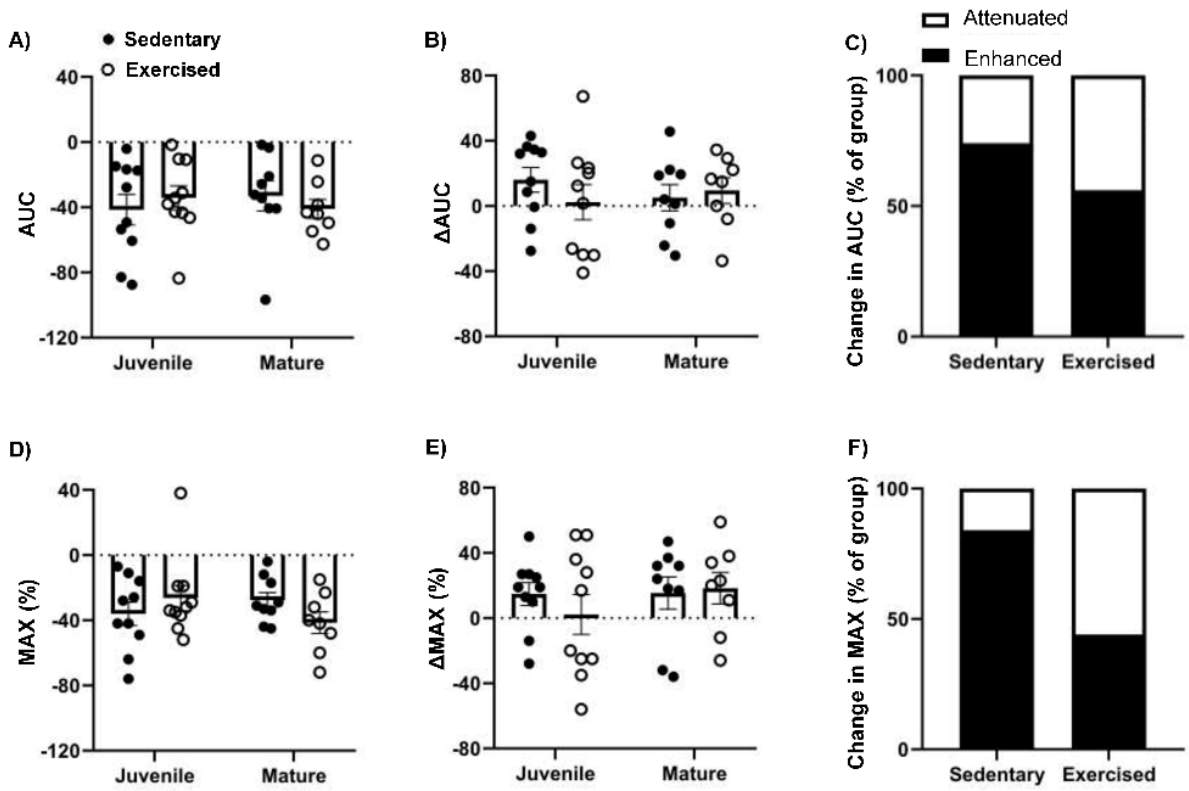

Figure 8. Vasoreactivity to ET1 following SOD pretreatment. A&B) AUC and ΔAUC (responses from SOD condition–untreated) derived from the ET1 dose-response curve in juvenile and mature sedentary (filled circles; N=9-10) and exercise trained (open circles; N=8-10) groups. C) Proportion of animals that SOD pretreatment either enhanced (filled portion) or attenuated (open portion) cumulative ET1-induced vasoconstriction. D&E) Maximal response (MAX) and ΔMAX (responses from SOD condition–untreated) derived from the ET1 dose-response curve in juvenile and mature sedentary and exercise trained groups. F) Proportion of animals that SOD pretreatment either enhanced or attenuated maximal ET1-induced vasoconstriction. Data were analyzed using a two-way ANOVA (A, B, D and E) and Fisher's exact test (C&F).

