Biological Actions of the Nutraceutical L-citrulline in Experimental Obesity

by

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Abstract

L-citrulline is an organic α-amino acid supplement that has been shown to produce a number of salutary actions on whole-body physiology, which includes reducing muscle wasting and augmenting exercise/muscle performance. The latter has been suggested to arise from elevations in mitochondrial function. Because enhancing mitochondrial function has been proposed as a novel strategy to mitigate insulin resistance, our goal was to determine whether supplementation with L-citrulline also could improve glycemia in an experimental mouse model of obesity. We hypothesized that L-citrulline treatment would improve glycemia in obese mice, and this would be associated with elevations in skeletal muscle mitochondrial function. 10-week old C57BL/6J mice were fed either a low-fat (10% kcal from lard) or high-fat (60% kcal from lard) diet, while receiving drinking water supplemented with either vehicle or L-citrulline (100 mg/kg) for 15 weeks. Glucose homeostasis was assessed via glucose/insulin tolerance testing, while in vivo metabolism was assessed via indirect calorimetry, and forced exercise treadmill testing was utilized to assess endurance. As expected, obese mice supplemented with L-citrulline exhibited an increase in exercise capacity, which was associated with an improvement in glucose tolerance. Consistent with augmented mitochondrial biogenesis/function, we observed an increase in whole body oxygen consumption rates in obese mice supplemented with L-citrulline. Suprisingly, L-citrulline supplementation revealed a trend to worse insulin tolerance, and a trend to reduction in insulin signaling in obese mice. Taken together, although L-citrulline supplementation improves both glucose tolerance and exercise capacity in obese mice, one should exercise caution with its broad use as a nutraceutical due to potential deteriorations in insulin sensitivity.

Preface

This thesis document is an original work by Amina Eshreif under the supervision of Dr. John Ussher. The majority of the work was conducted in the Ussher lab (2-055 Katz) in the Faculty of Pharmacy and Pharmaceutical Sciences. Some of the experiments were also conducted at various core facilities present at the University of Alberta.

Dedications

To my mam, Loutfyiah, who devoted her life to see us the better and taught me to love the education and sciences

To my dad, Miloud, who never knew about me and my work who passed away, when I was three months old

To my beloved husband, Shamsy, and our brilliant and beautiful kids, Marya and Muhammad, without whom I would be nothing

To my brothers and sisters who always trust so much in my abilities to be better

To all my family who is the symbol of love and giving, my friends who encourage and support me, and all the people in my life who touch my heart

My apology to, my Mam and Dad, my Brothers and Sisters, because I could not complete their dream and my dream to obtain a PhD degree, as I did not know if I can do it after I lost this chance, but I will try to keep my dream alive!

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List of Abbreviations and Symbols

°C	Celsius
^{31}P MRS	Phosphorous 31magnetic resonance spectroscopy
AAA	Aromatic amino acids
ADP	Adenosine diphosphate
Akt/PKB	Protein kinase B
ALA	Alpha-linolenic acid
APS	Ammonium persulfate
ARG	Arginine
ARGase	Arginase
ASC	Alanine-serine-cysteine system
ASL	Arginosuccinate lyase
ASS	Arginosuccinate synthase
ATP	Adenosine triphosphate
ATPase	ATP synthase
AUC	Area under plasma concentration
BCAA	Branch chain amino acids
bid	Twoic dialy
BMI	Body weight index
BSA	Bovine serum albumin
Ca^{+2}	Calcium ion
cDNA	Complementary DNA
CIT	Citrulline
Cmax	Maximum plasma concentration
Cmin	Minimal plasma concentration
COPD	Chronic obstructive pulmonary disease
CoQ	Ubiquinone
CV	Cardiovascular
CV	Caloric value
Cyt C	Cytochrome C
DHA	Docosahexaenoic acid
DM	Diabetes mellitus
DMEM	Dulbecco’s modified eagle’s medium
Drp 1	Dynamin related protein 1

