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ABSTRACT

Effects of Insulin Sensitivity Modulators on the Mitochondrial Fate of Oxygen in Skeletal Muscle

by Daniel A. Kane

April, 2010

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Increasingly, reactive oxygen species (ROS) are implicated in the development of insulin resistance. To test the hypothesis that modulators of insulin sensitivity (i.e., metformin, ovarian sex steroids and exercise training) affect the fate of oxygen in skeletal muscle, mitochondrial H_2O_2 emission (mE_{H2O2}) and respiratory O_2 flux (JO_2) were measured in saponin-permeabilized myofibers from rodents and women. Concommitant with improved glucose tolerance, complex I-linked mE_{H2O2}, but not JO_2, was reduced in metformin-treated obese rats to rates near or below those in the lean animals. *Ex vivo* dose-response experiments revealed that metformin inhibits complex I-linked mE_{H2O2} at a concentration ~2 orders of magnitude lower than that required to inhibit JO_2. To determine if estradiol or progesterone directly affect mitochondrial function, saponin-permeabilized vastus lateralis myofibers biopsied from women in the menstrual cycle follicular phase were incubated breifly in luteal phase serum concentrations of estradiol, progesterone, or both. While progesterone alone inhibited respiration, the effect was absent in the presence of estradiol. Progesterone, alone or in combination with estradiol increased complex I-linked mE_{H2O2}. Complex I-linked mE_{H2O2} measured in permeabilized myofibers from insulin sensitive and resistant women correlated significantly with serum progesterone in these subjects. Moreover, mE_{H2O2} was more than 80% greater in the insulin resistant women. Regular exercise

is known to improve insulin sensitivity. To determine the effects of exercise training on mitochondrial function, mE_{H2O2} and JO_2 were measured in saponin-permeabilized vastus lateralis myofibers from lean (BMI < 30) and obese (BMI > 30) women before (Pre) and after (Post) 8 weeks of exercise training (8WT = stationary cycling, 1 h/d, 5 d/w at heart rate corresponding to 70-75% VO_{2peak}). Interestingly, while Pre-Post there were no changes in JO_2 supported by multiple substrates or calculated ratios of respiratory control, there was a reduction in the potential for complex I-linked mE_{H2O2} following training in the lean women. Altogether, the results of this project support the notion that modulators of insulin sensitivity may do so through their ability to affect complex I-linked mE_{H2O2}, but not necessarily JO_2 in skeletal muscle.

EFFECTS OF INSULIN SENSITIVITY MODULATORS ON THE MITOCHONDRIAL FATE OF OXYGEN IN SKELETAL MUSCLE

A Dissertation

Presented To

The Faculty of the Department of Exercise and Sport Science

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

by

Daniel A. Kane

April, 2010

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DEDICATION

I dedicate this dissertation to my wonderful parents.

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the committee, I always felt comfortable garnering advice, discussing ideas, or soliciting helpful feedback.

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LIST OF SYMBOLS, ABBREVIATIONS, & DEFINITIONS

8WT:	eight weeks of exercise training, 1 hour per day at 70-75% $\dot{V}O_{2peak}$, 5 days per week
ACR	adenylate control ratio, uncoupled respiration/state 3 respiration
ADP	adenosine diphosphate
+ADP	"plus exogenous adenosine diphosphate"
Ag/AgCl	Silver/Silver Chloride
Akt	protein kinase B
Amplex red:	*N*-acetyl-3,7-dihydroxyphenoxazine, a redox-sensitive fluorescent dye
AMPK	adenosine monophosphate-activated protein kinase
ANCOVA	analysis of covariance
ANOVA	analysis of variance
ANT	adenine nucleotide translocase
ANT1	skeletal muscle/cardiac-specific isoform of adenine nucleotide translocase
Antimycin A	inhibitor of the cytochrome *c* reductase portion of complex III
ATP	adenosine triphosphate
Atractyloside	inhibitor of the adenine nucleotide translocase
AUC	area under curve
AW	African American women
BCA	bicinchoninic acid, used to determine total level of protein in solution
BMI	body mass index, $kg \cdot (m^2)^{-1}$
%BF	percent body fat

Bongkrekic acid	inhibitor of adenine nucleotide translocase
BTS	*N*-Benzyl-*p*-toluene sulphonamide, an inhibitor of myosin II
Buffer X	physiological relaxing buffer; permeabilization buffer
CPT1	carnitine palmitoyl transferase 1
+cyt *c*	"plus exogenous cytochrome *c*," to verify mitochondrial intactness
Cytochrome *c*	a small heme protein loosely bound to the outer surface of the inner mitochondrial membrane which transfers electrons from complex III to IV
%CV	ratio of the standard deviation to the mean multiplied by 100, a normalized measure of dispersion of a probability distribution
Complex I	NADH:ubiquinone oxidoreductase
Complex II	succinate dehydrogenase
Complex III	coenzyme Q : cytochrome *c* — oxidoreductase
Complex IV	cytochrome *c* oxidase
Complex V	mitochondrial ATP synthase
CW	caucasian women
DCF	2′,7′-dichlorodihydrofluorescein, relatively non-specific peroxide-sensitive fluorophore
$\Delta\Psi$	mitochondrial membrane potential, delta psi, millivolts
DEXA	dual energy X-ray absorptiometry; used to determine percent body fat
dL	deciliter, 10 liters
$\Delta\mu_{H+}$	proton electrochemical gradient ($kJ\cdot mol^{-1}$)
DTT	dithiothreitol, a strong chemical reductant
E2	17β-estradiol (a.k.a., estradiol), an ovarian sex steroid hormone
E2+P4	estradiol plus progesterone

ELISA	enzyme-linked immunosorbant assay
EDTA	ethylenediaminetetraacetic acid, a polyamino carboxylic acid used as a chelating agent
ECG	electrocardiograph, transthoracic interpretation of the electrical activity of the heart over time
ESR or EPR	electron spin resonance/electron paramagnetic resonance spectroscopy, a non-invasive technique for studying chemical species with one or more unpaired electrons
ETF	electron transfer flavoprotein, involved in transferring electrons from β-oxidation of fatty acids to the mitochondrial electron transfer flavoprotein dehydrogenase
ETS	mitochondrial electron transport system (a.k.a. electron transport chain, ETC)
ETFDH	electron transfer flavoprotein dehydrogenase
$FADH_2$	flavin adenine dinucleotide, a redox cofactor
+FCCP	"plus exogenous carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone"
FCCP	carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone, a lipophilic ionophore used to experimentally uncouple oxidative phosphorylation in mitochondria
FMN	flavin mononucleotide, a prosthetic group in the mitochondrial complex I
x g	multiple of the force of the Earth's gravitational pull at sea-level
g	gram
GLUT	glucose transporter protein
G/M	glutamate plus malate, a substrate combination supporting complex I-linked respiration
+Gp	"plus exogenous glycerophosphate, the substrate for the mitochondrial glycerophosphate dehydrogenase"

GSH	reduced glutathione
GSSG	oxidized glutathione
h	hour
H_2O	water
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, an organic chemical buffering agent
HO•	hydroxyl radical, the neutral form of the hydroxide ion
HOMA-IR	Homeostatic model assessment of insulin resistance, calculated from fasting plasma or serum glucose (mg/dL) and insulin (μU/mL) by the equation: HOMA-IR = (glucose • insulin) • 405^{-1}
HyPer:	genetically encoded fluorescent sensor capable of detecting intracellular hydrogen peroxide
i.p.	intraperitoneal
IR	insulin resistant, HOMA-IR > 3.6
IRS	insulin receptor substrate
IS	insulin sensitive, HOMA-IR < 3.6
IVGTT	intravenous glucose tolerance test
JO_2	respiratory oxygen flux ($pmol \cdot s^{-1} \cdot mg$ dry wt^{-1})
kDa	1000 Daltons, 1000 unified atomic mass units, or the approximate mass of a 1000 hydrogen atoms
KCl	potassium chloride
K_2EGTA	potassium ethylene glycol tetraacetic acid, a chelating agent
kg	kilogram, $1 \cdot 10^3$ grams
K_m	the substrate concentration at half the maximum velocity of an enzymatic reaction or reactions of an enzymatic reaction or reactions

K-MES	potassium- 2-(*N*-morpholino)ethanesulfonic acid, a physiological buffering agent
K_{50app}	the apparent substrate concentration at half the maximum velocity of an enzymatic reaction or reactions
K_2HPO_4	potassium phosphate, dibasic
L6	a continuous, cloned line of myoblasts originally derived from the thigh muscle of a newborn rat
LKB1	a kinase capable of phosphorylating adenine monophosphate-activated protein kinase
M-21	synthetic inhibitor of the adenine nucleotide translocase
Malonate	competitive inhibitor of succinate dehydrogenase
MAPK	mitogen-activated protein kinase, serine/threonine-specific protein kinases involved in the cellular response to extracellular stimuli
MDA	malondialdehyde, an often-measured end product of lipid peroxidation
mE_{H2O2}	mitochondrial H_2O_2 emission ($pmol \cdot min^{-1} \cdot mg\ dry\ wt^{-1}$), H_2O_2 produced by the mitochondria minus that which is scavenged by the mitochondria
mg	millisecond, $1 \cdot 10^{-3}$ grams
$MgCl_2$-$6H_2O$	hydrated magnesium chloride
mg	milligram, $1 \cdot 10^{-3}$ grams
mGpDH	mitochondrial glycerophosphate dehydrogenase, located on the outer surface of the inner mitochondrial membrane
µg	microgram, $1 \cdot 10^{-6}$ grams
µM	micromolar, $1 \cdot 10^{-6}$ molar concentration
µs	microsecond, $1 \cdot 10^{-6}$ seconds
µU	microunits, $1 \cdot 10^{-6}$ SI units of a substance

min	minute, 60 seconds
mL	milliliter, $1 \cdot 10^{-3}$ liters
mM	millimolar, $1 \cdot 10^{-3}$ molar concentration
MnSOD	manganese-containing superoxide dismutase, a mitochondrial enzyme catalyzing the dismutation of O_2^- to H_2O_2
mol	mole, a unit of quantity defined as $6.022 \cdot 10^{23}$ atoms or molecules of a substance
mOsm	milliosmolarity, $1 \cdot 10^{-3}$ moles of solute particles per liter of solution
ms	millisecond, $1 \cdot 10^{-3}$ seconds
Myxathiazol	inhibitor of the cytochrome bc1 of the mitochondrial complex III, blocking electron transfer to the Rieske iron-sulfur protein
N_2	diatomic nitrogen
Na^+	sodium
Na^+ fluoride	sodium fluoride
$NAD(P)H{:}NAD(P)^+$	the ratio of reduced to oxidized nicotinamide adenine dinucleotide and/or nicotinamide adenine dinucleotide-phosphate
NADH	reduced nicotinamide adenine dinucleotide
NDUFV1	the 51-kD subunit of complex I
NDUFS1	the largest of the 75 kD subunits of complex I, exhibiting NADH dehydrogenase activity and oxidoreductase activity.
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells, a protein complex which controls the transcription of DNA and is involved in cellular stress responses
nm	nanometers, $1 \cdot 10^{-9}$ meters
nM	nanomolar, $1 \cdot 10^{-9}$ molar concentration
O_2	diatomic oxygen, or dioxygen

O_2^-	superoxide anion, the product of one-electron reduction of dioxygen
OAA	oxaloacetate
Obesity	body mass index > 30 kg · $(m^2)^{-1}$
OGTT	oral glucose tolerance test
Oligomycin	inhibitor of the mitochondrial ATP synthase, complex V
OS	ovarian steroid hormones
P4	progesterone (pregn-4-ene-3,20-dione), an ovarian sex steroid hormone
P4:E2	ratio of serum progesterone concentration (pM) to serum estradiol concentration (pM)
p53	tumor protein 53, a tumor suppressor protein and regulator of the cell cycle
PCC	propionyl CoA carboxylase
P-C/M	25 µM palmitoyl-carnitine + 1 mM malate
P-C/M+G	25 µM palmitoyl-carnitine, 1 mM malate + 2 mM glutamate
P-C/MG+S	25 µM palmitoyl-carnitine, 1 mM malate, 2 mM glutamate + 3 mM succinate
P-C/MGS+Gp	25 µM palmitoyl-carnitine, 1 mM malate, 2 mM glutamate, 3 mM succinate + 10 mM glycerophosphate
PCr	phosphocreatine
pH	potentiometric hydrogen ion concentration, the negative logarithm (base 10) of the dissolved hydrogen ions, a measure acidity or basicity of a solution
Piericidin A	a complex I inhibitor
pmol	picomol, $1 \cdot 10^{-12}$ moles
PTPs	protein tyrosine phosphatases

PVDF	polyvinylidene fluoride, a highly non-reactive and pure thermoplastic fluoropolymer
PDK2	pyruvate dehydrogenase kinase isoform 2
PI3-K	phosphoinositide 3-kinase, an enzyme involved in the insulin signaling pathway
pK_a	the negative logarithm (base 10) of the acid dissociation constant, K_a
pM	picomolar, $1 \cdot 10^{-12}$ molarity
P:O	the ratio of adenosine triphosphate formed per oxygen consumed, the measure of which oxidation is coupled to phosphorylation in mitochondria
Pre	pre- eight weeks of exercise training
Protein-SH:protein-SS-R	ratio of reduced to oxidized protein thiols
PTP1B	protein tyrosine phosphatase 1B
Post	post- eight weeks of exercise training
PTEN	phosphatase and tensin homolog, a protein tyrosine phosphatase
PTP	permeability transition pore
Q	oxidized quinone
QH_2	reduced quinone
RCR	respiratory control ratio, quotient of state 3 to state 4 respiration
1/RCR	inverse of the quotient of state 3 to state 4 respiration
REF	reverse electron flow, backflow of electron through complex I
RER	respiratory exchange ratio (VCO_2/VO_2)
RMR	resting metabolic rate
RNA	ribonucleic acid

RNS	reactive nitrogen species, reactive molecules primarily derived from nitric oxide
ROS	reactive oxygen species, reactive molecules derived from dioxygen
s	seconds
S	succinate, a complex II substrate
+S	"plus exogenous succinate, a complex II substrate"
Saponin	a relatively mild nonionic detergent derived from the Soap bark tree (*Quillaja saponaria*)
SEM	standard error of the mean (standard deviation · $(n^{0.5})^{-1}$)
SDH	succinate dehydrogenase, or complex II
SOD	superoxide dismutase
SR	sarcoplasmic reticulum
State 1	respiration supported by mitochondria alone
State 2	respiration supported by ADP alone
State 3	respiration supported by substrates and ADP, actively phosphorylating respiration
State 4	non-phosphorylating respiration
State 5	anoxia
Stigmatellin	inhibitor of quinol oxidation site on complex III
SUIT	substrate-uncoupler-inhibitor-titration protocol
TBARS	thiobarbituric acid reactive substances
Teflon	polytetrafluoroethylene
TMPD	*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, an artificial substrate for complex IV
Triton X-100	a nonionic surfactant

Tyr	tyrosine
UCR	uncoupling control ratio, the quotient of FCCP-uncoupled respiration to oligomycin-inhibited, state 4 respiration
VDAC-1	voltage dependent anion channel, a component of the mitochondrial permeability transition pore
Vitamin C	ascorbate
Vitamin E	tocopherols and tocotrienols
V_{max}	the maximum velocity of an enzyme or enzymes
$\dot{V}O_{2peak}$	the peak velocity of minute-average oxygen uptake ($mL\ O_2 \cdot min^{-1} \cdot kg\ body\ mass^{-1}$)
$\dot{V}O_{2max}$	the maximal velocity of oxygen uptake ($mL\ O_2 \cdot min^{-1} \cdot kg\ body\ mass^{-1}$)
wt	weight
Zucker *fa/fa* rat	a genetically obese rat model, hyperphagic due to hypothalamic leptin receptor deficiency

CHAPTER 1: REVIEW OF LITERATURE

INSULIN-STIMULATED GLUCOSE UPTAKE

Insulin is a peptide hormone produced by the pancreatic beta cells which is capable of affecting many known acute metabolic and anabolic processes. One of the most important metabolic effects of insulin is the stimulation of glucose uptake into peripheral tissues. The actions of insulin on its target tissues begins with its binding to its cell surface transmembrane receptor, a tetramer composed of two extracellular insulin-binding α-subunits and two transmembrane β-subunits (177). When insulin binds to the α-subunits, activation and autophosphorylation of the intracellular β-subunits occurs. Autophosphorylation signals the recruitment of additional intracellular signaling proteins, beginning with the insulin receptor substrate (IRS) proteins, which contain Src-homology 2 (SH2) domains that serve as docking sites for even more signaling proteins (177). The signaling pathway thereafter increases in complexity as it diverges to bring about the myriad effects of insulin within the cell (177). With regard to insulin-stimulate glucose uptake, the signaling continues with recruitment of the heteromeric enzyme phosphatidyl 3-kinase (PI3-K) to the IRS/Insulin-receptor complex, resulting in activation of PI3-K. PI3-K catalyzes the phosphorylation of phosphoinositides at the 3-position of the inositol ring, resulting primarily in phosphatidyl 3,4,5-triphosphate (177). The events downstream of PI3-K are thought to involve activation of protein kinase B (Akt) via interaction with phosphatidyl 3,4,5-triphosphate and phosphorylation by phosphinositide-dependent protein kinases. Additionally, the atypical protein kinase C λ ζ isoforms, which may also be activated by phosphoinositides, are also likely also involved downstream of PI3-K in events leading to insulin-stimulated glucose uptake (177). Under basal conditions, glucose entry

into the cell is mediated by the ubiquitously expressed GLUT1 protein, one in a family of homologous glucose transporter (GLUT) proteins responsible for facilitated diffusion of glucose into mammalian cells. Upon stimulation by insulin, however, there is a rapid and massive increase in glucose entry into the cell. Although GLUT1 levels at the plamsa membrane are increased in response to insulin, the effect pales in comparison to GLUT4-mediated glucose transport in response to insulin (184). GLUT4 is expressed predominantly in peripheral insulin-target tissues: i.e., adipose and skeletal muscle (184). In response to acute insulin stimulation, GLUT4 is translocated rapidly from intracellular storage compartments to the plasma membrane, coincident with the rapid increase in glucose entry into the cell.

SKELETAL MUSCLE INSULIN RESISTANCE

Insulin resistance describes a situation in which there is a subnormal response by a tissue to a given level of insulin, and is typically referred to with regard to the ability - or inability, of target tissues like skeletal muscle to respond to insulin by taking up glucose from the circulation. The insulin resistance that precedes and occurs during overt type 2 diabetes is therefore often accompanied by fasting hyperglycemia (247). After an overnight fast, hepatic sources provide a steady input of glucose, even in healthy individuals (286). This is essential to offset the glucose cleared by target tissues like skeletal muscle and is vital for the central nervous system, which relies almost exclusively on glucose (226). Excessive hepatic glucose production contributes to the fasting hyperglycemia associated with type 2 diabetes (276). However, this defect is principally noticeable only when individuals transition from impaired glucose tolerance to overt diabetes mellitus (71, 193). The reason for this may be due to the ability of elevated basal insulin in the pre-diabetic condition to limit hepatic glucose production to near normal rates (193).

Indeed, insulin inhibits gluconeogenesis by suppressing the expression of phosphoenolpyruvate carboxykinase (unidirectional, rate-limiting enzyme of gluconeogenesis, which converts oxaloacetate to phosphoenolpyruvate) and glucose-6-phosphatase (unidirectional enzyme which dephosphorylates glucose-6-phosphate in the final steps of gluconeogenesis) (226). Therefore, maintenance of the fasting blood glucose concentration is due to a combination of glucose production (liver) and glucose clearance (primarily the peripheral tissues). However, evidence indicates that impaired peripheral insulin-stimulated glucose uptake accounts for the fasting hyperglycemia associated with pre-diabetic insulin resistance (71, 193). Moreover, by virtue of its high percentage of total body mass and high metabolic activity, skeletal muscle may contribute more quantitatively to whole-body glucose homeostasis that any other tissue (201). Therefore, modulators of insulin sensitivity in skeletal muscle are likely to affect whole-body glucose homeostasis and may represent important factors when studying insulin resistance, a hallmark of type 2 diabetes. Indeed, the initial events of insulin resistance involve a decrease in the rates of glucose uptake from the periphery including adipose, but predominately skeletal muscle (293).

The sensitivity of target tissues like skeletal muscle to insulin-stimulated glucose uptake are typically investigated through glucose clamping techniques (73). Often referred to as the "gold standard" for evaluating insulin sensitivity, the hyperinsulinemic-euglycemic clamp method involves a steady intravenous infusion of insulin in one arm. Serum glucose is then "clamped" at normal fasting levels by administering a variable intravenous glucose infusion into the other arm. Blood samples are drawn at regular intervals to monitor blood glucose in order to maintain the steady fasting level of glucose. Because the infused insulin suppresses hepatic glucose output, this technique measures the sensitivity of peripheral insulin-target tissues (mainly

skeletal muscle) to insulin-stimulated glucose uptake, with the rate of glucose uptake during the clamp being proportional to the inverse of the degree to which the periphery is insulin resistant (179). Because the hyperinsulinemic-euglycemic clamp is quite labor intensive, other minimalist approaches have been employed, two of which are the oral glucose tolerance test (OGTT) and the intravenous glucose tolerance test (IVGTT). The OGTT has been a mainstay in the diagnosis of impaired glucose tolerance and type 2 diabetes in humans and animal models. It involves patient ingestion of a body-mass-determined glucose dose, followed by the subsequent sampling of blood at regular time intervals over a 2-4 hour period following glucose ingestion. Insulin sensitivity is then estimated by calculating the area under the curve (AUC) for glucose and insulin values measured in the blood samples (179). The IVGTT technique is similar to the OGTT, with the primary difference being that the glucose is administered intravenously. A particular drawback to the OGTT method is that it does not specifically assess insulin resistance of the peripheral tissues, as hepatic glucose production is not suppressed (179). While less labor intensive than the clamp method, the IVGTT still requires as many as 25 blood samples to be collected over the 2-4 hour period, with subsequent computer-assisted mathematical analyses often performed. Alternatively, one can estimate insulin resistance from fasting blood glucose and insulin by homeostatic model assessment (HOMA). HOMA estimates for insulin resistance (HOMA-IR) and beta cell function were developed using data from physiological studies to develop mathematical equations describing glucose regulation as a feedback loop (296). In practical terms, the HOMA estimation can be calculated from fasting plasma or serum glucose (mg/dL) and insulin (μU/mL) by the equation: HOMA-IR = (glucose • insulin) • 405^{-1} (191). The use of HOMA has since been validated against a variety of physiological methods, and correlates with euglycemic clamp method (Pearson r-value range from six published reports: 0.58-0.88,

reviewed in (296)). Ideally, a normal-weight person aged < 35 years has a HOMA value of 1.0. (191). Clear HOMA cutoff values for insulin resistance in larger population studies have been employed, and typically fall anywhere from 2.5 (159), to 4.65 (265). In the present study used the decision criteria outlined by Stern et al (265): an individual tests positive for insulin resistance if HOMA-IR > 4.65 or if HOMA-IR > 3.60 and BMI > 27.5 kg/m^2.

SKELETAL MUSCLE INSULIN RESISTANCE AND MITOCHONDRIA

It has been argued that the recent increase in type 2 diabetes world-wide constitutes our greatest current health problem (136). Skeletal muscle is generally considered the primary site of insulin-stimulated glucose disposal in healthy humans (72, 88), and skeletal muscle is the primary site of insulin resistance associated with type 2 diabetes (72, 212). It therefore follows that skeletal muscle insulin resistance, the primary cause of type 2 diabetes, has received intense investigation. Beginning in 2001, the team of Kelley and co-workers (125, 157) reported that the skeletal muscle of individuals with type 2 diabetes contained fewer mitochondria than age-matched subjects who were insulin sensitive. These findings were used to explain a hypothetical model in which reduced mitochondrial content in skeletal muscle results in reduced capacity for fatty acid oxidation, and subsequent lipid accumulation within the skeletal muscle. They went on to hypothesize that intramuscular lipid accumulation leads to insulin resistance (125, 157), which was later supported by additional research from multiple laboratories (32, 203, 221), and has since garnered apparent general acceptance (186, 275). However, the notion that reduced mitochondrial content plays a causal role in the development of insulin resistance in skeletal muscle has not gone unchallenged (reviewed in (136)), and is underscored in a recent report from

the group of Holloszy of increased, rather than reduced skeletal muscle mitochondrial contents in animals fed a high fat diet, concomitant with reduced insulin sensitivity (120).

Increasingly, the role of oxidative stress has been implicated in the etiology of insulin resistance in multiple tissues, including skeletal muscle (reviewed in (16, 219)). Oxidative stress has been defined as an imbalance between production of oxidants and the antioxidant defense (21). While many oxidants exist in nature, it is generally accepted that reactive chemical species derived primarily from nitric oxide (i.e., reactive nitrogen species, or RNS) and perhaps more importantly reactive oxygen species (ROS) contribute, seemingly paradoxically, to both normal insulin signaling and insulin resistance in target tissues (reviewed in (16, 219)). Reactive oxygen species, or "ROS," is an umbrella term used to describe chemicals formed through the incomplete electrochemical reduction of diatomic oxygen, O_2. These include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\bullet$). Due to their relative instability and small size, ROS are generally quite difficult to study, a fact highlighted by the historical lack of widely available tools to measure ROS (267). In fact, the only way to directly detect or measure free radicals is through the use of electron spin resonance/electron paramagnetic resonance (ESR or EPR) spectroscopy (241), a technique for studying chemical species with one or more unpaired electrons (e.g., free radicals). Over 20 years ago, ESR spectroscopy was used to show that free radical production increased after exhaustive exercise in rat skeletal muscle homogenates (67). However, this, and other studies conducted using ESR spectroscopy are now thought to have detected primarily the ubisemiquinone involved in normal mitochondrial electron transport (241). In addition to the lack of specificity associated with ESR spectroscopy in the study of free radical production, lack of sensitivity to the levels of free

radicals typically associated with biological systems represent another limitation to the technique (241).

Because high levels of ROS often leave detectable traces of oxidatively modified molecules, studies examining the effects of ROS and RNS typically assess an array of "damaged" biological molecules. These can include oxidized proteins, lipids, low-density lipoproteins, carbohydrates (i.e., glycated products) or nucleic acids/bases (219). The vast majority of studies have assessed levels of the molecular by-products associated with lipid peroxidation (241), which can include alkanes measured in expired air, but more often tissue or fluid levels of malonyldialdehyde (MDA) via reactivity with thiobarbituric acid to assess so-called thiobarbituric acid reactive substances (TBARS) (241). However, an increasing number of reports show that ROS are involved in signal transduction that may not involve oxidative stress, per se, and that perhaps they are in fact required for normal cell function and signal transduction (38, 100). Considerable challenges exist to studying ROS, especially with regard to the limitations of tools available with which to study the role of ROS in cellular signaling. Fluorescent detection of the oxidation of the widely used 2′,7′-dichlorodihydrofluorescein (DCF) can gives a sense of ROS burst, for example (195). However, DCF is notorious for its relative lack of specificity toward ROS and is subject to auto-oxidation and photo-oxidation (195).

The non-radical ROS, H_2O_2, a relatively mild oxidant, is increasingly being recognized for its role as a biological second messenger (reviewed in: (23, 80, 90, 98, 116, 233, 234, 267)). An accumulating body of literature suggests that ROS produced by the mitochondria in skeletal muscle lead to the development of insulin resistance in this tissue (29). Indeed, aided by the availability of better tools with which to detect specific and biologically relevant levels of ROS, H_2O_2 produced by the mitochondria has recently been implicated by our group in the etiology of

dietary fat-induced insulin resistance in skeletal muscle (5). Specifically, these observations were made using the redox-sensitive dye, *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex red). First described by Mohanty et al (200), Amplex red has since been the mainstay in the detection of physiological levels of H_2O_2 for the past ten years. Unlike its predecessor scopoletin, Amplex red is a colorless and nonfluorescent derivative of dihydroresorufin, which is oxidized by H_2O_2 via horseradish peroxidase (1:1 reaction stoichiometry) to the highly fluorescent resorufin, with an excitation/emission maxima of 563 and 587 nm, respectively (314). The specificity of Amplex red for detection of H_2O_2 is evidenced by inhibition of the reaction when catalase is present (292). The more recent design of new and highly selective H_2O_2 sensors operate by a mechanism in which H_2O_2-mediated removal of a boronated protecting group on the probe confers fluorescence (195), and can be visualized by confocal microscopy in real-time to answer questions about the spatio-temporal nature of cellular H_2O_2 production. Additionally, recently developed H_2O_2-sensing probes, such as HyPer, can be genetically targeted to specific cellular compartments (e.g., cytosol, mitochondria, nulecei), permitting additional control over the cellular localization in which H_2O_2 is measured (19). The commercial availability of these new tools is expected to lead to exciting advancements in our knowledge regarding cellular H_2O_2 signaling.

H_2O_2 AS A SIGNALING MOLECULE

H_2O_2 has several unique qualities that make it a suitable signaling molecule, not the least of which is its relatively long half life (1 ms for H_2O_2 vs. 1 μs for O_2^- in lymphocytes) (233). Superoxide, on the other hand, is both less stable than H_2O_2, and is also unable to diffuse through membranes due to its negative charge. While the highly reactive $HO^\bullet$ reacts indiscriminately and

in the vicinity of its production, diffusion of H_2O_2 may be regulated by membrane permeability and is even thought to travel through specific aquaporins (22, 23). H_2O_2 is believed to directly affect the cellular redox environment via alterations in, for example, the $NAD(P)H:NAD(P)^+$, protein-SH:protein-SS-R and GSSG:GSH ratios, thereby regulating and influencing the function of myriad proteins (23). Furthermore, it has been determined that the specificity with which H_2O_2 reacts with key cysteine (Cys) residues on target proteins is far greater than other ROS molecules (63), and the effects of H_2O_2 on disulfide bond formation *in vitro* has been shown to be both concentration dependent and differentially targeted to proteins involved in translation and energy production, at least in the cytosol of neuronal cell cultures (62). Indeed, redox regulation of Cys residues by H_2O_2 can achieve such selectivity and specificity by virtue of the fact that not all cysteines are equally reactive; the reactivity of a given Cys residue being determined by a) its solvent-exposed localization, and b) its ionization state (i.e., thiol vs. thiolate) (63). The ionization state, and in turn the reactivity of a given Cys residue is determined by the intracellular pH and the pK_a of the Cys thiol moiety, with the thiolate typically being more susceptible to oxidation by H_2O_2 than the protonated form (304). However, certain Cys thiols may gain stability as the thiolate anion via electrostatic interactions with positively charged residues nearby (63). Simply put: in general, the lower the pK_a, the greater the Cys thiol is likely to react (63). Another quality that makes Cys ideal for redox signaling is that it is, in many cases, reversible. This allows for additional temporal control of redox signaling by H_2O_2 (63). As a particularly poignant example of redox regulation of proteins that may be involved in mammalian insulin signaling/insulin resistance, consider the protein tyrosine phosphatases (PTPs). PTPs are thiol-dependent enzymes that dephosphorylate key Tyr residues involved in myriad cell functions (267). Thiol moieties at the active sites of PTPs react with H_2O_2 more

readily than the average protein Cys thiol moiety, due in part to their low pK_as (e.g., pK_a 5.4 for PTP1B) (74, 304). Examples of PTPs that are implicated in insulin resistance include protein tyrosine phosphatase 1B (PTP1B) and phosphatase and tensin homologue (PTEN). Oxidative modification of active site Cys residue thiols in both PTP1B and PTEN has been demonstrated to decrease their activity (111, 181). Oxidative inactivation of PTP1B and PTEN should, in theory, improve the insulin signaling cascade by releasing the inhibitory effects of the PTPs on the PI3-kinase pathway. The empirical discrepancies noted in the literature between oxidative modification of PTPs and insulin action may speak more to the complexity of redox regulation than positivist conclusions about oxidative stress. Indeed, evidence suggests a role for low levels of endogenously-generated ROS in the normal activity of the PI-3 kinase signaling, but it is clear that chronic oxidative stress has a deleterious effect on the pathway (181). Furthermore, obesity-associated nitrosylation of components of the PI3-kinase – Akt pathway constitute one of the best-supported mechanisms of oxidative suppression of insulin signaling in skeletal muscle (44, 154, 310). To complicate matters further, the literature is replete with contradictory conclusions about the very nature of mitochondrial ROS ascertained from studies in intact cells versus isolated mitochondria (addressed in (10)). While efforts are being made to reconcile these observations (10), it is clear that more research is warranted in the area of redox regulation as it applies to the regulation of complex pathways like insulin-stimulated glucose uptake.

REDOX-SENSITIVE MITOCHONDRIAL PROTEIN MODIFICATIONS

The high mitochondrial matrix pH (~8) and proximity of mitochondrial proteins to the major ROS production sites predicts that mitochondrial protein thiols should be particularly susceptible to oxidation by ROS and RNS (144, 182). Mitochondrial electron transport chain

proteins are rich with thiols (142, 143); however, it is within complex I that the reactive/regulatory protein thiols believed to confer physiological function are primarily located (18, 66, 152, 247, 307). Many of these thiols are associated with non-heme iron centers, while others on the surface of the complex I are prime targets for redox modification. As an example, *S*-nitrosylation of complex I thiols has been shown to correlate with a significantly reduced activity of the enzyme, an effect that was readily reversible with thiol reductants (66). Moreover, this *S*-nitrosylation was also associated with an increased complex I superoxide (O_2^-) formation (66). Evidence also suggests that complex I is susceptible to glutathionylation by GSSG in the presence of the mitochondrial thiol transferase glutaredoxin 2 (Grx2) (18). Manipulating the redox milieu with an oxidized GSH:GSSG ratio lead to a dramatic loss of complex I activity (18). Indeed, complex I activity is inhibited by *S*-glutathionylation occurring in both 75-kDa (NDUFS1) and 51-kDa (FMN-binding subunit/ NDUFV1) subunits of isolated complex I upon addition of excess GSSG (18, 152, 182, 307), which can result in rapid production of O_2^- (18). Oxidative modification of complex I can result in self-inactivation and decreased electron transfer activity, resulting in greater O_2^- generation in a phenomenon often referred to in the literature as a "vicious cycle" of ROS-induced ROS production (203) . Experimental treatments with both exogenous and endogenous H_2O_2 challenges in isolated bovine heart mitochondria resulted in glutathionylated 75-kDa subunit Cys-531 and Cys-704 in isolated complex I, which also correlated with loss of complex I NADH oxidation activity (146). It was subsequently determined that O_2^-induces mixed intra-molecular disulfide bond formation in three cysteine pairs (i.e., Cys125/Cys142, Cys187/Cys206, and Cys142/Cys206) of the 51-kDa subunit of isolated bovine heart mitochondrial complex I (247). The formation of these disulfide bonds was inhibited by superoxide dismutase (SOD), indicating that O_2^- removal can prevent oxidative

redox modification of complex I (247). In addition, the ND3 subunit Cys39 becomes accessible to chemical modification in the de-active form of the complex I but is not available in the active form of the enzyme (95). This site was hypothesized to be modified by *S*-nitrosylation (95). In addition to mitochondrial respiratory chain proteins, important ROS antioxidant enzymes, such as peroxiredoxin III (154) and $NADP^+$-dependent isocitrate dehydrogenase (160, 314) also undergo redox-dependent thiol modification. Additional oxidative stress-sensitive thiols were identified by differential redox electrophoresis of isolated rat heart mitochondrial protein after treating with either low levels of exogenous H_2O_2 or RNS (145). Importantly, this was not a bulk thiol response, but rather a response effecting only specific protein thiols (145). The functional grouping of these redox-modified proteins were found to belong to those involved in β-oxidation and in the regulation of pyruvate dehydrogenase complex; they include mitochondrial creatine kinase, carnitine acetyltransferase, voltage dependent anion channel 1 (VDAC-1), acyl-CoA dehydrogenase (very long chain), mitochondrial acyl-CoA thioesterase 2, enoyl-CoA hydratase (short chain 1, mitochondria), propionyl-CoA carboxylase (α chain) (PCC), pyruvate dehydrogenase kinases 2 (PDK2) and pyruvate dehydrogenase E3 binding protein (145). Importantly, experimentally induced low endogenous ROS was sufficient to induce thiol modifications of PCC and PDK2, and these thiol modifications corresponded to reduced enzyme activity (145). Additionally, the regulation of mitochondria protein assembling (163), fission and fusion (149, 224) have also been shown to be redox sensitive. To summarize, it appears that ROS production at the level of the mitochondrial electron transport system (ETS) can affect its own enzymatic functions, conferring redox-level regulation of substrate metabolism. Importantly, ROS-induced ROS production via oxidative modification of ROS producing components of the mitochondrial ETS may lead to a vicious cycle of oxidant emission.

THEORY AND PRACTICE OF STUDYING MITOCHONDRIAL RESPIRATION

In 1961, Peter Mitchell published a unique hypothesis regarding cellular bioenergetic conservation (197), for which he was later awarded the Nobel Prize for chemistry in 1978. Termed the "chemiosmotic theory of oxidative phosphorylation," at its core is the notion of coupling hydrogen and electron transfer through an energy-conserving membrane to the phosphorylation of ADP to ATP. In mitochondria, there is an ETS consisting of several multi-polypeptide protein complexes embedded in the inner mitochondrial membrane that receive electrons from mitochondrial dehydrogenases. These electrons are then transferred through a series of electron carriers in the ETS, ordered in such a way that their redox potentials (tendency to give up electrons) progressively drop from NADH (high redox potential) or $FADH_2$ to O_2 (low redox potential, high tendency to accept electrons), ultimately reducing $^{1}/_{2}O_2$ to H_2O. In three of these complexes (I, III and IV), the fall in redox potential across the oxidation-reduction reactions within the complex is sufficient to drive the translocation of protons from the matrix to the intermembrane space. This creates a proton gradient across the inner membrane ($\Delta\tilde{\mu}_{H^+}$) that is, by convention, converted to units of electrical potential (i.e., millivolts) and referred to as "the membrane potential," $\Delta\Psi$. The essence of the chemiosmotic theory is that the electrical-chemical potential created by the accumulation of $\Delta\Psi$ is sufficient to drive the synthesis of ATP as protons flow back through the ATP synthase (a.k.a., Complex V) into the matrix. Proton leak reactions constitute an alternative means of re-entry for protons and account for the majority of respiration under basal conditions (239). In fact, in non-phosphorylating mitochondria, the rate of proton leak is directly proportional to the respiratory rate (166). Because respiration accounting for basal proton leak is not coupled to ATP synthesis, it contributes to a lower yield of ATP (P)

generated at complex V per oxygen (O) consumed at complex IV. The ratio of P generated to O consumed (P:O ratio) describes, by definition, the relative degree of coupling in a given mitochondrion or mitochondrial population.