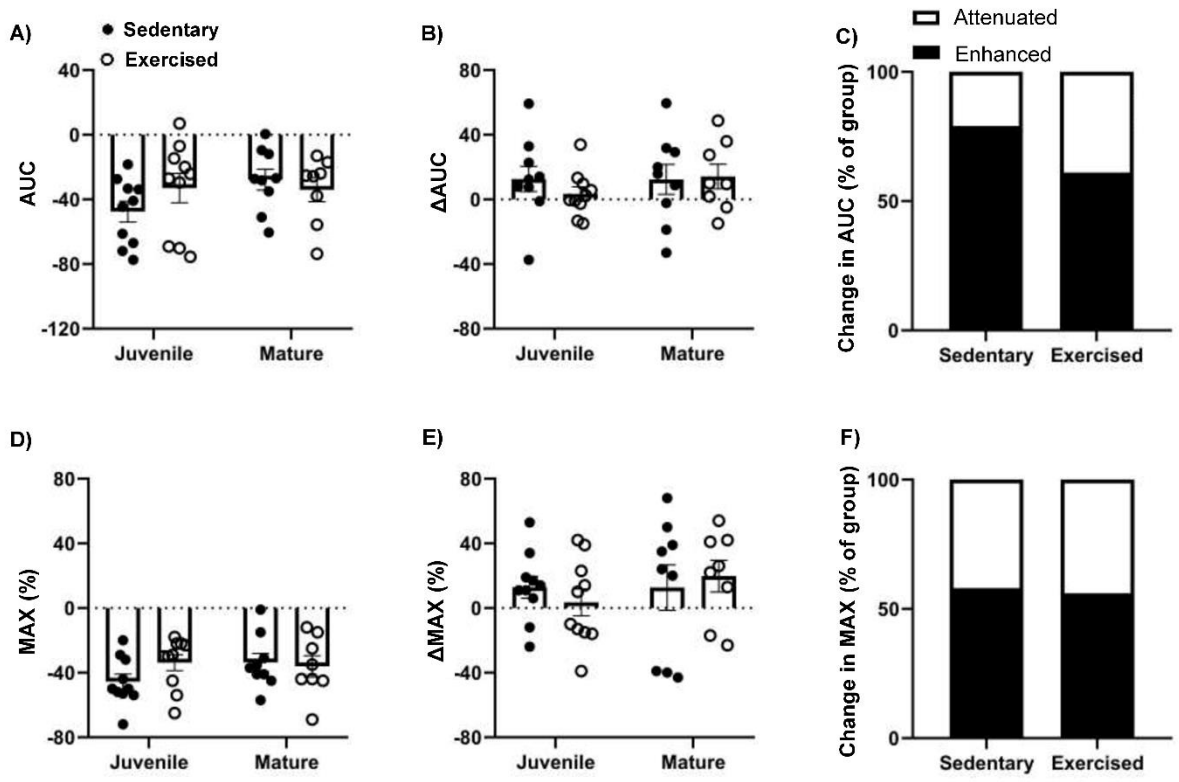

Figure 9. Vasoreactivity to ET1 following NOX pretreatment. A&B) AUC and ΔAUC (responses from NOX condition–untreated) derived from the ET1 dose-response curve in juvenile and mature sedentary (filled circles; N=9-10) and exercise trained (open circles; N=8-10) groups. C) Proportion of animals that NOX pretreatment either enhanced (filled portion) or attenuated (open portion) cumulative ET1-induced vasoconstriction. D&E) Maximal response (MAX) and ΔMAX (responses from NOX condition–untreated) derived from the ET1 dose-response curve in juvenile and mature sedentary and exercise trained groups. F) Proportion of animals that NOX pretreatment either enhanced or attenuated maximal ET1-induced vasoconstriction. Data were analyzed using a two-way ANOVA (A, B, D and E) and Fisher's exact test (C&F).

5 Discussion

A major finding of the current study was that sedentary pigs displayed selective insulin resistance, evidenced by insulin-stimulated pial arteriolar vasoconstriction. Acute SOD mimetic or NOX inhibition pretreatment attenuated insulin-stimulated vasoconstriction; highlighting the potential upstream modulatory role of ROS in cerebrovascular selective insulin resistance. The absence of a main effect of age in vasomotor responses to insulin suggests there is a uniform pathophysiology of selective insulin resistance at early and later stages of development. Another major finding of the current study was that juvenile and mature exercise trained pigs exhibited pial arteriolar insulin-stimulated vasodilation. However, unexpectedly, insulin-stimulated cerebral vasodilation was attenuated under the SOD mimetic and NOX inhibitor pretreatment conditions in a significant proportion of exercise trained pigs. Conceivably, basal, or insulin-stimulated ROS may have contributed to insulin-stimulated vasodilation exhibited by exercise trained animals. Contrary to our conjecture, cerebral vasomotor responses to ET1 were not significantly different between sedentary and exercise trained pigs, at either developmental stage, and the comparisons were unaffected by SOD mimetic or NOX inhibitor pretreatment. Collectively, these data indicate that by impairing the vasodilatory actions of insulin (i.e., PI3K/Akt/NO pathway) excessive ROS accumulation is a likely contributor to selective insulin resistance. HIIT-induced improvements in insulin-stimulated vasodilation may be related to improved ROS regulation. Paradoxically, ROS production may serve a role in realizing the vasodilatory actions of insulin in the cerebrovasculature of exercise-trained animals.