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentanoic acid
ERRα	Estrogen related receptor
$FinCO_2$	Carbon dioxide fraction at input
$FinO_2$	Oxygen fraction at input
$FoutCO_2$	Carbon dioxide fraction at output
$FoutO_2$	Oxygen fraction at output
g/kg	Gram per killo gram
g/L	Gram per litter
GLN	Glutamine
GLU	Glutamate
GLUT	Glucose transporter proteins
GSK3β	Glycogen synthase kinase 3 β
GTT	Glucose tolerance test
H^+	proton
H_2O	Water
HCl	Hydrochloric acid
HCO3	bicarbonate
HFD	High fat diet
hFis1	Human mitochondrial fission 1
hr	Hour
HS	Horse serum
IDT	Integrated DNA technology
IP	Intraperitoneal
IR	Immediate release
IRS	Insulin responsive substrates
Kcal	Kilo calorie
Km	Substrate concentration at half the maximum velocity
LFD	Lean fat diet
LPM	Litter per minute
m/min	Meter per minute
MAP Kinase	Mitogen-activated protein kinase
mDNA	Mitochondrial DNA
MELAS	Mitochondrial encephalomyopathy, lactic

	acidosis & stroke syndrome
Mfn1&2	Mitofusin 1 & 2
mg	Milli gram
mg/kg	Milli gram per killo gram
mg/L	Milli gram per litter
min	Minute
min-1	Per minute
mL	Milliliter
mL/kg/hr	Milliliter per kilogram per hour
mM	Milli molar
mmol/g	Milli mole per gram
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
mTORC1	Mammalian target of rapamycin complex 1
N_2	Nitrogen
Na^+	Sodium ion
ng	Nano gram
NH3	Ammonia
nM	Nano molar
nm	Nano meter
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
Nrf1&2	Nuclear respiratory factor 1 & 2
O_2	Oxygen
OAT	Ornithine aminotransferase
OCT	Ornithine carbamyltransferase
OPA 1	Optic atrophy 1
ORN	Ornithine
P5CS	Pyrroline-5-carboxylate synthase
PCR	Polymerase chain reaction
PGC-1 α	Peroxisome proliferator activated receptor gamma coactivator-1 α
Pi	Inorganic phosphate
PI3K	Phosphatidylinositol 3-kinae
PIPD 1&2	PI dependent protein kinase 1&2

PKC	Protein kinase C
pmol µg -1	Picomoles per micro gram
PRC	PGC-1 related coactivator
RCR	Respiratory control ratio
RER	Respiratory exchange ratio
RIP140	Receptor interacting protein 140
ROS	Reactive oxygen species
rpm	Revolutions per minute
rRNA	Ribosomal RNA
S6K	S6 kinase = 70S6 kinase
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
Sec	Second
SEM	Standard error of the mean
SH2	Src-homology-2 domain proteins
SN1	System N transporter 1
SR	Sustained release
T2D	Type 2 diabetes
TBST	Tween tris buffer saline
TEMED	Tetramethylethylenediamine
Tfam	Mitochondrial transcription factor A
Tfb1m	Mitochondrial transcription factor B 1
Tfb2m	Mitochondrial transcription factor B 2
Tmax	Time to reach maximum concentration
tRNA	Transfer RNA
U/Kg	Unit per kilo gram
VC	Vehicle control
VCO_2	Carbon dioxide production
Vin	Input ventilation rate
Vmax	Maximum rate of metabolism
VO_2	Oxygen consumption
VO_2max	Maximal oxygen uptake
vol/vol	Volume per volume
Vout	Output ventilation rate
WHO	World Health Organization

wt/vol	Weight per volume
β	Beta
µg	Micro gram
µL	Micro liter
µM	Micro molar

Chapter 1: Introduction

1. Introduction

1.1 Nutraceuticals

As human health can be highly dependent on diet and food (Kaur, 2016), in the last few decades there has been a number of dietary supplements have been introduced to the drugstores and can be purchased from pharmacies (Esther Bull, 2000). The term nutraceutical has been derived from two words, nutrition and pharmaceutics (Nasri, Baradaran, Shirzad, & Rafieian-Kopaei, 2014). Also there are many different terms that are used such as; medical foods, designer foods, phytochemicals, functional foods and nutritional supplements. This could lead to confusion, as Stephen De Felice, founder and chairman of the Foundation for Innovation in Medicine, an American organization which encourages medical health research used term 'nutraceuticals' in 1989 and defined it as a "food, or parts of a food, that provide medical or health benefits, including the prevention and treatment of disease'' (Esther Bull, 2000). The Bureau of Nutritional Sciences of food directorate of health Canada classified a nutraceutical as a product isolated or purified from food that is generally sold in medical forms not usually associated with food (G. o. Canada, 2002). Nutraceuticals should also demonstrate physiological benefit and improve overall health (Power et al., 2007). Many studies have been done to study the clinical use of the dietary supplements to prevent a number of diseases and improve the health, as well as, to develop them as medicine to treat many diseases (Lockwood, 2011). Hence, these days, the boundaries between pharmaceutical drugs and food products have faded, and nutraceuticals and functional foods patent publications become comparable with other pharmaceuticals (Curran & Leker, 2011).