The thermodynamic disequilibrium that exists between the redox potential spans across the proton-translocating regions of the ETS and ΔΨ constitutes the fundamental factor in respiratory control (209). Measuring the rate of mitochondrial respiratory oxygen consumption is accomplished by a number of methods, including oxygen-dependent quenching of porphyrin-based phosphors (303) and amperometic oxygen sensors (105). While phosphorescent probes are growing in use for *in vitro*/*in situ* respiratory measurements (e.g., the Seahorse biosciences XF analyzer) and are particularly useful for *in vivo* measurements of oxygen pressures (302), the amperometric approach represents the most popular with respect to *in vitro* investigations of mitochondrial respiration, with the Clark electrode having been used to investigate respiratory control for over 50 years. Named for inventor Leland Clark, the Clark electrode contains a gold or platinum cathode and a Ag/AgCl anode separated by a concentrated aqueous solution of KCl. A voltage is applied to these two half-cells, which are separated from the experimental solution by a membrane of oxygen-permeant material (e.g., PVDF). Oxygen diffuses through the membrane from the experimental solution and is reduced to water by electrons at the cathode, yielding H_2O_2. The H_2O_2 then oxidizes the Ag of the Ag/AgCl anode, which generates an electrical current. The resultant electrical current is then converted mathematically to an electrical potential that is proportional to the partial pressure, and in turn concentration, of O_2 in the experimental solution (105). Changes in the concentration of O_2 in the experimental solution therefore correspond to the inverse of the respiratory rate of a biological sample (e.g., mitochondria), and allow for quantifiable determination of respiratory O_2 flux (JO_2) under

various experimental conditions.

RESPIRATORY CONTROL AND THE STEADY-STATE CONVENTIONS

In 1956, Chance and Williams (49) published their definitions of respiratory steady-states they observed in experiments conducted on isolated mitochondria with a Clark electrode (50). Some of these steady-state conventions (e.g., states 3 and 4) remain in use today, forming the basis of communicating information regarding respiratory control in mitochondrial experiments. To illustrate how the steady-states were determined, consider a suspension of isolated mitochondria in which the partial pressure of oxygen is continuously monitored in a sealed chamber by a Clark-type electrode. The term "state 1 respiration" describes the rate of oxygen consumption when de-energized mitochondria are in the sealed chamber alone, and typically corresponds to artifacts of instrumental background (105). In the presence of ADP only, a steady-state rate of oxygen consumption will commence, which is substrate-limited and thus very low. This basal rate of respiratory oxygen flux (JO_2) was termed *state 2* respiration. This state 2 described by Chance and Williams (49) is thus not equivalent to the "state 2" found in the more recent *Bioenergetics3* textbook reference (209), in which state 2 is described as the respiratory steady-state achieved when substrate alone has been added (103, 209). This referential mismatch may owe to the fact that many mitochondrial experiments employ subtrate-uncoupler-inhibitor-titration (SUIT) protocol regimes that favor the sequential steady-state terminology defined by Nicholls and Ferguson in *Bioenergetics3* (209) (i.e., substrate is added to a mitochondrial preparation, followed by ADP). Returning to the Chance and Williams (49, 50) steady-state derivations: if substrate is then added to the chamber, respiration increases to match the drop in $\Delta\Psi$ that occurs as a consequence of rapid proton re-entry via ATP synthesis at

complex V. The JO_2 that is achieved upon addition of substrate was termed *state 3* respiration. However, the terminology applied in numerous published reports illustrates the current convention of using “state 3” to describe any situation in which both ADP and substrates are present (e.g., (5, 52, 89)), and is therefore synonymous with a state in which mitochondria are engaged in appreciable oxidative phosphorylation. Eventually, the added ADP will become exhausted (i.e., phosphorylated to ATP), and a new basal JO_2 will be achieved: *state 4* respiration. This state 4 respiration is empirically equivalent to the state 2 defined by Nicholls and Ferguson (209). During state 4, the rate of respiration (electron flow through the ETS) is matched to the proton leak that occurs as H^+ re-enter the matrix. It is important to note that states 2 and 4 respiration are often used synonymously to refer to basal (i.e., non-phosphorylating) respiration, but that “state 4” predominates as the current convention with which to describe a state in which mitochondria are not engaged in oxidative phosphorylation (e.g., (5, 52, 89)), and may also include respiratory steady-states achieved with inhibitors of mitochondrial oxidative phosphorylation, such as carboxyatractyloside/atractyloside/bongkrekic acid/M-21 (inhibitors of the mitochondrial adenine nucleotide transporter, ANT), rotenone/piericidin A (inhibitor of mitochondrial complex I) during respiration supported by complex I substrates, oligomycin (inhibitor of the mitochondrial ATP synthase/complex V), malonate (inhibitor of complex II) during respiration supported by succinate alone, or antimycin A/stigmatellin/myxothiazol (inhibitor of the mitochondrial complex III), to name a few. Finally, *state 5* describes the situation reached when most or all of the oxygen has been depleted from the experimental chamber by the mitochondria and/or the electrode, which should, by definition be no rate at all (i.e., zero JO_2). Therefore, in a true state 5 condition, observed rates of JO_2 are artifactual by nature, and most likely the result of back-diffusion of oxygen from an incompletely sealed

chamber or by the use of inappropriate materials (e.g., Teflon or plastic) (105). Conventionally, the respiratory control ratio (RCR), defined as the quotient of state 3 respiration to that of state 4, is used as an index of mitochondrial coupling. Indeed, a positive linear relationship between the inverse respiratory control ratio (1/RCR) and P:O ratios has been demonstrated in isolated mitochondria (101). Publications have also used RCR as an index of mitochondrial bioenergetic functional integrity for isolated mitochondrial preparations (e.g., (168, 231)), and a relationship between RCR and cytochrome *c* conservation (indicator of outer mitochondrial membrane intactness) has been demonstrated in mitochondria isolated from skeletal muscle (231). However, RCR is only useful when substrates for oxidative phosphorylation are not limiting, as this will underestimate state 3 respiration (103).

The overall flux of oxygen consumption by the mitochondria can be divided into many individual pathways that exert different flux control coefficients upon the overall respiratory metabolism. The flux control coefficient is defined as the fractional change in flux, divided by the fractional change in the amount of enzyme as the change tends towards zero (209). An example would involve altering the activity of an enzyme (e.g., ANT), in a metabolic pathway (e.g., respiration) and measuring the fractional change in respiration that occurs as a function of the fractional alteration in ANT activity. If a 50% change in ANT activity, for example, resulted in a 50% change in respiration, then the flux control coefficient for the ANT over respiration would be 1. On the other hand, if altering the activity of the ANT by 50% resulted in only a 10% change in respiration, then the flux control coefficient would be 0.2 (10%/50%) (209). During uncoupled respiration, the ATP turnover reactions and proton leak exert essentially zero respiratory flux control (209). Therefore, conclusions can be made regarding limitations of the phosphorylation system and respiratory enzyme content when the uncoupled respiratory rate is

compared to the maximal state 3 rate. Put another way, if uncoupled JO_2 is higher than maximal state 3 JO_2 (coupled), then a limitation to flux is exerted by the phoshorylation system and there is thus an excess capacity of electron transport (102).

MITOCHONDRIAL PRODUCTION OF REACTIVE OXYGEN SPECIES

As mentioned previously, discrepancies exist in the literature as to the how mitochondrial ROS leads to oxidative/redox stress. This is due primarily to inferences made from studies using either *in vitro* mitochondrial preparations as opposed to cell or *in vivo* work (10). With regard to the former, O_2^- production is favored when the components of the mitochondrial electron transport system are reduced; i.e., when the rate of electron flow through the chain is at its slowest and, by definition, $\Delta\Psi$ is at its highest (i.e., resting respiration) (166, 209). The use of specific respiratory chain inhibitors exploits this concept as blocking electron flow increases the redox state of ETS components upstream of the inhibitory site while components downstream become more oxidized. When rotenone, a complex I inhibitor, is added to mitochondria respiring exclusively on the complex II substrate succinate, H_2O_2 formation decreases (15, 183), providing evidence that complex I is a site of O_2^- formation when electrons enter the ETS beyond complex I (i.e., via $FADH_2$-linked respiratory complexes such as complex II) due to reverse electron flow from complex II back into complex I. $FADH_2$ is oxidized downstream of complex I by mitochondrial glycerol 3-phosphate dehydrogenase (mGpDH), succinate dehydrogenase (complex II), and during β-oxidation of fatty acids - in the case of the latter, donating electrons to the electron transferring flavoprotein (ETF) (209).

In contrast to the paradigm offered by the results of *in vitro* mitochondrial experiments mentioned above, studies conducted using intact cells observe high rates of mitochondrial ROS

during high rates of respiration, when $\Delta\Psi$ is at its lowest, and in theory, the ETS should least likely to produce ROS. These seemingly contradictory notions of mitochondrial ROS governance were recently reconciled in experiments by Aon et al (10) using both cardiomyocytes and isolated mitochondria from guinea pig hearts, upon which the authors propose a model in which an optimized ROS balance is maintained by the redox couple of the cell/mitochondria. The model predicts that high levels of mitochondrial ROS will be observed on either extreme of the overall cellular energy charge, and that high rates of mitochondrial ROS production are observed in isolated mitochondria due to the fact that, even though the redox couple that supplies the energy for the antioxidant defense (e.g., $NADPH/NADP^+$) will be at its maximum during state 4 conditions, the ROS production will be so great as to overwhelm these defenses. Conversely, in intact cells treated with low doses of mitochondrial uncouplers, rates of mitochondrial ROS production at the level of the ETS will be relatively low; however, the antioxidant redox couples will be at their lowest as well, permitting oxidative/redox stress via the small amounts of ROS that are produced (10). Therefore, under conditions of redox homeostasis, the two opposing forces (i.e., ROS and the antioxidant defense) are poised to balance one another. Physiological ROS signaling is therefore thought to occur in a narrow range of net oxidant emission from the mitochondria, further supporting the notion of physiological, and in turn, acute pathophysiological redox signaling in biological systems, and not necessarily oxidative stress per se.

MEASUREMENTS OF MITOCHONDRIAL FUNCTION IN SITU: THE PERMEABILIZED MYOFIBER APPROACH

To measure the function of the mitochondrial respiratory complexes working together, intact mitochondria must be studied (174). To this end, oxygen consumption and the net release of H_2O_2 can and has been measured in mitochondria isolated from tissues including skeletal muscle (e.g., (243, 279, 288)). Isolation of mitochondria is typically achieved by differential centrifugation of tissue and/or cell homogenates and allows valid characterization of mitochondrial function (93, 174). However, the disadvantages of the isolated mitochondria approach (described in (174)) include: 1) artifacts of damage conferred to the mitochondrial function by the isolation process (174, 223); 2) biased selection of certain mitochondrial populations in a given sample (e.g., less dense mitochondrial populations excluded from the differential centrifugation step) (174, 223); 3) relatively large sample sizes ($>200 \bullet 10^6$ cells, or >500 mg wet wt of tissue) are necessary for optimal isolated mitochondrial yields (93, 174); and 4) The mitochondrial interactions known to be occur *in vivo* (i.e., microcompartmentalization, metabolic channeling and intracellular energy transfer) are disrupted in isolated mitochondria (156, 174, 196, 246), thus altering the functional properties of isolated mitochondria relative to those *in vivo* (172, 174, 196, 246). Of course, *in vivo* approaches are the most physiologically relevant means by which to study mitochondrial function. Indeed, near-infrared spectroscopy and fluorescent imaging methodologies have been used to study mitochondrial redox states (121), membrane potential (311) and calcium handling (199) *in vivo* (174). However, the scope within which mitochondrial functions can be analyzed *in vivo* are limited due to the fact that many exogenously added treatments (e.g., effectors such ADP) do not readily penetrate cell membranes (174). Efforts to both circumvent the limitations associated with isolated mitochondrial preparations, and at the same time maintain the experimental freedoms conferred by the methodology have lead to established protocols involving chemically permeabilized cells

(289) and muscle fibers (174, 287). Selective permeabilization of cell membranes can be achieved with various chemical agents, such as digitonin, filipin, α-solanine, α-tomatine and β-escin (174). However, with regard to skeletal and cardiac muscle, the most widely used permeabilizing agent is quillaia saponin, a relatively mild detergent (e.g., compared to digitonin (306)) derived from the Soap bark tree (*Quillaja saponaria*) and referred to in the literature as simply "saponin". The chemical permeabilization of skeletal and cardiac muscle fibers with saponin to study mitochondrial function was first reported in 1987 (287), and has since been used in numerous studies (e.g., (5-7, 32, 172-174, 244-246, 278, 287)). Saponins owe their detergent properties to hydrophilic glycoside moieties attached to lipophilic triterpene derivatives (139), which gives saponin its high affinity for cholesterol (174). Because plasma membranes contain more than 7 times as much cholesterol (~0.5 mol cholesterol per mol phospholipid) as do mitochondria (0.07 and 0.01 mol cholesterol per mol phospholipid for the mitochondrial outer and inner membranes, respectively) (55, 165), very small concentrations of saponin (e.g., 50 $\mu g \cdot mL^{-1}$) will selectively complex with the sarcolemmal cholesterol of skeletal muscle fibers, forming pores on the order of 8 nm in diameter (14, 176), whilst leaving the mitochondrial membranes intact (174). Additional intracellular structures containing little or no cholesterol, such as the sarcoplasmic reticulum (SR) and the contractile apparatus will also remain intact upon saponin treatment (246). Furthermore, saponin concentrations as low as 50 $\mu g \cdot mL^{-1}$ have been shown to increase Ca^{+2} loss from the SR of mammalian cardiac and skeletal muscle (127, 175), and to also reduce the ability of the SR to load Ca^{+2} (176). Because mitochondria are known to readily take up Ca^{+2} (39), the loss of Ca^{+2} from the SR during permeabilization is beneficial to studies involving mitochondrial function. The selective permeabilization also permits "cytosolic washout" involving the removal of cytosol and all solutes therein, including

soluble cytosolic enzymes like lactate dehydrogenase (174), followed by rapid equilibration of the intracellular space with the external medium. This equilibration between the medium and the intracellular space allows for the use of experimental additions much as are routinely used in functional analyses involving isolated mitochondria (174). Importantly, the normal mitochondrial morphology, including intact inner and outer mitochondrial membranes have been verified by electron microscopy in saponin permeabilized cardiac fibers (287), and the saponin-permeabilization preparation has been shown to liberate not more than 4% of total muscle citrate synthase as determined by citrate synthase activity assay of saponin permeabilization and washing solutions (278), indicating that the majority of mitochondria in the sample are intact and remain in the fiber bundles during the mitochondrial function measurements. When compared with isolated mitochondria, the advantages of the permeabilized fiber approach to study muscle mitochondria (described in (174)) include: 1) The permeabilized myofiber approach more closely retains the morphological design that exists *in vivo* than does isolated mitochondria. By preserving the native mitochondrial architecture, the intracellular interactions between the mitochondrial reticulum and, for example, the contractile apparatus are retained; 2) in contrast to the large amounts of tissue sample required for isolated mitochondria studies (typically no less than 50 mg, e.g., (279, 301)), only a relatively small sample (<35 mg wet weight) is required for mitochondrial function measurements involving the permeabilized fiber approach. Thus, the permeabilized fiber approach is more suitable than isolated mitochondria for human clinical studies. Moreover, research involving skeletal muscle biopsies on humans can afford to relinquish small portions of the total biopsy for mitochondrial function measurements in permeabilized fibers, and still keep enough of the remainder for biochemical analyses, satellite cell isolation for culture of myocytes, etc. In spite of the virtues associated with measuring

mitochondrial function in permeabilized fibers, the literature has recorded notable differences in the apparent K_m for ADP (i.e., the concentration of ADP eliciting a half-maximal rate of oxygen consumption) between fibers and isolated mitochondria (174). In fact, at least four publications show that the apparent K_m for ADP exceeds that of isolated mitochondria for the same tissue by over one order of magnitude (173, 174, 244-246). However, this disparity may owe solely to the preparation itself. Maximal aerobic capacity is limited by oxygen supply *in vivo* (75, 235). Similarly, the respiratory capacity of mitochondria is limited by oxygen kinetics, such that saturating concentrations of oxygen *in vitro* are requisite for determining respiratory capacity (102). An appropriate analogy is that of oxidative phosphorylation in studies using isolated mitochondria, where saturating concentrations of ADP are necessary for assessment of maximal state 3 respiration. While partial pressures of oxygen, and therefore concentrations of oxygen in experimental aqueous media well below that of sea-level air saturation (e.g., 20 μM) are not limiting to respiration in isolated mitochondria and small cells (reviewed in (102, 248)), even at air saturation (i.e., ~200 μM O_2), oxygen is limiting to respiration in permeabilized muscle fibers (104). This is illustrated by a 100-fold increase in the sensitivity to oxygen concentration in permeabilized rat soleus and heart fibers (104). This sensitivity to changes in already relatively high oxygen concentrations is thought to be due, at least in part, to diffusion limitations resulting from the fiber bundle (104). At low oxygen concentrations, an “anoxic core” of the fiber is thought to occur. Limitations of respiration due to insufficient oxygen levels in the experimental setup involving permeabilized muscle fibers has been reduced or prevented by maintaining oxygen concentrations above air saturation in the range of 200-500 μM (11, 32). It has been suggested that the experimental oxygen limitations may account, at least in part, for the disparate

low and high maximal state 3 respiratory rates reported for experiments involving permeabilized fibers (102).

REVERSE ELCTRON FLOW AND MITOCHONDRIAL REACTIVE OXYGEN SPECIES

Aside from questions surrounding mitochondrial versus extramitochondrial sources of ROS production, the exact location and topology of ROS production at the level of the mitochondrial electron transport system remain to be conclusively determined. It is generally accepted, however, that the respiratory chain complex I and III account for the majority of superoxide generated by the mitochondria (33, 169). As mentioned previously, the production of ROS at these complexes has been revealed experimentally using the inhibitors rotenone (complex I) and antimycin (complex III) (reviewed in (283)). While the ROS production at these sites has been associated with metabolic perturbations (5, 7), their physiological roles remain poorly understood. Reverse electron flow/flux (REF) through complex I was first reported nearly 50 years ago by Chance and Hollunger (46, 47). REF involves the transfer of electrons through complex I in a manner opposite to the conventional notion of 1) oxidation of NADH to 2) reduction of quinone. The experimental conditions necessary to observe appreciable REF have most often involved high concentrations of succinate added to isolated mitochondrial preparations (is in (46)). Indeed, under these experimental conditions, reduction of NAD^+ to NADH at complex I has been observed using spectrophotometry, concurrent with succinate oxidation at complex II (succinate dehydrogenase) and a paradoxical increase in the mitochondrial $\Delta\Psi$ (17, 46). REF has also been observed using glycerophosphate (Gp) as substrate, which is oxidized at the outer surface of the mitochondrial inner membrane (206). Production of ROS linked to REF has been shown (166, 171, 291). However, the physiological

relevance of REF-associated ROS production has been questioned due to the experimental conditions necessary to demonstrate the phenomenon (i.e., high $\Delta\Psi$ and reduced natural antioxidant capacity (126, 166, 251)). Moreover, induction of REF using succinate (S) alone as mitochondrial substrate does not reflect the physiological situation in which multiple substrates feed into at least both complexes I and II simultaneously (126). Regardless, complex I has been increasingly implicated in mitochondrial ROS production, and this is thought to involve both forward and reverse electron flows through complex I (169, 290). Rotenone blocks the transfer of electrons from the iron-sulfur center N2 to quinone (216), which inhibits the flow of electrons into the quinone-cytochrome bc1 of complex III. However, reduction of molecular oxygen to superoxide anion can still occur at the FMN site of complex I. Indeed, the use of the complex I inhibitor rotenone has been shown to both inhibit REF-mediated ROS production, and to increase ROS production when electron flow from complex I substrate (i.e., those generating NADH) (reviewed in (36)).

Additional evidence against the physiological relevancy of succinate alone as substrate comes from studies examining the regulation of succinate dehydrogenase, (SDH, complex II) (2, 45, 85, 284). When S is added to a mitochondrial preparation without rotenone, complex I is free to generate NAD^+ in the mitochondrial matrix. NAD^+ is a cofactor involved in three of the reactions of the Krebs cycle, one of which is the oxidation of malate to oxaloacetate (OAA) by malate dehydrogenase. Because OAA cannot permeate the mitochondrial inner membrane, it accumulates in an isolated mitochondrial preparation in the absence of a source of acetyl CoA with which to condense to citrate (103). Even at low concentrations, OAA is a more potent competitive inhibitor of SDH than malonate, the commercially available tool used to experimentally inhibit SDH (17). This concept is supported by the experimental observation of

increased respiratory O_2 consumption upon addition of rotenone to a mitochondrial suspension in which S, or S + malate are substrates (78). The concept of feedback inhibition of SDH by OAA may therefore illustrate an elegant system of respiratory control, whereby efforts to maintain mitochondrial $\Delta\Psi$ by the proton translocating function of complex I is favored under states of high demand (e.g., high ADP, lowered $\Delta\Psi$). Conversely, the model dictates that under situations of low demand (e.g., low ADP), complex I activity will be less-favored; which will result in reduced NAD^+ turnover, malate dehydrogenase activity, and in turn, malate oxidation. The net effect would then be a decreased formation of OAA and consequently increased SDH activity. In a recent study exploring the effect of multi-substrate (i.e., substrates for both complex I and II) combinations in mitochondria isolated from mouse skeletal muscle, it was suggested that OAA inhibition of SDH activity may actually constitute a deliberate adaptation to minimize REF-mediated O_2^- production (204). The concept of complex I-activity - associated OAA inhibition of SDH illustrates yet another reason to interpret the results of *in vitro* mitochondrial experiments within the context of what are essentially unique metabolic situations that may in fact never present themselves physiologically.

METFORMIN: A PHARMACOLOGICAL STRATEGY TO TREAT INSULIN RESISTANCE

Dimethylbiguanide, popularly known as metformin, is among the most widely prescribed drugs for the treatment of type 2 diabetes. Improved glycemic control with metformin is generally attributed to both increased muscle glucose clearance and decreased hepatic glucose production. However, despite the routine prescription of metformin, its exact mode of action remains unclear. Two potential cellular targets of metformin have been identified: adenosine monophosphate-activated protein kinase (AMPK) (314), a major regulator of cellular glucose

and lipid metabolism, and complex I of the mitochondrial respiratory chain (17, 215). AMPK activity is increased in skeletal muscle and liver in response to either *in vitro* or *in vivo* exposure to metformin (25, 207, 314). However, cell free assays indicate that metformin does not activate AMPK directly nor alter the phosphorylation state of AMPK by upstream kinases (124). Metformin does bind to and inhibit complex I of the mitochondrial ETS (40, 43, 44, 215), but the degree of inhibition is mild and does not appear to affect overall cellular energy charge (124). Thus, although there is considerable circumstantial evidence, a direct link between AMPK and the insulin sensitizing action of metformin has not been established (123).

Metformin treatment has also been reported to reduce oxidative stress in target tissues (17, 30, 140, 214), raising the alternative possibility that its mechanism of action may be related to ROS generation and/or neutralization. Batandier et al (17) recently reported that metformin decreases complex I-linked H_2O_2 production during succinate-supported respiration in mitochondria isolated from rat liver. As mentioned previously, S induces high rates of ROS production by REF through complex I which dramatically accelerates electron leak and O_2^- formation (6, 183). While respiration supported exclusively by succinate is clearly non-physiological, the findings imply that substrate/respiration conditions *in vivo* that result in a greater proportion of reducing equivalents feeding into the electron transport system beyond complex I may lead to elevated ROS production; e.g., during basal (resting) respiration supported by fatty acids (7, 262). In support of this contention, elevated mitochondrial-derived H_2O_2 emission has recently been shown by our group to be a primary factor in the etiology of dietary fat-induced skeletal muscle insulin resistance (5). These findings raise the intriguing hypothesis that the insulin sensitizing actions of metformin *in vivo* may be mediated by its ability to attenuate complex I-mediated H_2O_2 emission induced by REF.

MITOCHONDRIAL FUNCTION AND THE RACIAL METABOLIC DISPARITY IN THE UNITED STATES

In the United States, African-American women (AW) are more likely to be overweight or obese, and are more than twice as likely to develop types 2 diabetes as Caucasian women (CW) (69). This not only suggests that AW may be genetically predisposed to metabolic disease in this country, but also suggests that studying AW may lead to a greater understanding of the etiology of insulin resistance and type 2 diabetes. The metabolic factors that precede and/or contribute to this racial disparity may involve a reduction in basal energy expenditure. Supporting this notion are reports that AW have a significantly lower resting metabolic rate (RMR) than CW (91, 147); findings that have also been observed in prepubertal African American girls compared to age and weight-matched Caucasian girls (155, 309). Skeletal muscle represents ~45% of total body mass (13) and accounts for ~25% of the body's RMR (238). In fact, at rest, approximately 25% of basal metabolic rate is due to respiration required to support proton leak (i.e., non-phosphorylating, or "idling" respiration) in skeletal muscle mitochondria (269). This implies that even subtle differences in the rate of proton leak in skeletal muscle mitochondria can profoundly influence overall metabolic control and energy balance. Furthermore, this proton leak is linked to ROS. As mentioned, mitochondrial ROS emission is evident under state 4 (resting) conditions, and increases exponentially as proton conductance decreases (i.e., $\Delta\Psi$ increases) (166). This is germane as ROS have been linked causally to the development of insulin resistance (140), the hallmark of type 2 diabetes.

THE FEMALE MENSTRUAL CYCLE

The menstrual cycle describes a periodic pattern of physiological changes that normally occur over the course of about 28 days in fertile females. The cycle is necessary for reproduction and is governed by the endocrine system. The menstrual cycle is divided into three phases: 1) the follicular phase, 2) ovulation, and 3) the luteal phase. In humans, Menstrual cycles are counted from the first day of overt menstruation, which involves blood flow from the uterus through the vagina and typically occurs once during each menstrual cycle (268). During the follicular phase, estradiol (E2) secretion by the ovaries progressively increases and circulating estrogen levels rise gradually. Overt menstruation ceases, follicles in the ovaries develop and the lining of the uterus thickens. On or about day 14, a surge in luteinizing hormone results in ovulation. Unless fertilized within the next 24 hours, the dominant follicle in the ovary becomes the corpus luteum, which produces large amount of progesterone (P4). The rising levels of P4 begin the luteal phase, and the endometrium changes in preparation for implantation of an embryo. In the absence of implantation, involution of the corpus luteum commences, resulting in sharp declines in levels of P4 and E2, and shedding of the uterine lining once again. A teleological explanation for menstruation in humans proposed by Strassmann (268) posits that the uterine endometrium is shed whenever implantation fails over the course of an ovulatory cycle because this cyclical renewal of the endometrium is less energetically costly than maintaining it in the state required for implantation. Indeed, the O_2 consumption by the human endometrium declines by seven-fold during endometrial regression (268). The cyclicity in metabolic rate matches the cyclicity of the ovarian hormones (e.g., E2 and P4), which govern the menstrual cycle (268). The effects of the ovarian hormones on metabolic rate during the normal menstrual cycle are evident in nonendometrial tissues as well (e.g., skeletal muscle) (268). The net result is that metabolic rate is more than 7% lower during the follicular phase compared to the luteal phase of the menstrual

cycle (268). The estimated energy savings during four cycles are therefore equivalent to the caloric content in roughly six days worth of food, or about 53 megajoules (268). In the context of the results of studies comparing the basal metabolic rate between AW and CW, it would seem prudent to consider ovarian hormone status. Moreover, studies examining the metabolic consequences of various effectors in women would do well to consider, control, or test ovarian hormones, of which E2 and P4 appear to exert particular influence. In the context of the current study, the role of E2 and P4 on skeletal muscle mitochondrial function was addressed.

THE EFFECTS OF PROGESTERONE AND ESTRADIOL ON PERFORMANCE AND THE METABOLIC RESPONSE TO EXERCISE

Nearly forty years ago, Reinke et al (232) observed a significant increase in free fatty acids in normal women specifically during the luteal phase, when the P4 levels in the blood were greatest. Since then, many studies have examined the effects of menstrual cycle on metabolism. Lipid oxidation has also been shown to be greatest during exercise performed in the luteal phase (313). There is no consensus in the literature as to the effect(s) of menstrual cycle on exercise performance or the metabolic response to exercise. While many published reports conclude that menstrual cycle has either no or only subtle effects on the metabolic response to exercise (96, 138, 153) or exercise performance (70, 79), others report differently (27, 64, 210). During low to moderate intensity (35-60%) exercise, women in the mid-luteal phase have been shown to oxidize lipid more, and carbohydrate less when compared to the respective exercise response during the mid-follicular phase (117). It has also been shown that the core body temperature is elevated during submaximal exercise performed during the luteal compared to the follicular phase (264). Interestingly, the effects of menstrual cycle on exercise performance may only

present themselves in nonathletes, as one study found the luteal phase of the menstrual cycle induced increases in ventilatory drives and exercise ventilation in both athletes and controls, but the athletes, in contrast to controls, demonstrated no significant decrease in exercise performance in the luteal phase (250). Further confounding the aims of the original research goals in the current study was the use of synthetic steroidal contraceptives by some of the subjects. Indeed, the metabolic response to exercise has been shown to be affected by synthetic steroidal contraceptives, with those taking oral contraceptives exhibiting a blunted rise in E2, P4, and cortisol during exercise compared to controls (28). In a study involving stable isotope dilution and indirect calorimetry, d'Eon et al (64) were able to measure glucose uptake and estimate skeletal muscle glucose oxidation during exercise while manipulating the blood levels of E2 and P4 in healthy women. They discovered opposing actions of E2 and P4, the former reducing estimated muscle glycogen utilization and the rate glucose disappearance from the blood. On the other hand, increasing blood levels of P4 in addition to E2 increased the estimated muscle glycogen utilization, but not the rate of glucose disappearance from the blood. Such a description of the complementary effects of E2 and P4 in skeletal muscle substrate utilization frame the logic of testing both hormones in the current study.

ESTRADIOL, PROGESTERONE AND INSULIN SENSITIVITY

The volume of published data demonstrating that sex steroids affect the sensitivity of tissues to insulin in animal models are substantial (reviewed in (184)). More relevant to the current project are reports that show women with high serum levels of sex steroids are at a greater risk of developing type 2 diabetes (e.g., (180)). Premature adrenarche has also been shown to reduce insulin sensitivity in girls, and may also be at a greater risk of developing

polycystic ovarian syndrome in later adulthood (294). Normal pregnancy is associated both with high circulating levels of both estrogens and P4 and also reduced insulin sensitivity (134). Similarly, a fall in insulin sensitivity has been reported in normal women during the luteal phase of the menstrual cycle, when serum P4 and E2 levels are both at their greatest (184). When ethinyl estradiol was administered to male transsexuals, a reduced peripheral glucose uptake was observed without any change in endogenous glucose production, indicating that estrogens may have a peripheral site of action (225). Because skeletal muscle is responsible for the majority of peripheral glucose disposal, it would appear that sex steroids have a direct effect on skeletal muscle insulin sensitivity. However, despite evidence relating sex steroids and insulin resistance (184), the exact nature of the link is unclear.

NON-GENOMIC EFFECTS OF ESTROGEN AND PROGESTERONE ON MITOCHONDRIAL FUNCTION

As early as 1963, Chance and co-workers (51) reported that high concentrations (i.e., mM) of P4 exhibited a "rotenone-like" effect on respiration and pyridine nucleotide reduction in mitochondria isolated from pigeon heart. A review (263) of the this, and subsequent publications regarding posttranslational effects of supraphysiological experimental steroid hormone concentrations on the function of isolated mitochondria casted doubt upon the physiological relevance of these studies. More recently, studies examining the effects of E2 and P4 on mitochondrial function have employed treatment designs that increase the physiological relevancy of their results. In a study of the effects of *in vivo*-administered P4 and E2 on mitochondria isolated from mouse liver, it was found that state 3 respiration supported by complex II substrate (succinate) was reduced after three hours of treatment with either P4 alone,

or in combination with E2 (114). When the mitochondria were supplied exclusively with complex I substrates (glutamate + malate), both state 3 and state 4 respiration were significantly lowered by treatment with either P4 alone, or in combination with E2 compared to controls (114). Interestingly, while the RCR calculated with succinate as substrate in mice treated with E2 or E2 + P4 was reduced from controls, neither the RCR or P:O ratio could be determined for mice treated with either P4 or E2, such was the inhibition of state 3 respiration (114). These effects of the female sex steroid hormones on mitochondrial respiration were not observed during TMPD + ascorbate respiration, which supplies electrons exclusively to complex IV (114). This suggests complex I as one of the sites of action by P4, and possibly the P4 + E2 combination as well on mitochondrial respiration. When mitochondria isolated from male rat livers were incubated briefly (1 min) with 30 μM E2, both state 3, and FCCP-uncoupled respiration supported by the complex I substrates glutamate + malate were significantly reduced from controls (202). The effect of E2 was also manifest in a lower RCR (glutamate + malate). However, E2 treatment had no effect on the mitochondrial $\Delta\Psi$ (202). Furthermore, no effect of E2 on mitochondrial H_2O_2 production was observed, with or without rotenone present (202). This is in contrast to findings of increased mitochondrial ROS in cultured cells treated for 15 minutes with very high (i.e. > 360 nM) E2 (86). Even more recently, it was shown that adding P4 to preparations of isolated rat liver mitochondria during the experimental measurements decreased the $\Delta\Psi$, calcium retention capacity and the capacity for complex I-linked state 3 respiration (84). However, as with most of the investigations into the non-genomic effects of female sex steroids on mitochondrial function, the P4 concentrations used were supraphysiological - in this case, anywhere from 80-150 μM, or over 1000 times greater than the luteal phase serum P4 concentration in women (237). In the current study, luteal phase serum

concentrations of E2 and P4 were used to test the acute effects of E2 and P4 on mitochondrial function.

EXERCISE, SKELETAL MUSCLE INSULIN SENSITIVITY AND MITOCHONDRIAL FUNCTION

During exercise, insulin-independent and -dependent glucose uptake by human skeletal muscle is enhanced (124). Furthermore, exercise training improves insulin sensitivity in the Zucker rat model of obesity-associated peripheral insulin resistance (53). After a single bout of exercise, insulin sensitivity increases primarily in the muscles involved in the physical activity, an effect which may last for up to two days (124, 305). This acute effect of exercise on skeletal muscle insulin sensitivity corresponds to glycogen replenishment in the exercised muscles (305). Though improved insulin sensitivity with acute exercise may be short-lived and likely involves improvements in GLUT4 content and/or trafficking rather than improved insulin receptor signaling (77, 124), evidence exists for metabolic adaptations which sustain whole-body muscle insulin sensitivity with exercise training via enhanced insulin signaling involving the PI3-K pathway and AMPK (reviewed in (124)).

First reported by John Holloszy in 1967 (135), the notion of increased mitochondrial oxygen consumption and respiratory activity in skeletal muscle with exercise training has since become dogma, with the generally accepted explanation being an increase in the transcriptional/posttranscriptional activities involved in mitochondrial biogenesis following skeletal muscle contractions associated with exercise (reviewed in (137, 185)). As mentioned previously, the observation of reduced mitochondrial content in the skeletal muscle of individuals with type 2 diabetes (125, 157) lead to the hypothesis that such a reduction in

mitochondrial content was responsible for skeletal muscle insulin resistance due to the accumulation of lipid within the tissue (32, 203, 221). This hypothesis was subsequently extended to the effects of exercise on skeletal muscle insulin sensitivity (167). However, the notion that exercise exerts its insulin-sensitizing effect via removal of intramuscular lipid was confounded by the fact that highly trained, insulin-sensitive individuals similarly exhibit high intramuscular lipid content, a phenomenon termed the “athlete paradox” (113). Moreover, Tuominen et al (281) describe an acute phenomenon of insulin resistance following prolonged exercise, they called the “postmarathon paradox” of insulin resistance in otherwise healthy subjects the day following a marathon run. Additionally, the postmarathon paradox also involves impaired glycogen resynthesis that is not related to any decrease in muscle GLUT4 content (12). Therefore, perturbances to the insulin signaling pathway in skeletal muscle are likely to blame for the postmarathon paradox, and may involve secondary inflammation associated with exercise-induced muscle damage (54), or possibly even reactive oxygen species (194).