5.1 Discrepant Vascular Insulin Signalling

In the presented data, sedentary pigs displayed insulin-stimulated vasoconstriction, which was treatable with pharmacological SOD mimetic and NOX inhibition, implicating aberrant ROS signalling in cerebrovascular dysfunction. It has been reported that glucotoxicity are major contributors to impaired vascular function in obese populations (38). An abundance of glucose present in the blood, which was exhibited by pigs in the current investigation, can attenuate endothelium-dependent vasodilation (50). At the molecular level, excess circulating glucose can stimulate ROS overproduction in the endothelium, and subsequently augment scavenging of NO

(94). Although the source of ROS was not examined in current experiments, evidence indicates hyperglycemia is conjunctional with surplus O^{2-}, derived from mitochondrial NOX2 activity (10, 104) as well as membrane bound NOX1 and 2 activity (62). Excess O^{2-}, beyond which can be catalyzed to H_2O_2 by SOD, scavenges NO and forms $ONOO^-$ (101). In addition to decreasing NO bioavailability, $ONOO^-$ can enhance eNOS uncoupling, thereby reducing NO production in favor of O^{2-} (13). This cycle of excessive ROS production, resulting in decreased NO signalling, may be compounded by insulin-stimulation, and in the current study, the role of ROS was investigated under insulin-stimulated conditions. In total, both hyperglycemia and insulin-stimulated NOX-mediated production of O^{2-}, culminating in excessive $ONOO^-$, may have served a role in the manifestation of selective insulin resistance observed in the sedentary animals.

Despite similar levels of hyperglycemia as sedentary animals, the exercise trained groups displayed insulin-stimulated vasodilation. In the majority of exercise-trained pigs, contrasted by sedentary pigs, a SOD mimetic and NOX inhibition negatively affected the vasodilatory actions of insulin. The attenuation of insulin-stimulated vasodilation while inhibiting ROS may be related to differential oxidative stress regulation in the exercise-trained groups. Whereby, enhanced insulin-stimulated or basal SOD activity favours the catalyzation of O^{2-} to H_2O_2, instead of the accumulation of O^{2-} and subsequent formation of $ONOO^-$ (101). Whereas O^{2-} impairs vasodilation, evidence indicates H_2O_2 elicits vasodilation (35, 55, 95). More specifically, Lacza and colleagues demonstrated that treatment with the H_2O_2 scavenger catalase blunts endothelial-dependent, K^+ channel-mediated, vasodilation in pig cerebral arteries (55). In addition, H_2O_2 is also implicated in facilitating phosphorylation and activation of IRS-1 and Akt by inhibiting PTP1B and PP2A, respectively (59). Thus, H_2O_2 may contribute to insulin-stimulated vasodilation directly through enhanced K^+-channel activity and indirectly by facilitating increased PI3K/Akt/NO signalling. However, it is important to note, in the presence of Fe^{2+}, excess H_2O_2 can be decomposed to form $^{\bullet}OH$ which may cause vasoconstriction (70). Moreover, the SOD mimetic TEMPOL, which was used in the present experiments, can also mimic the catalytic activity of $MbFe^{III}$ and enhance H_2O_2 dismutation, thereby reducing H_2O_2 content (91). Taken together, in the current study, it is possible both the SOD mimetic and NOX inhibitor pretreatments dysregulated ROS in exercise-trained pigs, resulting in attenuated insulin-stimulated vasodilation.