Besides the nutritional value of nutraceuticals, *in-vitro* and *in-vivo* studies suggest their potential health benefits, and their protective effects against chronic diseases (Gonzalez-Suarez,

Martin, Hoeng, & Peitsch, 2016). As nutraceuticals are considered generally safe at dietary doses, there is no concern about their toxicity (Gonzalez-Suarez et al., 2016). At Guy's hospital, London, there was a five-year study of the side effects of traditional remedies and food supplements (e.g. single and multiple vitamin and mineral preparations and amino acid preparations) between 1991 and 1995 in the medical toxicology unit. This study concluded that these products have a low risk and are considered to be safe (Shaw, Leon, Kolev, & Murray, 1997). However, consumption of supra-dietary doses of nutraceuticals could cause certain side effects including allergic reactions, insomnia, cardiac arrhythmias, interactions with other nutraceuticals and therapeutic drugs, excessive blood thinning, genomic changes in target tissues and other serious conditions (Singh, 2016). In addition, some nutraceuticals exert drug-like actions which may lead to interaction with other medications (Singh, 2016). Because nutraceuticals are a form of self-medication, all these situations may occur. Therefore, nutraceuticals should be tightly regulated like prescription drugs and their safety should be ensured like over the counter medicines (Singh, 2016).

1.2 Commonly Used Nutraceuticals

Nutraceuticals are often used by individuals as a dietary measure to combat many health problems, including allergy, Alzheimer's, cardiovascular disease, cancer, diabetes, eye disorders, immune and inflammatory conditions, or Parkinson's disease (Singh, 2016). Common examples of nutraceuticals include:

1.2.1. L-carnitine

L-carnitine is known chemically, 3-carboxy-2-hydroxy-N,N,N-trimethyl-1-propanaminium (Figure 1.1) (Eskin, 2006). It is a small, water soluble compound (Eskin, 2006). Although L-carnitine is an amino acid synthesized endogenously, our bodies may also acquire it

from the diet (Eskin, 2006). The majority of L-carnitine on the market is chemically synthesized (Eskin, 2006). L-carnitine plays crucial role in transporting long chain fatty acid into mitochondria to produce the energy (Power et al., 2007). Hence, it is used to treat a number of critical diseases, including heart disease, hemodialysis, and Alzheimer's disease (Cederbaum et al., 1984; Breningstall, 1990; Seim et al., 2001; Eskin, M., et al. 2005). Atherosclerotic rats treated with L-carnitine can be protected not only by significantly reducing lipid-peroxidation levels in their hearts, but also restoring the levels of enzymatic oxidants, superoxide dismutase, catalase, glutathione peroxidase, and glucose 6 phosphate dehydrogenase, and antioxidant vitamins C, E, and B6 (Dayanandan, Kumar, & Panneerselvam, 2001). Administration of L-carnitine with doxorubicin has a protective effect by improving cardiac- energy metabolism and reducing lipid peroxidation production (Luo, Reichetzer, Trines, Benson, & Lehotay, 1999). Male infertility is another application of L-carnitine, as a research demonstrates that L-carnitine therapy was effective in increasing semen quality. The usage of L-carnitine for this application still needs to larger clinical trial and in-vitro studies to be reported (Lenzi et al., 2003).