It is generally accepted that during exercise, high rates of oxygen flux in skeletal muscle increase the rates of ROS production, the source of which is been attributed primarily to the mitochondria (reviewed in (189)). However, this is based upon the misconception that mitochondrial ROS accounts for ~2% of total O_2 consumed. The early work of Britton Chance and co-workers demonstrated that this high percentage of mitochondrial ROS is only evident under resting (i.e., state 4) conditions (48). Indeed, with regard to the effects of exercise on REF-mediated mitochondrial H_2O_2 production, Sahlin et al (243) very recently published a study that examined the effects. In mitochondria isolated from the vastus lateralis muscles of males athletes participating in ultra-endurance exercise, the rate of state 4, succinate-supported (i.e., due to REF) H_2O_2 emission detected by Amplex red was increased 73% immediately post-exercise

from pre-exercise rates (243). As expected, the rates of H_2O_2 emission from mitochondria isolated from a biopsy performed 28 hours after the exercise returned to pre-exercise rates (243). As mentioned previously, during state 3 respiration, mitochondrial ROS production drops precipitously. Because studies have shown that intense or exhaustive exercise is associated with markers of oxidative stress (189, 241), it has since been proposed that extramitochondrial or extracellular sources of ROS are involved , possibly due to ROS generated by xanthine oxidase (112). Regardless of the oxidant source, an accumulating body of evidence employing both direct (i.e., ESR spectroscopy) and indirect methodologies (e.g., assay of oxidatively modified macromolecules) strongly suggests that free radicals generated during mild to moderate endurance exercise actually constitute a stimulus mechanism for adaptations to exercise, including mitochondrial biogenesis in skeletal muscle (reviewed in (241)). An example illustrating this view comes from a very recent study in which severely overtrained athletes exhibited elevated levels of lipid peroxidation products and protein carbonylation compared to control athletes, both at rest and after exhaustive exercise (274). While exercise-associated redox stress has been shown to affect signaling pathways that include the PI3-kinase/Akt, p53 and heat shock proteins, the mitogen-activated protein kinase (MAPK) and nuclear factor (NF) κB pathways are thought to be major players in the cellular reaction to ROS (150). The NFκB protein complex was first suggested to be redox-responsive when Sen et al (255) demonstrated that NFκB activation in L6 muscle cells was responsive to H_2O_2 treatment and controlled by intracellular GSH:GSSG status. Upon direct reaction with H_2O_2 or other ROS, NFκB is translocated to the nucleus, where it binds to a number of target gene promoters, initiating activation of various target genes, one of which is the manganese-containing superoxide dismutase (MnSOD), a mitochondrial enzyme which converts superoxide to H_2O_2. Indeed, it was

shown that the expression of MnSOD increased significantly after a single, 1 –hour bout of exhaustive exercise in rat skeletal muscle (133), and further confirmed that acute exercise increases the NFκB signaling pathway in rat skeletal muscle (151). Observations made by these and others regarding the adaptive response to exercise-association redox perturbations (227, 241) may therefore account, at least in part, for the often unimpressive results from studies examining the effects antioxidant supplementation on adaptation to exercise (reviewed in (38)) and improvements to diabetes (reviewed in (256)). In fact, a very recent study found that supplementation with the antioxidant vitamins C and E actually prevented the exercise-associated benefits on insulin sensitivity in humans (236). As expected, the RNA transcripts for the endogenous antioxidant enzymes superoxide dismutase and glutathione peroxidase were increased in the control subjects after 4 weeks of exercise training in the control group; however, no change was observed in the group taking antioxidant supplements (236). Taken as a whole, the literature suggests that the improvements in whole-body insulin sensitivity with exercise training may owe to adaptations associated with attenuating mitochondrial ROS. Whether this involves an increase in the antioxidant defense, a decrease in the production of ROS, or both, remains to be clarified.

CONCLUSIONS

Characterized by skeletal muscle insulin resistance, type 2 diabetes constitutes a rising health concern in the modern industrialized world. An accumulating body of literature suggests that ROS produced by mitochondria in skeletal muscle leads to the development of insulin resistance in the tissue. Indeed, ROS has been linked causally to the development of insulin resistance; more specifically, we recently demonstrated the potential importance of complex I-

linked H_2O_2 to the etiology of dietary fat-induced skeletal muscle insulin resistance (5). In light of this recent work, and because many of the drugs used to treat hyperglycemia (e.g., berberine, thiazolidinediones, metformin) also bind to and/or inhibit complex I of the mitochondrial respiratory chain (40, 282), it was reasoned that a common mechanism may exist by which mitochondria effect insulin sensitivity involving complex I – or more specifically, complex I-linked H_2O_2. In the current study, it was hypothesized that three known modulators of peripheral insulin sensitivity (i.e., progesterone/estradiol, metformin and exercise training) would affect complex I-linked respiration and H_2O_2 emission in concert with their effects on insulin sensitivity, without necessarily affecting the capacity for respiratory O_2 flux in skeletal muscle.

CENTRAL HYPOTHESIS

Taken together, the evidence described above supports the notion that modulators of insulin sensitivity may do so through their ability to affect the mitochondrial complex I-linked fate of O_2 in skeletal muscle. Furthermore, preliminary data suggest that insulin sensitivity modulators may be more specific for complex I-linked H_2O_2 induced by reverse electron flow than for complex I-linked respiration. Therefore, the central hypothesis of this project is that known modulators of insulin sensitivity (i.e., metformin, estradiol & progesterone and exercise training) alter the potential for complex I-linked mitochondrial H_2O_2 emission while having little to no effect on respiration in skeletal muscle.

SPECIFIC AIM #1. Determine the effects of metformin treatment on skeletal muscle mitochondrial complex I-linked respiration, H_2O_2 emission and insulin sensitivity.

The insulin-sensitizing drug metformin has been shown to bind to and inhibit complex I of

the mitochondrial ETS (40, 44, 215). More specifically, Batandier et al (17) recently demonstrated that ROS production induced by reverse electron flow at complex I in isolated liver mitochondria is inhibited by metformin, while respiration was not significantly affected. To test the hypothesis that the mechanism of metformin action at therapeutic concentrations (i.e., micromolar) may be related to the attenuation of complex I-linked ROS generation, the following questions were addressed:

a) In addition to improved glycemic control (OGTT), how does oral metformin treatment affect complex I-linked respiration and H_2O_2 emission in the skeletal muscle of an animal model of obesity-associated peripheral insulin resistance?
b) What is the *in vitro* dose-response relationship between metformin concentration and complex I-linked respiration and how does it compare to the dose-response relationship between metformin and complex I-linked H_2O_2 emission in skeletal muscle?

SPECIFIC AIM #2. Determine the influence of progesterone and estradiol on skeletal muscle mitochondrial JO_2, H_2O_2 emission and insulin sensitivity.

Preliminary data suggested that serum progesterone (P4) concentration correlates positively with the potential for complex I-linked H_2O_2 emission in skeletal muscle from lean and obese women at various stages of their normal menstrual cycle; that acute, *ex vivo* P4 treatment inhibits complex I-linked respiration in human skeletal muscle; and that acute, *ex vivo* incubation with progesterone increases the potential for complex I-linked mitochondrial H_2O_2 emission in human skeletal muscle. Given that insulin sensitivity decreases during the luteal phase of the normal menstrual cycle (83, 230, 285) when sex steroid levels are at their highest (190), it is hypothesized that a connection exists between P4 and/or estradiol (E2) and peripheral insulin

sensitivity. To test the hypothesis that P4 and/or E2 are related to insulin sensitivity and that this effect is similarly manifest in skeletal muscle mitochondrial function, the following questions were addressed:

a) What is the relationship between serum progesterone, estradiol and insulin sensitivity of lean and obese premenopausal women?

b) What is the relationship between serum P4, E2 and mitochondrial complex I-linked respiration and H_2O_2 emission in the skeletal muscle of premenopausal women?

c) What is the *in vitro* effect of upper luteal phase serum concentrations of progesterone (60 nM (237)) and estradiol (1.4 nM (237)) on complex I-linked respiration and H_2O_2 emission in skeletal muscle during a multi-substrate titration protocol?

SPECIFIC AIM #3. To determine the effects of exercise training on skeletal muscle mitochondrial complex I-linked respiration, H_2O_2 emission and insulin sensitivity in lean and obese women.

Regular exercise has long been recognized as an effective therapeutic modality to improve insulin sensitivity. The membrane potential-dependent ROS generated by reverse electron flow at complex I is reduced exponentially as proton leak across the mitochondrial inner membrane (i.e., mild uncoupling) increases (166, 198). Exercise training has been shown to increase the mitochondrial content of known contributors to basal proton leak (e.g., uncoupling proteins), which would, in theory, decrease membrane potential-dependent ROS generation at complex I (198). To test the hypothesis that exercise training improves insulin sensitivity concurrent with increased mitochondrial respiration and reduced complex I-linked H_2O_2 emission in skeletal muscle, the following questions were addressed:

a) How does 8 weeks of exercise training affect insulin sensitivity (HOMA-IR) in lean and obese premenopausal women?

b) How does 8 weeks of exercise training affect complex I-linked respiration vs. complex I-linked H_2O_2 emission in lean and obese premenopausal women?

c) What is the relationship between improved insulin sensitivity and changes in both mitochondrial respiration and complex I-linked H_2O_2 emission following 8 weeks of exercise training in lean and obese premenopausal women?

METHODOLOGICAL CONSIDERATIONS

Preliminary data indicated that in lean AW, the maximal rate of respiration induced by fully uncoupling the mitochondria with the lipophilic iononophore carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) exceeded the maximal rate achieved during ADP-stimulated respiration (state 3). This suggested that in AW there is a limitation to respiration by the phosphorylation system, of which the adenine nucleotide translocase (ANT) is a major component. The ANT catalyzes the exchange of ADP for ATP across the mitochondrial inner membrane, is the most abundant mitochondrial protein, and was recently found to be responsible for $^1/_2$ to $^2/_3$ of the basal respiratory proton conductance in skeletal muscle by the research group of Martin Brand (37). Moreover, the skeletal muscle/cardiac-specific isoform of ANT (ANT1) protein content increases with regular exercise training (87), suggesting a potential mechanism by which an increase in physical activity ameliorates obesity-associated insulin resistance. Preliminary data indicated that a difference in the content of ANT1 in rectus abdominus was greater in CW vs. AW (Appendix A, Figure 13). Preliminary data further demonstrated that ten days of exercise training could increase ADP-stimulated respiration in skeletal muscle

mitochondria from AW. Thus, the central hypothesis of the project was initially that AW possess skeletal muscle mitochondria with lower ANT1 content, which limits state 3 respiration, decreases basal respiration and predisposes them to conditions favoring mitochondrial ROS emission, and that ten days of exercise training ameliorates these racial mitochondrial disparities. However, during the course of data collection, it was discovered that the female subjects in the current study were not controlled for menstrual cycle status – i.e., the relative phase of the female menstrual cycle did not dictate the day upon which any given subject was sampled. Moreover, the current study also failed to exclude subjects that were taking synthetic steroidal (i.e., exogenous progestin or progestin + estrogen) contraceptives at the time of their enrollment in the study.

In addition to being predisposed towards obesity and type 2 diabetes, AW are also more likely than CW to suffer breast cancer mortality (208), which may be related to the way in which they metabolize estrogen (99, 272). There is an overwhelming body of literature describing the primarily genomic effects of estrogens on skeletal muscle function (reviewed in (82)) and the effects of sex steroids on insulin sensitivity/resistance (reviewed above). Therefore, an aim of the current project shifted away from the effects of race to investigate what was a confounding variable in the original proposal: the effects of menstrual cycle ovarian steroid hormones, estradiol (E2) and progesterone (P4), on skeletal muscle mitochondrial function as it relates to insulin resistance. Initial plans to perform western blots for the skeletal/cardiac muscle specific isoform of ANT (ANT1) were decided against, based in part upon the results of Too et al (280). They found that a single dose injection of E2 in female rats resulted in a more than 3-fold increase in RNA transcripts for ANT1 in cardiac muscle, beginning as early as 1 hour to as long as 24 hours after treatment (280). While the increase was certainly impressive in these female

rats, the effects of E2 on the expression of ANT1 was interestingly absent in male rats (280). In light of the well established variations in E2 known to occur over the course of the female menstrual cycle (described above), conclusions made about the effect of race or obesity on the expression/content of ANT1 in skeletal muscle in a group of subjects for whom the menstrual cycle was not controlled would be *a priori* dubious at best. Moreover, failure to exclude subjects taking oral contraceptives has the potential to confound any study examining insulin sensitivity (107, 109, 164), carbohydrate (108, 298) and/or lipid metabolism (106, 295) in women, even when the contraceptives are taken in low doses (220, 259). In fact, it was due in part to studies of women taking oral contraceptives containing synthetic estrogens and progestins (107, 109, 220), the most common ingredient in such forms of birth control, that lead to the speculation that P4 and/or E2 are responsible for the decrease in insulin sensitivity reported in the literature to accompany the luteal phase of the menstrual cycle (184).

An additional methodological hurdle involved tailoring the permeabilized myofiber approach to female subjects. Pilot data collected with permeabilzed myofibers from female subjects using standard protocols (174) exhibited abnormally low rates of JO_2. This was accompanied by a more than 40% increase in complex I-linked JO_2 (i.e., glutamate + malate substrates) after addition of 10 μM cytochrome *c* (Appendix A, Figure 14), indicating disruption of the outer mitochondrial membranes. Because the permeabilization process involves saponin complexes with cholesterol, we therefore suspected that the saponin concentration used in all publications employing the saponin permeabilzed myofiber approach (i.e., 50 μg/mL) was too high for these female subjects. Indeed, it has been shown in rat hepatocytes and human chondrocytes for example, that the membrane fluidity differs between males and females (20, 252). Because the cholesterol content dictates membrane fluidity, for example in human

erythrocytes (56), perhaps the sarcolemmal and/or mitochondrial cholesterol contents differ between men and women. Preliminary testing confirmed that reducing the saponin concentration was beneficial to the mitochondrial preparations from female skeletal muscle. Moreover, it was demonstrated that 30 μg/mL saponin resulted in optimal permeabilzed myofiber preparations for mitochondrial function analysis (Appendix B, Figure 15). Indeed, it was determined that the percent coefficient of variation (%CV) for repeated measurements of respirometric O_2 flux (JO_2) during state 3 respiration in 4 myofibers from one woman permeabilized with 30 μg/mL saponin was less than half the %CV of the JO_2 measured under the same conditions, but permeabilzed with the standard 50 μg/mL. This novel finding may therefore explain the anecdotal accounts of increased variability of JO_2 determinations in saponin-permeabilized myofibers from women and the concomitant propensity for cytochrome c responses (personal correspondence).

CHAPTER 2: METFORMIN SELECTIVELY ATTENUATES MITOCHONDRIAL H_2O_2 EMISSION WITHOUT AFFECTING RESPIRATORY CAPACITY IN SKELETAL MUSCLE OF OBESE RATS

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Abstract: Metformin is a widely prescribed drug for treatment of type 2 diabetes, although no cellular mechanism of action has been established. In isolated mitochondria from liver, metformin has been shown to partially inhibit respiration as well as attenuate H_2O_2 production associated with reverse electron flux at complex I. To determine whether *in vivo* metformin treatment alters mitochondrial function in skeletal muscle of an obese rodent model of insulin resistance, respiratory O_2 flux and H_2O_2 emission were measured in saponin-permeabilized myofibers from lean and obese (*fa/fa*) Zucker rats treated for 4 wks with metformin. Succinate supported respiration generated >2-fold higher rate of reverse electron flux, complex I- mediated H_2O_2 emission in myofibers from untreated obese versus lean rats, providing evidence of an obesity-associated increased mitochondrial oxidant emitting potential. In conjunction with improved glycemic control, metformin treatment significantly ($P < 0.05$) reduced H_2O_2 emission

in muscle from obese rats to rates near or below those observed in lean rats during both succinate- and palmitoyl-carnitine- supported respiration. Surprisingly, metformin treatment did not affect basal or maximal rates of O_2 consumption under a variety of substrate conditions in muscle from obese or lean rats. *Ex vivo* dose-response experiments on control myofibers revealed that metformin inhibits complex I-linked H_2O_2 emission at a concentration ~2 orders of magnitude lower than that required to inhibit respiratory O_2 flux. These findings suggest that therapeutic concentrations of metformin normalize mitochondrial H_2O_2 emission by blocking reverse electron flow without affecting forward electron flow or respiratory O_2 flux in skeletal muscle.

INTRODUCTION

Dimethylbiguanide, popularly known as metformin, is among the most widely prescribed drugs for the treatment of type 2 diabetes. However, despite the routine prescription of metformin, its exact mode of action remains unclear. Two potential cellular targets of metformin have been identified: adenosine monophosphate-activated protein kinase (AMPK) (314), a major regulator of cellular glucose and lipid metabolism, and complex I of the mitochondrial respiratory chain (17, 215). AMPK activity is increased in skeletal muscle and liver in response to either *in vitro* or *in vivo* exposure to metformin (25, 207, 314). However, cell free assays indicate that metformin does not activate AMPK directly nor alter the phosphorylation state of AMPK by upstream kinases (124). Metformin does bind to and inhibit complex I of the mitochondrial electron transport chain (40, 43, 44, 215), but the degree of inhibition is mild and does not appear to affect overall cellular energy charge (124). Thus, although there is considerable circumstantial evidence, a direct link between AMPK and the insulin sensitizing action of metformin has not been established (123).

In addition to increasing insulin-stimulated glucose uptake in skeletal muscle (97), metformin treatment has also been reported to reduce oxidative stress in target tissues (17, 30, 140, 214), raising the alternative possibility that its mechanism of action may be related to reactive oxygen species (ROS) generation and/or neutralization. Batandier et al. (17) have recently reported that metformin decreases complex I-linked H_2O_2 production during succinate-supported respiration in mitochondria isolated from rat liver. Succinate, a complex II substrate, induces high rates of ROS production by generating a backflow of electrons through complex I which dramatically accelerates electron leak and superoxide formation (6, 183). While respiration supported exclusively by succinate is unphysiological, the findings suggest that

substrate/respiration conditions *in vivo* that result in a greater proportion of reducing equivalents feeding into the electron transport system beyond complex I may lead to elevated ROS production (132); e.g., during basal (resting) respiration supported by fatty acids (7, 262). In support of this contention, elevated mitochondrial-derived H_2O_2 emission has recently been shown to be a primary factor in the etiology of dietary fat-induced skeletal muscle insulin resistance (5). In the present study, we examined whether treatment of obese Zucker (*fa/fa*) rats with metformin daily for 4 weeks alters mitochondrial H_2O_2 emission and/or O_2 respiration in the skeletal muscle of this animal model of obesity-associated peripheral insulin resistance (61, 158). Our findings reveal that, in addition to improved whole-body glycemic control, oral metformin treatment dramatically reduces the potential for complex I-linked mitochondrial H_2O_2 emission without affecting O_2 respiratory capacity in skeletal muscle. Moreover, dose-response experiments conducted *ex vivo* on control myofibers demonstrate that complex I-linked mitochondrial H_2O_2 emission is far more sensitive than complex I-linked respiration to inhibition by metformin.

METHODS

ANIMALS

All animal studies were approved by the East Carolina University Institutional Animal Care and Use Committee. Thirty-two age-matched male Zucker rats (16 lean, 16 *fa/fa* obese rats; Harlan Laboratories, Inc.) were housed in single cages in a temperature (22°C) and light-controlled (12:12 hour light-dark cycle) room with *ad libitum* access to standard chow and water

for the duration of the study. Sprague-Dawley rats (Charles River Laboratories, Inc.) were used in control dose-response experiments and were housed as described above.

METFORMIN TREATMENT AND ORAL GLUCOSE TOLERANCE TESTING

At 9-10 weeks of age, obese and lean Zucker rats were randomly assigned to receive either control (water) or metformin (320 mg/kg/day) by gavage for four weeks (n = 8/group). At the end of the fourth week, rats were fasted 10 h and an oral glucose tolerance test (OGTT; 2 g/kg BW by oral gavage of dextrose) was performed 17 h after the last dose of metformin. Blood glucose (glucose oxidase method, One Touch Ultra glucose analyzer; Lifescan, Milpitas, CA) and insulin levels (ELISA, Linco Research, St. Charles, MO) were determined in the fasting condition and at time 30, 60, and 120 min after dextrose administration. After an additional three days of treatment, rats were anesthetized (ketamine:xylazine, 10 mg/0.1 kg of 9:1 mixture, i.p.) ~4 h after metformin treatment, the gastrocnemius muscle was removed and red portions of the muscle dissected and separated for preparation of fiber bundles (mitochondrial function studies) or quick-frozen in liquid N_2. All rats were fasted 10 h prior to sacrifice.

PREPARATION OF PERMEABILIZED MYOFIBERS

This technique is partially adapted from previous methods (173, 277) and has been thoroughly described (5-7). Briefly, after dissection, connective tissue was removed and fiber bundles were separated with fine forceps under binocular dissecting microscope in ice cold buffer X, containing (in mM): 60 K-MES, 35 KCl, 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 Imidazole, 0.5 DTT, 20 Taurine, 5.7 ATP, 15 PCr, 6.56 $MgCl_2$-$6H_2O$ (pH 7.4, 295 mOsm). After separation, myofiber bundles were placed in buffer X containing 50 μg/mL saponin for 30

minutes and then were washed in ice-cold buffer Z containing (in mM) 110 K-MES, 35 KCl, 1 EGTA, 10 K_2HPO_4, 3 $MgCl_2$-$6H_2O$, 5 mg/ml BSA, 0.1 glutamate and 0.05 malate (pH 7.4, 295 mOsm) until analysis (<1 hour). Fibers used in the H_2O_2 emission experiments were briefly washed in cold buffer Z containing 10 mM pyrophosphate prior to analysis to prevent Ca^{+2}-independent contraction.

MITOCHONDRIAL RESPIRATION AND H_2O_2 EMISSION MEASUREMENTS IN PERMEABILIZED MYOFIBERS FROM LEAN AND OBESE ZUCKER RATS

O_2 consumption rate was measured by high resolution respirometry (Oroboros O_2K Oxygraph, Innsbruck, Austria) at 30°C in Buffer Z + 20 mM creatine hydrate and 50 µM *N*-Benzyl-*p*-toluene sulphonamide (BTS, an inhibitor of myosin II) under the following two protocols: *Respirometric protocol A* - 2 mM glutamate + 1 mM malate (complex I substrates) followed by sequential additions of 2 mM ADP, 3 mM succinate (complex II substrate), 10 µg/mL oligomycin (inhibitor of mitochondrial ATP synthase), and finally 2 µM carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP, a protonophoric uncoupler); *Respirometric protocol B* - 25 µM palmitoyl-carnitine + 1 mM malate followed by sequential additions of 2 mM glutamate and 3 mM succinate. H_2O_2 emission was measured at 30°C in Buffer Z during state 4 respiration (1 mM atractyloside to inhibit adenine nucleotide translocase) by continuously monitoring oxidation of Amplex Red using a Spex Fluoromax 3 (Jobin Yvon, Ltd.) spectrofluorometer under the following two protocols: *Fluorometric protocol A* - 25 µM palmitoyl-carnitine + 1 mM malate followed by sequential additions of 2 mM glutamate and 3 mM succinate; Fluorometric *protocol B* - succinate titration (in mM), 0.1, 0.25, 0.5, 0.75, 1.5 and 3.0. At the conclusion of each experiment, permeabilized fiber bundles were washed in distilled

H_2O to remove salts and freeze-dried in a lyophilizer (LabConco). Mitochondrial respiration rates are expressed as pmol·s^{-1}·mg^{-1} dry weight and H_2O_2 emission rates as pmol·min^{-1}·mg^{-1} dry weight.

WHOLE MUSCLE PROTEIN EXTRACTION AND MEASUREMENTS OF CITRATE SYNTHASE ACTIVITY

Frozen red gastrocnemius muscle samples (50-80 mg) were homogenized in ice-cold lysis buffer [50 mM HEPES, 50 mM Na^+ pyrophosphate, 100 mM Na^+ fluoride, 10 mM EDTA, 10 mM Na^+ orthovanadate, 1% Triton X-100, and protease and phosphatase (1 and 2) inhibitor cocktails (Sigma, St. Louis, MO)]. Homogenates were sonicated for 10 sec then rotated for 2 h at 4°C. After centrifugation for 25 min at 15,000 x g, supernatants were extracted and protein concentration was determined (BCA protein assay, Pierce, Rockford, IL) and individual homogenate volumes were separated into 50 µg of protein aliquots, frozen in liquid nitrogen and stored at -80°C. Citrate synthase activity was determined using the methods of Srere (261).

ACUTE METFORMIN INCUBATIONS

Skeletal muscle samples were obtained from adult male Sprague-Dawley rats and processed as described above with the exception that fiber bundles were washed (Buffer Z) without or with metformin (0.1, 1, 5 or 10 mM) for 20 min. Maximal respiration rate was determined in the presence of 5 mM glutamate, 2 mM malate, 5 mM pyruvate and 8 mM ADP. Maximal H_2O_2 emission was determined under state 4 respiration (10 µM oligomycin) supported by 3 mM succinate.

STATISTICAL ANALAYSES

Data are presented as mean ± SEM. Statistical analyses were performed with GraphPad Prism (GraphPad Software, Inc.) using a two-way ANOVA with Bonferroni post-hoc analysis of significance in experiments involving Zucker rats; one-way ANOVA with Tukey's post-hoc analysis for experiments involving Sprague-Dawley rats; and a curve-fitting model for kinetic analyses of the succinate titrations. The alpha level for significance was established *a priori* at $P \leq 0.05$.

RESULTS

GLUCOSE TOLERANCE

Oral glucose tolerance tests were performed to determine whole body glycemic control in lean and obese Zucker rats after four weeks of daily oral metformin treatment. As expected, obese Zucker rats were characterized by greater (main effect, $P < 0.0001$) area under the curve (AUC) for blood glucose (Figure 1*A*) and insulin (Figure 1*B*) compared with lean Zucker rats, consistent with peripheral insulin resistance in the obese rats. Also as expected, metformin treatment in the obese rats significantly ($P < 0.05$) reduced both glucose and insulin AUC, demonstrating improved glycemic control with the drug. No difference in glucose or insulin AUC was observed in lean rats treated with metformin. Metformin treatment did not affect body weight (Figure 2) suggesting that improvements in glucose tolerance occurred independent of body mass.

MITOCHONDRIAL RESPIRATION IN PERMEABILIZED MYOFIBERS

Previous reports indicate that metformin causes a mild to moderate decrease in oxygen consumption in isolated mitochondria or intact cells (17, 40, 215), although the effect appears to require high concentrations (millimolar) and/or long exposure time. To determine if four weeks of oral metformin treatment inhibits mitochondrial respiration in skeletal muscle of lean and obese Zucker rats, we measured respiratory O_2 flux in permeabilized fibers from the red gastrocnemius muscle. Metformin treatment did not affect complex I-linked (glutamate/malate) basal (state 4) or ADP-stimulated (state 3) respiration, either alone or in combination with the complex II substrate succinate, nor maximal uncoupled, FCCP-stimulated respiration in either lean or obese animals (Figure 4*A*). Metformin also had no effect on palmitoyl-carnitine supported respiration under any of the conditions tested (Figure 4*B*). Taken together, these results indicate that oral metformin treatment does not inhibit complex I-linked respiration (i.e., forward electron flux) in skeletal muscle of lean or obese Zucker rats under the conditions employed in the current study.

Maximal O_2 consumption was significantly greater in skeletal muscle of obese verses lean rats under nearly all respiratory conditions (Figure 4). Citrate synthase activity, a marker of mitochondrial content, tended to be higher ($P = 0.061$) in the muscle from obese rats, consistent with reports of increased mitochondrial content in muscle of rats under elevated lipid load (120, 131). However, citrate synthase activity was not different between metformin treated and control Zucker rats (Figure 3), indicating the effects of metformin treatment were not due to a change in mitochondrial content.

MITOCHONDRIAL H_2O_2 EMISSION IN PERMEABILIZED MYOFIBERS

Metformin has been shown to reduce ROS production at complex I in isolated mitochondria obtained from rat liver pre-perfused with 10 mM metformin (17). To determine if metformin attenuates reverse electron flux-mediated ROS production at complex I in skeletal muscle, mitochondrial H_2O_2 emission was measured in permeabilized fibers from red gastrocnemius muscle of lean and obese Zucker rats treated for four weeks with metformin. Titration of succinate to induce reverse electron flux revealed a more than 2-fold greater ($P < 0.05$) potential for complex I-linked H_2O_2 emission in skeletal muscle from untreated obese verses lean rats (53.2 ± 2.3 vs. 22.8 ± 2.3 pmol $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$dry wt, respectively, Figure 5*A*). Metformin treatment significantly ($P < 0.05$) reduced maximal succinate-induced H_2O_2 emission in the obese Zucker rats (30.9 ± 2.5 pmol $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$dry wt) to levels near that of untreated leans. In parallel experiments, when respiration was supported by palmitoyl-carnitine, reduced mitochondrial H_2O_2 emission was also evident in muscle from obese Zucker rats treated with metformin, particularly upon addition of succinate (Figure 5*B*). These findings clearly indicate that: 1) obesity in the Zucker rat model is associated with a marked increase in the potential for skeletal muscle mitochondrial H_2O_2 emission and 2) oral metformin treatment significantly attenuates mitochondrial H_2O_2 emission associated with reverse electron flux at complex I.

ACUTE EX VIVO EFFECTS OF METFORMIN ON MITOCHONDRIAL FUNCTION IN PERMEABILIZED MYOFIBERS

To examine the direct effects of metformin on forward (respiration) and reverse (H_2O_2 emission) electron flux at mitochondrial complex I in skeletal muscle, fiber bundles from the red portion of the gastrocnemius muscle of Sprague-Dawley rats, after permeabilization, were pre-incubated (~20 min) in increasing concentrations of metformin (0-10 mM). Maximal succinate-

generated H_2O_2 emission was reduced ($P < 0.01$) by ~50% at the lowest concentration of metformin tested (100 μM) and was further inhibited ($P < 0.001$) to <25% of the maximal rate at 10 mM metformin (Figure 6). In stark contrast, maximal ADP-stimulated O_2 consumption was maintained at the lower concentrations of metformin (0.1 and 1.0 mM) and inhibited ($P < 0.001$) only at a metformin concentration of 10 mM (Figure 6). These findings demonstrate that metformin inhibits reverse electron flux-mediated H_2O_2 emission at complex I of the mitochondrial electron transport chain in skeletal muscle at concentrations approximately two orders of magnitude lower than that required to inhibit electron flux in the forward direction.

DISCUSSION

The results of the present study demonstrate a marked reduction in the potential for mitochondrial H_2O_2 emission with metformin treatment in skeletal muscle in the obese Zucker *fa/fa* rat, concurrent with improved whole-body glycemic control. Although previous reports have provided evidence that metformin mildly inhibits complex I-supported respiration (17, 40, 44, 215) and increases AMPK activity in muscle (207, 314), we found no difference in ADP-stimulated submaximal or maximal O_2 consumption or AMPK activation (in press, (24)) in red gastrocnemius myofibers from the same lean and obese Zucker rats treated with metformin in the current study. Interestingly however, *ex vivo* experiments using control myofibers incubated with increasing concentrations of metformin revealed that metformin is indeed capable of inhibiting ADP-stimulated respiration, but at concentrations two orders of magnitude greater than that required to inhibit complex I-linked H_2O_2 emission. Thus, in view of recent data linking mitochondrial H_2O_2 emission in skeletal muscle to the development of high fat diet-

induced insulin resistance (5), the findings of the present study raise the possibility that the insulin-sensitizing actions of metformin in muscle may be mediated by the drug's ability to inhibit complex I-linked H_2O_2 emission while minimally affecting complex I-linked respiration.

Mild to moderate inhibition of the respiratory system by metformin was first reported nearly a decade ago and thus raised the prospect that the anti-diabetic actions of metformin may be somehow related to inhibition of mitochondrial respiration (17, 215). However, the effect requires very high concentrations (>10 mM) of metformin in isolated mitochondria (17, 40, 44) or extended exposure time in intact cells (215). This time-dependency of metformin action is attributed to the slow membrane potential-driven rate at which metformin accumulates in the mitochondrial matrix (81). Complex I has been identified as a potential site of metformin action based on the finding that respiration is inhibited by metformin only when supported by complex I but not complex II substrates (17, 40, 44). However, even at high concentrations, the degree of inhibition of respiration by metformin is only a fraction of that observed with the classical complex I inhibitor rotenone (17). Nevertheless, it has been proposed in liver that even mild inhibition of mitochondrial respiration, which has a high flux-control coefficient over gluconeogenesis, may represent a mechanism by which metformin reduces hepatic glucose output (215).

As might be predicted by a partial inhibition of respiratory function, metformin has also been shown to activate AMPK in multiple cell types (130, 170, 314, 316), including skeletal muscle (207, 314). As a target for metformin, AMPK is an attractive candidate given that it is activated by decreases in cellular energy charge and, once phosphorylated, coordinates the simultaneous activation of catabolic and inactivation of anabolic pathways by phosphorylating key metabolic enzymes and transcription factors (122). In liver, AMPK and its upstream kinase,

LKB1, appear to be required for both the glucose- and lipid- lowering effects of metformin (25, 312). However, the mechanism by which metformin may activate AMPK is less clear as cellular energy charge (i.e., ATP:ADP ratio) does not appear to be affected by metformin treatment in hepatocytes, and metformin does not activate AMPK in cell-free systems indicating that there is no direct interaction between metformin and AMPK (124). In skeletal muscle, decreased cellular energy charge and increased AMPK activity have been reported with metformin treatment (207, 314), but a definitive link has not been established. Moreover, treatment with oral metformin in rats requires at least 5 hours before an increase in AMPK phosphorylation in skeletal muscle is observed (271), and metformin appears to increase phospho-AMPK specifically in fast twitch, glycolytic fiber-type muscle groups (24). Thus, the mechanism by which metformin activates AMPK, and whether AMPK-induced signaling constitutes the mechanism responsible for the insulin-sensitizing actions of metformin in skeletal muscle, remain unknown.

An alternative mechanism of action for metformin recently emerged from studies of the mitochondrial permeability transition pore (PTP). The PTP is a large conductance channel within the inner mitochondrial membrane that opens in response to a number of physiological factors and various forms of cellular stress (e.g., large increases in intracellular [Ca^{+2}]), triggering collapse of the protonmotive force and release of pro-apoptotic factors (118). Although the molecular composition of the PTP is unknown, complex I is thought to comprise at least part of the pore complex (17). Leverve and co-workers (17) recently found that metformin inhibits PTP opening in permeabilized cells in response to Ca^{2+} overload and in intact cells in response to high glucose concentrations or the oxidizing agent t-butyl hydroperoxide. In fact, metformin was found to be nearly as effective as cyclosporin A in preventing PTP opening in

intact and permeabilized cells (17). Inclusion of the antioxidant N-acetyl-L-cysteine prevents hyperglycemia-induced opening of the PTP (17), suggesting that oxidative stress may represent the underlying link between high glucose, PTP opening and the therapeutic effects of metformin. Indeed, metformin has been reported to reduce oxidative stress in many (17, 30, 140, 214) though not all (9, 44) studies. Notably, Batandier et al. (17) found that ROS production induced by succinate-supported reverse electron flux at complex I in isolated liver mitochondria is inhibited by metformin in a manner similar to rotenone, implying that complex I is the source of free radical production associated with hyperglycemia. While respiration supported exclusively by succinate is clearly unphysiological, the findings suggest that substrate/respiration conditions *in vivo* that result in a greater proportion of reducing equivalents feeding into the electron transport system beyond complex I may lead to elevated ROS production (132); e.g., during basal (resting) respiration supported by fatty acids (7, 262). In support of this contention, elevated mitochondrial-derived H_2O_2 emission has recently been shown to be a primary factor in the etiology of dietary fat-induced skeletal muscle insulin resistance (5). However, it should also be noted that a very recent report by Schonfeld et al (251) demonstrated no complex I reverse electron flow acitivity in mitochondria respiring on activiated fatty acids (e.g., palmitoyl-carnitine). Thus, the effects of metformin on complex I may not necessarily be due to succinate-driven, or even fatty acid-driven reverse electron flow alone. *In vivo*, multiple substrates enter the mitochondrial ETS simultaneously. This multifactorial substrate combination may therefore be effected by the inhibitory effects of metformin on complex I reverse electron flow in ways that are not fully replicated in mitochondrial experiments involving singular substrate conditions. Nevertheless, the results of the current study clearly demonstrate the effects of metformin on inhibiting the flow of electrons in the reverse direction (mE_{H2O2}) vs. the forward direction (JO_2).

The present study also demonstrates that the maximal rate of mitochondrial H_2O_2 emission induced by succinate-driven reverse electron flux at complex I is more than 2-fold greater in skeletal muscle from obese vs. lean Zucker rats (Figure 5). This difference is nearly identical to the increase in H_2O_2 emitting potential previously observed in skeletal muscle of both rats and mice fed a high fat diet or humans after consuming a high fat meal (5). Moreover, in rats injected with a mitochondrial-targeted antioxidant, or in mice genetically-engineered to express catalase in their muscle mitochondria, mitochondrial H_2O_2 emitting potential was restored to normal and rodents were protected against high fat diet-induced insulin resistance (5). In the present study, metformin had nearly an identical effect, decreasing complex I-derived H_2O_2 emitting potential in the muscle of obese rats to a rate nearly equal to that observed in lean rats in conjunction with improving glucose tolerance.

Metformin did not affect the rate of O_2 consumption during basal (state 4) respiration supported by either glutamate/malate or palmitoyl-carnitine/malate, indicating that the decrease in H_2O_2 emission induced by metformin was not due to an uncoupling effect. Surprisingly, in contrast to the fairly well-established mild inhibitory action of metformin on ADP-stimulated respiration (17, 40, 44, 215), we found no evidence of impaired ADP-stimulated, complex I-supported respiration in muscles from metformin treated lean or obese rats (Figure 4). To further explore this discrepancy, we tested *in vitro* the concentration-dependent effects of metformin on mitochondrial H_2O_2 emission and O_2 consumption in parallel. Interestingly, we found metformin is capable of suppressing mitochondrial H_2O_2 emission at concentrations two orders of magnitude lower than concentrations needed to inhibit respiration (Figure 6). In the context of metformin's action *in vivo*, these findings are significant as they suggest that at therapeutic doses

(i.e., micromolar range) (97), metformin is capable of suppressing muscle mitochondrial H_2O_2 emission with little to no affect on mitochondrial respiration.