In the current study, vasoreactivity to ET1 was similar between groups, suggesting differences between sedentary and exercise-trained pigs in vasomotor responses to insulin were

independent of disparate vascular sensitivity to ET1. As previously outlined, enhanced insulin-stimulated vasodilation could stem from an enhancement of NO signalling as well as an attenuated influence or reductions in ET1 production and secretion (86). Indeed, in obese rodents, chronic wheel running augments insulin-stimulated vasodilation in cerebral arteries by increasing the contribution from NO and diminishing the contribution of ET1 to cerebrovascular insulin signalling (78). Additional evidence reveals exercise training decreases contractile responses to ET1 in pig coronary arteries (12). Although the present data do not preclude the possibility of differences in insulin-stimulated ET1 signalling, it can be deduced that, unlike the previous report in coronary arteries, contractile responses to ET1 were not influenced by exercise training. Combined with evidence that neither SOD mimetic nor NOX inhibition influenced vasoreactivity to ET1, these data point to a potential enhancement in insulin-stimulated NO production rather than an attenuation of ET1 signalling with exercise training. Therefore, it is important to interrogate further mechanisms that modulate insulin-stimulated NO signalling with exercise training.

5.2 Potential Exercise Mechanism of Action

Improvements in insulin-stimulated vasodilation observed in the present study may reflect an adaptation to repeated bouts of exercise-induced hyperemia during HIIT. Current evidence indicates that exercise training imparts a beneficial effect on vascular insulin sensitivity in a localized rather than a systemic manner (76). That is, exercise training-induced improvements in vascular insulin sensitivity appear to be restricted to vascular beds engaged during the exercise bout. Because both insulin-stimulated vasodilation and vascular shear stress engage the PI3K/Akt/NO pathway, researchers speculate the exercise hyperemic response primes the endothelium to become more sensitive to insulin. Of note, cerebral blood flow increases during acute interval aerobic exercise, raising the possibility that in the present study, by repeatedly engaging the PI3K/Akt/NO pathway, the HIIT program improved insulin-stimulated cerebral vasodilation. However, as discussed in the literature review, to realize the full functional benefits, increases in NO production must coincide with improved ROS regulation, otherwise NO may be scavenged, thereby decreasing NO bioavailability, and subsequently reduce NO production through eNOS uncoupling.

Exercise-induced hyperemia and vascular shear stress can augment ROS production, while exercise training can enhance oxidative stress defences; thus presenting another paradoxical

relationship between exercise and oxidative stress regulation (2, 109). Briefly, although shear stress has been shown to increase O^{2-} production (43), it has also been shown to upregulate SOD2 and eNOS expression, in endothelial cell culture studies (29, 45). Indeed, likely related to this acute phase response, chronic exercise training leads to an attenuation of NOX and an augmentation of SOD in the cerebrovasculature (31). Thus, conjunctional with the NO-stimulating, priming effect of exercise-induced hyperemia and vascular shear stress, enhanced regulation of oxidative stress with exercise training may represent an integrative mechanism underlying improved vascular insulin signalling observed in the exercise trained animals from the current investigation.

5.3 Exercise Prescription Considerations

Identifying clinically relevant training modalities that amplify hyperemic responses to the brain may be critical for devising appropriate exercise interventions to alleviate cerebrovascular selective insulin resistance. Although blood flow to contracting skeletal muscle can increase by up to 800% during exercise, more modest increases (10-30%) are observed in brain blood flow (97). With respect to selecting the optimal exercise intensity to increase cerebral blood flow, an investigation in pigs demonstrated that running at 70% and 100% of VO_{2max} (equivalent to moderate-high and maximal exercise intensity, respectively) exhibited increases in flow to brain regions responsible for locomotion, maintenance of equilibrium, and cardiorespiratory control (27). In humans, one study demonstrated that participants that performed 3 separate 8-minute bouts of cycling at target heart rates of 90-, 120-, and 180-beats per minute exhibited increases in middle cerebral artery blood flow velocity at 120- and 180-, but not 90-beats per minute (69). These data suggest that the transition from rest to moderate or vigorous but not mild exercise could elicit a greater shear-stress stimulus in cerebral circulation. Furthermore, when compared with work-matched continuous aerobic exercise, evidence indicates HIIT elicits similar increases blood flow velocity values during exercise, but greater increases over the whole exercise session on account of the hyperemic response observed during recovery periods between intervals. This raises the possibility that HIIT could impart superior benefits on cerebrovascular insulin signalling by virtue of maximizing exercise-induced hyperemia in the cerebral circulation. Currently, our data provides the only available evidence for chronic HIIT-induced adaptations in vascular insulin signalling in the cerebral circulation. Interrogating shear stress coupled with enhanced ROS regulation as a

unifying mechanism for augmented vascular insulin sensitivity with HIIT is an indispensable, imminent undertaking.