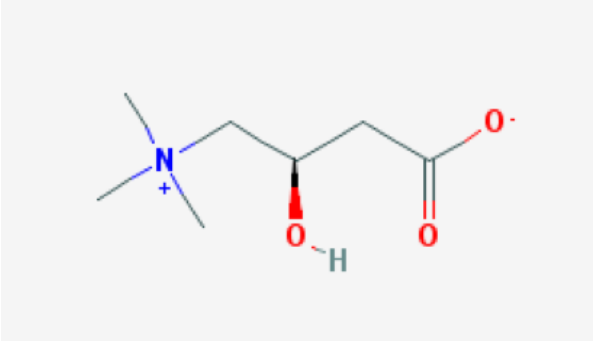

Figure 1.1: L-carnitine Structure

1.2.2. Omega-3 fatty acid Supplements

Omega-3 fatty acids, one of two major classes of long-chain polyunsaturated fatty acids, are the precursors of prostaglandins, thromboxanes, and leukotrienes, chemical messengers that control cell growth and division, blood pressure and clotting, immune reactions, and inflammation (De Caterina & Basta, 2001; Eskin, 2006). There are three main types of omega-3 fatty acids, alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) ("Omega-3 Fatty Acids," 2018). ALA, an essential fatty acid, found mainly in plant oils such as flaxseed, soybean, and canola oils while DHA and EPA are found in fish and other seafood ("Omega-3 Fatty Acids," 2018). Omega-3 fatty acids are important components of the membranes that surround each cell in our bodies, as well as, they provide us with energy (Eritsland J, 1995). Therefore, increasing omega-3 intake is essential for individuals with heart or circulatory problems. As it has been reported a 19 percent reduction in triglyceride levels when fed 4 g of fish (Eritsland J, 1995; Eskin, 2006), this reduction decreases the risk of heart attacks. Epidemiological studies suggest that in societies where diets are high in fish, heart attacks, strokes, and circulatory problems are relatively rare. Moreover, supplements containing EPA (171 mg) and DHA (114 mg) can reduce the amount of nonsteroidal, anti-inflammatory drugs (NSAIDs) needed in patients with rheumatoid arthritis (Lau CS, 1993). There was also a significant reduction in tender-joint counts and morning stiffness compared to the control group after a year of treatment (Eskin, 2006; Kremer et al., 1995). The level of interleukin- 1beta decreased significantly in patients consuming fish oils, suggesting that omega-3 fatty acids may reduce the underlying disease process (Eskin, 2006). Many other areas found the importance of omega-3 fatty acids, including a possible protection for smokers against chronic obstructive pulmonary disease (COPD), a reduction of prostaglandin and leukotriene synthesis, inhibition of migration of proinflammatory neutrophils into the lung, and reduction of the lung's response to

allergens (Britton, 1995; Eskin, 2006). The importance role of omega-3 fatty acids in the synthesis of prostaglandins and other inflammatory mediators has been shown to lessen itching and inflammation in psoriasis. Also, essential fatty acids exhibit anti-inflammatory action in infantile seborrheic dermatisis and diaper dermatitis (Eskin, 2006). More recent research is studying the possible use of omega-3 fatty acids for reducing the relapse in the inflammatory disease of the gastrointestinal tract in Crohn's disease. Besides all the previous benefits, many studies suggest that omega-3 fatty acids exert a protective effect against common cancers, such as breast and colon cancers (Eskin, 2006; Maillard et al., 2002; Rose & Connolly, 1999). Animal studies provided convincing evidence that omega-3 fatty acids inhibited mammary-tumor growth and metastasis. Hardman reviewed the evidence for omega-3 fatty acids as an anticancer agent, suggesting it may augment cancer therapy (Hardman, 2002). A DHA showed significant tumor suppressing activities on the growth of colorectal cancer (Kato et al., 2002).

1.2.3. Garlic

Garlic is a member of the Alliaceae family, and it is also one of the more economically critical cultivated spices (John A. Milner, 2007). The consumption of Fresh garlic is the most; however, it is also found as dehydrated flakes, and salts in a variety of food preparations (John A. Milner, 2007). Also, garlic can be purchased as a dietary supplement in different forms as essential oils, garlic-oil macerate, garlic powder, or garlic extract (John A. Milner, 2007). The carbohydrates provide about 33% of garlic's weight, whereas protein accounts for another 6.4% (John A. Milner, 2007). However, sulfur components have the most important health benefits, besides other constituents, including arginine, selenium, oligosaccharides and flavonoids (Eskin, 2006; Milner, 1996). γ-glutamyl-S-alk(en)yl-L-cysteines and S-alk(en)yl-L-cysteine sulfoxides are the primary sulfur-containing constituents in garlic bulbs (John A. Milner, 2007).

Garlic medicinal properties have been reported for centuries (Eskin, 2006). However, several publications reveal its effect of decreasing risk of heart diseases and cancers in recent years (Wildman, 2007). The ability of garlic and related components to serve as antioxidants, influence immune-competence, and possibly mental function suggests its health implications may be extremely widespread (John A. Milner, 2007). Garlic is pharmacologically active against microbial infection, thrombosis, hypertension, hyperglycemia, hyperlipidemia, and cancer (Eskin, 2006). Two studies suggest the anti-mutagenic effects of garlic extract through suppression chromosomal aberrations in cyclophosphamide-treated (a well-known mutagen) mice (Sengupta, Ghosh, & Das, 2002; Shukla & Taneja, 2002). Garlic extract appeared to be useful for reducing the risk of gastric cancer by suppressing Helicobacter pylori-induced gastritis in Mongolian gerbils, as the development of stomach cancer has associated with the infection induced by this organism (Iimuro et al., 2002). Durak et al demonstrated significant improvements in patients with benign prostate hyperplasia and prostate cancer, as well as a reduction in urinary frequency while increasing in the rate of urine flow after a month of consuming an aqueous garlic extract (1 mL/kg weight) (Durak, Yılmaz, Devrim, Perk, & Kaçmaz, 2003). In addition, research has observed the beneficial effects of garlic extract in reducing risk of cardiovascular disease, because of its cardioprotective effects (Banerjee & Maulik, 2002; Kendler, 1987) and antihypertensive effect (Baluchnejadmojarad, Roghani, Homayounfar, & Hosseini, 2003; Öztürk, Aydin, Koşar, & Can Başer, 1994; Sharifi, Darabi, & Akbarloo, 2003).