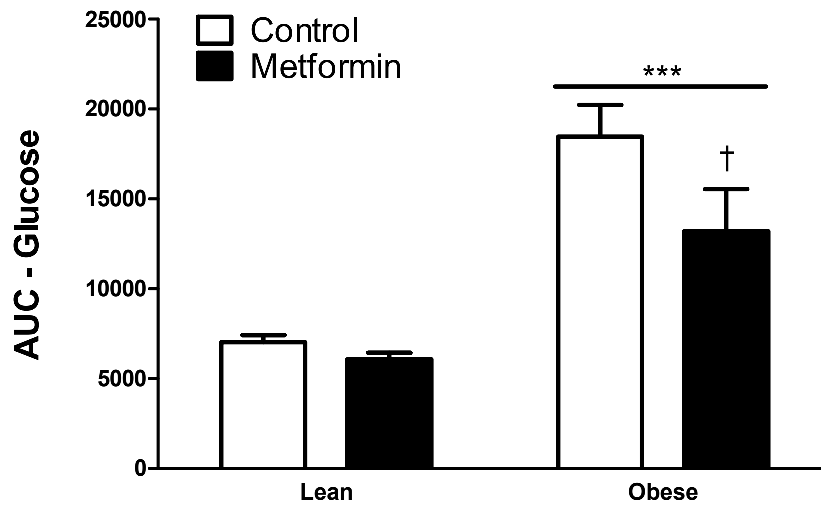
It is proposed that the unique ability of metformin to limit reverse electron flux-associated superoxide production at complex I, while minimally affecting forward electron flux (i.e., respiration), supports this model as the potential insulin-sensitizing mechanism of action of the drug in skeletal muscle (Figure 7). Although impaired insulin action specific to skeletal muscle has been demonstrated in the obese Zucker *fa/fa* rat (61, 158); and 100 μM metformin has been shown increase insulin-stimulated glucose uptake in human skeletal muscle (97), further research will be necessary to determine the exact nature of the effect of metformin on muscle-specific insulin sensitivity as it relates to mitochondrial oxidant emission *in vivo*, as well as the subsequent intermediary steps that may link reduced mitochondrial oxidant emission to restored insulin sensitivity.

ACKNOWLEDGEMENTS

This study was supported by U.S. National Institute of Health grants R01 [DK074825] & [DK073488] (PDN) and [DK061314] (RNC).

FIGURE 1. EFFECTS OF METFORMIN TREATMENT ON ORAL GLUCOSE TOLERANCE IN LEAN AND OBESE ZUCKER RATS.

Area under the curve (AUC) from an oral glucose tolerance test performed on obese and lean male Zucker controls following four weeks of metformin treatment. Results reveal significant main effects for obesity (***$P < 0.0001$) on AUC for both glucose (A) and insulin (B). Metformin treatment (black bars) in the obese rats improved glucose tolerance compared with control (white bars), reducing blood glucose (A) and plasma insulin (B) concentrations (*$P < 0.05$). The data represent the means ± SEM ($n = 8$/group).


A.
25000
20000
15000
10000
5000
0
AUC - Glucose
Control
Metformin
***
†
Lean
Obese

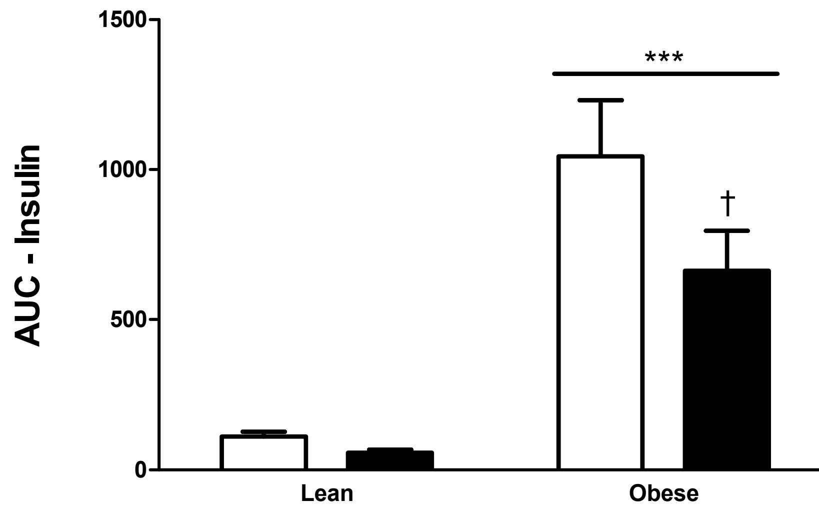

B.
1500
1000
500
0
AUC - Insulin
***
†
Lean
Obese

FIGURE 2. BODY MASS CHANGES IN LEAN AND OBESE ZUCKER RATS OVER THE COURSE OF FOUR WEEKS OF METFORMIN TREATMENT.

Body mass of lean (circles) and obese (squares) Zucker rats measured over four weeks in metformin treated (open symbols) and control groups (black symbols). A significant difference in the body mass of the obese vs. lean rats was observed (***$P < 0.001$) However, there were no differences in either the obese or lean groups with metformin treatment. The improved insulin sensitivity with metformin treatment was therefore independent of body mass. The data represent the means ± SEM ($n = 8$/group).

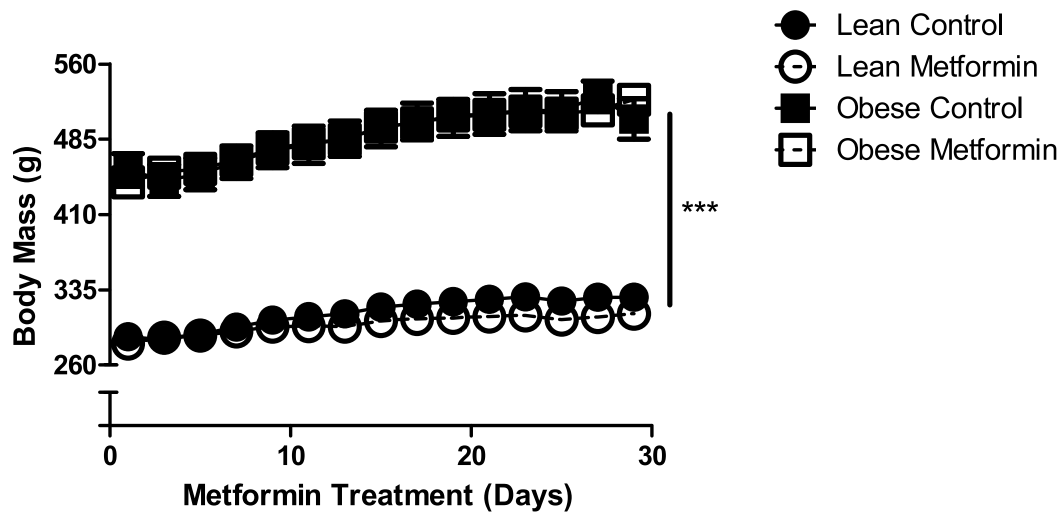
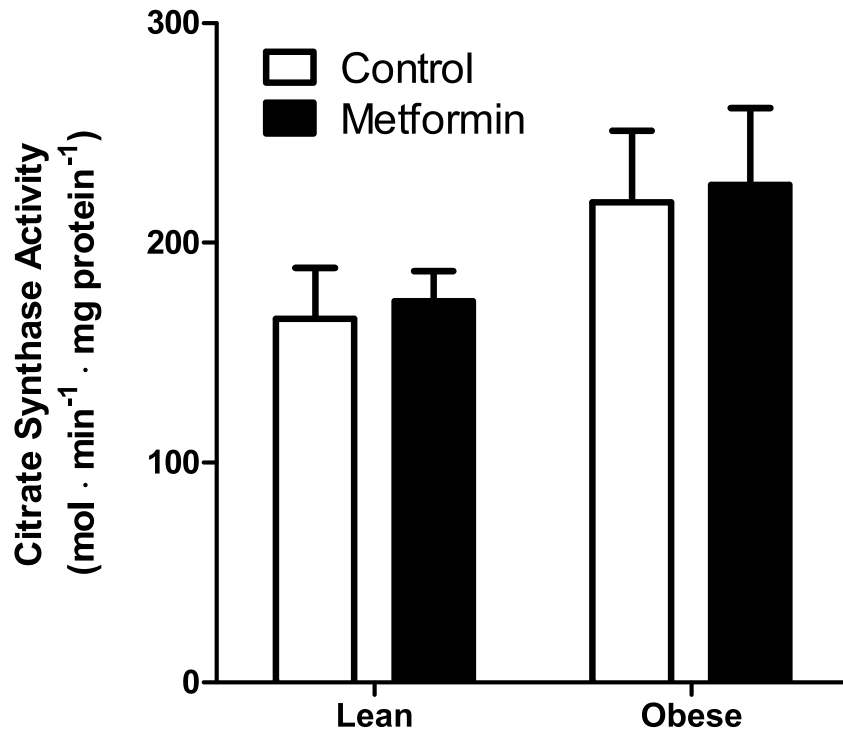

Lean Control
Lean Metformin
Obese Control
Obese Metformin
560
485
410
335
260
Body Mass (g)
***
0
10
20
30
Metformin Treatment (Days)

FIGURE 3. EFFECTS OF METFORMIN TREATEMENT ON CITRATE SYNTHASE ACTIVITY IN SKELETAL MUSCLE FROM LEAN AND OBESE ZUCKER RATS.

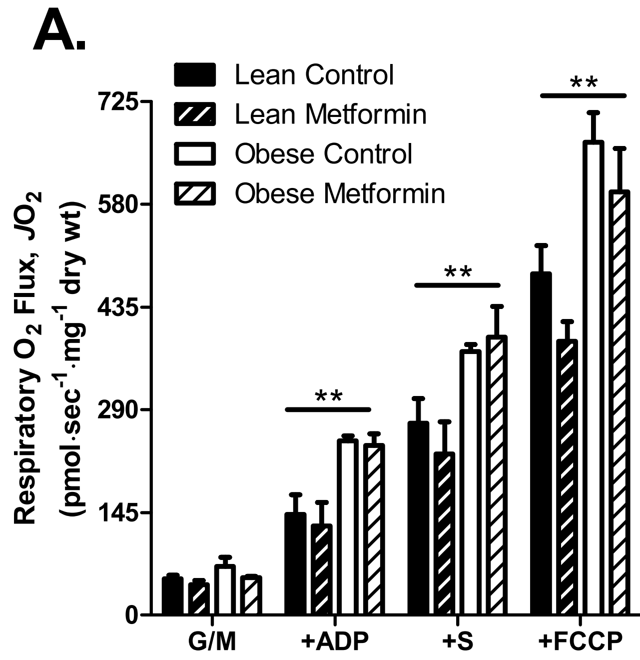
Citrate synthase activity, a marker of mitochondrial content, tended to be higher (P = 0.061) in the muscle from obese rats. However, citrate synthase activity was not different between metformin treated (black bars) and control (white bars) Zucker rats, indicating the effects of metformin treatment were not due to a change in mitochondrial content. The data represent the means ± SEM (n = 4/group).



300
200
100
0
Control
Metformin
Citrate Synthase Activity
(mol · min-1 · mg protein-1)
Lean
Obese

FIGURE 4. EFFECTS OF ORAL METFORMIN TREATMENT ON SKELETAL MUSCLE RESPIRATORY O_2 FLUX MEASURED IN PERMEABILIZED MYOFIBERS FROM LEAN AND OBESE ZUCKER RATS.

A: Respiratory O_2 flux supported by the complex I-linked substrates glutamate/malate (G/M, 2/1 mM) was greater in obese rats in the presence of maximal ADP (2 mM), upon addition of the complex II succinate (+S, 3 mM), and during maximally uncoupled respiration (+FCCP, 2 μM + 10 μg/ml oligomycin to block ATP synthase). The data represent means ± SEM (n = 4/group). ** Main effect for obesity, $P < 0.01$. B: Respiratory O_2 flux supported by the activated fatty acid palmitoyl-carnitine and malate (P-C/M, 25 μM/1 mM) alone or in the presence of ADP (+ADP) was greater in the obese rats, as was the ADP-stimulated O_2 flux after adding the complex I substrate glutamate (+G, 2 mM) and the complex II substrate succinate (+S, 3 mM) (*main effect for obesity, $P < 0.05$; **$P < 0.01$). No effect of metformin treatment on respiratory O_2 flux was observed in A or B. The data represent the means ± SEM (n = 4/group).


A.
Lean Control
Lean Metformin
Obese Control
Obese Metformin
Respiratory O2 Flux, JO2
(pmol·sec-1·mg-1 dry wt)
725
580
435
290
145
0
**
**
**
G/M
+ADP
+S
+FCCP

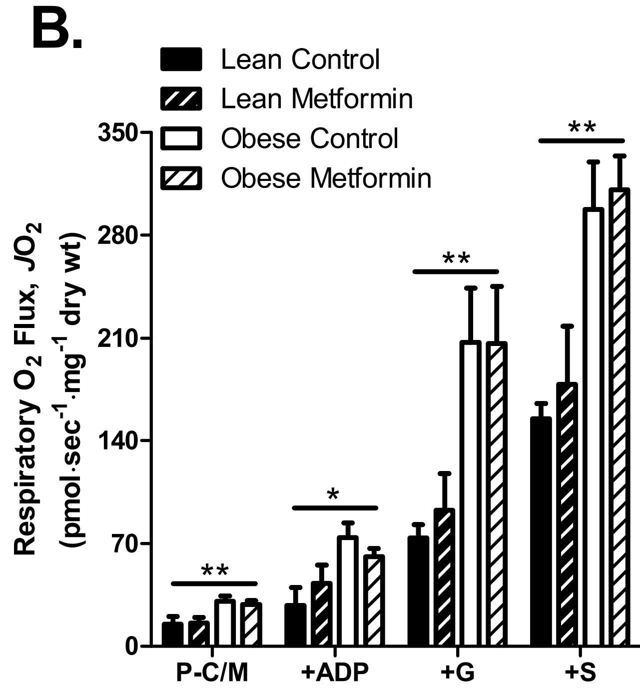
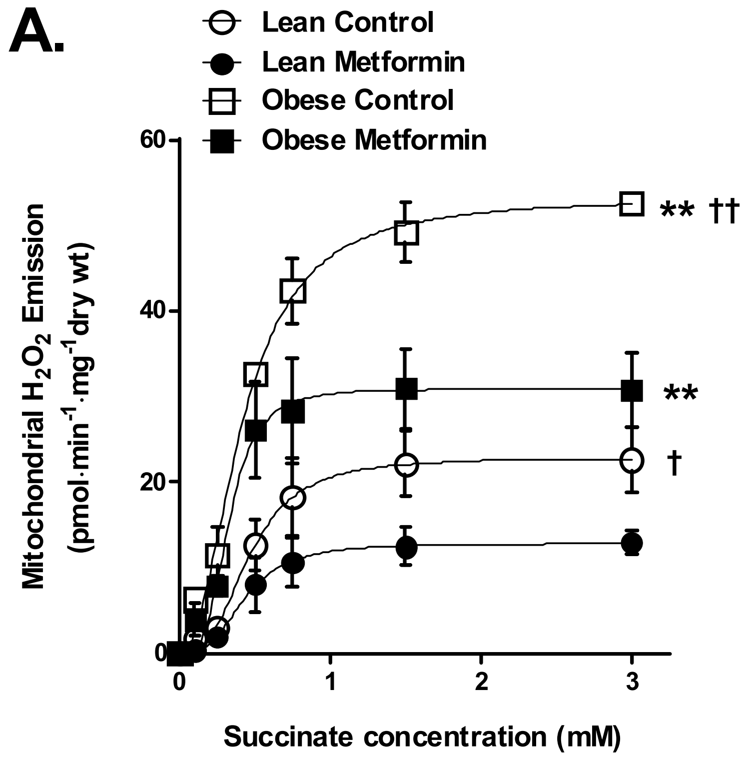

B.
Lean Control
Lean Metformin
Obese Control
Obese Metformin
Respiratory O2 Flux, JO2
(pmol·sec-1·mg-1 dry wt)
350
280
210
140
70
0
**
*
**
**
P-C/M
+ADP
+G
+S

FIGURE 5. EFFECTS OF ORAL METFORMIN TREATMENT ON SKELETAL MUSCLE MITOCHONDIRAL H_2O_2 EMSSION IN PERMEABILIZED MYOFIBERS FROM LEAN AND OBESE ZUCKER RATS.

A: H_2O_2 emission rates during atractyloside-inhibited (1 mM) basal (i.e., state 4) respiration during titration of the complex II substrate succinate revealed no differences in the sensitivity (i.e., K_{50app}) for reverse electron flow-mediated superoxide generation at complex I. However, the V_{max} for succinate-supported mitochondrial H_2O_2 emission was significantly greater in the obese control (**$P < 0.01$) and metformin treated (*$P < 0.05$) Zucker groups yet significantly reduced by metformin treatment in both the lean ($^{\dagger}P < 0.05$) and obese ($^{\dagger\dagger}P < 0.01$) animals. B: H_2O_2 emission rates during atractyloside-inhibited (1 mM) basal (i.e., state 4) respiration supported by the activated fatty acid palmitoyl-carnitine and malate (P-C/M, 25 μM/1 mM) were significantly reduced by metformin treatment, as were the rates of H_2O_2 emission supported by the complex I-linked substrate glutamate (+G, 2 mM) and the complex II substrate succinate (+S, 3 mM) (†Main effect for metformin treatment, $P < .05$, $^{\dagger\dagger}P < 0.01$; **within group effects of metformin treatment, $P < 0.01$, ***$P < 0.001$). Moreover, the rate of H_2O_2 emission +S was significantly affected by obesity (‡‡‡main effect for obesity, $P < 0.001$). The data represent the means ± SEM (n = 4/group).


A.
Lean Control
Lean Metformin
Obese Control
Obese Metformin
Mitochondrial H_2O_2 Emission
(pmol·min^{-1}·mg^{-1}dry wt)
** ††
**
†
Succinate concentration (mM)

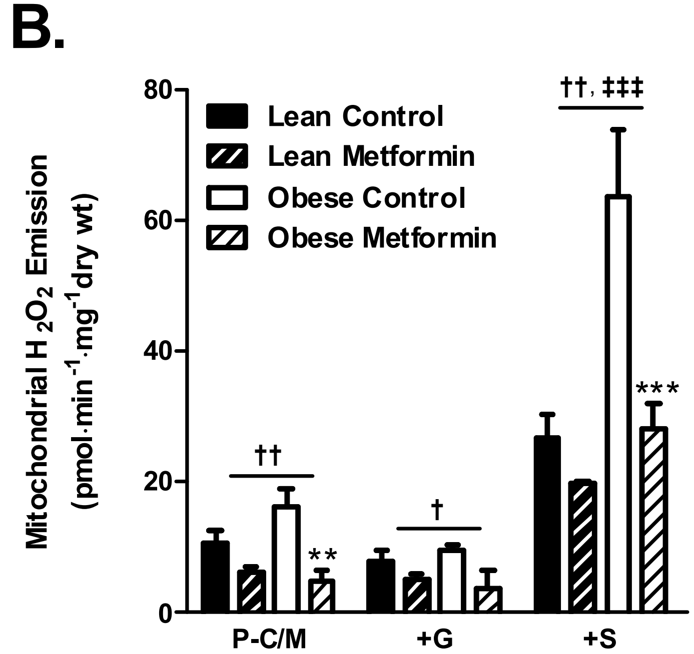

B.
Lean Control
Lean Metformin
Obese Control
Obese Metformin
Mitochondrial H_2O_2 Emission
(pmol·min^{-1}·mg^{-1}dry wt)
††, ‡‡‡
***
††
**
†
P-C/M
+G
+S

FIGURE 6. ACUTE, DOSE-DEPENDENT METFORMIN EFFECTS ON RESPIRATORY O_2 FLUX AND MITOCHONDRIAL H_2O_2 EMISSION IN PERMEABILIZED MYOFIBERS.

Acute (~20 min), dose-dependent (0-10 mM) metformin effects on respiratory O_2 flux and mitochondrial H_2O_2 emission in permeabilized red gastrocnemius myofibers from Sprague-Dawley rats. Respiratory O_2 flux (white bars) supported by complex I-linked substrates glutamate/pyruvate/ malate (5/5/2 mM) in the presence of ADP (8 mM) was not significantly affected by treatment with 0.1 or 1 mM metformin compared control myofibers (no metformin). Only in 10 mM metformin were the respiratory O_2 fluxes significantly reduced to less than 11% of control ($^{\dagger\dagger\dagger}P < 0.001$). Interestingly, metformin treatment significantly reduced state 4 (i.e., oligomycin-inhibited), succinate-supported (3 mM) mitochondrial H_2O_2 emission (black bars) at the lowest concentration of the drug tested (i.e., 0.1 mM, $^{**}P < 0.01$); and further inhibited H_2O_2 emission at metformin concentrations 1 and 10 mM to less than 50 and 80% of control, respectively ($^{***}P < 0.001$). Taken together, it appears that the inhibitory effects of metformin on complex I of the respiratory chain are more specific for reverse (H_2O_2) than forward (O_2) electron flows. The data represent the means ± SEM (n = 3-4/group).

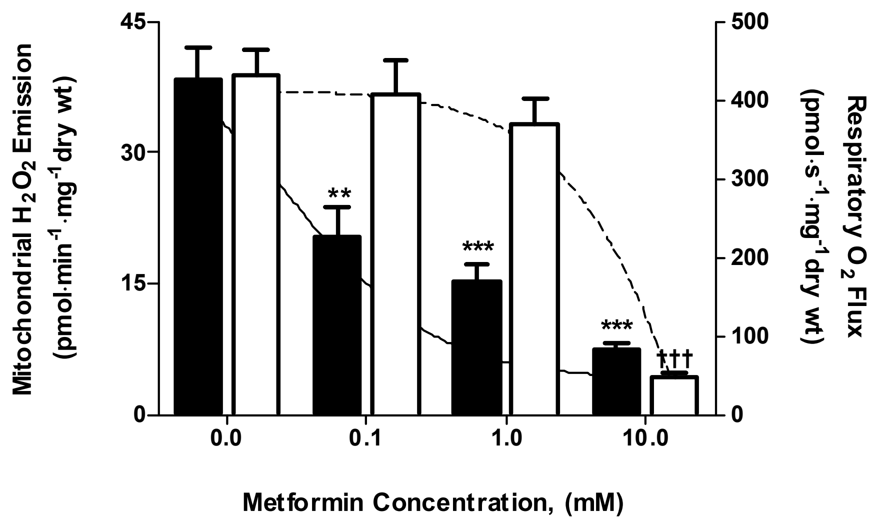
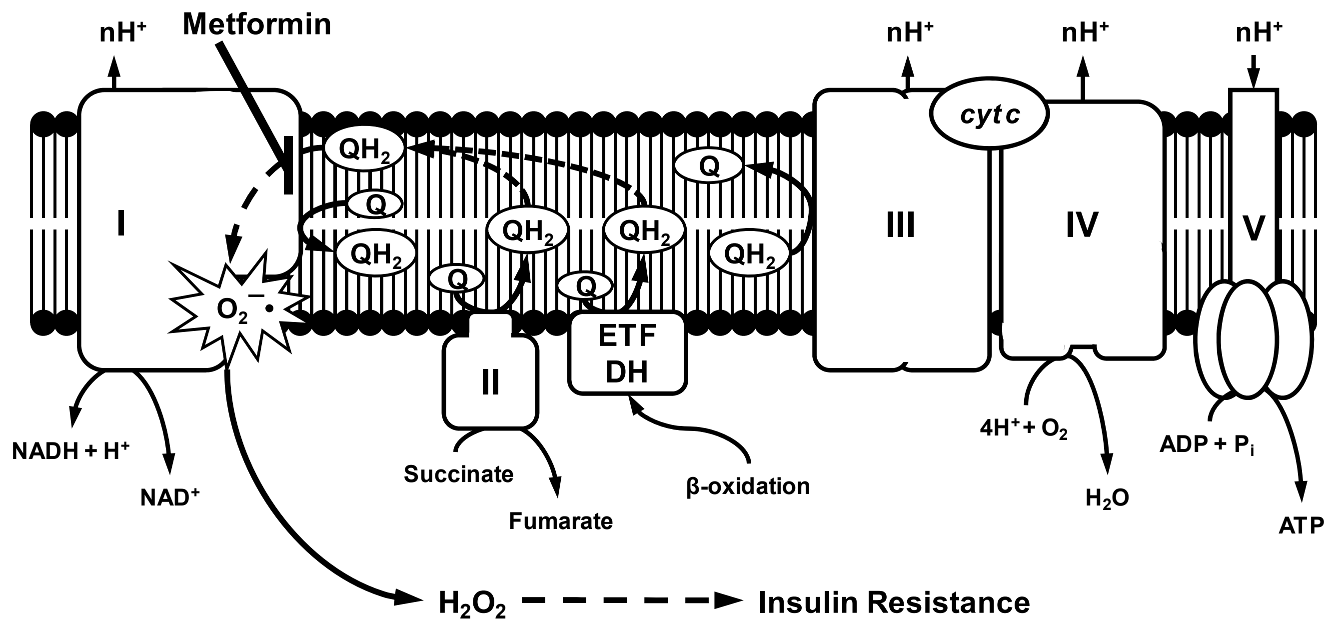

Mitochondrial H_2O_2 Emission
(pmol·min^{-1}·mg^{-1}dry wt)
45
30
15
0
500
400
300
200
100
0
Respiratory O_2 Flux
(pmol·s^{-1}·mg^{-1}dry wt)
**
***
***
†††
0.0
0.1
1.0
10.0
Metformin Concentration, (mM)

FIGURE 7. DIAGRAMMATIC REPRESENTATION OF THE MITOCHONDRIAL ELECTRON TRANSPORT SYSTEM AND THE PROPOSED MODE OF ACTION FOR METFORMIN IN SKELETAL MUSCLE MITOCHONDRIA.

Figure 4. Diagrammatic representation of the mitochondrial electron transport system and the proposed mode of action for metformin in skeletal muscle mitochondria. During forward electron flow, NADH is oxidized at complex I, succinate at complex II, and $FADH_2$ from β-oxidation at the electron transferring flavoprotein dehydrogenase (ETFDH). Oxidized quinone (Q) accepts electrons from complex I, complex II, and ETFDH, converting to the reduced form (QH_2). QH_2 then shuttles electrons to complex III where they subsequently pass to complex IV via cytochrome *c* and finally to oxygen, reducing it to water. The model proposes that during periods of low energy demand (i.e., state 4 respiration), reducing equivalents feeding into the electron transport system at complex II (experimentally induced by succinate) or via ETFDH (during fatty-acid supported respiration) reduces more of the quinone pool, generating the potential for reverse electron flow into complex I and accelerating complex I-linked superoxide (O_2^{-}•) production leading to elevated mitochondrial H_2O_2 emission and subsequently insulin resistance. The results of the current study suggest that metformin inhibits reverse electron flow-associated mitochondrial H_2O_2 emission at complex I, potentially accounting for the insulin-sensitizing effects of the drug.


nH+
Metformin
nH+
nH+
nH+
cyt c
QH2
Q
Q
QH2
QH2
QH2
QH2
Q
Q
I
III
IV
V
O2−•
II
ETF
DH
NADH + H+
NAD+
Succinate
Fumarate
β-oxidation
4H+ + O2
H2O
ADP + Pi
ATP
H2O2
Insulin Resistance

CHAPTER 3: MITOCHONDRIAL H_2O_2 LINKS OVARIAN SEX STEROIDS TO INSULIN RESISTANCE IN WOMEN

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[1]The East Carolina Diabetes and Obesity Institute, [2]Department of Exercise and Sport Science, [3]Departments of Pharmacology & Toxicology, and [4]Department of Physiology, East Carolina University, Greenville, North Carolina

Abstract: The luteal phase of the female menstrual cycle and pregnancy are associated with both 1) elevated levels of serum progesterone (P4) and estradiol (E2), and 2) insulin resistance. Recently, we demonstrated elevated rates of mitochondrial H_2O_2 emission (mE_{H2O2}) in skeletal muscle following a high-fat diet, concomitant with insulin resistance. To determine if either P4 or E2 exert a direct effect on mitochondrial function, saponin-permeabilized vastus lateralis myofibers biopsied from women in the menstrual cycle follicular phase were incubated (1-2 h) in luteal phase serum concentrations of P4 (60 nM), E2 (1.4 nM), or both E2+P4. P4 alone inhibited state 3 JO_2 supported by multisubstrate combination ($P < 0.01$). E2 alone however, or in combination with P4 had no effect on JO_2. In contrast, during state 4 respiration supported by substrates known to generate reactive oxygen species via reverse electron flow at complex I (i.e., succinate and glycerophosphate), mE_{H2O2} was increased with P4 alone or in combination with E2 compared to either E2 alone or control ($P < 0.01$). To test the hypothesis that serum levels of P4

and/or E2 are related to mitochondrial function, mE_{H2O2} and JO_2 were measured in permeabilized myofibers from both insulin sensitive (IS, $n = 24$) and resistant (IR, $n = 7$) women (IR = HOMA-IR > 3.6). Serum P4 (log-transformed) correlated strongly with succinate-supported mE_{H2O2} (r = 0. 53; $P < 0.01$), demonstrating an extension of the acute *ex vivo* P4 incubation results. Surprisingly, after adjusting for % body fat, succinate-supported mE_{H2O2} was more than 80% greater in the IR vs. IS women ($P < 0.01$). However, no differences were observed in JO_2 or ratios of respiratory control between IS and IR women ($P < 0.05$). Additionally, we compared the JO_2 and mE_{H2O2} from one subject who learned she was pregnant in the days following biopsy to the IS and IR subjects. Interestingly, the succinate-supported mE_{H2O2} from this pregnant subject was outside of the 99% confidence interval of the mean for the IS, but not the IR women ($P < 0.01$), suggesting that the insulin resistance associated with pregnancy may be linked to elevated mE_{H2O2}, and perhaps in turn, elevated E2 and/or P4. Altogether, the results of this study suggest that at physiologically relevant concentrations, 1) P4 alone inhibits JO_2 and increases mE_{H2O2}; 2) E2 counteracts the effects of P4 on JO_2, but may even increase mE_{H2O2} in combination with P4; 3) E2 alone has no effect on JO_2 or mE_{H2O2}; and 4) P4 is related to the mE_{H2O2} linked to skeletal muscle insulin resistance. A causative connection between elevated ovarian sex steroids, mE_{H2O2} and insulin resistance during the luteal phase and pregnancy is proposed, but will require further research to verify.

INTRODUCTION

The current and increasing epidemic of type 2 diabetes constitutes one of the greatest health concerns in the industrialized world. In skeletal muscle, reduced mitochondrial content and intramuscular accumulation of lipid is associated with insulin resistance in this tissue (186). Increasingly, the role of oxidative stress has been implicated in the etiology of insulin resistance in multiple tissues, including skeletal muscle (reviewed in (16, 219)); and a recent study by our group demonstrated a link between high dietary fat intake and insulin resistance involving elevated mitochondrial H_2O_2 emission (mE_{H2O2}) in skeletal muscle (5).

The volume of published data demonstrating that ovarian sex steroids affect the sensitivity of tissues to insulin in animal models are substantial (reviewed in (184)). While fewer data exist regarding the effects of the ovarian sex hormones in women, most suggest a negative relationship with insulin sensitivity (184). Normal pregnancy is associated with high circulating levels of both estrogens and progesterone and also reduced insulin sensitivity (134). Similarly, a fall in insulin sensitivity has been reported in normal women during the luteal phase of the menstrual cycle when serum progesterone and estrogen levels are both at their greatest (184). Because skeletal muscle is responsible for the majority of peripheral glucose disposal, it would appear that sex steroids have a direct effect on skeletal muscle insulin sensitivity. However, despite evidence relating sex steroids and insulin resistance (184), the exact nature of the link is unclear.

Nearly 50 years ago, very high concentrations of progesterone were shown to inhibit complex I-linked respiration in the mitochondria isolated from pigeon hearts *in vitro* (51). More recently, studies examining the effects of estradiol (E2) and progesterone (P4) on mitochondrial function have employed treatment designs that increase the physiological relevancy of data

supporting the inhibitory effects of sex steroids on mitochondrial respiration (114). Nevertheless, most studies continue to employ supraphysiological concentrations of sex steroids to investigate the non-genomic effects of female sex steroids on mitochondrial function (84, 263). In a very recent study, for example, it was shown that adding P4 to preparations of isolated rat liver mitochondria during the experimental measurements decreased the mitochondrial membrane potential, calcium retention capacity and the capacity for complex I-linked state 3 respiration (84). However, the P4 concentrations used were supraphysiological with respect to women; in this case, anywhere from 80-150 μM, or over 1000 times greater than the luteal phase serum P4 concentrations in women (237).

In the current study, it was hypothesized that a link between skeletal muscle mE_{H2O2}, insulin sensitivity and/or the menstrual cycle hormones E2 and P4 would exist in women. To this end, premenopausal female subjects donated small muscle samples in the follicular phase for *ex vivo* incubation experiments in luteal phase concentrations of E2, P4, or both. Additionally, serum E2 and P4 was measured in a larger group of insulin resistant and insulin sensitive subjects on the same day they were biopsied for skeletal muscle mitochondrial function analyses. Our findings reveal that serum levels of P4 influence the mE_{H2O2} linked to insulin resistance. This effect may be an acute, posttranslational phenomenon whereby an E2+P4 combination, as occurs *in vivo*, promotes an increase in mE_{H2O2}, but has little or no effect on JO_2.

METHODS

SUBJECTS

In the United States, the incidence of obesity and type 2 diabetes is greater among African American women (AW) compared to Caucasian women (CW) (258). The initial purpose of the this study was to explore a potential link between mitochondrial respiration, mitochondrial reactive oxygen species and insulin resistance with regard to race (i.e., African American vs. Caucasian) and obesity (BMI > 30) in women. However, it was quickly determined that race played no role in any of the variables measured. Surprisingly, obesity also had no effect on the main outcome variables when adjusted for body composition (i.e., % body fat). Therefore, the AW and CW were pooled and divided by insulin resistance.

All subjects were premenopausal female U.S. citizens of mixed ancestry, between the ages of 22 and 45 (subject characteristics presented in Tables 1 and 2). All participants were nonsmokers with no history of metabolic disease. The first set of female subjects (Group A, N = 5; Table 1) were lean, healthy, with no history of metabolic disease (e.g., HOMA-IR < 3.0) and not taking medications known to alter carbohydrate or lipid metabolism. All subjects in Group A were scheduled for biopsy such that the procedure would occur during the early follicular phase of their menstrual cycle (days 1-10), when estradiol and progesterone levels are lowest (190). Biopsies from subjects in the group A were used in hormone incubation experiments.

The second set of subjects (Group B, N = 33; Table 2) consisted of self-described African American (AW) and Caucasian women (CW) of varying body compositions and menstrual cycle status. After confirming that neither race, nor % body fat-adjusted obesity (i.e., BMI > 30) exerted an effect on any of the major outcome variables measured in the current study, AW, CW, obese and lean women were pooled and divided by estimated insulin resistance (see below).

On a day preceding the biopsy, the percent body fat (% BF) was determined for each subject by dual energy X-ray absorptiometry (DEXA). These protocols were approved by the

East Carolina University Policy and Review Committee on Human Research in accordance with the Declaration of Helsinki principles. Informed consent was obtained from each subject after both written and oral information was presented about the procedure. One lean (BMI = 20.7; 22 yrs old), healthy individual learned she was pregnant in the days following skeletal muscle biopsy. The pregnant individual mistook the idiopathic vaginal bleeding commonly associated with pregnancy (119, 308) for overt menstruation, as it occurred on or about her predicted period of menstruation. Upon learning of the subject's pregnancy, she was disenrolled from the study, and no additional data were obtained from the subject (i.e., IVGTT). Because the biopsy was performed and mitochondrial function data collected before learning of her pregnancy, the data were compared to group B subjects.

PROCEDURE

On the day of the skeletal muscle biopsy, subjects reported to the obesity research clinic at East Carolina University between the hours of 0630-0900 following overnight fast (approximately 12 hours). Body mass and height were recorded for body mass index determination (BMI), and a fasting venous blood sample was obtained prior to the skeletal muscle biopsy for subsequent analysis. With regard to the subjects in group B, plasma and serum were separated from the blood for subsequent analysis of glucose (YSI 2300 STAT Plus Glucose and Lactate Analyzer, YSI Inc; Yellow Springs, OH), serum insulin, 17β-estradiol and progesterone (Access Immunoassay System, Beckman-Coulter; Fullerton, CA). A homeostasis model assessment value for insulin resistance (HOMA-IR) was calculated (HOMA-IR = (glucose, $mg \cdot dL^{-1}$ • insulin, $\mu U \cdot mL^{-1}$) • 405^{-1}; (191)). Subjects from group B were divided by presence of insulin resistance as defined by Stern et al (265). Group B subjects were therefore

described as insulin sensitive (IS, HOMA-IR < 3.60) or insulin resistant (IR, HOMA-IR > 3.60; Table 2).

Skeletal muscle biopsies were obtained from the lateral aspect of the vastus lateralis by the percutaneous needle biopsy technique with constant suction under local subcutaneous anesthesia (1% lidocaine). A portion of each biopsy sample was flash frozen in liquid N_2 for subsequent protein analysis as part of another study. The remaining portion of the biopsy (~50 mg wet wt) was transferred to ice-cold physiological relaxing buffer (buffer X) for transport, on ice, to the laboratory (< 5 min) for dissection, permeabilization, and mitochondrial function assays.

PREPARATION OF PERMEABILIZED HUMAN MYOFIBERS

This technique is partially adapted from previous methods (173, 277) and has been thoroughly described elsewhere (5-7). Briefly, after dissection, connective tissue was removed and fiber bundles were separated with fine forceps under binocular dissecting microscope in ice cold buffer X, containing (in mM): 60 K-MES, 35 KCl, 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 Imidazole, 0.5 DTT, 20 Taurine, 5.7 ATP, 15 PCr, 6.56 $MgCl_2$-6H_2O (pH 7.4, 295 mOsm). After separation, myofiber bundles were placed in 4° C buffer X containing 30 μg/mL saponin for 30 minutes and then were washed individually in ice-cold Buffer Z containing (in mM) 110 K-MES, 35 KCl, 1 EGTA, 10 K_2HPO_4, 3 $MgCl_2$-6H_2O, 5 mg/ml BSA (pH 7.4, 295 mOsm) until analysis (< 1 hour). To determine the acute effects of 17β-estradiol (E2) and progesterone (P4) on mitochondrial function, washes for the permeabilized myofibers obtained from subjects in group A contained hormone treatments: two of the Z washes contained 60 nM P4, 2 contained 1.4 nM E2, 2 contained 60 + 1.4 nM P4 + E2. Because P4 and E2 stocks were dissolved in

dimethyl sulfoxide (DMSO), 2 Z washes contained similar amounts of DMSO (< 1.5%) and served as controls. Fibers from both groups A and B used in the H_2O_2 emission experiments were briefly washed in cold buffer Z containing 10 mM pyrophosphate prior to analysis to prevent Ca^{+2}-independent contraction.