5.4 Clinical Perspective

Presently, there are no exercise training recommendations to optimize brain vascular or brain health. Notably, cerebrovascular insulin resistance and oxidative stress dysregulation frequently accompany several neurodegenerative disorders, such as dementia and Alzheimer's disease (19, 85). Indeed, it has been posited that alleviation of oxidative stress in the setting of selective insulin resistance may normalize cerebral perfusion and protect against neurodegenerative diseases (46). Herein, our findings indicated that HIIT improved oxidative stress regulation leading to enhanced cerebrovascular insulin sensitivity; hence, it is reasonable to speculate that this training modality could preserve and improve brain function in clinical settings.

The manifestation of selective insulin resistance in juvenile sedentary pigs represents another critical consideration for the clinical setting. Whereby, the contemporary rise in childhood obesity, reaching >14% in Canada (84), is an uncharted territory for the impending prevalence of cerebrovascular disease and selective insulin resistance. The vast majority cohorts studied in the presented scientific literature consisted of adult subjects; therefore, little is known about the etiology of cerebrovascular selective insulin resistance in juvenile populations. Importantly, Olver and colleagues demonstrated that indices of cerebrovascular and brain insulin resistance develop concomitantly and early on in the development of obesity in juvenile Ossabaw miniature-pigs. Assuredly, if cerebrovascular selective insulin resistance is not managed at an early stage, the deleterious outcomes on individual and societal levels may be amplified. Other groups have demonstrated that exercise training can enhance endothelium-dependent vasodilation in obese children (66); therefore, exercise may enhance vascular insulin sensitivity following the development of selective insulin resistance in childhood obesity. Ultimately, we emphasize the protective role of acute exercise and chronic training on cerebrovascular function, and by extension, normal brain health; more specifically, attention should be drawn to this prospect at early stages of development.

5.5 Limitations

There are some experimental design limitations in the present study that need to be taken into consideration. As a function of deficient Ossabaw miniature-pig colonies in Canada, we were unable to obtain enough animals for a true control group; wherein pigs would be euthanized upon

acquisition, prior to exercise training, for the purpose of determining baseline pial arteriolar vasomotor responses to insulin or ET1. Moreover, owing to the challenging nature of conducting a blood draw on pigs without sedation, we did not assess changes in circulating biomarkers over the course of the study. As a result, comparisons were made between groups at a terminal end point, rather than assessing changes over time. Future studies on the subject may benefit from employing a baseline group and longitudinal blood draws, allowing for determination of developmental aspects of this study, including changes in vasomotor responses and circulating biomarkers, respectively. Moreover, while the functional vascular responses allowed for inferences relating to insulin signalling and oxidative stress regulation, further confirmatory studies of genomic and proteomic differences could enhance the internal validity of our findings. Consequently, additional pial arteriolar segments were isolated and stored for mRNA sequencing and Western blotting, at a later date. Additionally, it is important to account for the possibility of variance in vascular responses to insulin, between whole-organism, *in vivo* blood flow control and *ex vivo,* isolated blood vessel experiments. Although the latter may be superior for isolating the independent effects of insulin stimulation on vasomotor control, it may not be representative cerebrovascular responses to insulin in conscious, living animals. Furthermore, although the Ossabaw miniature-pig may be a representative model for human disease, investigations in human subjects, employing appropriate measures (i.e., transcranial Doppler and functional magnetic resonance imaging) are necessary to confirm the translational relevance of these findings. Finally, we acquiesce the potential for researcher bias as this study was conducted during the COVID-19 pandemic and that restricted our group to a small number of study personnel, which disallowed us from establishing formal blinding from experimental groups during data acquisition. Coordination of additional study personnel in future investigations is requisite to establish blinding, and thus mitigate potential researcher bias and ensure appropriate data interpretation.