1.2.4. Curcumin

Curcumin is one of the active ingredients of turmeric, which belongs to the group called curcuminoids. Diferuloylmethane is chemical name of curcumin (Javeri & Chand, 2016).

Moreover, curcumin have most important derivatives, demethoxycurcumin, and bisdemethoxycurcumin, which also belong to the same group as shown in Figure 1.2 (Javeri & Chand, 2016). Turmeric powder contains around 3.14% curcumin in average (Tayyem, Heath, Al-Delaimy, & Rock, 2006). Turmeric has been valued for its medicinal properties for centuries as it was used in India as a medicine as far back as 5,000 years ago. Curcumin's therapeutic utility revealed against a wide range of human diseases over the past three decades (Javeri & Chand, 2016).

Many studies have demonstrated its potential benefit for many diseases, including liver disease, cancer, Alzheimer's disease, Parkinson's disease, and even AIDS, because of its ascribed properties like antioxidant, anti-inflammatory, antibacterial, and antiviral properties (Javeri & Chand, 2016). For example, due to its property as a detoxifying agent (antioxidant, anti-inflammatory, antibacterial, and antiviral), it can stimulate the immune system and lowers the high level of cholesterol (Javeri & Chand, 2016). Curcumin can also modulate multiple cell-signaling molecules such as pro-inflammatory cytokines (tumor necrosis factor-α, interleukin-1β, interleukin-6), apoptotic proteins, cyclooxygenase-2, endothelin-1, malondialdehyde, C-reactive protein, prostaglandin E2, glutathione S-transferase, prostate-specific antigen, vascular cell adhesion molecule, glutathione, pepsinogen, phosphorylase kinase, transferrin receptor, total cholesterol, transforming growth factor-β, triglyceride, creatinine, antioxidants, aspartate transaminase, and alanine transaminase (Javeri & Chand, 2016). This makes it work with more than one way to cure many diseases in our bodies (Javeri & Chand, 2016).

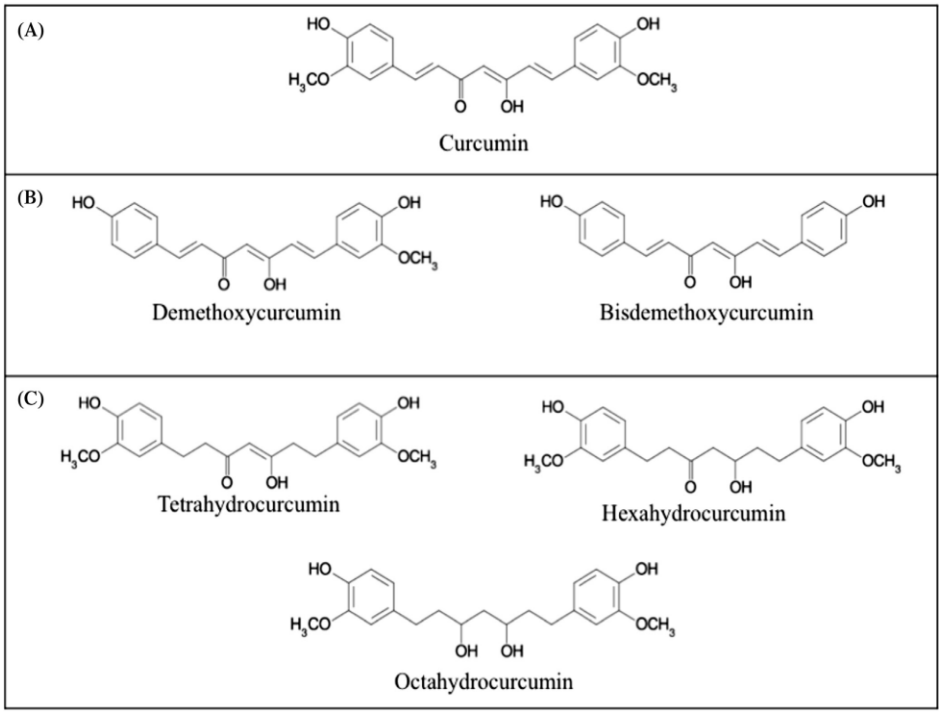

Figure 1.2: Curcumin and its Derivatives Structure (Javeri & Chand, 2016)