MITOCHONDRIAL RESPIRATION AND H_2O_2 EMISSION MEASUREMENTS IN PERMEABILIZED HUMAN MYOFIBERS

O_2 consumption rate was measured by polarographic high-resolution respirometry (Oroboros O_2K Oxygraph, Innsbruck, Austria) at 30°C in air-saturated (~220–150 μM O_2) Buffer Z + 20 mM creatine hydrate and 50 μM *N*-Benzyl-*p*-toluene sulphonamide (BTS, an inhibitor of myosin II) under the following protocol: 25 μM palmitoyl-carnitine + 1 mM malate followed by sequential additions of 2 mM ADP, 10 μM cytochrome *c*, 2 mM glutamate, 3 mM succinate, 10 μg/mL oligomycin (inhibitor of mitochondrial ATP synthase), and finally 2 μM carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP, a protonophoric uncoupler). With regard to the acute E2 and P4 incubation experiments, neither oligomycin nor FCCP were added due to time constraints associated with multiple testing.

H_2O_2 emission was measured at 30° C in Buffer Z during state 4 respiration (10 μg/mL oligomycin) by continuously monitoring oxidation of Amplex red (excitation/emission λ = 563/587 nm) using a Fluorolog-3 (Horiba Jobin Yvon, Ltd; Edison, NJ) spectrofluorometer under the following protocol: 25 μM palmitoyl-carnitine + 1 mM malate followed by sequential additions of 2 mM glutamate, 3 mM succinate, and 10 mM glycerophosphate. At the conclusion of each experiment, permeabilized fiber bundles were washed in distilled H_2O to remove salts and freeze-dried in a lyophilizer (LabConco). Mitochondrial respiration rates (JO_2) are

expressed as pmol·s^{-1}·mg^{-1} dry weight and H_2O_2 emission rates (mE_{H2O2}) as pmol·min^{-1}·mg^{-1} dry weight.

As with the buffer Z washes, respective treatments of P4 and/or E2 conditions were also created by adding the hormones (dissolved in DMSO) to the respective experimental chamber/cuvette (final DMSO concentration < 2.0%). A parallel volume of DMSO alone was added to the control chamber/cuvette (i.e., final DMSO concentration < 2.0%). Neither O_2 consumption nor Amplex red fluorescence (standard curve) were differentially affected by any of the treatment conditions in the absence of biological sample.

STATISTICS

Data are presented as mean ± SEM. Statistical analyses were performed with GraphPad Prism (GraphPad Software, Inc.) using 2-way ANOVA (as appropriate) with Bonferroni post hoc method for analysis of significance among groups. Pearson bivariate correlations and variable adjustments for % BF were performed using ANCOVA with SPSS 17 software (SPSS, Inc.). The α-level of statistical significance was set a priori at $p < 0.05$.

RESULTS

ACUTE EX VIVO EFFECTS OF PROGESTERONE AND ESTRADIOL ON MITOCHONDRIAL FUNCTION IN PERMEABILIZED MYOFIBERS

To test the acute, *ex vivo* effects of E2 and P4 on mitochondrial function, skeletal muscle fibers were incubated in either 1.4 nM E2, 60 nM P4, or both for 1-2 hours after permeabilization, but before and during the experimental measurements. As Figure 8 illustrates,

a trend manifest in significant main effects for steroid hormones on JO_2 was present in the respirometric experiments. Compared to controls (DMSO), the results indicate that P4 alone significantly inhibited JO_2 during state 3 respiration supported by palmitoyl-carnitine/malate + glutamate (P-C/M+G; $P < 0.05$) and P-C/MG + succinate (P-C/MG+S; $P < 0.01$; Figure 8). This suggests that P4 exerts an inhibitory affect on complex I (+G) and possibly also complex II (+S). Interestingly, when combined with E2, P4 (i.e., E2+P4) did not significantly inhibit JO_2 (Figure 8). When combined with the results of E2 treatment alone (i.e., no effect on JO_2), the respirometric data with E2+P4 suggest that E2 may prevent the inhibitory effects of P4 on JO_2.

When rates of mE_{H2O2} were measured in the group B muscle fibers treated with P4 and/or E2 acutely post-permeabilization, significant differences were observed after the addition of succinate and also glycerophosphate (Figure 9). Compared to control (DMSO), E2+P4 treatment resulted in significantly greater rates of mE_{H2O2} during state 4 respiration supported by either P-C/MG + succinate (+S; $P < 0.05$) and P-C/MGS + glycerophosphate (+Gp; $P < 0.01$; Figure 9). Moreover, P4 alone significantly increased mE_{H2O2} compared to DMSO during P-C/MGS+Gp ($P < 0.01$; Figure 9). Interestingly however, E2 alone did not increase mE_{H2O2} (Figure 9). Because additions of succinate and glycerophosphate are known to elicit reverse electron flow – mediated superoxide production at complex I (206), these data suggest that P4 increases the potential for complex I-linked mitochondrial H_2O_2 production. Furthermore, the protection conferred by E2 with regard to the inhibitory effects of P4 on JO_2 (Figure 8) was not paralleled in the mE_{H2O2} measurements (Figure 9). Taken together, these data support a model whereby E2 prevents P4 inhibited complex I-linked, and possibly complex II-linked JO_2; and conversely, a model whereby E2 does not attenuate a P4-mediated increase in complex I-linked mE_{H2O2}.

MITOCHONDRIAL H_2O_2 EMISSION AND RESPIRATORY O_2 FLUX IN PERMEABILZED MYOFIBERS FROM INSULIN SENSITIVE AND INSULIN RESISTANT SUBJECTS

Because the rates of P-C/MG+S - supported mE_{H2O2} were affected by E2+P4 in the acute *ex vivo* incubation experiments (Figure 9), we hypothesized that serum levels of E2 and/or P4 would exert an influence on mE_{H2O2} and/or HOMA-IR in the Group B subjects. Interestingly, only serum P4 concentration (nM, log-transformed) correlated with P-C/MG+S - supported mE_{H2O2} ($r = 0.53$; $P < 0.01$; Figure 10). This further supports P4 as the sex steroid responsible for increasing mE_{H2O2}, and not E2.

When adjusted for %BF (ANCOVA), the rates of P-C/MG+S - supported mE_{H2O2} in permeabilized myofibers from the IR women were more than 80% greater than that of the IS women ($P < 0.01$; Figure 11*A*). Not only does this further support a link between mE_{H2O2} and insulin resistance, but it also demonstrates a role for P4 in this link. Furthermore, when expressed relative to JO_2, the rate of mE_{H2O2} was still significantly greater in the IR compared to IS women ($P < 0.01$; Figure 11*B*), suggesting that the increase mE_{H2O2} with IR was independent of differences in JO_2.

To examine whether the link between mE_{H2O2} and insulin resistance might be mirrored in mitochondrial respiration and/or coupling, we measured JO_2 in premeabilized fibers from IS and IR women, and subsequently calculated ratios of respiratory control (Table 3). While no differences in JO_2 were detected with insulin resistance, only after adjusting for %BF and serum P4:E2 ratio was a difference in the uncoupling control ratio (UCR, ratio of uncoupled JO_2 to oligomycin-inhibited JO_2) detected (Table 3; $P < 0.05$). This suggests that mitochondrial coupling may be affected by or affecting insulin resistance.

PREGNANT SUBJECT

Mitochondrial function data (i.e., JO_2 and mE_{H2O2}) for one lean (BMI = 20.7; 22 yrs old), healthy woman who later learned she was pregnant at the time of biopsy were compared to the IS and IR subjects from group B. As Figure 12*A* illustrates, this pregnant subject exhibited P-C/MG+S - supported mE_{H2O2} that exceeded the 99% confidence interval of the mean for IS women under similar conditions by more than 50% (Figure 12*A*; $P < 0.01$). This suggests that pregnancy may be associated with an increased potential for mE_{H2O2} in skeletal muscle. Normal pregnancy is associated with reduced insulin sensitivity (134). During pregnancy, serum levels of P4 and E2 increase (190). Therefore, the effects of pregnancy on mE_{H2O2} may be related to the rise in ovarian sex steroids. However, no discernable difference in JO_2 was observed between the pregnant subject and IS or IR women. It was therefore surprising to find that mE_{H2O2} from the pregnant subject, expressed as a percentage of JO_2 ($pmol \cdot min^{-1} \cdot mg$ dry wt^{-1}), was within the 95% confidence interval of the mean for the IS, but not the IR women (Figure 12*B*; $P < 0.05$). This suggests that pregnancy may be associated with increased mE_{H2O2}, but that it is proportional to JO_2 in skeletal muscle.

DISCUSSION

As mentioned previously, the initial purpose of the study in Chapter 3 was to explore a potential link between mitochondrial respiration, mitochondrial reactive oxygen species and insulin resistance with regard to race (i.e., African American vs. Caucasian women) and obesity in women. Surprisingly however, race did not affect any of the outcome variables measured in the current study. AW and CW were therefore pooled and divided by insulin resistance.

In this study, it was hypothesized that in women, the ovarian steroid hormones estradiol (E2) and progesterone (P4) influence insulin sensitivity via alterations in the production of mitochondrial H_2O_2 in skeletal muscle.

The results presented here demonstrate that E2 and P4 can directly affect mitochondrial function. Furthermore, a clear relationship emerged between serum P4 and mE_{H2O2}. Finally, these results provide further evidence for a link between skeletal muscle mE_{H2O2} and insulin resistance.

As early as 1963, Chance and co-workers (51) reported that high concentrations of P4 (i.e., mM) exhibited an inhibitory, "rotenone-like" effect on complex I-linked respiration and pyridine nucleotide reduction in mitochondria isolated from pigeon heart. A review (263) of the this, and subsequent publications regarding posttranslational effects of supraphysiological experimental steroid hormone concentrations on the function of isolated mitochondria questioned the physiological relevance of these studies. In the current study, we incubated permeabilized myofibers from women in the follicular phase with luteal phase serum concentrations of E2 (1.4 nM) and P4 (60 nM) (237). In agreement with the results of reports demonstrating an inhibitory effect of P4 on mitochondrial respiration (51, 84, 114), we observed a significantly lower JO_2 in fibers incubated with P4 alone, but not when combined with E2. In light of the results of our recent study linking mE_{H2O2} to skeletal muscle insulin resistance (5), the observed increase in mE_{H2O2} after acute *ex vivo* treatment with P4 and E2+P4 (but not E2) provides a potential link between the ovarian sex steroids and the reduced insulin sensitivity reported during the luteal phase of the menstrual cycle and pregnancy when P4 and E2 levels are naturally high (184). Furthermore, only when adjusting for serum E2 and P4 were we able to resolve clearly the differences in skeletal muscle mE_{H2O2} between IS and IR women. Additionally, when the

mitochondrial function data from one subject who later learned she was pregnant were compared to non-pregnant IS and IR women, mE_{H2O2} supported by P-C/MG+S elevated relative to the IS women. This provides further evidence that mE_{H2O2} is increased in skeletal muscle during conditions of elevated P4 and/or E2, both hormones being known to increase during pregnancy (190). However, when mE_{H2O2} was normalized to JO_2 (no discernable difference in JO_2, data not shown), the % H_2O_2 for the pregnant subject was outside of the 95% confidence interval of the mean for the IR, but not the IS women. Therefore, the disparity in mE_{H2O2} vs. $\%H_2O_2$ in the pregnant subjects may constitute a fundamental difference between the insulin resistance associated with pregnancy, and the insulin resistance associated with positive energy balance (e.g., obesity).

The results of the current study support the notion of an inhibitory effect of P4 alone on respiration, but not in combination with E2. In slight contrast, a study of the effects of P4 and E2 administered *in vivo* on mitochondria isolated from mouse liver found that state 3 JO_2 supported by succinate (complex II substrate) was reduced after three hours of treatment with either P4 alone, or in combination with E2 (114). When the mitochondria were supplied exclusively with glutamate + malate (complex I substrate), both state 3 and state 4 JO_2 were significantly lowered by treatment with either P4 alone, or in combination with E2 compared to controls (114). These effects of the P4 or E2+P4 on mitochondrial JO_2 were not observed during TMPD + ascorbate respiration, which supplies electrons exclusively to complex IV (114), suggesting complex I as one of the sites of action by P4, and possibly the E2+P4 combination. In another study, when mitochondria isolated from male rat livers were incubated briefly (1 min) with 30 μM E2, both state 3 and FCCP-uncoupled JO_2 supported by the complex I substrates glutamate + malate were significantly reduced from controls (202). However, E2 treatment had no effect on the

mitochondrial membrane potential ($\Delta\Psi$) (202). Furthermore, no effect of E2 on mitochondrial H_2O_2 production was observed with or without rotenone present (202). This is in contrast to findings of increased mitochondrial ROS in cultured cells treated for 15 minutes with very high (i.e. > 360 nM) E2 (86). Even more recently, it was shown that adding P4 to preparations of isolated rat liver mitochondria during experimental measurements decreased the $\Delta\Psi$, calcium retention capacity and the capacity for complex I-linked state 3 JO_2 (84). However, as with most of the investigations into the non-genomic effects of female sex steroids on mitochondrial function, the P4 concentrations used were unphysiological; in this case, anywhere from 80-150 μM, or over 1000 times greater than the luteal phase serum P4 concentration in women (237).

In the current study, luteal phase serum concentrations of E2 and P4 were used to demonstrate an inhibitory effect of P4 alone on mitochondrial JO_2. P4 is one of the female reproductive hormones most associated with pregnancy, as even its namesake implies - *proge*stational *ster*oidal ket*one* (4). While extending the findings of the current study to the increase in P4 during the luteal phase of the menstrual cycle and pregnancy (190) might predict a decrease in the basal metabolic rate accompanying the luteal phase or pregnancy, the results of the current study also demonstrate that E2 can prevent the inhibitory effects of P4 on mitochondrial JO_2. Because E2 also increases during the luteal phase and during pregnancy (190), this may explain why basal metabolic rate does not decrease in the face of increasing P4 during the luteal phase (25, 257, 299) or pregnancy (92), even when adjusting for maternal and fetal mass (129). The potential ability for E2 to counteract the inhibitory effects of P4 on respiration may also explain why, in the current study, we found no relationship between serum P4, E2 or the P4:E2 ratio and JO_2 in the group B subjects (data not shown).

Many reports describe a reduction in insulin sensitivity by the ovarian sex steroids in both humans and animal models (184). In the current study, concentrations of serum P4 and E2 were measured in subjects for whom menstrual cycle did not dictate the day upon which skeletal muscle biopsies were performed and the subsequent mitochondrial function assays performed. A significant correlation between succinate-supported mE_{H2O2} and serum P4 was observed in the current study (Figure 10). Because both succinate and glycerophosphate are known to stimulate reverse electron flow-mediated superoxide production at complex I (206), these results demonstrate a relationship between P4 and complex I-mediated H_2O_2 in skeletal muscle mitochondria. While changes in the serum concentrations of E2 and P4 in women may be dictated by factors such as smoking (317), dietary fiber (240) and fat (110), they are primarily associated with the menstrual cycle (190). Moreover, insulin sensitivity is shown to decrease during both the luteal phase and during pregnancy, two conditions in which P4 and E2 levels are elevated. If in fact high levels of E2 and P4 link conditions such as pregnancy and the luteal phase of the menstrual cycle to skeletal muscle insulin resistance, the next relevant question is which are responsible: E2, P4, or both? In a study involving stable isotope dilution and indirect calorimetry, d'Eon et al (64) were able to measure glucose uptake and estimate skeletal muscle glucose oxidation during exercise while manipulating the blood levels of E2 and P4 in healthy women. They discovered opposing actions of E2 and P4, the former reducing estimated muscle glycogen utilization and the rate glucose disappearance from the blood. On the other hand, increasing blood levels of P4 in addition to E2 increased the estimated muscle glycogen utilization, but not the rate of glucose disappearance from the blood. Such a description of the complementary effects of E2 and P4 in skeletal muscle substrate parallels some of the findings in the current study. Indeed, the results of the current study show that, with regard to skeletal

muscle mitochondrial JO_2, P4 alone significantly reduced respiration supported by the multisubstrate combination P-C/MGS (Figure 8). However, even when P4 was present, E2 preserved JO_2 (Figure 8). This is in agreement with literature reports that E2 preserves mitochondrial function in neuronal cells challenged with proapoptotic factors (192), inhibitors of succinate dehydrogenase (297), high calcium (211) and oxidative stress (192, 297). While further research will be necessary to reveal the exact mechanism by which E2 exerts its effects on mitochondrial maintenance of function, studies indicate that E2 exerts a direct antioxidant effect on isolated mitochondria (31), and there is even evidence from studies involving isolated mitochondria that E2 can directly enhance the activity of the manganese-containing superoxide dismutase (217). The results of the current study do not support the notion of E2 as a direct antioxidant when in combination with progesterone (Figure 9). Alone, however, E2 did not increase the rate of mE_{H2O2} (Figure 9). Therefore, the results of the current study suggest that P4 may be related to the insulin resistance observed during conditions of elevated sex steroids. If mE_{H2O2} is linked to insulin resistance in skeletal muscle (5), the finding that acute exposure of permeabilized myofibers to P4 or P4+E2 increased mE_{H2O2} (Figure 9) permits speculation about potential mechanisms whereby P4 influences skeletal muscle insulin sensitivity via mE_{H2O2}. Because the women in the present study were fasted, their serum P4 and E2 levels may actually reflect how their skeletal muscle will respond to metabolic challenges known to reduce insulin sensitivity, such as consuming a large meal (148), prolonged fasting (e.g., 1-2 days (76)), sleep restriction (260) or marathon running (281). During the protocols used in the current study, rates of mE_{H2O2} were in fact stimulated progressively through the addition of various substrates used in mitochondrial oxidative phosphorylation, and not in the absence, or progressive lowering of substrates. If the measurements of mE_{H2O2} in the current study are viewed not as a resting-level

enzymatic activity assay, but rather as how the mitochondria in skeletal muscle will respond to an influx of substrate, the implications may be more physiologically relevant. Perhaps the effects of P4/E2+P4 on skeletal muscle are pleiotropic, conditionally specific, and evident experimentally only under extreme conditions, such as a high rate of mitochondrial substrate flux. From an evolutionary perspective, P4/E2+P4 might serve as a regulator of substrate provision associated with pregnancy. Indeed, when a woman becomes pregnant, the rise in E2 and P4 that accompanies the luteal phase continues and increases during gestation, as often does insulin resistance (134). Teleologically, the rise in P4 or E2+P4 during pregnancy and an increase in skeletal muscle mE_{H2O2}, and perhaps in turn, muted insulin sensitivity, may have more to do with satisfying the energetic needs of the developing fetus than any pathological condition in carbohydrate metabolism. The rise in P4/E2+P4 may therefore set the stage for a means to divert substrate away from the mother's skeletal muscle following a meal.

To conclude, the results of the current study support a model in which physiologically relevant levels of P4 increase mE_{H2O2} and decrease JO_2 in skeletal muscle, and in which E2 removes the inhibitory effects of P4 on JO_2, but not mE_{H2O2}. Furthermore, the results of this study clearly demonstrate a link between mE_{H2O2} and insulin resistance in women. Whether the model can explain a causative role for ovarian sex steroids in the etiology of insulin resistance and type 2 diabetes will require further research.

ACKNOWLEDGEMENTS

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TABLE 1. GROUP A SUBJECT CHARACTERISTICS.

Age (y), weight (kg), body mass index (BMI = kg/m^2) and percent body fat (% BF) determined for 5 lean women recruited for acute *ex vivo* estradiol and progesterone incubation experiments.

N	Age (y)	Weight (kg)	BMI	% Body Fat
5	22.4 ± 1.4	67.2 ± 3.8	22.8 ± 1.2	32.6 ± 2.2

Data are mean ± SEM

FIGURE 8. ACUTE *EX VIVO* EFFECTS OF PROGESTERONE AND/OR ESTRADIOL ON MITOCHONDRIAL RESPIRATORY O_2 FLUX IN PERMEABILIZED HUMAN FEMALE MYOFIBERS.

Rates of mitochondrial respiratory O_2 flux (JO_2, pmol·s^{-1}·mg dry wt^{-1}) and H_2O_2 emission (mE_{H2O2}) measured in saponin-permeabilized myofibers from lean, healthy women in the menstrual cycle follicular phase (days 1-10). Permeabilized fibers were incubated in either DMSO (< 1.5%, vehicle), 1.4 nM estradiol (E2), 60 nM progesterone (P4) or both (E2+P4). Substrate conditions were: 25 μM palmitoyl-carnitine + 1 mM malate (P-C/M); P-C/M + 2 mM ADP (+ADP); P-C/M, state 3 + 10 μM cytochrome *c* (+cyt *c*); P-C/M, State 3 + 2 mM glutamate (+G); P-C/M, G, State 3 +3 mM Succinate (+S). Results are mean ± SEM (n = 4). * = Less than control (DMSO), $P < 0.05$; ** = $P < 0.01$; † = less than E2, $P < 0.05$; ‡ = less than E2+P4, $P < 0.05$.

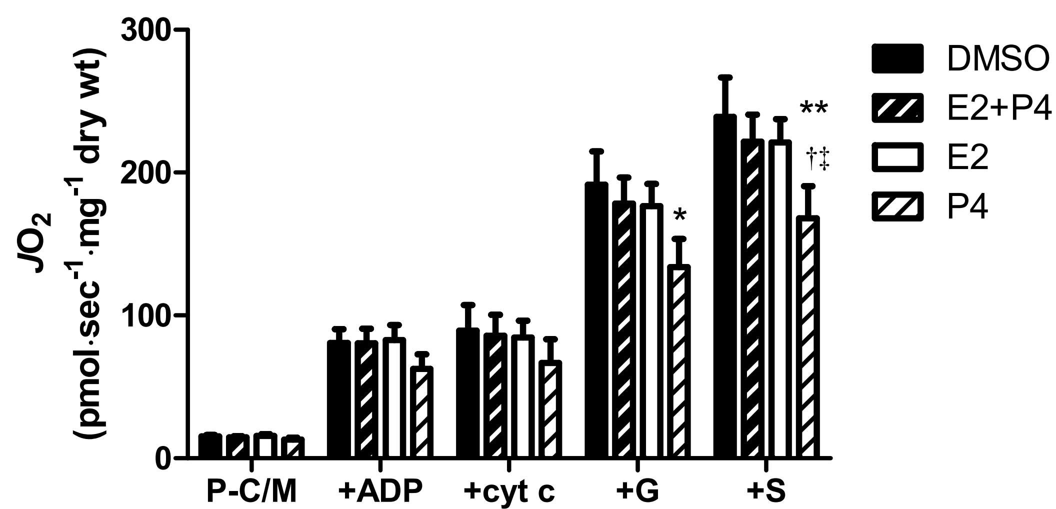

300
200
100
0
JO_2
(pmol·sec^{-1}·mg^{-1} dry wt)
P-C/M
+ADP
+cyt c
+G
+S
*
**
†‡
DMSO
E2+P4
E2
P4

FIGURE 9. ACUTE *EX VIVO* EFFECTS OF PROGESTERONE AND/OR ESTRADIOL ON MITOCHONDRIAL H_2O_2 EMISSION IN PERMEABILIZED HUMAN FEMALE MYOFIBERS.

Rates of mitochondrial H_2O_2 emission (mE_{H2O2}) measured in saponin-permeabilized myofibers from lean, healthy women in the menstrual cycle follicular phase (days 1-10). Permeabilized fibers were incubated in either DMSO (< 1.5%, vehicle), 1.4 nM estradiol (E2), 60 nM progesterone (P4) or both (E2+P4). Substrate conditions were, in the presence of 10 µM oligomycin: 25 µM palmitoyl-carnitine + 1 mM malate (P-C/M); P-C/M + 2 mM glutamate (+G); P-C/MG + 3 mM Succinate (+S); and P-C/MGS + 10 mM glycerophosphate (+Gp). Results are mean ± SEM (n = 5). * = Less than control (DMSO), $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; † = less than E2, $P < 0.05$.

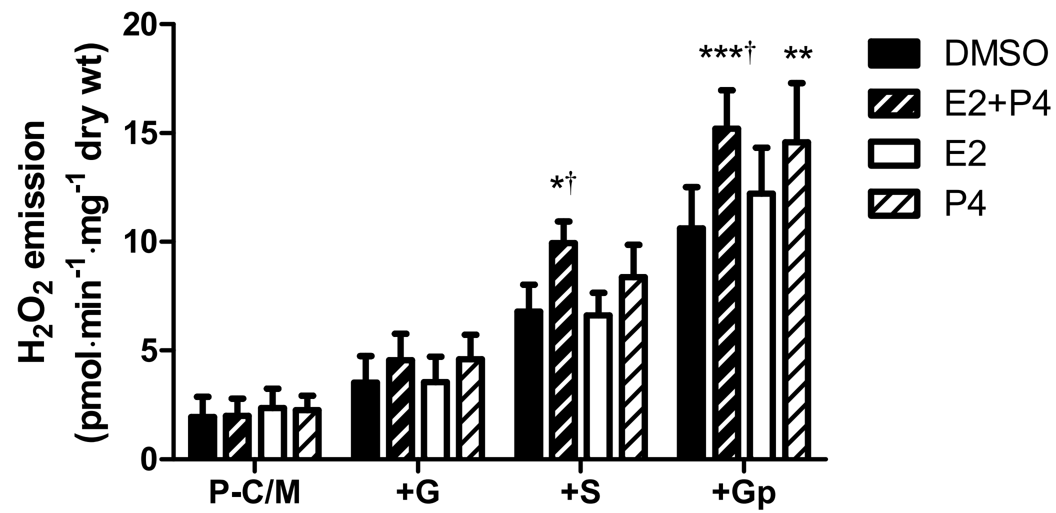

H2O2 emission
(pmol·min-1·mg-1 dry wt)
20
15
10
5
0
P-C/M
+G
+S
+Gp
*†
***†
**
DMSO
E2+P4
E2
P4

TABLE 2. GROUP B SUBJECT CHARACTERISTICS.

Age (y), body mass index (BMI = $kg \cdot (m^2)^{-1}$), percent body fat (% BF), homeostatic model assessment for insulin resistance (HOMA-IR), serum estradiol (E2, pM), serum progesterone (P4, nM), and ratio of P4, pM to E2, pM (P4:E2) determined for 32 women of varying insulin sensitivity. Thus, they are categorized as insulin sensitive (IS, HOMA-IR < 3.6; $n = 24$) or insulin resistant (IR, HOMA-IR > 3.6; $n = 8$). Results are mean ± SEM. *$P < 0.05$ vs. IS; ***$P < 0.0001$ vs. IS.

	Insulin Sensitive	Insulin Resistant
Age (y)	31.6 ± 1.4	35.1 ± 2.4
BMI (kg/m^2)	30.2 ± 1.4	36.5 ± 2.4*
Body fat (%)	44.5 ± 1.5	47.3 ± 2.6
HOMA-IR	1.7 ± 0.2	4.6 ± 0.3***
E2 (pM)	407.2 ± 61.1	313.1 ± 113.1
P4 (nM)	8.8 ± 2.8	15.2 ± 5.2
P4:E2	29.3 ± 6.8	42.4 ± 12.6

FIGURE 10. RELATIONSHIP BETWEEN SERUM PROGESTERONE AND MITOCHONDRIAL H_2O_2 EMISSION IN WOMEN.

Rates of mitochondrial H_2O_2 emission (mE_{H2O2}) plotted against the log-transformed serum concentrations of P4 (nM) from 32 women in group A. A significant correlation was present with respect to the substrate conditions: 10 µM oligomycin + 25 µM palmitoyl-carnitine + 1 mM malate + 2 mM glutamate + 3 mM Succinate (P-C/MGS; $r = 0.53$, $P < 0.01$).

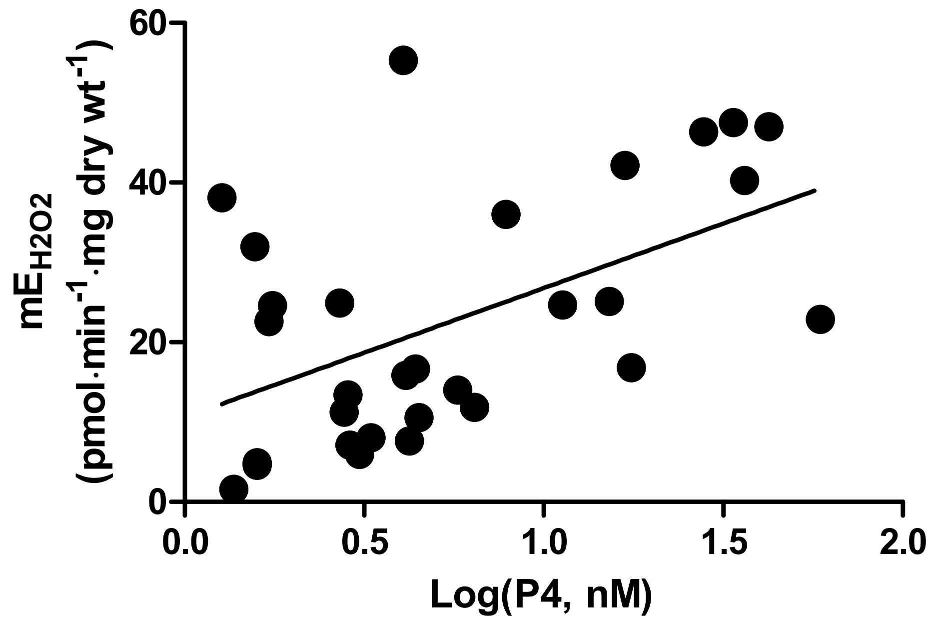
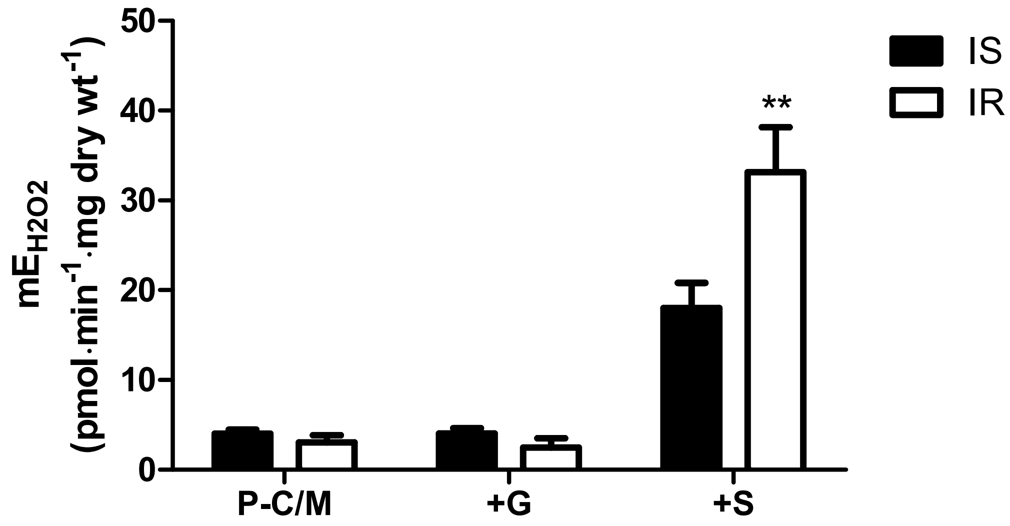

60
40
20
0
0.0
0.5
1.0
1.5
2.0
Log(P4, nM)
mEH2O2
(pmol·min-1·mg dry wt-1)

FIGURE 11. MITOCHONDRIAL H_2O_2 EMISSION AND INSULIN RESISTANCE IN WOMEN.

Mitochondrial H_2O_2 emission (mE_{H2O2}) measured in permeabilzed myofibers and fractional mE_{H2O2} ($mE_{H2O2} \cdot JO_2^{-1}$, %) adjusted for % body fat in insulin sensitive (IS, n = 22) and insulin resistant (IR, n = 7) women. ***A.*** Substrate conditions were, in the presence of 10 μM oligomycin: 25 μM palmitoyl-carnitine + 1 mM malate (P-C/M); P-C/M + 2 mM glutamate (+G); and P-C/MG + 3 mM Succinate (+S). ***B.*** Fractional mE_{H2O2} is expressed as a percentage of the JO_2 (pmol · min^{-1} · mg dry wt^{-1}) measured in parallel substrate conditions (i.e., P-C/MGS + 10 μM oligomycin). ** $P < 0.01$ vs. IS.


A.
50
40
30
20
10
0
mE$_{H2O2}$
(pmol·min^{-1}·mg dry wt^{-1})
**
P-C/M
+G
+S
IS
IR

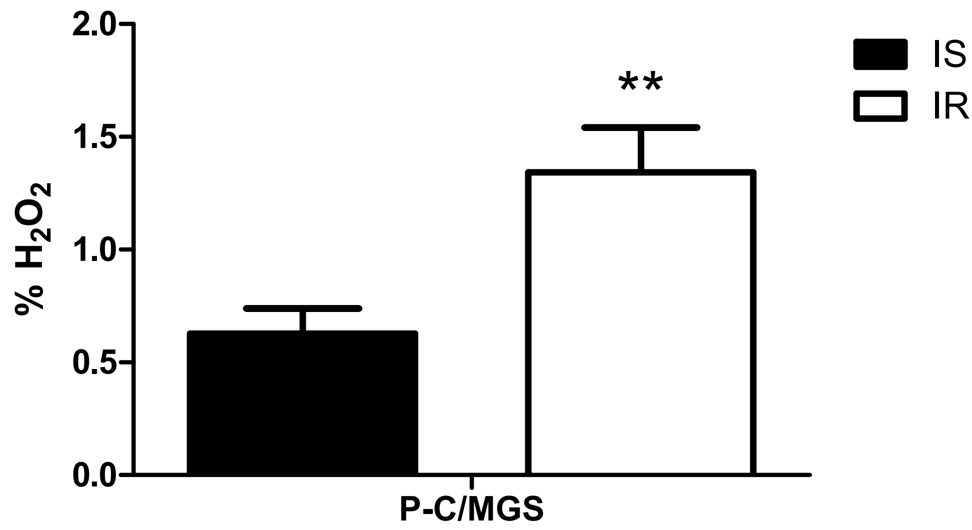

B.
2.0
1.5
1.0
0.5
0.0
% H_2O_2
**
P-C/MGS
IS
IR

TABLE 3. RESPIRATORY O_2 FLUX AND CONTROL IN INSULIN SENSITIVE AND INSULIN RESISTANT WOMEN.

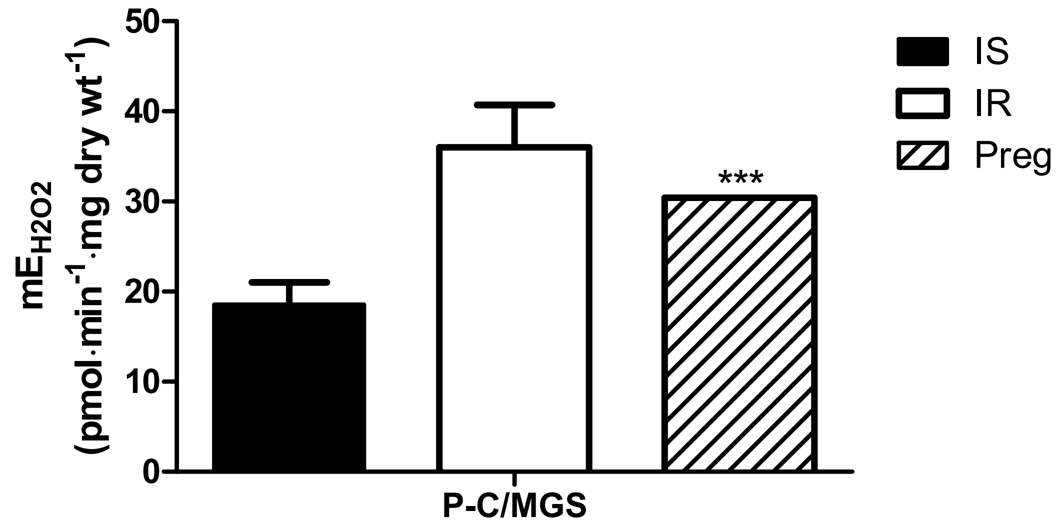
Rates of mitochondrial respiratory O_2 flux (JO_2) measured in saponin-permeabilzed myofibers and calculated ratios of respiratory control adjusted for % body fat (ANCOVA) and ratio of serum P4:E2 measured in insulin sensitive (IS, n = 23) and insulin resistant (IR, n = 8) women. Substrate conditions in the JO_2 measurements were: 25 μM palmitoyl-carnitine + 1 mM malate (P-C/M4); P-C/M + 2 mM ADP (P-C/M3); P-C/M, State 3 + 2 mM glutamate (P-C/MG); P-C/M, G, State 3 +3 mM Succinate (P-C/MGS); P-C/MGS + 10 μM oligomycin (P-C/MGSO); P-C/MGSO + 2 μM FCCP (P-C/MGSOU). Ratios of respiratory control were calculated as follows: RCR, respiratory control ratio = (JO_2, P-C/M3)·(JO_2, P-C/M4)$^{-1}$; ACR, adenylate control ratio = (JO_2, P-C/MGSOU)·(JO_2, P-C/MG+S)$^{-1}$; UCR, uncoupling control ratio = (JO_2, P-C/MGSOU)·(JO_2, P-C/MGSO)$^{-1}$. *$P < 0.05$ vs. Insulin sensitive.