5.6 Summary

The present study provided novel insight into cerebrovascular insulin signalling and the modulatory roles of oxidative stress in sedentary and exercise-trained states, in juvenile and mature Ossabaw miniature-pigs. Whereas sedentary pigs displayed insulin-induced vasoconstriction, exercise-trained pigs exhibited insulin-induced vasodilation. Likewise, although SOD mimetic or NOX inhibitor pretreatments improved insulin-stimulated vasodilation in sedentary pigs, they attenuated insulin-stimulated vasodilation in exercise trained pigs. These data implicate aberrant

oxidative stress regulation in the setting of selective insulin resistance, but a beneficial contribution of ROS to insulin-stimulated vasodilation in exercise-trained states. Furthermore, despite the lack of improvement in hyperglycemia or hyperinsulinemia, these data provide support for the use of HIIT as an intervention for improving ROS regulation and insulin sensitivity in the cerebrovasculature.

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Appendix

Table 3. Pig feed mix.

Ingredients list	% of mix
Spring grain wheat	27
Soybean meal	23
Sucrose	21
Beef tallow	12
Jefo dairy fat	12
Lysine HCl	1
Limestone	1
Dicalcium phosphate	1
Salt (sodium chloride)	1
di-Methionine	1
L-Threonine	>1
GFC USask Swine Starter 4	>1
L-Tryptophan	>1

Table 4. Absolute diameters of pial arteries during experiments.

Vessel inner diameter (μm)	Juvenile		Mature	
	SED (N=10)	EX (N=10)	SED (N=9)	EX (N=8)
Untreated Condition				
Pre-insulin	125±14	123±14	159±14	138±15
1e-9	117±10	131±10	145±11	142±12
1e-8	120±12	131±12	128±13	141±14
1e-7	117±11	135±11	138±11	152±12
1e-6	116±11	136±11	144±11	148±12
Pre-ET1	135±12	151±12	185±12	167±13
1e-12	130±14	152±14	178±15	164±16
1e-11	122±15	144±13	165±15	156±14
1e-10	107±15	127±14	170±14	143±15
1e-9	77±16	116±11	143±17	108±13
1e-8	70±14	101±16	111±14	99±16
Ca^{2+}-free wash	201±17	190±20	242±15	218±18
SOD mimetic				
Pre-insulin	140±11	159±10	166±13	167±8
1e-9	142±11	164±10	167±13	167±10
1e-8	147±13	167±9	167±12	174±10
1e-7	146±11	161±10	163±10	175±9
1e-6	150±10	161±12	163±12	177±12
Pre-ET1	173±12	176±15	174±13	182±14
1e-12	170±15	176±14	170±15	184±16
1e-11	166±15	164±12	155±18	176±14
1e-10	149±14	160±13	155±14	152±11
1e-9	117±17	136±12	135±12	140±13
1e-8	101±14	118±14	127±12	114±13
Ca^{2+}-free wash	215±26	251±21	249±30	251±35
NOX inhibition				
Pre-insulin	158±13	155±8	232±14	192±12
1e-9	144±13	154±8	223±14	190±12
1e-8	147±12	160±10	223±13	186±10
1e-7	153±11	163±9	222±11	196±10
1e-6	158±12	162±11	221±12	200±11
Pre-ET1	177±15	170±15	249±12	202±10
1e-12	174±15	170±14	256±15	198±16
1e-11	169±13	170±13	253±11	202±16
1e-10	154±14	154±11	232±16	184±12
1e-9	128±15	135±14	201±15	163±14
1e-8	97±12	117±15	166±9	141±15
Ca^{2+}-free wash	239±20	226±26	333±23	269±41

Table 5. Effect size analyses.

		Effect Size	Description
AUC	Insulin-stimulated vasodilation		
	Sedentary vs. Exercise trained	1.7	Large positive effect of exercise
	Juvenile vs. Mature	-0.3	Small negative effect of age
	ET1-induced vasoconstriction		
	Sedentary vs. Exercise trained	0.3	Small positive effect of exercise
	Juvenile vs. Mature	0.1	Small positive effect of age
MAX	Insulin-stimulated vasodilation		
	Sedentary vs. Exercise trained	1.5	Large positive effect of exercise
	Juvenile vs. Mature	-0.4	Small negative effect of age
	ET1-induced vasoconstriction		
	Sedentary vs. Exercise trained	0.4	Small positive effect of exercise
	Juvenile vs. Mature	-0.1	Small negative effect of age

Effect size: <0.5 is a small effect: ≥0.5, <0.8 is a medium effect; ≥0.8 is a large effect; + denotes a positive effect and – denotes a negative effect