1.3. L-citrulline as Nutraceutical

L-citrulline, a nonessential organic α-amino acid, presents in mammals and also in each living organism (S. N. Kaore & Kaore, 2016). It is synthesized mainly in the intestine and kidney from L-ornithine and carbamoyl phosphate in the Urea Cycle via ornithine carbamoyltransferase (S. N. Kaore & Kaore, 2016). Also, L-citrulline is a key byproduct of the nitric oxide synthase (NOS) reaction that produces nitric oxide (NO), but it is also involved in the biosynthesis of nitric oxide. Therefore, it has an important role in stress and diseases (S. N. Kaore & Kaore, 2016). However, citrulline is also found naturally in certain foods like watermelons, garlic, onion, cucumbers, pumpkins, muskmelons, bitter melons, squashes, and gourds (S. N. Kaore & Kaore, 2016). Its name was derived from the Latin word for watermelon *Citrullus vulgaris*, as the first isolation of citrulline was from watermelon (J. K. Collins et al., 2007). Because it is restricted in

some diets, it can be purchased and taken as dietary supplement tablets and used in many health and disease conditions (S. N. Kaore & Kaore, 2016).

1.3.1. Chemistry of L-citrulline

L-citrulline is an α-amino acid and is known chemically as (2-amino-5-ureidovaleric acid) (S. N. Kaore & Kaore, 2016). L-citrulline has a molecular formula of C6H13N3O3 and molecular weight of 175.19 g/mol (S. N. Kaore & Kaore, 2016). Figure 1.3 shows its structure.

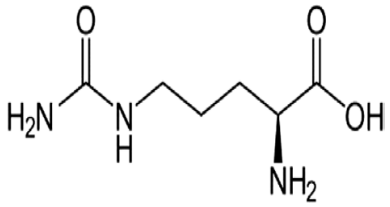

Figure 1.3: L-citrulline Structure (S. N. Kaore & Kaore, 2016)

1.3.2. Physicochemical Properties of L-citrulline

L-citrulline exists in white powder form. It is soluble in water and used in the human body in the L-form (Johnson, 2017). For storage, L-citrulline is stable under ordinary storage conditions as solid or liquid oral dosage forms (Johnson, 2017).

1.3.3. Pharmacokinetics of L-citrulline

Several research studies of pharmacokinetics of L-cituline have compared it with L-arginine, as it is an endogenous precursor of L-arginine (Romero, Platt, Caldwell, & Caldwell, 2006). Around 80% of citrulline is synthesized in enterocytes and is metabolized to arginine in the kidneys (Oliverius M, 2010). Bailly-Botuha et al. revealed clinical relevance of decreased citulline levels with enterocyte functional mass (Bailly-Botuha et al., 2009). A systematic review confirms the negative correlation between plasma citrulline concentrations and disease severity in intestinal enteropathies (Fragkos & Forbes, 2018). It has been reported that plasma flux of L-

citrulline is not affected by fasting or L-arginine free diets, meanwhile L-arginine levels are substantially reduced. Hence, regardless of the dietary state, the effect of L-citrulline supplementation is supposed to be the same (Romero et al., 2006).

L-citrulline supplementation shows a dose-dependent increase in area under the curve (AUC) and maximal plasma concentration (Cmax) of both citrulline and arginine as well as the minimal plasma concentration (Cmin) of arginine and improved the arginine-to-ADMA (asymmetric dimethylarginine) ratio were significantly increased only with the highest dose of L-citrulline (Table 1.1 and 1.2) (Schwedhelm et al., 2008). The half-life of L-citrulline is ~ 60 min (Johnson, 2017). Another study conducted with Berthe et al. compared the sustained release (SR) citrulline formulation with immediate release (IR) citrulline. This study appeared the benefits of using SR citrulline formulation than IR citrulline for the treatment of atherosclerosis (Berthe et al., 2011). In addition, studies in human also showed that oral administration of L-citrulline increases plasma L-citrulline and L-arginine levels more than L-arginine itself (Romero et al., 2006).