	Insulin Sensitive	Insulin Resistant
JO_2 (P-C/M4)	8.8 ± 1.2	8.4 ± 2.2
JO_2 (P-C/M3)	49.7 ± 2.7	42.2 ± 4.9
JO_2 (P-C/MG3)	145.5 ± 7.8	147.8 ± 14.1
JO_2 (P-C/MGS3)	207.8 ± 9.7	198.0 ± 17.7
JO_2 (P-C/MGSO)	49.7 ± 2.8	40.3 ± 5.0
JO_2 (P-C/MGSU)	253.6 ± 12.0	245.9 ± 21.8
RCR (P-C/M)	7.2 ± 0.8	6.3 ± 1.5
ACR	1.2 ± 0.0	1.3 ± 0.1
UCR	5.2 ± 0.2	6.4 ± 0.4*

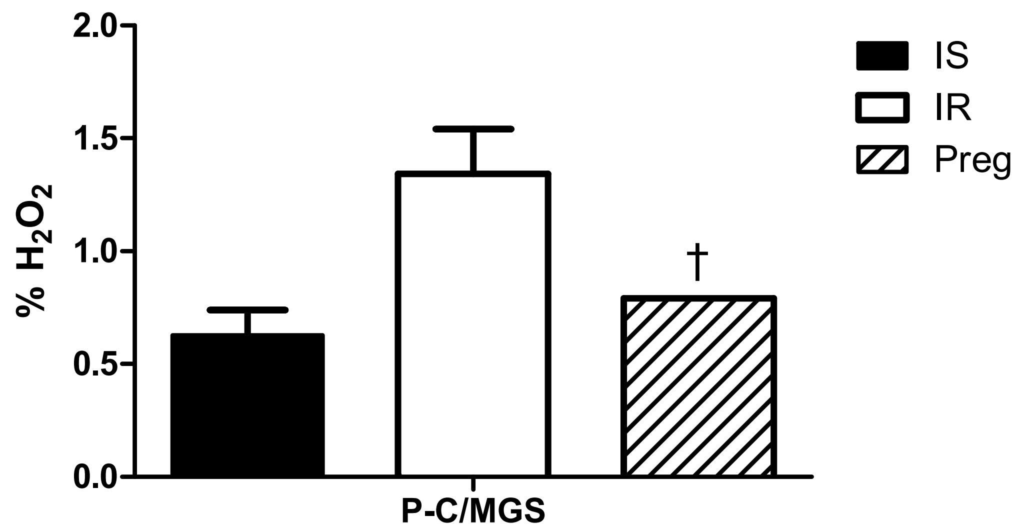
FIGURE 12. MITOCHONDRIAL H_2O_2 EMISSION AND FRACTIONAL H_2O_2 IN ONE PREGNANT WOMAN.

Mitochondrial H_2O_2 emission (mE_{H2O2}) and fractional mE_{H2O2} (%H_2O_2) for one individual who learned post hoc that she was pregnant at the time of biopsy were compared to values for insulin sensitive (IS, n = 22) and insulin resistant (IR, n = 6) women (group B subjects). *A*. Substrate conditions for mE_{H2O2} measurements were, in the presence of 10 μM oligomycin: 25 μM palmitoyl-carnitine + 1 mM malate + 2 mM glutamate + 3 mM Succinate (P-C/MGS). *B*. mE_{H2O2} expressed as a percentage of JO_2 (pmol·min^{-1}·mg dry wt^{-1}) under similar conditions (i.e., P-C/MGS + 10 μM oligomycin). ***value was outside of the 99.9% confidence interval of the mean for IS women. †value was outside of the 95% confidence interval of the mean for IR women.

A.



B.



CHAPTER 4: REDUCED CAPACITY FOR MITOCHONDRIAL H_2O_2 EMISSION FOLLOWING 8 WEEKS OF EXERCISE TRAINING IN WOMEN

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Abstract: Regular exercise has long been recognized as an effective therapeutic modality to improve overall health, including insulin sensitivity. Recently, we demonstrated that skeletal muscle mitochondrial H_2O_2 emission (mE_{H2O2}) links high-fat diet to insulin resistance. To test the hypothesis that exercise training is associated with reduced potential for mitochondrial H_2O_2 production, we measured mE_{H2O2} in and respiratory O_2 flux (JO_2) in saponin-permeabilized vastus lateralis myofibers from lean (BMI < 30) and obese (BMI > 30) women before (Pre) and after (Post) 8 weeks of exercise training (8WT = stationary cycle ergometer, 1 h/d, 5d/w at heart rate corresponding to 70-75% $\dot{V}O_{2peak}$). Compared to Pre, Post $\dot{V}O_{2peak}$ was significantly greater ($P < 0.05$), and respiratory exchange ratio (RER) at exercise intensity equivalent to 75% Pre $\dot{V}O_{2peak}$ was significantly lower ($P < 0.001$). However, no changes in body composition (BMI or % body fat), estimated insulin resistance (HOMA-IR) or serum levels of estradiol or progesterone were observed Pre-Post. Interestingly, while Pre-Post there were no changes in JO_2 supported by multiple substrates or calculated ratios of respiratory control, there was a

significant reduction in succinate-supported rates of mE_{H2O2} ($P < 0.05$) following training, even when expressed as a percentage of JO_2 ($P < 0.05$). Absent changes in both body composition and insulin sensitivity at rest, the results of this study suggest that reduced capacity for mE_{H2O2} in skeletal muscle after 8WT in lean and obese women may constitute adaptations to exercise training that parallel improvements in cardiorespiratory fitness and reliance on energy derived from fat during exercise.

INTRODUCTION

During exercise, insulin-independent and -dependent glucose uptake by human skeletal muscle is enhanced After a single bout of exercise, insulin sensitivity increases primarily in the muscles involved in the physical activity, an effect which may last for up to two days (124, 305). Though improved insulin sensitivity with acute exercise may be short-lived and likely involves improvements in GLUT4 content and/or trafficking rather than improved insulin receptor signaling (77, 124), evidence exists for metabolic adaptations which sustain whole-body muscle insulin sensitivity with exercise training via enhanced insulin signaling (reviewed in (124)).

First reported by John Holloszy in 1967 (135), the notion of increased mitochondrial oxygen consumption and respiratory activity in skeletal muscle with exercise training has since become dogma, with the generally accepted explanation being an increase in the transcriptional/posttranscriptional activities involved in mitochondrial biogenesis following skeletal muscle contractions associated with exercise (reviewed in (137, 185)).

It is generally accepted that during exercise, high rates of oxygen flux in skeletal muscle increase the rates of ROS production, the source of which has been attributed primarily to the mitochondria (reviewed in (189)). *In vitro* however, high rates of mitochondrial ROS production are only evident under resting (i.e., state 4) conditions (48). This mitochondrial membrane potential ($\Delta\Psi$) - dependent ROS is reduced exponentially as proton flux back into the mitochondrial matrix increases due to leak (i.e., mild uncoupling) increases (198) or during the transition to state 3 respiration, mitochondrial ROS production drops precipitously (198). Exercise training has been shown to increase the mitochondrial content of known contributors to basal proton leak (e.g., uncoupling proteins) (37, 87), which should in theory, decrease $\Delta\Psi$ - dependent ROS, favoring the benign consumption of O_2 at the terminus of the respiratory chain.

Because studies have shown that intense or exhaustive exercise is associated with markers of oxidative stress (189, 241), it has since been proposed that extramitochondrial or extracellular sources of ROS are involved, possibly due to ROS generated by xanthine oxidase (112). Regardless of the oxidant source, an accumulating body of evidence employing both direct (i.e., ESR spectroscopy) and indirect methodologies (e.g., assay of oxidatively modified macromolecules) strongly suggests that free radicals generated during mild to moderate endurance exercise actually constitute a stimulus mechanism for adaptations to exercise, including mitochondrial biogenesis in skeletal muscle (reviewed in (241)). Observations made regarding the adaptive response to exercise-associated redox perturbations (227, 241) may therefore account, at least in part, for the often unimpressive results from studies examining the effects antioxidant supplementation on adaptation to exercise (reviewed in (38)) and improvements to diabetes (reviewed in (256)). Taken as a whole, the literature suggests that the improvements in whole-body insulin sensitivity with exercise training may owe to adaptations associated with attenuating mitochondrial ROS. Whether this involves an increase in the antioxidant defense, a decrease in the production of ROS, or both, remains to be clarified.

Here, we report a reduction in the rate of mE_{H2O2} in saponin-permeabilized myofibers from lean and obese women biopsied before and after 8 weeks of exercise training that was not accompanied by a change in mitochondrial respiration. It does not appear that the training adaptations related to reduced mE_{H2O2} involve improved insulin sensitivity, absent changes in body mass or composition.

METHODS

SUBJECTS

In the United States, the incidence of obesity and type 2 diabetes is greater among African American women (AW) compared to Caucasian women (CW) (258). To explore exercise training as a viable therapy, the initial purpose of the current study was to examine the effects of 8 weeks of exercise training on mitochondrial respiration, mitochondrial reactive oxygen species and insulin resistance with regard to race (i.e., African American vs. Caucasian) and obesity (BMI) in women. However, it was determined that race played no role in any of the outcome variables measured. Therefore, the AW and CW were pooled into lean (BMI < 30) and obese (BMI > 30) groups.

Additionally, these subjects were sampled at random with regard to their respective menstrual cycle phase. The volume of published data demonstrating that ovarian sex steroids affect the sensitivity of tissues to insulin in both animal and humans are substantial (reviewed in (184)). A fall in insulin sensitivity has been reported in normal women during the luteal phase of the menstrual cycle, when serum progesterone and estrogen levels are both at their greatest (184). Because skeletal muscle is responsible for the majority of peripheral glucose disposal, it would appear that sex steroids have a direct effect on skeletal muscle insulin sensitivity. In light of the evidence relating sex steroids and insulin resistance (184), we measured serum concentrations of estradiol and progesterone in the event that they represented a factor in potential adaptations to exercise training.

All subjects were premenopausal female U.S. citizens of mixed ancestry, between the ages of 22 and 45 (subject characteristic presented in Table 4). All participants were nonsmokers with no history of metabolic disease. Subjects consisted of self-described African American (AW) and Caucasian women (CW). After confirming that race did not exert an effect on any of

the major outcome variables measured in the current study, AW and CW were pooled into lean (BMI < 30, n = 5) and obese (BMI > 30, n = 7) groups. On a day preceding each biopsy, the percent body fat (%BF) was determined for each subject by dual energy X-ray absorptiometry (DEXA). The protocol was approved by the East Carolina University Policy and Review Committee on Human Research in accordance with the Declaration of Helsinki principles. Informed consent was obtained from each subject after both written and oral information was presented about the procedure.

DESIGN

Vastus lateralis skeletal muscle biopsies were performed before (2 d), and after 8 weeks of exercise training (24-48 h after the last exercise bout). Peak aerobic capacities (VO_{2peak}) were determined from expired air analysis during an incremental exercise protocol on an electronically-braked cycle ergometer (Lode, Diversified, CA). Rates of oxygen consumption were measured using open circuit spirometry with a metabolic cart (ParvoMedics, OH); and heart rate (HR) was simultaneously recorded via 12-lead electrocardiogram (ECG). VO_{2peak} was determined before (Pre) and after (Post) training as the greatest average oxygen consumption rate during the incremental exercise test. The exercise regimen lasted 8 weeks and consisted of 5 days of exercise per week, for one hour per day at a heart rate corresponding to 70-75% VO_{2peak}. VO_{2peak} was measured every two weeks to readjust training workloads as the subjects improved their cardiorespiratory and muscle oxidative capacities. During the training protocol, each subject was intermittently monitored for oxygen consumption to assure the workload was maintained at the predetermined intensity. During the 7th week of training, subjects performed an exercise bout at the intensity equivalent to 75% of their pretraining VO_2 peak during which

respiratory exchange ratio (RER, minute VCO_2/VO_2) was determined.

PROCEDURE

On the day of the skeletal muscle biopsy, subjects reported to the obesity research clinic at East Carolina University between the hours of 0630-0900 following an overnight fast (approximately 12 hours). Body mass and height were recorded, and a fasting venous blood sample was obtained the day prior to the skeletal muscle biopsy for subsequent analysis. From this blood sample, plasma and serum were separated from the blood for subsequent analysis of glucose (YSI 2300 STAT Plus Glucose and Lactate Analyzer, YSI Inc; Yellow Springs, OH) and serum insulin. Additionally, 17β-estradiol and progesterone were measured (Access Immunoassay System, Beckman-Coulter; Fullerton, CA) to account for potential menstrual cycle effects. A homeostasis model assessment value for insulin resistance (HOMA-IR) was calculated (HOMA-IR = (glucose, $mg \cdot dL^{-1} \cdot$ insulin, $\mu U \cdot mL^{-1}) \cdot 405^{-1}$; (191)).

Skeletal muscle biopsies were obtained from the lateral aspect of the vastus lateralis by the percutaneous needle biopsy technique with constant suction under local subcutaneous anesthesia (1% lidocaine). A portion of each biopsy sample was flash frozen in liquid N_2 for subsequent protein analysis as part of another study. The remaining portion of the biopsy (~50 mg wet wt) were transferred to ice-cold physiological relaxing buffer (buffer X) for transport, on ice, to the laboratory (< 5 min) for dissection, permeabilization, and mitochondrial function assays.

PREPARATION OF PERMEABILIZED HUMAN MYOFIBERS

This technique is partially adapted from previous methods (173, 277) and has been thoroughly described (5-7). Briefly, after dissection, connective tissue was removed and fiber bundles were separated with fine forceps under binocular dissecting microscope in ice cold buffer X, containing (in mM): 60 K-MES, 35 KCl, 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 Imidazole, 0.5 DTT, 20 Taurine, 5.7 ATP, 15 PCr, 6.56 $MgCl_2$-$6H_2O$ (pH 7.4, 295 mOsm). After separation, myofiber bundles were placed in 4° C buffer X containing 30 µg/mL saponin for 30 minutes and then were washed individually in ice-cold Buffer Z containing (in mM) 110 K-MES, 35 KCl, 1 EGTA, 10 K_2HPO_4, 3 $MgCl_2$-$6H_2O$, 5 mg/ml BSA (pH 7.4, 295 mOsm) until analysis (< 1 hour). Fibers used in the H_2O_2 emission experiments were briefly washed in cold buffer Z containing 10 mM pyrophosphate prior to analysis to prevent Ca^{+2}-independent contraction.

MITOCHONDRIAL RESPIRATION AND H_2O_2 EMISSION MEASUREMENTS IN PERMEABILIZED HUMAN MYOFIBERS

O_2 consumption rate was measured by polarographic high-resolution respirometry (Oroboros O_2K Oxygraph, Innsbruck, Austria) at 30°C in air-saturated (~220–150 µM O_2) Buffer Z + 20 mM creatine hydrate and 50 µM *N*-Benzyl-*p*-toluene sulphonamide (BTS, an inhibitor of myosin II) under the following protocol: 25 µM palmitoyl-carnitine + 1 mM malate (P-C/M, State 4) followed by sequential additions of 2 mM ADP (P-C/M, State 3), 10 µM cytochrome *c* (indicator of outer mitochondrial membrane intactness), 2 mM glutamate (P-C/MG), 3 mM succinate (P-C/MGS), 10 µg/mL oligomycin (inhibitor of mitochondrial ATP synthase) (P-C/MGSO), and finally 2 µM carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP, a protonophoric uncoupler) (P-C/MGSOFCCP). Subsequently, ratios of respiratory

control were calculated as follows: $RCR_{P\text{-}C/M}$, respiratory control ratio = (JO_2, P-C/M, State 3)·(JO_2, P-C/M, State 4)$^{-1}$; $UCR_{P\text{-}C/MGS}$, uncoupling control ratio = (JO_2, P-C/MGSOFCCP)·(JO_2, P-C/MGSO)$^{-1}$; $ACR_{P\text{-}C/MGS}$, adenylate control ratio = (JO_2, P-C/MGSOFCCP)·(JO_2, P-C/MGS)$^{-1}$.

H_2O_2 emission was measured at 30° C in Buffer Z during state 4 respiration (10 µg/mL oligomycin) by continuously monitoring oxidation of Amplex red (excitation/emission λ = 563/587 nm) using a Fluorolog-3 (Horiba Jobin Yvon, Ltd; Edison, NJ) spectrofluorometer under the following protocol: 25 µM palmitoyl-carnitine + 1 mM malate (P-C/M) followed by sequential additions of 2 mM glutamate (P-C/MG) and 3 mM succinate (P-C/MGS). At the conclusion of each experiment, permeabilized fiber bundles were washed in distilled H_2O to remove salts and freeze-dried in a lyophilizer (LabConco). Mitochondrial respiration rates are expressed as pmol·s^{-1}·mg^{-1} dry weight and H_2O_2 emission rates as pmol·min^{-1}·mg^{-1} dry weight.

STATISTICS

Data are presented as mean ± SEM. Statistical analyses were performed with SPSS 17 (SPSS, Inc.) using 2-way ANOVA with repeated measures and covariates as indicated (ANCOVA). Bonferroni post hoc tests were used to determine significance among groups. The α-level of significance was set *a priori* at $p < 0.05$.

RESULTS

EFFECTS OF 8 WEEKS EXERCISE TRAINING ON BODY COMPOSITION, INSULIN SENSITIVITY AND OVARIAN STEROID HORMONES

To asses the responses to 8 weeks of exercise training *in vivo*, BMI, % body fat (% BF), serum estradiol (E2), progesterone (P4) and HOMA were determined both before (pre) and after (post) training (Table 4). Peak oxygen consumption (VO_{2peak}) and the respiratory exchange ratio (RER) at 75% VO_{2peak} during exercise were also determined (Table 4). Not surprisingly, BMI and % BF differed between the lean and obese groups as this is how the subjects were grouped (i.e., lean vs. obese, BMI < 30 vs. BMI > 30, respectively). Interestingly, the only variables affected by 8 weeks of exercise training were those determined during exercise. As expected, VO_{2peak} increased with training in the lean group indicative of improved cardiorespiratory fitness, whereas RER at the exercise intensity equivalent to 75% of the Pre VO_{2peak} decreased in the lean and obese groups. The decrease in exercise RER represents an improved reliance on fat as substrate for a given absolute exercise intensity with training, and was expected. However, there was no improvement in insulin sensitivity (HOMA) with training (Table 4). Therefore, these data demonstrate an improvement in cardiorespiratory fitness and ability to oxidize fat during exercise, but not improved insulin sensitivity at rest.

EFFECTS OF 8 WEEKS EXERCISE TRAINING ON MITOCHONDRIAL RESPIRATION AND H_2O_2 EMISSION IN PERMEABILIZED MYOFIBERS.

To assess the response to 8WT in skeletal muscle mitochondria, mitochondrial respiratory O_2 flux (JO_2) and H_2O_2 emission (mE_{H2O2}) were measured in saponin-permeabilized myofibers from lean and obese women before (Pre) and after (Post) 8WT. Because differences were not observed when covarying for any of the ovarian sex steroids or their ratios, neither E2, P4 or P4:E2 ratio was included as a covariate. As Table 5 indicates, no training-induced changes in JO_2 during a multisubstrate titration were observed in either lean or obese subjects, even when

adjusted for the difference in Pre-Post BMI, %BF, or HOMA-IR (ANCOVA). Similarly, no changes in respiratory (RCR), uncoupling (UCR) or adenylate (ACR) control ratios were observed Pre-Post (Table 5), suggesting that 8WT had no effect on coupling (RCR and UCR) or limitations to JO_2 exerted by the phosphorylation system (ACR). In contrast, 8WT resulted in a significantly lower succinate-supported mE_{H2O2} when the effect of Pre-Post HOMA-IR difference was removed (Table 6; $P < 0.01$). Surprisingly however, the rates of mE_{H2O2} were greater in the lean compared to the obese subjects ($P < 0.05$). When the rates of mE_{H2O2} were expressed as a percentage of the JO_2 (pmol·min^{-1}·mg dry wt^{-1}) supported by identical substrates (% mE_{H2O2}), a training-induced effect remained (Table 6; $P < 0.05$). This suggests that 8WT affected the proportion of mE_{H2O2} relative to JO_2, and that the change in mE_{H2O2} did not simply parallel a change in JO_2. Interestingly, when adjusted for the difference in Pre-Post mE_{H2O2}, no difference in HOMA-IR with 8WT was observed, though statistical significant was approached ($P = 0.61$; data not shown). This suggests that mE_{H2O2} affects insulin sensitivity and not the other way around.

DISCUSSION

Initially, the current study sought to explore the effects of exercise training on the mitochondrial fate of O_2 in the context of the racial metabolic disparity between AW and CW in the United States. Thus, mitochondrial JO_2, mE_{H2O2} and insulin resistance were studied in lean and obese AW and CW, Pre and Post 8WT. However, contrary to the previously published decrement in fat oxidation in AW during exercise relative to CW (128), it was determined that race played no role in any of the outcome variables measured in Chapter 4, including RER at

75% VO_{2peak}. Therefore, the AW and CW were pooled into lean (BMI < 30) and obese (BMI > 30) groups.

In the current study, we hypothesized that 8 weeks of exercise training would result in improved insulin sensitivity, concomitant with a reduction in the potential for mitochondrial reactive oxygen species production, an increase in mitochondrial respiration, and a reduction in ratios of respiratory control. The results presented here do not support the notion of improved insulin sensitivity absent any potential for acute effects during or following a bout of exercise or changes in body composition (% BF or BMI). However, the results demonstrate an improvement in cardiorespiratory fitness (VO_{2peak}), fat oxidation during exercise (RER at 75% VO_{2peak}), and a reduced potential for succinate-supported mE_{H2O2} ($>$ 30 % in both groups). Furthermore, the respirometric experiments conducted on permeabilized fibers do not support that the reduction in mE_{H2O2} with exercise training is necessarily the result of increased (or decreased) state 4 JO_2. Indeed, when expressed relative to JO_2 (% mE_{H2O2}), mE_{H2O2} was still significantly lower after training (Table 6).

It is generally accepted that during exercise, high rates of JO_2 in skeletal muscle increase the rates of ROS production (reviewed in (189)), and the source of this exercise-associated ROS has been attributed primarily to the mitochondria (189). However, the early work of Britton Chance and co-workers demonstrated that this high percentage of mitochondrial ROS is only evident during basal, state 4 respiration supported by succinate (48) for example, which induces a high rate of mE_{H2O2} via reverse electron flow (REF) at complex I of the mitochondrial respiratory chain (206). With regard to the acute effects of exercise on REF-mediated mE_{H2O2}, Sahlin et al (243) very recently published a study that examined these effects. In mitochondria isolated from the vastus lateralis muscles of male athletes participating in ultra-endurance

exercise, the rate of state 4, succinate-supported (i.e., due to REF) mE_{H2O2} detected by Amplex red was increased 73% immediately post-exercise from pre-exercise rates (243). As expected, the rates of H_2O_2 emission from mitochondria isolated from a biopsy performed 28 hours after the exercise returned to pre-exercise rates (243). *In vitro*, during state 3 respiration, mitochondrial ROS production drops precipitously (198). Because studies have shown that intense or exhaustive exercise is associated with markers of oxidative stress (189, 241), it has since been proposed that extramitochondrial or extracellular sources of ROS are involved, possibly due to ROS generated by xanthine oxidase (112). However, a very recent report by the group of O'Rourke have synthesized an elegant hypothesis from experimental observations in isolated cardiac mitochondria and cardiomyocytes that may explain how mitochondria can produce significant rates of ROS during exercise (10). Termed "Redox-Optimized ROS balance," the idea holds that during conditions of either greatly reduced or oxidized redox environments, the net ROS emitted from the mitochondria will be elevated (10). During mild uncoupling *in vivo*, rates of ROS released from the mitochondria may therefore actually increase due to a decrease in the redox couple (e.g., $NADH/NAD^+$) that supplies electrons for the ROS scavenging system (10). Under this paradigm of redox-optimized ROS balance, the rates of mE_{H2O2} generated in the *in vitro* experiments conducted in the current study would occur at the more reduced end of the redox spectrum, when the superoxide generated at the level of the respiratory chain by exogenous substrates overwhelms the antioxidant defense. Viewed in this context, our data therefore suggest an improvement in the antioxidant defense after 8WT.

Conventionally, the respiratory control ratio (RCR), defined as the quotient of state 3 respiration to that of state 4, is used as an index of mitochondrial coupling. Indeed, a positive linear relationship between the inverse respiratory control ratio (1/RCR) and P:O ratios has been

demonstrated in isolated mitochondria (101). In the current study, there was also no effect of the exercise training on any of the calculated respiratory control ratios (Table 5). Mitochondrial production of ROS increases exponentially as respiration slows to resting state 4 conditions, and by definition, the mitochondrial membrane potential (ΔΨ) is at its highest (166). Because this ΔΨ – dependent mitochondrial ROS is very sensitive to even mild uncoupling (198), logic follows that an increase in metabolic rate and or uncoupling agents (e.g., uncoupling proteins) in the mitochondria with exercise should reduce the potential for mitochondrial ROS production. Indeed, exercise has been shown to increase the expression of known contributors to the basal proton leak across the mitochondrial inner membrane (87), such as the cardiac/skeletal muscle-specific isoform of the adenine nucleotide translocase, ANT1 (37). However, in light of the absence of a change in the RCR or UCR (Table 5), it is deduced that the decrease in the potential for mE_{H2O2} with 8 weeks of exercise training in the current study are not due to uncoupling per se. The possibility that these changes in mE_{H2O2} are instead mediated by an increase in the ROS scavenging systems are supported in the literature (243). Indeed, an accumulating body of evidence employing both direct (i.e., ESR spectroscopy) and indirect methodologies (e.g., assay of oxidatively modified macromolecules) strongly suggests that free radicals generated during mild to moderate endurance exercise actually constitute a stimulus mechanism for adaptations to exercise, including mitochondrial biogenesis in skeletal muscle (reviewed in (241)). However, in accordance with Hans Selye's classic theorem of general adaptation to a given stressor (254), even exercise training will result in deleterious effects if the body is not allowed to adapt. An example illustrating this concept with regard to exercise-associated oxidative stress in the extreme comes from a very recent study in which severely overtrained athletes exhibited

elevated levels of lipid peroxidation products and protein carbonylation compared to control athletes, both at rest and after exhaustive exercise (274).

Another possible explanation for the reduction in mE_{H2O2} with exercise training may owe to redox modifications at the level of the mitochondrial electron transport chain. Indeed, it appears that ROS production at the level of the mitochondrial electron transport system (ETS) can affect its own enzymatic functions, conferring redox-level regulation of both substrate metabolism and superoxide (O_2^-) generation. The high mitochondrial matrix pH (~8) and proximity of mitochondrial proteins to the major ROS production sites makes mitochondrial protein thiols particularly susceptible to oxidation by ROS (144, 182). Mitochondrial electron transport chain proteins are rich with thiols (142, 143); but it is within complex I that the reactive/regulatory protein thiols believed to confer physiological function are primarily located (18, 66, 152, 247, 307). Many of these thiols are associated with non-heme iron centers, while others on the surface of the complex I are prime targets for redox modification. As an example, *S*-nitrosylation of complex I thiols has been shown to correlate with a significantly reduced activity of the enzyme, an effect that was readily reversible with thiol reductants (66). Moreover, this *S*-nitrosylation was also associated with an increased complex I O_2^- formation (66). Evidence also suggests that complex I is susceptible to glutathionylation by GSSG in the presence of the mitochondrial thiol transferase glutaredoxin 2 (Grx2) (18). Manipulating the redox milieu with an oxidized GSH:GSSG ratio leads to a dramatic loss of complex I activity (18). Moreover, complex I activity is inhibited by *S*-glutathionylation occurring in both 75-kDa (NDUFS1) and 51-kDa (FMN-binding subunit/ NDUFV1) subunits of isolated complex I upon addition of excess GSSG (18, 152, 182, 307), which can result in rapid production of O_2^- (18). Similarly, oxidative modification of complex I results in self-inactivation, decreased electron transfer

activity, and in turn resulting in more O_2^- generation, a phenomenon often referred to in the literature as a "vicious cycle" of ROS-induced ROS production (203) . In the current study, mE_{H2O2} induced by addition of succinate differed Pre-Post 8 weeks of exercise training (Table 6). Because succinate stimulates superoxide generation via reverse electron flow at complex I (206), the results of the current study therefore suggest that exercise training may also attenuate complex I-linked mE_{H2O2}, and that perhaps exercise/exercise training interrupts a vicious cycle of ROS-induced ROS production associated with oxidative modification of the O_2^- - generating components of the mitochondrial ETS.

At the mechanistic level, exercise-associated redox stress has been shown to affect signaling pathways that include the PI3-kinase/Akt, p53, heat shock proteins, the mitogen-activated protein kinase (MAPK) and nuclear factor (NF) κB (150). Upon direct reaction with H_2O_2 or other ROS, NFκB is translocated to the nucleus, where it binds to a number of target gene promoters, initiating activation of various target genes, one of which is the mitochondrial manganese-containing superoxide dismutase (MnSOD), an enzyme which converts superoxide to H_2O_2. Indeed, it was shown that the expression of MnSOD increased significantly after a single, 1 –hour bout of exhaustive exercise in rat skeletal muscle (133), and further confirmed that acute exercise increases the NFκB signaling pathway in rat skeletal muscle (151). Observations made by these and others regarding the adaptive response to exercise-association redox perturbations (227, 241) may therefore account, at least in part, for the often unimpressive results from studies examining the effects of antioxidant supplementation on adaptation to exercise (reviewed in (38)) and improvements to diabetes (reviewed in (256)). In fact, a very recent study found that supplementation with the antioxidant vitamins C and E actually prevented the exercise-associated benefits on insulin sensitivity in humans (236). Taken as a whole, the literature

suggests that the improvements in exercise performance and whole-body insulin sensitivity with exercise training may owe to adaptations associated with attenuating mitochondrial ROS. Whether this involves an increase in the antioxidant defense, a decrease in the production of ROS, or both, will require further research. The results of the current study do not support that increased proton flux (i.e., lowered $\Delta\Psi$) during basal respiration accounts for the observed decrease in mE_{H2O2}. Moreover, the results of the current study do not even support the long-held notion of improved insulin sensitivity with exercise training, absent any changes in body mass or composition. In a study investigating the effect of 12 weeks of exercise training (cycle ergometer 4 h/wk at 70% $\dot{V}O_{2max}$) on insulin sensitivity (euglycemic-hyperinsulinemic clamp) and glucose metabolism (indirect calorimetry) in lean, obese and diabetic men, Segal et al (253) made conclusions corroborating the results of the current study with regard to the effects of exercise training on insulin sensitivity. Indeed, while the subjects improved their cardiorespiratory fitness, no improvements in insulin sensitivity were observed after the exercise training (253). Because they also controlled for energy balance by refeeding the energy expended in each training session, they concluded that exercise does not improve insulin sensitivity independent of changes in body mass or composition (253). In the current study, exercise training did not result in a significant change in body mass or % body fat (Table 4). In fact, the only variables observed *in vivo* to change over the course of the 8 weeks of exercise training in the present study were $\dot{V}O_{2peak}$ and the RER at 75% $\dot{V}O_{2peak}$. The decrease in RER with training suggests that during exercise, the subjects relied more on fat for energy during exercise after the 8 weeks of training.

With regard to the permeabilized fiber experiments in the current study, mE_{H2O2} was reduced following training, but there was no change in JO_2. Because insulin sensitivity did not similarly improve with 8 weeks of exercise training, the changes in mE_{H2O2} observed here may

therefore relate more to adaptations conferred as a specific response to the exercise stressor, paralleling the typical improvements in cardiorespiratory fitness (60) and fat oxidation (222) associated with exercise training in general, and also observed in the current study. In support of this contention, when first first-degree relatives of type 2 diabetic patients were tested pre- and post- 10 weeks of exercise training, the improvements in VO_{2max} did not correlate with improvements in insulin sensitivity (213). The authors therefore concluded that improvements in insulin sensitivity may be dissociated from the adaptations to exercise training in skeletal muscle in first degree relatives of type 2 diabetic patients (213). Since then, additional reports support the notion that exercise training does not improve insulin sensitivity independent of changes in body mass or composition (300), which by definition represents a negative energy balance situation.

The fact that all the subjects in the current study were women may help explain some of the results. In contrast to men (8), women who regularly exercise are reported to remain weight-stable, even if the exercise incurs significant energy deficits (205). It has therefore been suggested that women are better able to adapt to changes in energy balance associated with endurance exercise training (26). While the ovarian hormones have been suggested to play a role in the metabolic response to exercise in women (64) the results of the current study do not support that a change in either E2 or P4 can account for any of the results after 8 weeks of exercise training (Table 4). When the effects of voluntary wheel running on energy balance, linear growth and body composition were compared between male and female rats over a nine-week period, it was determined that both the male and female rats were in negative energy balance versus control (57). However, despite running greater distances (50-80%) than the male

rats, the female rats did not lose weight (57). These data agree with the results of the current study, which found no change in body mass or % body fat after 8 weeks of exercise training.

In the current study, post hoc analysis revealed that mE_{H2O2} expressed relative to JO_2 (i.e., % mE_{H2O2}) was significantly reduced Pre-Post in the lean group following training (Table 6). Moreover, it was noted that the mean pre- and post-training mE_{H2O2} tended to be greater in the lean group compared to the obese group, albeit not significantly so. One potential explanation for these observations has to do with the lifestyles characteristics for which these subjects were recruited; namely, they were selected because they were self-described sedentary women. Because these women were certainly not bedridden individuals, having arrived to the research center under their own power, it follows that the work associated with average daily tasks would be greater in the obese subjects, who by definition have greater body mass to move. Literature further supports the contention that energy expenditure is greater in obese compared to lean women (228). In the current study, the skeletal muscle biopsies were performed in the subjects' vastus lateralis, a component of the quadriceps muscle group. A recent study confirmed that in adolescents, obesity has no effect on quadriceps muscle function (188). If quadriceps function (e.g., force production, fatigueability) are presumed to be similar between the lean and obese subjects in the current study, and the energy expenditures greater in the obese subjects (228), the possibility may therefore exist that the obese subjects actually had better-trained vastus laterali before the current study commenced. If, similar to exercise training, reduced mE_{H2O2} in the musculature constitutes an adaptation to the activities of daily living (e.g., walking), then this might explain why the lean subjects did not have a lower mE_{H2O2} either pre- or post-training compared to the obese subjects.

During exercise, insulin-independent and -dependent glucose uptake by human skeletal muscle is enhanced. After a single bout of exercise, insulin sensitivity increases primarily in the muscles involved in the physical activity, an effect which may last for up to two days (124, 305). Many studies measure indices of insulin sensitivity after exercise training within 1-2 days following the final exercise bout (e.g., (59)). Three of the 12 subjects in the current study were biopsied 48 h after the last exercise bout, with the remaining 9 biopsied 24 h after the last bout. Thus, if improved insulin sensitivity with exercise training owes substantially to the acute (and diminishing) affects conferred within the 1-2 days following a bout of exercise (124, 305), then the fact that the subjects in the current study were in some cases tested ~2 days following the last bout of exercise may account for the lack of improved insulin sensitivity observed after 8 weeks of exercise training in the current study. Indeed, it was only after covarying for the Pre-Post difference in HOMA-IR that significant training effects were observed with regard to mE_{H2O2} (Table 6). This fact highlights the potential underlying training adaptations to 8WT, which clearly involve a reduced propensity for mE_{H2O2} in skeletal muscle. A relevant corollary question was what happens to estimated insulin resistance when mE_{H2O2} is controlled in the statistical model. Surprisingly, there HOMA-IR did not differ significantly Pre-Post, even when controlling for mE_{H2O2}. This suggests that the underlying mitochondrial adaptation to 8WT was therefore only revealed in our *in vitro* assay in which mitochondria are subjected to increasing amounts of substrates, at least one of which (succinate) is known to induce very high rates of complex I-mediated H_2O_2. The body mass/composition data indicate that the subjects in the current study were likely in a zero, or more probably positive energy balance following the 8WT. Under basal *in vivo* conditions in a positive energy balance situation, the mitochondrial metabolite flux pattern are expected to exhibit state 4 - like conditions vs. state 3, in turn masking the potential

training effects on, for example, HOMA-IR. If true, this would explain why statistically controlling for Pre-Post HOMA-IR revealed such marked differences in the capacity for substrate-stimulated mE_{H2O2} *in vitro*.

In conclusion, the results of this study demonstrate a significant reduction in the potential for mE_{H2O2} in skeletal muscle from women following 8 weeks of exercise training, with no change in mitochondrial JO_2. Absent acutely improved insulin sensitivity or changes in body mass or composition, these data suggest that the reduction in mE_{H2O2} may constitute an adaptation to exercise training which parallels improved cardiorespiratory fitness and improved reliance on fat during exercise, and not necessarily improved estimated resting glucose homeostasis.

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TABLE 4. EFFECTS OF 8 WEEKS OF EXERCISE TRAINING ON BODY MASS & COMPOSITION, SERUM OVARIAN SEX STEROIDS, INSULIN SENSITIVITY, AND EXERCISE PEAK OXYGEN CONSUMPTION AND FUEL METABOLISM.

Body mass index (BMI = $kg \cdot (m^2)^{-1}$), percent body fat (% BF), serum estradiol (E2, pM), serum progesterone (P4, nM), ratio of P4, pM to E2, pM (P4:E2), homeostatic model assessment (HOMA), VO_{2peak} ($mL \cdot kg^{-1} \cdot min^{-1}$) and respiratory exchange ratio (RER = VCO_2/VO_2) at 75% VO_{2peak} determined for 5 lean and 7 obese women before (Pre) and after (Post) 8 weeks of exercise training. $P < 0.05$ represents significant main effects; * = significantly different from pre-training value, $P < 0.05$; ** = $P < 0.01$.