Table 1.1: Kinetic parameters of citrulline in human plasma after 1 week of oral supplementation with either citrulline or arginine‡¶ adapted from (Schwedhelm et al., 2008)

Compound	Dose (mg)	*C*max (µmol l−1)	*T*max (h)	*C*min (µmol l−1)	AUC (µmol h l−1)
Citrulline	750 bid	163 ± 14	0.7 ± 0.1	9 ± 2	288 ± 35
Citrulline	1500 bid	350 ± 38*	0.8 ± 0.1	6 ± 1	566 ± 47*
Citrulline	3000 bid	864 ± 45*†	0.7 ± 0.1	9 ± 2	1486 ± 78*†

*$P < 0.01$ vs. citrulline 750 bid.
†$P < 0.01$ *vs*. citrulline 1500 bid.
‡Kinetic parameters are calculated for baseline–placebo corrected data. Data are given as mean ± SEM.
¶Kinetic parameters of citrulline in human plasma after arginine supplementation were not available (no increase of citrulline in human plasma over baseline). *C*max, Maximal plasma concentration; *T*max, time of reach *C*max; *C*min, minimal plasma concentration; bid, twice daily.

Table 1.2: Kinetic parameters of arginine in human plasma after 1 week of oral supplementation with either citrulline or arginine‡ adapted from (Schwedhelm et al., 2008)

Compound	Dose (mg)	*C*max (µmol l−1)	*T*max (h)	*C*min (µmol l−1)	AUC (µmol h l−1)
Citrulline	750 bid	54 ± 5	2.3 ± 0.7	19 ± 4	271 ± 38
Citrulline	1500 bid	79 ± 8*	1.6 ± 0.3	21 ± 4	421 ± 65*
Citrulline	3000 bid	149 ± 42*†	1.4 ± 0.1	45 ± 5*†	898 ± 67*†
Arginine SR	1600 bid	49 ± 6	3.7 ± 1.3§	19 ± 4	289 ± 50
Arginine lR	1000 tid	84 ± 9	0.7 ± 0.1	10 ± 3	283 ± 51

*$P < 0.01$ vs. arginine sustained-release (SR).
†$P < 0.01$ vs. arginine immediate-release (IR).
§$P = 0.03$ *vs*. arginine IR.
‡Kinetic parameters are calculated for baseline-placebo corrected data. Data are given as mean ± SEM. bid, twice daily.

1.4. Metabolism of L-citrulline

L-citrulline is produced mainly from dietary glutamine in the fed state and plasma glutamine in the fasting state in the small intestinal mucosa (Boelens, Leeuwen, Dejong, & Deutz, 2005; Curis et al., 2005; Wu et al., 2009). It is also synthesized in the liver (Curis et al., 2005). However, the small intestine contributes significantly to circulating L-citrulline levels whereas L-citrulline produced in the liver is considered as an intermediate of the urea cycle (Curis et al., 2005). Carbamoyl phosphate, another precursor involved in intestinal L-citrulline synthesis, is regulated by two enzymes; N-acetylglutamate synthase and carbamoyl-phosphate synthase I (Romero et al., 2006). As illustrated in Figure 1.4, the process starts from the conversion of glutamine to glutamate, then by the catalysis of pyrroline-5-carboxylate synthase (P5CS) and ornithine aminotransferase (OAT) subsequently to ornithine. L-citrulline synthesized from ornithine and carbamoyl phosphate via ornithine carbamoyltransferase (OCT) (Romero et al., 2006). L-citrulline production in the small intestine gradually increased while intestinal L-arginine decreased with the growth, because of the reduction of expression of the two cytosolic

enzymes responsible for the conversion of citrulline into arginine, argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) (S. Osowska, Moinard, Neveux, Loï, & Cynober, 2004). However, the conversion of citrulline to arginine via ASS and ASL gradually increased in the kidney as the compensatory mechanism (S. Osowska et al., 2004). As long as L-citrulline is synthesized in enterocytes, it goes to the portal circulation then to the liver (Curis et al., 2005). Without major metabolism, it is then exported to the circulation and is taken up and metabolized to arginine by the kidney in the proximal renal tubules (Curis et al., 2005). As reported by many studies, L-citrulline serves as substrate for the de novo synthesis of L-arginine in mammals, as the majority of the L-citrulline produced by enterocytes reaches the systemic circulation as L-arginine (Brosnan & Brosnan, 2004; Curis et al., 2005; Romero et al., 2006). Another source of L-citrulline can arise during the synthesis of NO from arginine via NOS reaction as illustrated in Figure 1.4. The L-citrulline formed in the NOS reaction goes back to arginine via ASS and ASL (Bahri et al., 2013). As many tissues do not possess citrulline transporters, they can synthesize L-citrulline and recycle it to arginine, but they do not take it up from the circulation (Brosnan & Brosnan, 2004).