	Lean, *n* = 5		Obese, *n* = 7		Main effects, *P*-value	
Variable	Pre	Post	Pre	Post	Training	Obesity
Age (y)	33.6 ± 4.3		30 ± 2.4		-	0.45
BMI	25.3 ± 0.8	25.6 ± 0.7	37.2 ± 1.8	37.9 ± 1.9	0.09	< 0.001
% BF	38.3 ± 1.5	37.6 ± 1.2	48.2 ± 1.8	49.0 ± 1.8	0.80	< 0.01
E2 (pM)	398.7 ± 140.6	517.6 ± 252.0	378.9 ± 94.3	353.0 ± 128.6	0.68	0.63
P4 (nM)	14.3 ± 5.4	12.7 ± 6.5	11.1 ± 5.9	9.1 ± 4.6	0.53	0.66
P4:E2	36.9 ± 14.1	33.1 ± 15.3	32.7 ± 12.0	34.6 ± 24.1	0.55	0.34
HOMA	2.7 ± 0.44	2.0 ± 0.3	5.2 ± 1.4	5.4 ± 1.4	0.56	0.10
VO_{2Peak} (mL•kg^{-1}•min^{-1})	21.3 ± 1.9	24.5 ± 1.3	16.4 ± 1.0	17.0 ± 0.4	< 0.05	< 0.01
RER (75%VO_{2Peak})	0.92 ± 0.02	0.88 ± 0.01**	0.91 ± 0.01	0.89 ± 0.01*	< 0.001	0.66

TABLE 5. EFFECTS 8 WEEKS OF EXERCISE TRAINING ON MITOCHONDRIAL RESPIRATION AND RATIOS OF RESPIRATORY CONTROL.

Rates of mitochondrial respiratory O_2 flux (JO_2, pmol·s^{-1}·mg dry wt^{-1}) measured in saponin-permeabilzed myofibers from vastus lateralis skeletal muscle biopsies from 5 lean and 7 obese women before (Pre) and after (Post) 8 weeks of exercise training. Substrate conditions were: 25 μM palmitoyl-carnitine + 1 mM malate (P-C/M, State 4); P-C/M + 2 mM ADP (P-C/M, State 3); P-C/M, State 3 + 2 mM glutamate (P-C/M+G); P-C/M, G, State 3 +3 mM Succinate (P-C/MG+S); P-C/MGS + 10 μM oligomycin (P-C/MGS+O); P-C/MGSO + 2 μM FCCP (P-C/MGSO+FCCP). Ratios of respiratory control were calculated as follows: $RCR_{P\text{-}C/M}$, respiratory control ratio = (JO_2, P-C/M, State 3)·(JO_2, P-C/M, State 4)$^{-1}$; $UCR_{P\text{-}C/MGS}$, uncoupling control ratio = (JO_2, P-C/MGSO+FCCP)·(JO_2, P-C/MGS+O)$^{-1}$; $ACR_{P\text{-}C/MGS}$, adenylate control ratio = (JO_2, P-C/MGSO+FCCP)·(JO_2, P-C/MG+S)$^{-1}$.

	Lean, *n* = 5		Obese, *n* = 7		Main effects, *P*-value	
JO_2 (pmol · s^{-1} · mg^{-1})	Pre	Post	Pre	Post	Training	Obesity
P-C/M (State 4)	8.9 ± 1.7	8.2 ± 2.0	6.6 ± 1.8	9.4 ± 2.6	0.66	0.77
P-C/M (State 3)	52.5 ± 4.4	53.1 ± 4.4	38.9 ± 4.2	51.1 ± 6.5	0.17	0.23
P-C/M+G	155.2 ± 15.1	163.7 ± 16.6	122.8 ± 14.8	163.0 ± 20.6	0.17	0.41
P-C/MG+S	219.1 ± 19.0	211.4 ± 18.0	178.7 ± 21.5	216.2 ± 31.1	0.55	0.51
P-C/MGS+O	47.13 ± 2.3	53.0 ± 7.5	44.3 ± 5.2	58.3 ± 9.2	0.20	0.87
P-C/MGSO+FCCP	269.8 ± 27.4	277.9 ± 23.8	220.9 ± 20.1	265.5 ± 37.3	0.36	0.35
$RCR_{P\text{-}C/M}$	7.2 ± 1.8	7.5 ± 1.3	8.8 ± 2.3	7.7 ± 1.8	0.84	0.64
$UCR_{P\text{-}C/MGS}$	5.7 ± 0.5	5.5 ± 0.6	5.3 ± 0.6	4.7 ± 0.2	0.31	0.32
$ACR_{P\text{-}C/MGS}$	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.0	0.74	0.63

TABLE 6. EFFECTS OF 8 WEEKS OF EXERCISE TRAINING ON MITOCHONDRIAL H_2O_2 EMISSION AND FRACTIONAL H_2O_2.

Rates of mitochondrial H_2O_2 emission (mE_{H2O2}, pmol·min^{-1}·mg dry wt^{-1}) measured in saponin-permeabilzed myofibers from vastus lateralis skeletal muscle biopsies from 5 lean and 7 obese women before (Pre) and after (Post) 8 weeks of exercise training. Substrate conditions were, in the presence of 10 μM oligomycin: 25 μM palmitoyl-carnitine + 1 mM malate (P-C/M); P-C/M + 2 mM glutamate (P-C/M+G); P-C/MG + 3 mM Succinate (P-C/MG+S). Additionally, mE_{H2O2} are expressed as a percentage of the respective rate of respiratory O_2 flux (JO_2) measured in parallel: P-C/M, State 4 (% mE_{H2O2} P-C/M); and P-C/MGS + 10 μM oligomycin (% mE_{H2O2} P-C/MGS). $P < 0.05$ represents significant main effects; * = significantly different from pre-training value.

mE_{H2O2} (pmol·min^{-1}·mg^{-1})	Lean, *n* = 5		Obese, *n* = 7		Main effects, *P*-value	
	Pre	Post	Pre	Post	Training	Obesity
P-C/M	4.0 ± 0.8	2.0 ± 1.1	2.2 ± 0.6	3.1 ± 0.9	0.12	0.66
P-C/M+G	4.2 ± 1.0	2.6 ± 1.1	2.7 ± 0.9	3.1 ± 0.9	0.54	0.50
P-C/MG+S	41.2 ± 8.1	14.0 ± 5.1*	10.3 ± 6.8	7.4 ± 4.3	< 0.01	< 0.05
% H_2O_2 P-C/MGS	1.5 ± 0.3	0.4 ± 0.1*	0.4 ± 0.3	0.2 ± 0.1	< 0.05	0.07

CHAPTER 5: INTEGRATED DISCUSSION

The current and rising epidemic in diabetes has lead to intense investigation into the mechanisms of skeletal muscle insulin resistance. In Chapter 1, review of the literature clearly implicates the mitochondrion and redox signaling in the etiology of insulin resistance. The studies described in Chapter 2-4 were all conducted with hypotheses governed by the same theme; each investigated the effect of a known modulator of insulin sensitivity on the potential for mE_{H2O2} in skeletal muscle. In Chapter 2, the effect of metformin, a well established pharmacological means of improving insulin sensitivity, was used to treat the Zucker rat model of obesity-associated peripheral insulin resistance. Confirming the overall hypothesis of the current work, metformin treatment both improved glycemic control, and at the same time reduced the potential for mE_{H2O2}. Subsequently, acute *ex vivo* metformin incubation experiments revealed that complex I is dose-dependently inhibited by metformin. It was further determined that the specificity by which metformin inhibits reverse electron flow-mediated mE_{H2O2} outweighed the effects of the drug on electron flow in the forward direction (i.e., JO_2). Batandier et al (17) reported a similar phenomenon in mitochondria isolate from rat liver incubated in a suprapharmacological concentration of metformin. The main contribution of the metformin data presented in the current work owes to its physiological relevancy. Indeed, the Zucker rats in the present work were treated with oral metformin dosed to body mass; and the mitochondria were tested in permeabilzed skeletal muscle fibers from these rats without exogenous metformin. The dose response experiments represent another unique aspect of this study. It is the first study to show that quantitatively, complex I-linked mE_{H2O2} is more sensitive to metformin that complex I-linked JO_2. Whether this phenomenon applies to humans or occurs *in vivo* will require further research.

As mentioned previously in Chapters 1, 3 and 4, the incidence of obesity and type 2 diabetes is greater among African American women (AW) compared to Caucasian women (CW) in the United States (258). This phenomenon has fueled considerable research aimed at elucidating the potential mechanisms which may account for this racial disparity (e.g., (34, 35, 41, 58, 68, 94, 128, 141, 162, 178, 187, 229, 249, 270, 273)). The initial purpose of the study in Chapter 3 was to explore a potential link between mitochondrial respiration, mitochondrial reactive oxygen species and insulin resistance with regard to race (i.e., AW vs. CW) and obesity (BMI > 30) in women. Surprisingly however, race did not affect any of the outcome variables measured in Chapter 3 or Chapter 4. In Chapter 3, the AW and CW were therefore pooled and divided by insulin resistance. It is important to note that in the current studies, only palmitoyl-carnitine, the activated form of the fatty acid was used to test JO_2 and mE_{H2O2}. Palmitoyl-carnitine readily enters the mitochondrial matrix, bypassing carnitine palmitoyl transferase (CPT1), the purported rate-limiting step in the oxidation of long-chain fatty acids (161). Because it is expected that respiration supported exclusively by palmitoyl-carnitine in experimental mitochondrial preparations is therefore rate-limited by the β-oxidative machinery within the matrix, and not CPT1, the possibility remains that we were unable to detect potential differences in fatty-acid supported JO_2 in skeletal muscle mitochondria between AW and CW because a/the biomolecular source(s) responsible for the racial disparity in fatty acid oxidation lies upstream of β-oxidation, i.e., the steps of activation and/or transport. Indeed, recent yet scarce research supports the notion that impaired activity/content of acyl coA synthetase, an important enzyme involved in the activation of fatty acids for oxidation and/or storage may represent a salient enzymatic deficiency in the skeletal muscle of AW (58, 229), which manifests in a reduced capacity for lipid oxidation and predisposition to obesity and type 2 diabetes.

A clear connection between insulin resistance and mE_{H2O2} in women was established in Chapter 3. Moreover, a significant relationship between P4 and mE_{H2O2} was demonstrated. Several studies have investigated the effects of E2 and/or P4 on mitochondrial physiology (3, 31, 51, 84, 114, 192, 202, 211, 266, 297). The design of the current study was unique in that the effects of physiologically relevant concentrations of E2 and P4 on mE_{H2O2} and JO_2 were demonstrated. First, saponin-permeabilized myofibers from women in the follicular phase of their menstrual cycle were incubated with luteal phase serum concentrations of either E2, P4, or both. Second, a strong relationship between serum levels of progesterone and mE_{H2O2} was established in women selected at random with regard to menstrual cycle status. These results from Chapter 3 demonstrate a model by which P4 promotes an increased potential for mE_{H2O2}, with or without E2 present; and also in which P4 inhibits JO_2, but not when E2 is present. Lastly, mE_{H2O2} in permeabilized myofibers from one woman who learned post hoc that she was pregnant at the time of the biopsy, revealed a markedly greater rate of mE_{H2O2} compared to insulin sensitive women. Altogether, the results from Chapter 3 implicate the rising OS during the luteal phase and pregnancy in a rise in mE_{H2O2}, which may be due to elevated P4. When the results from both Chapters 2 and 3 are considered together, evidence for a potential unifying mechanism emerges whereby complex I-linked mE_{H2O2} represents a convergence of two known modulators of insulin sensitivity. Metformin, a well established means of improving insulin sensitivity, reduces complex I-linked mE_{H2O2}. P4 on the other hand, a female sex hormone implicated in peripheral insulin resistance, increases complex I-linked mE_{H2O2}. It was therefore tempting to presume that exercise training would accomplish its generally accepted improvements in skeletal muscle insulin sensitivity through a similar mechanism involving complex I-linked mE_{H2O2}.

In Chapter 4, the effects of exercise training on insulin sensitivity and mE_{H2O2} in women were explored. Initially, the study in Chapter 4 sought to explore the effects of exercise training on the mitochondrial fate of O_2 in the context of the racial metabolic disparity between AW and CW in the United States. Thus, mitochondrial JO_2, mE_{H2O2} and insulin resistance were studied in lean and obese AW and CW, Pre and Post 8WT. However, contrary to the previously published decrement in fat oxidation in AW during exercise relative to CW (128), it was determined that race played no role in any of the outcome variables measured in Chapter 4, including RER at 75% VO_{2peak}. Therefore, the AW and CW were pooled into lean (BMI < 30) and obese (BMI > 30) groups.

A well established therapeutic modality in the treatment of diabetes, 8 weeks of exercise training (8WT) did not improve estimated insulin sensitivity in Chapter 4. 8WT did however reduce mE_{H2O2} in the lean subjects and improve the peak rates of oxygen consumption (VO_{2peak}) and fat oxidation during exercise (RER at 75% VO_{2peak}). Unexpectedly, JO_2 was not altered with training, nor were any of the ratios of respiratory control. Therefore, it appears likely that reductions in the potential for mE_{H2O2} associated with training may owe more to improvements in the antioxidant defense machinery or redox protein modifications at the level of the ETS, and not necessarily a reduction in mitochondrial $\Delta\Psi$ at rest. Increasingly, the literature points to exercise associated mitochondrial ROS as stimulus for training-induced adaptations in skeletal muscle (227). The results presented in Chapter 4 support that reduced potential for mE_{H2O2} constitutes an adaptation to exercise training, concurrent with improvements in cardiorespiratory fitness (increased VO_{2Peak}) and increased reliance on fat (reduced RER) as substrate during exercise. Aside from potential acute exercise affects that were likely not present following exercise training in the current study, a role for mE_{H2O2} in training associated improvements in

insulin sensitivity are not supported by the results presented in Chapter 4. These findings agree with previous studies which conclude that exercise training does not improve insulin sensitivity in the absence of changes in body weight or composition (i.e., sustained negative energy balance) (213, 253, 300). Moreover, the data from Chapter 4 regarding the lower pre-training rates of mE_{H2O2} in obese subjects is in agreement with at least one study (1) which demonstrated a lower mE_{H2O2} in isolated mitochondria of obese vs. lean individuals. The mechanisms accounting for the disparate observations of mE_{H2O2} with obesity (1, 5) await further research.

The results of Chapters 2-4 may better describe the acute effects of insulin sensitivity modulators on the potential for mE_{H2O2} in skeletal muscle. Indeed, when permeabilized myofibers were incubated briefly in metformin, mE_{H2O2} was reduced (Chapter 2). Conversely, when permeabilized myofibers were incubated briefly with P4, mE_{H2O2} increased (Chapter 3). Because the overall hypothesis of Chapter 4 did not involve the equivalent of an "acute incubation in exercise" with which to test the direct effects of exercise on mE_{H2O2} and JO_2, it is important comment on what such an experiment would add to our understanding about how exercise might directly affect insulin sensitivity. In Chapter 4, the subjects exercise trained on a cycle ergometer at 75 % of their respective VO_{2peak}. During exercise performed at 75% VO_{2max} on a cycle ergometer, Sahlin et al (242) observed that ADP and P_i concentrations (mmol/kg dry wt muscle) within the quadriceps increases significantly above those at rest by more than 5%. Therefore, the effects of exercise can be mimicked *ex vivo* in a permeabilized myofiber preparation by addition of exogenous ADP. As mentioned in Chapter 1, the physiological rate of mitochondrial ROS production is inversely proportional to the availability of ADP (42), with the mitochondrial $\Delta\Psi$-dependent ROS production being sensitive to even mild uncoupling (198). Therefore, it is not surprising that addition of exogenous ADP (100 μM) to isolated rat heart

mitochondria abolished the state 4 rate of mE_{H2O2} (166). Interestingly, even the mitochondrial-associated hexokinase is thought to confer "antioxidant" properties by generating ADP via enzymatic phosphorylation of glucose (65). It was demonstrated in rat brain mitochondria that stimulating the mitochondrial bound hexokinase with glucose and ATP decreased the rate of mitochondrial H_2O_2 production, concurrent with a reduction in the mitochondrial $\Delta\Psi$ (65). Conversely, additions of exogenous glucose 6-phosphate has the opposite effect, keeping ADP levels low and the $\Delta\Psi$ high, which increased the mitochondrial production of H_2O_2 (65). Because mE_{H2O2} produced during state 4 respiration is thought to come predominantly from complex I (206), the fact that ADP abolishes state 4 mE_{H2O2} *in vitro* implies that any condition in which ADP levels are increased may attenuate complex I-linked ROS production. By increasing cellular ADP, exercise is therefore expected to attenuate complex I-mediated mE_{H2O2}. While during an exercise bout and shortly thereafter, complex I-linked ROS may be attenuated, there is no reason to believe that a long term training effect would reduce the rate of mE_{H2O2}, absent a negative energy balance. The main-effect reduction in mE_{H2O2} following 8 weeks of exercise training described in Chapter 4 may therefore owe to residual effects remaining from the last exercise bout (124, 305), interrupted "vicious cycle" ROS-induced ROS release (203) during the regular exercise bouts, or improvements in the antioxidant defense (133). It should also be mentioned that mitochondria have been advocated as "ROS sinks," consuming ROS as well as producing it (115, 218, 315). If the exercise-associated ROS in skeletal muscle is indeed due to extramitochondrial sources (e.g., xanthine oxidase), then mitochondrial scavengers of this ROS during exercise could potentially be depleted (e.g., GSH to GSSG) with intense or prolonged exercise. This would therefore account for the findings of increased potential for REF-associated mE_{H2O2} in isolated skeletal muscle mitochondria following prolonged exercise (243). Despite

uncertainties surrounding potential training-associated, long term improvements in insulin sensitivity and/or mE_{H2O2}, the short term, acute improvements in insulin sensitivity with exercise and reduced mE_{H2O2} that occurs with ADP *in vitro* have been recognized for quite some time.

An outstanding observation common to Chapters 2-4 was a lack of discernible differences in JO_2 between groups, save for the acute, *in vitro* incubation experiments involving metformin and P4. Recall, no differences in JO_2 or ratios of respiratory control were observed between oral metformin treated rats vs. control; nor between IS and IR women or following 8WT. While these observations were overshadowed by the marked differences between these same groups with respect to mE_{H2O2}, the results from the respiratory experiments in permeabilized myofibers may not necessarily represent a negligible difference or change in the capacity for JO_2 between the *in vivo* groupings (i.e., oral metformin, IS vs. IR, and 8WT). As mentioned in Chapter 1, maximal aerobic capacity is limited by oxygen supply *in vivo* (75, 235). Similarly, the respiratory capacity of mitochondria is limited by oxygen kinetics, such that saturating concentrations of oxygen *in vitro* are requisite for determining respiratory capacity (102). An appropriate analogy is that of oxidative phosphorylation in studies using isolated mitochondria, where saturating concentrations of ADP are necessary for assessment of maximal state 3 respiration. While partial pressures of oxygen, and therefore concentrations of oxygen in experimental aqueous media well below that of sea-level air saturation (e.g., 20 µM) are not limiting to respiration in isolated mitochondria and small cells (reviewed in (102, 248)), even at air saturation (i.e., ~200 µM O_2), oxygen is limiting to respiration in permeabilized muscle fibers (104). This is illustrated by a 100-fold increase in the sensitivity to oxygen concentration in permeabilized rat soleus and heart fibers (104). This sensitivity to changes in already relatively high oxygen concentrations is thought to be due, at least in part, to diffusion limitations resulting

from the fiber bundle (104). At low oxygen concentrations, an "anoxic core" of the fiber is therefore thought to occur. Limitations of respiration due to insufficient oxygen levels in the experimental setup involving permeabilized muscle fibers has been reduced or prevented by maintaining oxygen concentrations above air saturation in the range of 200-500 μM (11, 32). It has been suggested that the experimental oxygen limitations may account, at least in part, for the disparate low and high maximal state 3 respiratory rates reported for experiments involving permeabilized fibers (102). Nevertheless, the data promoting the use of high oxygen in measurements of JO_2 capacity in permeabilized fibers continue to go largely ignored or unrecognized (e.g., (174)). In the studies described in Chapter 2-4 of the current project, all respiratory experiments were conducted in air saturated media (i.e., ~200 μM O_2). The potential for the oxygen limitations reported to occur at air-saturated media in permeabilized myofiber preparations (104) to mask differences or changes in mitochondrial capacity warrant recognition with regard to the results of the current project. While these experimental limitations may have contributed to the lack of observable differences between groups, the fact that differences in JO_2 were observed in the *in vitro* incubation experiments may therefore mean that the observed differences were understated. Hence, one could argue that the possibility cannot be ruled out that metformin's actions may indeed be mediated by an inhibition of complex I-linked respiration (i.e., forward electron flow), in addition to, or instead of inhibition solely of mE_{H2O2} linked to REF in complex I. However, counter to such an argument, when Batandier et al (17) incubated isolated rat liver mitochondria in 10 mM metformin, they observed a hampering of succinate-stimulated mE_{H2O2}, but no decrement whatsoever in JO_2 supported by complex I-linked substrates. It is important to note that the JO_2 data in our study (Chapter 2) were generated with an oxygraph identical to that used by Batandier et al (17). Because at air-saturated media, O_2 is

certainly not limiting to respiration in isolated mitochondria (102, 248), it would seem therefore likely that the preparation used in Chapter 2 to test the acute *ex vivo* effects of metfomin on JO_2 in permeabilized myofibers may actually have been more sensitive than that employed by Batandier et al (17), assuming that mitochondria from skeletal muscle and liver are affected by metformin similarly. Indeed, at a metformin concentration of 10 mM, complex I-linked JO_2 was potently inhibited in our experimental conditions (Chapter 2).

It should be noted however, that reducing limitations to respiration by maintaining oxygen concentrations above air saturation may not, relatively speaking, constitute the most important means of quality assurance in experiments involving permeabilized myofibers from women. Indeed, in experiments conducted in quadruplicate on myofibers from one obese women permeabilized with 30 µg/mL saponin, the calculated methodological variation, expressed as the %CV for JO_2 in state 4 (10 mM glutamate + 2 mM malate), maximal ADP (4 mM) and 10 µM cytochrome *c* conditions were 14.8, 7.0 and 7.6 %, respectively (Appendix B; Figure 14). This is comparable to the methodological variation determined by Tonkonogi et al (278) for JO_2 measured in permeabilized myofibers from men. They calculated %CV for state 4 JO_2 (5 mM pyruvate + 2 mM malate) and after stimulation with low (100 µM) and high ADP (1 mM) concentrations to be 18.0, 7.9 and 10.9 %, respectively, in only two fiber bundles from the same muscle biopsy (278). The results of the saponin concentration optimization experiments (Appendix B; Figure 14) clearly demonstrate the methodological pitfalls avoided by using 30 µg/mL vs. 50 µg/mL saponin to permeabilize myofibers from women. Future research involving permeabilized myofibers would do well to additionally rule out the potential confounding effects and methodological variation associated with assessing respiratory capacities under submaximal vs. maximal kinetic oxygen concentrations.

To summarize from the results of the current project and the literature, it appears that, with regard to complex I-linked, ΔΨ-dependent, state 4-associated ROS, 1) metformin decreases; 2) progesterone increases; and 3) exercise decreases the potential for mE_{H2O2} in skeletal muscle. If, as the results of Chapters 2 & 3 and other studies suggest, there is a causative relationship between mE_{H2O2} and the regulation/inhibition of insulin signaling, then with regard to insulin-stimulated glucose uptake, it would appear likely that 1) metformin improves; 2) progesterone reduces; and 3) exercise improves the sensitivity of skeletal muscle to insulin. Taken as a whole, the results presented here support that known modulators of insulin sensitivity have an effect on the potential for complex I - mediated mE_{H2O2} in skeletal muscle. Increasingly, the literature has demonstrated a diverse array of signaling and metabolic pathways that may be redox regulated. Absent observable changes in skeletal muscle insulin sensitivity (e.g., Chapter 4), a demonstrable link between mE_{H2O2} and known effectors of insulin signaling may also represent the involvement of these same effectors in mitochondrial redox signaling elsewhere. The physiological significance of these findings with regard to other potential consequent cellular responses to changes in mE_{H2O2} awaits further research.

Despite the current and increasing awareness of the well-established means of improving skeletal muscle insulin sensitivity to treat type 2 diabetes and its associated co-morbidities (e.g., exercise, diet, and insulin-sensitizing drugs), the prevalence of metabolic disease continues to increase in the industrialized world. While targeting mE_{H2O2} in novel ways to treat types 2 diabetes and the associated skeletal muscle insulin resistance may represent an attractive avenue for biomedical research, the line of reasoning governing this approach may not address the underlying issue at hand in the long-term. If insulin resistance represents a physiological response to a homeostatic perturbation (e.g., cellular redox imbalance), then confirming what is

already known about the relationship between metabolic imbalance and the physiological preservation of life in greater detail does not add anything in and of itself to slowing or even reversing the current and increasing epidemic in obesity and type 2 diabetes world-wide. It will be imperative to apply the knowledge gained from these and related studies to established therapeutic modalities used to treat insulin resistance so that they may be optimized to meet the needs of an ever-evolving culture.

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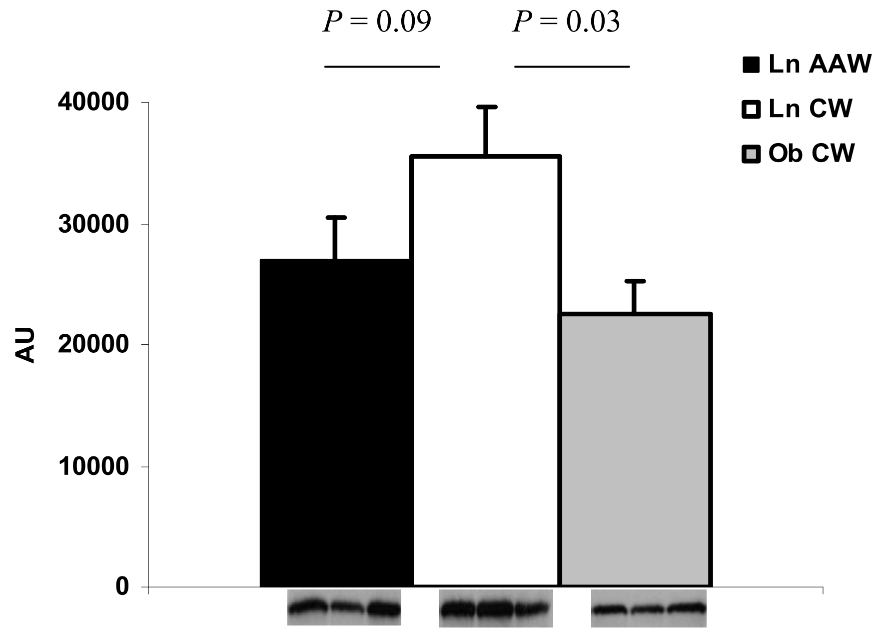
APPENDIX A. PRELIMINARY SUPPORT FOR RACIAL DIFFERENCES IN ADENINE NUCLEOTIDE TRANSLOCASE 1 CONTENT IN WOMEN.

FIGURE 13. WESTERN BLOT FOR ADENINE NUCLEOTIDE TRANSLOCASE 1 IN RECTUS ABDOMINUS FROM AFRICAN AMERICAN AND CAUCASIAN WOMEN.

Western blot of rectus abdominus (abdominal) muscle protein extracts from pre-menopausal women using adenine nucleotide translocase isoform 1 (ANT1) - specific polyclonal antibody (arbitrary units, AU). ANT1 content in rectus abdominus of three lean Caucasian women (Ln CW) was greater than the ANT-1 content in rectus abdominus of three lean African American women (Ln AAW; $P = 0.09$ vs. Ln CW) and three obese CW (Ob CW; $P = 0.03$ vs. Ln CW). These results illustrate a potential decrement in ANT1 of obese skeletal muscle and suggest that ANT1 content may be lower in AAW compared to CW.

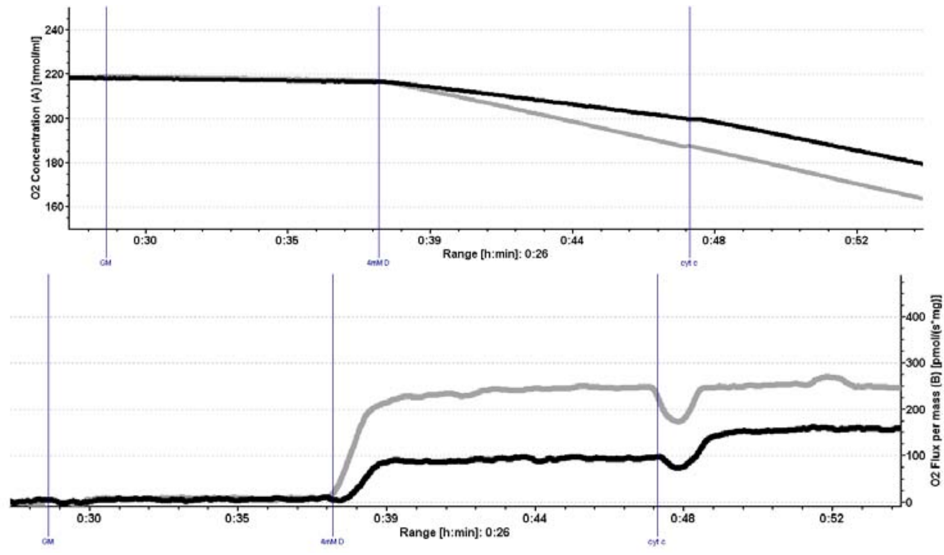

P = 0.09
P = 0.03
Ln AAW
Ln CW
Ob CW
40000
30000
20000
10000
0
AU

APPENDIX B: OPTIMIZATION OF THE SAPONIN-PERMEABILIZED MYOFIBER PREPARATION FOR HUMAN FEMALE SUBJECTS

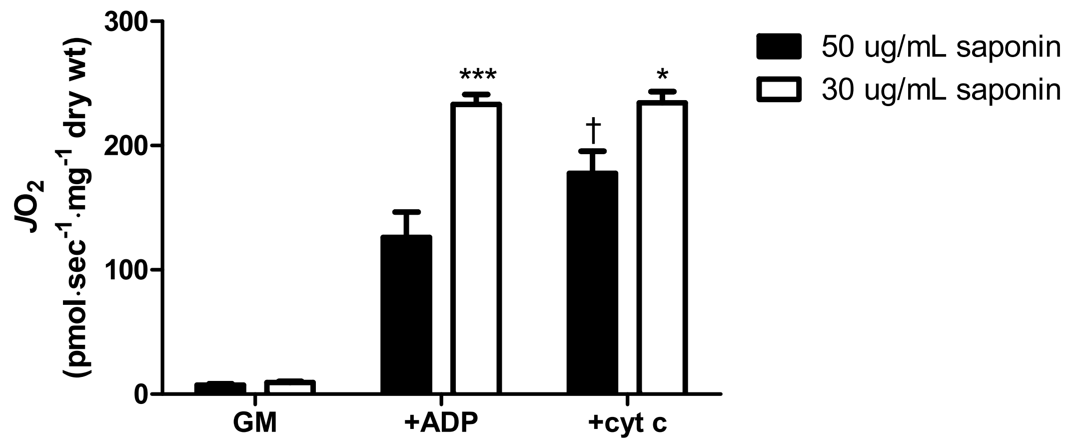
FIGURE 14. METHODOLOGICAL MODIFICATIONS MADE WITH RESPECT TO SAPONIN-PERMEABILIZED MYOFIBERS FROM WOMEN.

Comparison between the standard saponin concentration (50 µg/mL) and the saponin concentration (30 µg/mL) used in the current studies to permeabilzed vastus lateralis myofibers obtained from women. ***A***. Representative oxygraphic trace of O_2 concentration (top) in the experimental chamber with vastus lateralis myofibers from an obese woman (i.e., BMI > 30) permeabilized with either 50 µg/mL (black trace) or 30 µg/mL (gray trace) and the corresponding respiratory O_2 flux derivation (JO_2; bottom) from the oxygraphic trace. Substrate conditions during JO_2 measurements were: 10 mM glutamate + 2 mM malate (GM); GM + 4 mM ADP (+ADP); GM+ADP + 10 µM cytochrome *c* (+cyt c). *B*. Data are mean ± SEM from 4 separate myofibers obtained from one obese woman. Percent coefficient of variation (%CV) of the JO_2 for the myofibers permeabilized with 50 µg/mL saponin were 32.7, 32.7 and 20.1% for GM, +ADP and +cyt c conditions, respectively. The % CV of the JO_2 for the myofibers permeabilized with 30 µg/mL saponin were 14.8, 7.0 and 7.6 % for GM, +ADP and +cyt c conditions, respectively. $^{*}P < 0.05$ vs. 50 µg/mL; $^{***}P < 0.001$ vs. 50 µg/mL. $^{\dagger}P < 0.05$ vs. +ADP, 50 µg/mL.

A.



B.



APPENDIX C

INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

ANIMAL USE PROTOCOL APPROVAL LETTERS

East Carolina University.

1907-2007 CENTENNIAL

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

December 4, 2009

Darrell Neufer, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Neufer:

Your Animal Use Protocol entitled, "Mitochondrial Bioenergetics and Metabolic Disease - Mice," (AUP #Q237a) was reviewed by this institution's Animal Care and Use Committee on 12/3/09. The following action was taken by the Committee:

"Approved as submitted"

This AUP approves total animal numbers, strains of animals, and procedures that can be done in various combinations. **As each specific experiment is designed, please submit an amendment to the IACUC specifying number of animals per experimental group, strain(s), and procedures to be done.**

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

East Carolina University is a constituent institution of the University of North Carolina. An equal opportunity university.

East Carolina University.

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

December 4, 2009

Darrell Neufer, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Neufer:

Your Animal Use Protocol entitled, "Mitochondrial Bioenergetics and Metabolic Disease - Rats," (AUP #Q238a) was reviewed by this institution's Animal Care and Use Committee on December 3, 2009. The following action was taken by the Committee:

"Require the following modification to receive approval"

1. Please reconcile rat treadmill familiarization time. Page 7 indicates 5 minutes while page 15 indicates 10 minutes.

This AUP approves total animal numbers, strains of animals, and procedures that can be done in various combinations. **As each specific experiment is designed, please submit an amendment to the IACUC specifying number of animals per experimental group, strain(s), and procedures to be done.**

Please revise the original Animal Use Protocol to address this required modification and return to Ms. Janine Davenport (212 Life Sciences Building).

Please contact me if I can be of further assistance.

Sincerely yours,

Robert G. Carroll, Ph.D.

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

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East Carolina University.

1907-2007 CENTENNIAL

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

December 4, 2009

Darrell Neufer, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Neufer:

Your Animal Use Protocol entitled, "Breeding of Mice for Mitochondrial Bioenergetics and Metabolic Disease Studies," (AUP #Q285) was reviewed by this institution's Animal Care and Use Committee on 12/3/09. The following action was taken by the Committee:

"Approved as submitted"

Note: Please send a registration to the Biological Safety Committee for the breeding of transgenic/outcross animals.

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

East Carolina University is a constituent institution of the University of North Carolina. An equal opportunity university.

APPENDIX D

INSTITUTIONAL REVIEW BOARD

USE & CARE OF HUMAN SUBJECTS APPROVAL LETTERS

University and Medical Center Institutional Review Board
East Carolina University • Brody School of Medicine
600 Moye Boulevard • Old Health Sciences Library, Room 1L-09 • Greenville, NC 27834
Office **252-744-2914** • Fax **252-744-2284** • **www.ecu.edu/irb**
Chair and Director of Biomedical IRB: L. Wiley Nifong, MD
Chair and Director of Behavioral and Social Science IRB: Susan L. McCammon, PhD

TO: P. Darrell Neufer, PhD, Department of EXSS, ECU, Brody 6N-98

FROM: UMCIRB

DATE: December 16, 2009

RE: Full Committee Approval for Continuing Review of a Research Study

TITLE: "Linking Mitochondrial Bioenergetics to Muscle Insulin Sensitivity"

UMCIRB #08-0699

The above referenced research study was initially reviewed by the convened University and Medical Center Institutional Review Board (UMCIRB) on **11/26/08 & 12/10/08.** The research study underwent a subsequent continuing review for approval on **12/9/09** by the convened UMCIRB. The UMCIRB deemed this **NIH/NIDDK** sponsored study **more than minimal risk** requiring a continuing review in **12 months**. Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

Please note that this approval is contingent upon receipt of correspondence from the Identity Theft Protection Committee (ITPC) allowing for collection in use of social security numbers until such time said collection and use is prohibited. The IRB office requests that a note to file be submitted electronically once the ITPC and the investigator/coordinator have resolved this matter.

The above referenced research study has been given approval for the period of **12/9/09** to **12/8/10.** The approval includes the following items:

- Continuing Review Form (dated 11/13/09)
- Protocol (dated 12/10/08)
- Protocol Description (dated 2/21/08)
- Informed consent: Muscle Biopsy and IVGTT (dated 12/14/09)
- Informed consent: Mito Restoration (dated 12/14/09)
- Informed consent: Acute High Fat Study in Leans (dated 12/14/09)
- Informed consent: Acute High Carbohydrate Study in Leans (dated 12/14/09)
- Informed consent: Chronic High Fat Study in Leans (dated 12/14/09)
- Informed consent: Exercise in Obese (dated 12/14/09)
- Informed consent: High Fat Diet and Exercise in Leans (dated 12/14/09)
- International Physical Activity Questionnaire (dated 8/02)
- 3-Day Diet Record Guidelines

- STRRIDE 3-Day Diet Record
- Personal History Form
- Flyer

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research study:
R. Hickner

NOTE: The following UMCIRB members with a potential Conflict of Interest did not attend this IRB meeting:
None

The UMCIRB applies 45 CFR 46, Subparts A-D, to all research reviewed by the UMCIRB regardless of the funding source. 21 CFR 50 and 21 CFR 56 are applied to all research studies under the Food and Drug Administration regulation. The UMCIRB follows applicable International Conference on Harmonisation Good Clinical Practice guidelines.

APPENDIX E

INSTITUTIONAL REVIEW BOARD

APPROVED CONSENT FORMS FOR RESEARCH INVOLVING HUMAN SUBJECTS

Informed Consent

Title of Project: Impaired Acyl-CoA Synthetase-Muscle Lipid Oxidation in African-American Women (UMCIRB # 07-0135)
Subtitle: Effects of 8 Weeks of Exercise Training on Adipoctye Lipolytic Rate and Muscle Lipid Metabolism

Principal Investigator: Ronald N. Cortright, Ph.D.
Co-Investigators: Robert C. Hickner, Ph.D.; Joseph Houmard, Ph.D.; Hisham Barakat, Ph.D.; and James DeVente, M.D., PhD.

Institution: Human Performance Laboratory, East Carolina University
Address: 371 Ward Sports Medicine Building, Greenville, NC

Telephone: (252) Office: 737-4678, lab: 252-744.2934, or home: 756-7735

This consent document may contain words that you do not understand. You should ask the study doctor or the study coordinator to explain any words or information in this consent form that you do not understand.

Introduction: You have been asked to participate in a research study being conducted by **Dr. Ronald N. Cortright, Dr. Robert C. Hickner, Dr. Joseph Houmard, Dr. Hisham Barakat, Dr. James deVente** and fellow researchers at East Carolina University.

The **purposes** of this study are 1) to determine why African-American women have a greater tendency to gain weight and develop diabetes and 2) whether exercise can improve the ability of both African-American and Caucasian women to use fat for fuel.

Obesity has reached epidemic proportions in the United States and is threatening to become a global epidemic. Obesity represents a serious health threat because of the increased risk of developing chronic diseases such as diabetes and cardiovascular disease. According to recent estimates, the prevalence of obesity is greater among African-American than Caucasian women in the United States. African-American women gain weight at an earlier age and remain heavier than Caucasian women at the same age. This racial difference is important because obesity is strongly associated with skeletal muscle insulin resistance (inability of muscle to take in sugar from the blood) and supports the existing data demonstrating that African-American women have twice the incidence of type 2 diabetes compared with Caucasian women. Although environmental factors such as socioeconomic status, diet, and level of activity may influence the greater prevalence of obesity and diabetes, it is becoming increasingly evident that inherent physiological and biochemical differences underlie the increased incidence of these diseases in African-American women. We have demonstrated that obese African-American women have a reduced ability to release fat from sites of storage (the adipocyte) and to use fat by skeletal muscle to make energy when compared to Caucasian women of similar age and weight. This is fundamentally important because the reduced ability to release and "burn" fat can result in its increased accumulation within the fat and muscle cells, the latter which is strongly linked with insulin resistance in obese individuals. Newer information suggests that African-American women who are not obese may be more likely to gain weight when compared to non-obese Caucasian women. For example, our lab has noticed that non-obese African-American women have a greater difficulty using fat to make energy when they are at rest and during exercise.

Despite the negative implications of these findings for health however, the cellular mechanisms to explain this race/ethnic metabolic difference in the propensity toward obesity and diabetes has scarcely been studied in African-American women.
We hope to apply the knowledge gained from this research to better understand why obese women in general, and more so, African-American women, can not use fat as effectively for energy production as non-obese Caucasian women. These studies could lead to more specific (dietary, activity, or pharmacological) treatments for obesity and diabetes, especially for African-American women.

You should understand that you will be one of approximately 48 women (over 2 years) in the research study (ages 18 – 45 years). The study groups are comprised of 24 lean (BMI $\leq$ 26 kg/m^2) African-American (AAW; N=12) and Caucasian Women (CW; N=12) and 24 obese (BMI > 30 kg/m^2) AAW (N=12) and CW (N=12) who are premenopausal, non-diabetic (fasting plasma glucose < 7.0 mmol/l), sedentary (< 20 min of exercise/d, 1 day/week as determined by an activity questionnaire).

The study will include 2 days of assessment and nutritional education and eight weeks of aerobic exercise training. Your <u>first visit</u> will take approximately 90 minutes during which time we will determine your body composition (in terms of fat and lean weight) and your aerobic exercise capacity. On the same day, the researchers will also teach you how to select, measure, and record the foods that you eat for two-three day periods during the study. On another day of your choice and prior to beginning the exercise training, you will be asked to report to the office of The Leo Jenkins Cancer Center for a CT scan to determine your body's sites of trunk fat storage. A CT scan will also be performed at the end of the 8 week training study. The total hourly commitment for your participation in the study, is approximately 40 hours (assessments = ~ 1.5 hours; CT scan = ~ 2 hours; eight weeks/4 days per week for 1 hour each of aerobic exercise; 3 blood draws and 6 biopsies = ~ 4 hours).

On the <u>first, tenth, and last day of the exercise training protocol</u>, you will be asked to report in the morning (after an overnight fast-no food after 10:00 PM the night before) to room 2377 of the East Carolina Heart Institute (ECHI) for the muscle biopsy and blood sampling procedures. The first biopsy will take place on day 1, before you exercise and the second biopsy will take place 4 hours after you exercise.

You will be asked to exercise on a bicycle for <u>eight weeks</u> thereafter (total of 32 hours of exercise training). The training sessions will take place in the exercise facility known as the FITT building, adjacent to Minges Coliseum. Each exercise session will last 60 minutes. A trained exercise physiologist will assist you and will monitor your heart rate, blood pressure, and ability to use fat and carbohydrates for energy production during each exercise session.

Biopsies three and four will take place after 10 days of training and biopsies five and six will take place at the end of eight weeks. You will perform your first, 10th, and last exercise sessions in room 2377 of the East Carolina Heart Institute. Again, you will be asked to report to the ECHI Room 2377 and will be biopsied before and 4 hours after each exercise session, which will take place in the same room.

From the small biopsy samples (~ 75 milligrams each; the size of a pencil eraser) the investigators will determine your muscle's ability to burn fat before and after exercise training. We will also determine which muscle genetic factors change (gene expression) because of exercise and training. This information will help us to improve drug and physical activity strategies for individuals prone to obesity and diabetes.

In order to determine the effects of a single or repeated bouts of exercise on your ability to release fat so that the muscle can burn it, on days 1 & 10, and after 8 weeks, we will insert a small catheter into a vein in your arm and collect blood before, during, and after exercise. The total amount of blood collected each time will be small (about 40 milliliters = ~ 4 tablespoons). The total amount of blood collected across 8 weeks will be approximately 160 milliliters (~ 16 tablespoons).

Details of each procedure are described below.

Plan and Procedures

My participation will involve:

The following are screening procedures and assessment of metabolism, fitness, and body composition:

1. **Preliminary Assessments: First Visit.**

- **Health History and Other Forms (**FITT building). You will be asked to complete a health history questionnaire to help determine if you are suitable for this study (e.g., types of medications used if any). In addition, you will be asked to record the history of your recent menstrual cycle and whether you are currently taking birth control pills.

- **Diet Recording and Study Eating Habits.** Because different diets can affect the body's use of fat and carbohydrates as fuel, you will be asked to record your diet three times during the 8-week study study. Study personnel will explain how to measure the amount and select foods from a list provided to you based on what you normally eat. We will ask that you eat foods from the list and record your portion sizes for three days prior to the first exercise session. This procedure will allow us to determine each subject's amount of calories and diet composition. This way, we can rule out the possible variability in diet on the metabolism of your body. You will then be asked to eat the same or similar meals over days 7-10 and the last three days (end of eight weeks) of the study. These diets conform to nutritional health standards as suggested by the American Diabetic Association standards (60% carbohydrate, 25% fat, and 15% protein).

- **Body Composition** (FITT Building): **DEXA.** Your body composition (relative amounts of fat and lean tissue) will be determined by using an FDA-approved bone density instrument (Prodigy Advanced, GE Lunar Corp., Madison, WI). The procedure is called Dual Energy X-ray Absorptiometry (DEXA). A person trained for the use of the DEXA will perform all testing, and you will need to report to the FITT building for the scan. One benefit of this testing is that it provides the most accurate assessment of body composition available. You will be asked to lie face up, on a padded table for 7 minutes while the scanner arm of the DEXA machine passes over your entire body. The scanner will not enclose or touch you, and you can wear regular clothing (no metal allowed). The results of the DEXA Scan will be confirmed by skinfold determination of body composition. Several sites on your body will be measured for skinfold thickness. This is a painless procedure that involves the use of a caliper that determines the thickness of the skin and fat located under the skin at the site. The information gained from both procedures will allow us to use equations to estimate your percent body fat and percent lean body weight. Subjects will be exempt from post training

DEXA scans (eight week training study only) if pregnancy is detected. The procedure will be repeated at the end of the eight week training period.

- **CT or CAT Scan.** Computed (Axial) Tomography (CT) is a routine method that provides very clear pictures of structures inside the body. The CT scan device uses sophisticated computers and a safe amount of X-rays. It will be used to assess the regional fat content in your trunk area. An appointment will be made for you at The Leo Jenkins Cancer Center. The visit will take approximately one hour. The CT scan device looks like a giant donut. You will be asked to lay down on a table and an instrument will be used to scan your middle body area. The test takes approximately 30 minutes. The procedure will be repeated at the end of the eight week training period. This information will help us to interpret the lipid metabolism data gained from your blood and muscle biopsies. The CT scans are safe procedures for assessing body composition.

  Females with ANY chance of being pregnant should not undergo DEXA or CT scanning. If you become pregnant during the course of this study, you should immediately inform the staff.

- **Maximal Exercise Test. Fitness test (FITT Building - Human Performance Laboratory).** The procedure will determine your maximal ability to use the oxygen (air) you breathe to make energy from the food you eat. It will also allow the study investigators to set your workload for the submaximal exercise test described above. You will perform cycling (cyclists) exercise for ~10 minutes. You will begin cycling/running at light intensity (you will not breathe hard) for 2 minutes. The workload will be increased every 2 min. until you can no longer continue. This will allow us to determine your maximal exercise capacity. The procedure will be repeated at the end of the eight week training period.

2. **Exercise Training.** Exercise training will occur over 8 weeks. You will be asked to come to the FITT building near Minges Coliseum at a time that is convenient for you. At least one of the study personnel will always be present. Your weight will be measured and you will be fitted with a heart rate monitor. Exercise will consist of pedaling a stationary bicycle for 60 minutes at approximately three-fourths of your maximal capacity as determined from your maximal aerobic capacity test taken at your first visit. We would like for you to pedal continuously for the entire 60 minutes, but if that becomes difficult on any day, you may stop exercising and resume as soon as you feel ready. Water will be provided for you throughout the exercise session. Occasionally, you will be fitted with a mouthpiece and nose clips so we can measure your consumption of oxygen and production of carbon dioxide. This will allow us to determine your utilization of fat as fuel for your working muscles. We will also monitor your heart rate and blood pressure each session. After the exercise session is finished, you will be encouraged to pedal at a very light workload to "cool down" and let your heart rate, blood pressure, and breathing return to near resting levels.

3. **The muscle biopsy procedure.** The biopsy procedures will take place at the East Carolina Heart Institute. The procedure will occur under sterile conditions and a physician will be available during the entire time of the procedure. You will report to room 2377 at 7:30 AM following an overnight fast (no food after 10:00 PM the night before). For this procedure, a small amount of anesthesia (3 cc of 1% Lidocaine) will be injected in a ½ inch area under the skin of your thigh. A small (1/4 inch) incision will then be made through the skin, fat, and fibrous layer that lies over the muscle. A biopsy needle (about ½ the width of a pencil) is then inserted through the incision ½ to 1 inch into the muscle. A small piece of muscle (1/2 the size of an erasure at the end of a pencil) is then clipped out with the biopsy needle. The

needle is withdrawn and the muscle sample is prepared for analysis. You will undergo two muscle biopsies on day 0, two muscle biopsies on day 10, and two muscle biopsies after 8 weeks of the study. A separate incision will be made for each of the **six biopsies**. The first two biopsies will be taken from the left and right leg, the second two biopsies will be taken one from each leg and the last two biopsies will be taken, one from each leg. The muscle biopsies will be taken by Robert Hickner, Ph.D., Joseph Houmard, Ph.D., or Ronald N. Cortright, Ph.D. The muscle samples will be assessed for the ability to metabolize fat and to control the metabolism of energy by assessing the levels of certain muscle factors (the expression of mitochondria and certain genes that regulate fat metabolism in skeletal muscle; e.g, PGC-1, PPARs, uncoupling proteins, etc.). In addition, a portion of the muscle biopsy may be used to culture cells to understand the portion of lipid metabolism that is inherited.

4. **Blood samples** will be obtained on the day of each biopsy (days 0, 10, and after 8 weeks) and once midway (4th week) through the training period. Blood will be drawn immediately prior to, twice during exercise, and once after exercise for analysis of fat release from stored sites (adipocytes). Blood will be drawn from a small catheter placed in your arm vein. On each occasion, the total amount of blood will be approximately 40 ml (~ 4 tablespoons) and the total amount of blood obtained for the entire study will be approximately 160 ml (~ 16 tablespoons). By determining blood born fat components known as glycerol and fatty acids, the investigators can determine the extent of lipolysis (fat release) that occurs before, during and after exercise. In addition, we will measure blood insulin and glucose as well as hormones known as catecholamines that are involved in the fat releasing process. Other fat metabolism related hormones such as Leptin, Adiponectin, Ghrelin (hormones released by the fat cells which are associated with the regulation of fat and blood sugar) will also be measured. Determining blood lipids will also help us to determine the relationship between these blood variables and factors indicating your muscle's lipid metabolic capacity and diabetic status. The blood samples will be taken by Dr. Robert Hickner, Dr. Ronald Cortright, Dr. Joseph Houmard, or a trained research nurse either at the FITT building (Human Performance Lab) or the ECHI Room 2377.

Potential Risk and Discomforts

Certain risks and discomforts may be associated with this research. They include:

- The DEXA is a safe procedure for assessing body composition. The scanner will not enclose or touch you, and you can wear regular clothing (no metal allowed). You will be exposed to minimal radiation (DEXA: ~0.4 microSieverts per whole body scan) that is within an acceptable range as provided by "North Carolina Regulations for Protection Against Radiation". (30 miliSieverts) For example, one would receive radiation exposure of approximately 80 microSieverts on a transatlantic airline flight of 8 hours, 50 microSieverts living in Denver, Colorado, at an elevation of 5,000 feet for approximately 4 weeks, or 30 to 40 microSieverts during a typical chest x-ray. However, even this minimal exposure to X-ray radiation my have negative effects on the unborn fetus. Therefore, you will be screened for menstrual cycle status by questionnaire and queried to be sure they are not pregnant prior to commencing the study.
- For each CT scan, the amount of radiation (~75 miliSieverts per abdominal CT scan) that you will be exposed to falls within the national acceptable range for CT scans. A normal CT scan consists of multiple slices yielding a total expose of 500 -650 mGY (500-650 mSV). Therefore, you will be exposed to 1/20th of the typical diagnostic CT scan. However, even this minimal exposure to X-ray radiation my have negative effects on the unborn fetus. Therefore, you will be screened for menstrual cycle status by questionnaire and queried to be sure you are

not pregnant prior to commencing the study. You will be exempt from post training CT scans (eight week training study only) if pregnancy is detected. The CT scan will be performed in a way that will minimize your exposure to radiation.

•The total amount of blood drawn for lipid metabolism measurements (16 tablespoons) is very small compared to the total amount (about a gallon) of blood that you have. There is an extremely small risk of local bruising or infection associated with insertion of intravenous catheters (we draw blood through these) into your arm.

- Risks associated with the exercise protocols are dizziness, ventricular arrhythmia (odd heart

beats), and in very rare instances death. These risks are very small, with an incidence of fewer than 1 in 10,000 deaths in patients who are known to, or suspected of, having heart disease. The risk is expectedly much smaller than this in a group of younger, healthy subjects. To minimize this risk, we will have a physician present or on call and a heart (ECG) monitoring device will be used during the exercise tests. The physician will be trained to recognize heart problems during exercise and trained to revive people in the event of serious heart problems during the exercise test. The exercise tests will be stopped if you feel dizzy, are having chest pain, are having serious shortness of breath, or ask that the test be ended. The test will also be stopped if the physician detects (from the ECG) heart function that is not normal. All of the necessary emergency equipment (including crash cart for heart problems) will be in the room. If you experience a cardiovascular event or pass out, then Pitt County Emergency Services will be contacted.

• Dr. James deVente, M.D., PhD. or other attending physicians will be provided medical coverage for the maximal exercise test and the muscle biopsies performed at the East Carolina Heart Institute or the Human Performance Laboratory. With respect to the muscle biopsy procedure, there is a small risk of hematoma (bruise) or infection around the biopsy site. This risk will be minimized by using sterile procedures and applying pressure to the biopsy site for 10 minutes, or until bleeding is stopped if longer than 10 minutes, following biopsy. A steri-strip (thin bandage) will be applied over the incision and will remain in place for at least 4 days to close the incision during healing. A pressure wrap will also be placed around the biopsied limb and will remain for 8 hours following biopsy. There is an extremely remote risk of allergic reaction to the Lidocaine anesthesia. This risk will be minimized by using subjects who have had prior exposure to Lidocaine or Novocaine anesthesia; this precaution should eliminate this risk. Dr. James deVente, M.D., PhD (or other physicians associated with the study) will initiate any medical treatment necessary during or following any adverse event from the biopsy procedure.

Exclusions

To the best of your knowledge, you are not allergic to Novocaine. For example, you have not had an allergic reaction to an injection at the dentist's office. To your knowledge, you do not possess any condition which would result in excessive bleeding. You do not have known kidney disease, and you do not have know heart disease (i.e., had a heart attack). Other exclusion criteria include: individuals who are ill or taking medications. individuals who are known diabetics, individuals who currently smoke, African-Americans that are not of at least second generation African-American decent, individuals who are pregnant and, individuals who are exercise training or who have exercise trained regularly within the last 6 months.

Potential Benefits

1) You will receive information concerning your health risk due to your level of obesity and insulin resistance.

2) You will benefit from gaining knowledge of your body composition and aerobic fitness level.
3) You may receive muscle and cardiovascular-respiratory benefits from exercise training for 8 weeks.
4) You will receive information about your skeletal muscle fiber type.
5) Society and medical science may benefit from gaining the knowledge resulting form this investigation.

Termination of Participation

Your participation in this research study may be terminated without your consent if the investigators believe that these procedures will pose unnecessary risk to you. You may also be terminated from participation if you do not adhere to the study protocol.

Cost and Compensation

The policy of East Carolina University does not provide for compensation or medical treatment for subjects because of physical or other injury resulting from this research activity. However, every effort will be made to make the facilities of the School of Medicine/ECHI available for treatment in the event of such injury.

You will receive $400.00 for your time and efforts for participating in the exercise and muscle biopsy procedures. You will receive, free of charge, the body composition and maximal aerobic capacity analysis.

The remuneration is prorated as follows:

1. $50.00 per muscle biopsy (maximum 6 biopsies total)
2. $100.00 for exercise training for 8 weeks

You do not give up any legal rights as a research participant by signing this consent form.

Confidentiality

Only the investigators associated with this study will have access to the data obtained. The data gathered from the study will be stored on a computer hard drive which will be accessible only by the investigators or technical staff. Numeric coding will protect the identity of the subjects. No identifying information will be released. The information and insights gained from the study may be presented at scientific conferences and/or published. In both instances, you will not be identified by name.

Voluntary Participation

The nature and purpose of the procedures, the known risks involved, and the possibility of complications have been explained to you. No guarantee of assurance has been given by anyone as to the results that may be obtained. You know that being in this study is of your own free will. You know that you can decide not to be in this study after you have already started. You may stop at any time without losing benefits that you would have received before being in the research study.

Persons to Contact with Questions

The investigators will be available to answer any questions concerning this research, now and in the future. You may contact the investigators, **Ronald Cortright Ph.D**. (work: 737-4678/office or home: 756-7735), **Robert Hickner, Ph.D**. (work: 737-4677 or home: 353-5556), or **Joe Houmard, Ph.D.** (work: 737.4688/328-4617). Drs. Cortright, Hickner and Houmard are found in the Human Performance Laboratory, Ward Sports Medicine Building, ECU. Also, if questions arise about your rights as a research subject, you may contact the Chairman of the University and Medical Center Institutional Review Board at phone number 252-744-2914 (days).

Research Participant Authorization To Use And Disclose Information

Federal laws require that researchers and health care providers protect your identifiable health information. Federal laws also require that researchers get your permission to use collected health information for research. The identifiable information we will collect from subjects in this research project will include:

*General Medical History including: Family health history, medications, nutrition, physical activity levels, menstrual history, nutritional history, and body weight history.

* Muscle biopsy information, body composition information, blood levels of insulin, glucose, and other compounds related to muscle and fat cell lipid metabolism.

The members of our research team that will have access to your information will include the Principle Investigator, Co-investigators, as well as technical and nursing personnel involved in this project. Information about you will be used and released in such a way that will protect your identity as much as possible; however, confidentiality cannot be absolutely guaranteed. We will only share your information with those individuals listed above. If we need to share information with other individuals other than those listed, we will request your permission a second time.

You will be given a signed copy of your authorization to release medical information for your records. You can limit the amount and type of information that is shared and you must make this request in writing; however, the researcher is able to use any and all information collected prior to the request not to disclose information. Although you can limit the release of your medical information, withholding some information may cause you to become ineligible for this research project. Because research information continues to be looked at after a study is finished, it is difficult to say when the use of your information will stop. There is currently not an expiration date for the use and disclosure of your information for this study.

If you have questions related to the sharing of information, please call Ronald N. Cortright, Ph.D. at 252-737-4678/office. You may also telephone the University and Medical Center Institutional Review Board at 252-744-2914. In addition, if you have concerns about confidentiality and privacy rights, you may phone the Privacy Officer at Pitt County Memorial Hospital at 252-847-6545 or at East Carolina University at 252-744-2030.

FUTURE TESTING OF BLOOD/MUSCLE SAMPLES

Upon termination of this study, the blood and muscle samples collected for this study will be stored for up to 7 years to research scientific questions specifically related to obesity, the effects of exercise, muscle/fat cell lipid metabolism and insulin resistance/diabetes in African-American and Caucasian women. You will continue to be the owner of the samples and retain the right to have the sample material destroyed at any time during this study by contacting the

study principal investigator Ronald N. Cortright, at 252.737.4678/office. During this study, the samples will be stored with number identifiers only; however, the number identifier will be linked to a specific name and will be kept on file in the possession of the principal investigator. The linked file will be stored password protected on the Principal Investigator's computer with CD backup. No other individuals will have access to these identifying materials unless the principal investigator is required by law to provide such identifying information. Data will not be publicly available and participants will not be identified or linked to the samples in publication. If a commercial product is developed from this research project, you will not profit financially from such a product. Furthermore, there are no plans for the investigators to profit financially from such a product.

CONSENT TO PARTICIPATE

I have read all of the above information, asked questions and have received satisfactory answers in areas I did not understand. I willingly give my consent for participation in this research study. (A copy of this signed and dated consent form will be given to the person signing this form as the participant or as the participant authorized representative).

____

Participant's Name **(PRINT)** **Signature** **Date** **Time**

____

Guardian's Name **(PRINT)** **Signature** **Date** **Time**

WITNESS: I confirm that the contents of this consent document were orally presented, the participant or guardian indicates all questions have been answered to his or her satisfaction, and the participant or guardian has signed the document.

____

Witness's Name **(PRINT)** **Signature** **Date** **Time**

PERSON ADMINISTERING CONSENT: I have conducted the consent process and orally reviewed the contents of the consent document. I believe the participant understands the research.

____

Person Obtaining consent **(PRINT)** **Signature** **Date** **Time**

____

Principal Investigator's **(PRINT)** **Signature** **Date** **Time**

East Carolina University

Consent to Participate in Research that is Greater than Minimal Risk

Information to Consider Before Taking Part in This Research

Title of Research Study: Linking Mitochondrial Bioenergetics to Muscle Insulin Sensitivity: Muscle Biopsy and IVGTT

Principal Investigator: P. Darrell Neufer, Ph.D
Institution/Department or Division: Human Performance Laboratory & Brody School of Medicine
Address:363 Ward Sports Medicine Building, East Carolina University
Telephone #: 252-744-2780

Researchers at East Carolina University (ECU) study diseases, health problems, environmental problems, behavior problems and the human condition. Our goal is to try to find better ways to improve the lives of you and others. To do this, we need the help of people who are willing to take part in research.

The person who is in charge of this research is called the Principal Investigator. The Principal Investigator may have other research staff members who will perform some of the procedures.

The person explaining the research to you may be someone other than the Principal Investigator. The Study Coordinator may be asking you to take part in this study.

You may have questions that this form does not answer. If you do have questions, feel free to ask the person explaining the study, as you go along. You may have questions later and you should ask those questions, as you think of them. There is no time limit for asking about this research.

You do not have to take part in this research. Take your time and think about the information that is provided. If you want, have a friend or family member go over this form with you before you decide. It is up to you. If you choose to be in the study, then you should sign the form when you are comfortable that you understand the information provided below. If you do not want to take part in the study, you should not sign this form. That decision is yours and it is okay to decide not to volunteer.

This form explains why this research is being done, what will happen during the research, and what you will need to do if you decide to volunteer to take part in this research.

Why is this research being done?

The purpose of this research study is to determine how high calorie intake and low levels of daily physical activity may influence how your muscle cells function. In this experiment, you will first be asked to maintain your normal diet and weight for 3 consecutive days. On the morning of the fourth day, you will report to the laboratory after an overnight fast (no exercise on those mornings), have a resting muscle biopsy, and complete a 3 h intravenous glucose tolerance test (IVGTT). Depending on the study, you may be asked if you would like to participate in a follow up study requiring a second muscle biopsy and/or IVGTT.

We are asking you to take part in this research. However, the decision is yours to make. By doing this research, we hope to learn how mitochondria (the engines of the cell) are affected by metabolic balance and, in turn, influence insulin action in human skeletal muscle.

Why am I being invited to take part in this research?

You are being invited to take part in this research because you are a healthy, adult volunteer. If you volunteer to take part in this study, you will be one of about 40 people to do so.

Are there reasons I should not take part in this research?

You will not be able to participate in this study if you are allergic to "cain-type" anesthetics; for example, if you have had an allergic reaction to an injection at the dentists' office. You will also not be able to participate if you possess any condition that would result in excessive bleeding or poor healing, if you have had any type of cardiac event (i.e. heart attack), if your fasting glucose, total cholesterol, LDL cholesterol, or blood pressure exceed standard values, or if you are pregnant (or decline to take a pregnancy test).

What other choices do I have if I do not take part in this research?
You have the choice of not taking part in this research study.

Where is the research going to take place and how long will it last?
The research procedures will be conducted at the Brody School of Medicine. You will need to come to the 3rd floor of the Brody building to room 3S08 one or two times during the study. Each of those visits will take about 4 hrs. The total amount of time you will be asked to volunteer for this study is 4 to 8 hrs over the next 1-2 days

What will I be asked to do?
You are being asked to do the following: You will undergo the procedures listed below during this research project. You can ask the investigators at any time for further clarification on why these measurements are being taken and specifics about the procedures.

Initial Screening. The purpose of this visit is to obtain measurements of your health status and to make sure you qualify for the study. It will require an overnight fast.

- Complete a written *health history questionnaire*.

- Have your *weight*, *height*, and *resting blood pressure* measured.

- Have a *fasting blood sample* obtained from a vein in your arm. Blood lipids such as cholesterol, HDL, your blood sugar, and your insulin concentration will be measured.

You will have your *body composition* determined by a method known as dual x-ray absorptiometry (DEXA). DEXA is non-invasive and works somewhat like an X-ray. You will need to remove metal clothing accessories, jewelry, and your shoes as these can affect the scan results; however, you will otherwise remain fully clothed. You will be asked to lie on your back on the DEXA table. When you are properly positioned a trained technician will initiate the scan. The scanning arm of the machine will pass over your body taking measurements. It is important that you stay as still as possible during the procedure to ensure a clear, useful image. The scan takes about 10 minutes to complete. No anesthesia is required. The procedure is painless and radiation exposure is minimal. There are no restrictions to your normal activity following this procedure. The test will determine the amount of fat and muscle that you have.

Diet. Based on your diet history, you will consume a *weight standardized* diet consisting of ~50-55% carbohydrate and ~30-35% fat for 3 days prior to your first muscle biopsy. You will write down the food you consume during these three days so that the researchers will be able to analyze your diet. On the day before testing you will receive a standard meal which you will consume during the evening between 6:00-7:00 pm. At 7:00 am (12 hr fast) you will report to the laboratory for your first muscle biopsy and an intravenous glucose tolerance test.

Muscle Biopsy. You will have a muscle biopsy obtained from your thigh (vastus lateralis) on the morning a after your 12 hr fast. This biopsy will be obtained approximately 20 minutes after you come into the lab on your day of testing. You will have second muscle biopsy one week later after the first muscle biopsy and after following the same protocol as before. The second biopsy will be performed on the opposite leg. The muscle biopsy procedure consists of initially shaving a two by three inch square on your thigh and cleansing this area with iodine. The iodine may temporarily leave your skin with a yellowish tinge. A substance that becomes cold upon exposure to air (ethyl chloride) will then be sprayed on the biopsy site to numb the skin surface. A local anesthetic, much like that used at the dentists' office (lidocaine) will be injected just beneath the skin in an area the size of a nickel. This injection may feel like a bee sting, but will numb the skin. A small incision of approximately 1/2 inch will then be made in the numbed area. A sterile needle about the diameter of a pencil (1/4 inch) will then be inserted into your thigh muscle. A piece of muscle about half the size of an eraser at the end of a pencil (~100 mg) will be obtained. The time to insert, cut, and remove the muscle sample will be 3 to 5 seconds. You will then have pressure applied to the biopsy site for 15 minutes and a cold pack applied for 5 minutes. The incision will be closed with a steri-strip and a bandaid; a temporary pressure wrap will also be applied to your leg. The needle muscle biopsy technique is a research procedure designed to investigate characteristics in skeletal muscle; it has been performed on over 4000 occasions at ECU.

Intravenous Glucose Tolerance Test (IVGTT). This test measures your body's ability to clear sugar from your blood, a measure of insulin sensitivity. This test will be performed in the morning after an overnight fast at the ECU Diabetes/Obesity Center. The IVGTT involves having catheters (small flexible plastic tubes) placed into a vein in each arm. You will then have glucose (0.3 grams/kg body weight) injected into the catheter of one arm; blood samples (3 cc or about 1 teaspoon each) will then be taken every 1 to 2 minutes for 20 minutes. After the initial 20 minutes, a small amount of insulin (0.025 u/kg body weight) will be injected into the catheter. Blood samples (1 tsp) will be obtained at *minutes 2, 3, 4, 5, 6 , 8, 10, 12, 14, 16, 19, 22, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160*, and *180.* A small amount of sterile salt water (saline) will be injected into the catheter after each blood sample is obtained so that blood does not clot in the catheter and clog it. The total amount of blood taken during this 3 hour test is about 100 cc or about 4 ounces. This is about 25% of what is taken during a Red Cross blood donation. The minimal model is a common research procedure for measuring insulin sensitivity and has been performed over 1000 times at ECU.

What possible harms or discomforts might I experience if I take part in the research?

DEXA - The duel energy x-ray absorptiometry (DEXA) procedure is painless and radiation exposure to me is minimal (about 100 times less radiation than you would receive in an airplane flying across the Unites States or if you had an X-ray of your chest). You will simply lie still on the DEXA table while the scanning arm of the machine passes over me. There are no restrictions to your normal activity following this procedure. The effects of DEXA upon an unborn fetus is not known; if you are a women of child-bearing age, you can request a pregnancy test at the lab before you undergo the procedure or perform such a test yourself.

Blood Sampling - You may feel some pain during the insertion of the needle into your arm. Possible risks associated with blood sampling are nausea, bruising, and a small chance of infection. To minimize

risk, the procedure will be performed with sterile technique by qualified personnel in the Diabetes and Obesity Research Center, East Carolina University School of Medicine.

Muscle Biopsy Procedure - Possible risks are a slight chance of fainting, a small risk of infection, the possibility of injuring a blood vessel or nerve, and muscle soreness with bruising. You may have an allergic response to the injected anesthetic used in the current study if you do possess an allergy. You will also feel a stinging sensation during the injection of the anesthetic and may feel a sensation of pressure when the biopsy needle is inserted into the muscle. No stitches are required to heal the incision, but you will be left with a small scar at each site.

Intravenous Glucose Tolerance Test - You may feel some pain during the insertion of the plastic catheter into your arm. Possible risks associated with the test are nausea, bruising, and a small chance of infection. To minimize risk, the procedure will be performed using sterile glucose and insulin solutions and will be performed using sterile techniques by qualified personnel in the Diabetes and Obesity Research Center, East Carolina University School of Medicine.

Are there any reasons you might take me out of the research?

During the study, information about this research may become available that would be important to you. This includes information that, once learned, might cause you to change your mind about wanting to be in the study. We will tell you as soon as we can. This might include information about the side effects that are caused by taking part in this study. If that happens, we can tell you about these new side effects and let you decide whether you want to continue to take part in the research.

What are the possible benefits I may experience from taking part in this research?

We do not know if you will get any benefits by taking part in this study. There may be no personal benefit from your participation but the information gained by doing this research may help others in the future.

Will I be paid for taking part in this research?

We will pay you for the time you volunteer while being in this study. As a result of your time commitment, travel expenses, and physical inconveniences, you will be reimbursed at the most $400, with a check from East Carolina University, which will be mailed to you 1 to 2 months after completion of the study. If you elect to withdraw before this study is completed, you will be compensated $100 for each muscle biopsy and $100 for completing the intravenous glucose tolerance test. These procedures will involve no costs to you. The social security number and address of those participants will be collected who receive $600.00 or more per year for participating in this research study and their names will also be reported to the Internal Revenue Service (IRS).

What will it cost me to take part in this research?

It will not cost you any money to be part of the research. The sponsor of this research will pay the costs of: all procedures and analyses involved with this study.

Who will know that I took part in this research and learn personal information about me?

To do this research, ECU and the people and organizations listed below may know that you took part in this research and may see information about you that is normally kept private. With your permission, these people may use your private information to do this research:

- The research team, including the Principal Investigator, study coordinator, research nurses, and all other research staff.
- The sponsors of this study
- Any agency of the federal, state, or local government that regulates this research. This includes the Department of Health and Human Services (DHHS), the Food and Drug Administration (FDA), the North Carolina Department of Health, and the Office for Human Research Protections
- All of the research sites' staff. This includes the research and medical staff at each site.
- The ECU University & Medical Center Institutional Review Board (UMCIRB) and the staff who have responsibility for overseeing your welfare during this research, and other ECU office staff who oversee this research.
- People designated by PCMH and University Health System;
- Additionally, the following people and/or organizations may be given access to your personal health information and they are:None

How will you keep the information you collect about me secure and how long will you keep it?

The investigators will review all the data that is collected and all information about you will be maintained electronically, encrypted and password protected, and kept in strict confidence. Your name will be assigned a randomly generated code that will be used in all documents related to the research. The subject key document will also be encrypted and kept under the direct control of the Principle Investigator. You will not be identified by name or any other distinguishable way in any part of this research if it is published by the doctors that are doing this study.

What if I decide I do not want to continue in this research?

Participating in this study is voluntary. If you decide not to be in this research after it has already started, you may stop at any time. You will not be penalized or criticized for stopping. You will not lose any benefits that you should normally receive.

What if I get sick or hurt while I am in this research?

If you need emergency care:

If you need emergency care: Call 911 or Moahad Dar, M.D. at phone numbers 744-2873 (days) or 413-4456 (pager) (nights and weekends) for help. It is important that you tell the doctors, the hospital or emergency room staff that you are taking part in a research study and the name of the Principal Investigator. If possible, take a copy of this consent form with you when you go.

Call the principal investigator as soon as you can. He/she needs to know that you are hurt or ill. Contact Darrell Neufer at 744-2780 (days) or 203-641-0589 (nights and weekends) or Moahad Dar, M.D. at phone numbers 744-2873 (days) or 413-4456 (pager) (nights and weekends).

If you do NOT need emergency care, but have been hurt or get sick:
Contact Darrell Neufer at 744-2780 (days) or 203-641-0589 (nights and weekends) or Moahad Dar, M.D. at phone numbers 744-2873 (days) or 413-4456 (pager) (nights and weekends).
Call the principal investigator as soon as you can. As necessary, go to your regular doctor. It is important that you tell your regular doctor that you are participating in a research study. If possible, take a copy of this consent form with you when you go.

The ECU Medical Clinics may be able to give you the kind of help you need. However, you may need to get help from a different type of medical facility and your Principal Investigator will know best what you should do.

If you are harmed while taking part in this study:
If you believe you have been hurt or if you get sick because of something that is done during the study, you should call Contact Darrell Neufer at 744-2780 (days) or 203-641-0589 (nights and weekends) or Moahad Dar, M.D. at phone numbers 744-2873 (days) or 413-4456 (pager) (nights and weekends) immediately. There are procedures in place to help attend to your injuries or provide care for you. Costs associated with this care will be billed in the ordinary manner, to you or your insurance company. However, some insurance companies will not pay bills that are related to research costs. You should check with your insurance about this. Medical costs that result from research-related harm may also not qualify for payments through Medicare, or Medicaid. You should talk to the Principal Investigator about this, if you have concerns.

Who should I contact if I have questions?

The people conducting this study will be available to answer any questions concerning this research, now or in the future. You may contact the Principal Investigator, Darrell Neufer at 744-2780 (days) or 203-641- (nights and weekends).
If you have questions about your rights as someone taking part in research, you may call the ECU Institutional Review Board Office at phone number 252-744-2914 (days). If you would like to report a complaint or concern about this research study, you may call the Director of UMCIRB Office, at 252-744-1971

Is there anything else I should know?
No

I have decided I want to take part in this research. What should I do now?
The person obtaining informed consent will ask you to read the following and if you agree, you should sign this form:

- I have read (or had read to me) all of the above information.
- I have had an opportunity to ask questions about things in this research I did not understand and have received satisfactory answers.
- I understand that I can stop taking part in this study at any time.
- By signing this informed consent form, I am not giving up any of my rights.
- I have been given a copy of this consent document, and it is mine to keep.

____

Participant's Name (PRINT) **Signature** **Date**

Person Obtaining Informed Consent: I have conducted the initial informed consent process. I have orally reviewed the contents of the consent document with the person who has signed above, and answered all of the person's questions about the research.

____

Person Obtaining Consent (PRINT) Signature Date

____

Principal Investigator (PRINT) Signature Date
(If other than person obtaining informed consent)