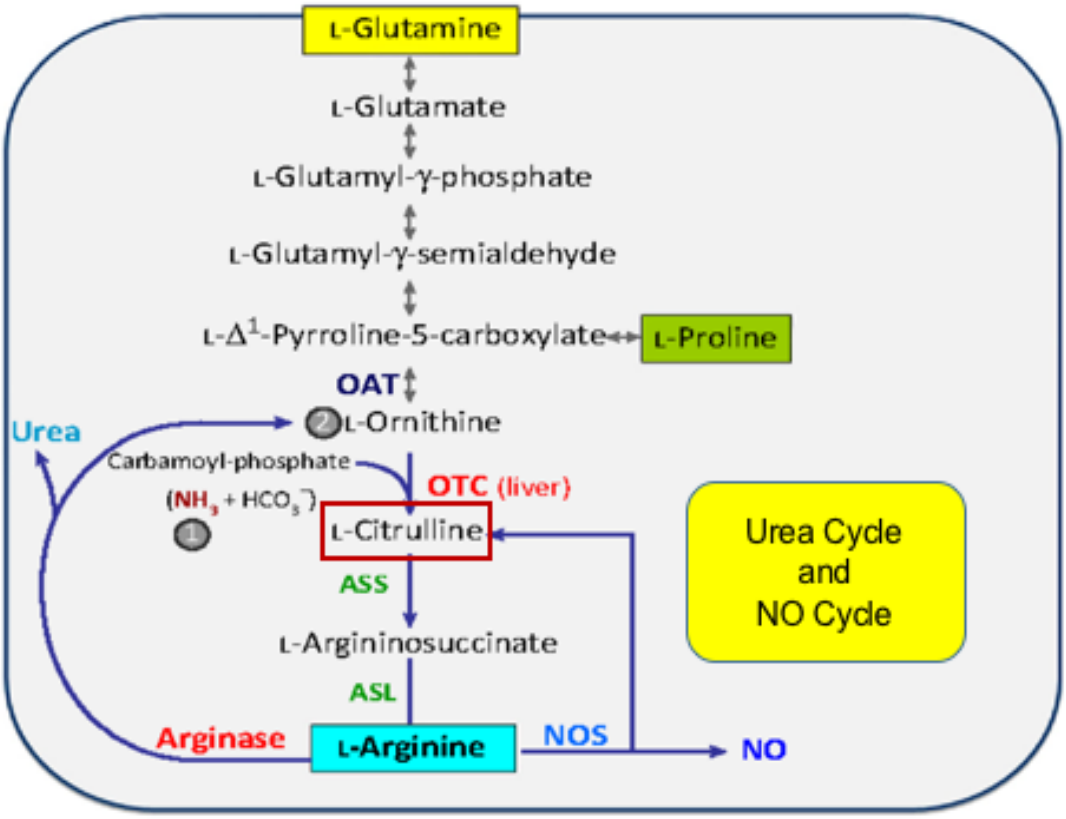

Figure 1.4: L-citrulline Metabolism (Caldwell, Toque, Narayanan, & Caldwell, 2015) The pathway for synthesis of L-arginine from L-glutamine, the reversible pathway between L-ornithine and L-glutamine, and the recycling of L-citrulline into L-arginine. ASL, Aminosuccinate lyase; ASS, Aminosuccinate synthase; NOS, Nitric oxide synthase; OAT; Ornithine aminotransferase; ODC, Ornithine decarboxylase; OTC, ornithine transcarbamylase.

1.4.1. Normal Levels of L-citrulline in the Plasma

In healthy individuals, L-citrulline is mainly produced by enterocytes of the small bowel, and reaches the circulation in concentration of 30-40 mol/L (Crenn, Messing, & Cynober, 2008) with some racial variation (less in Chinese) (Papadia, Osowska, Cynober, & Forbes, 2018). However, L-citrulline supplementation increases plasma citrulline levels 20 fold during the first hour and then reaches a plateau (Jourdan et al., 2015).

1.4.2. L-citrulline Transporters

L-citrulline is absorbed at the middle to lower ileum of the small intestine (Romero et al., 2006). Vadgama and Evered described that L-citrulline transports through a Na^+-dependent active transport system for neutral amino acids (Vadgama & Evered, 1992). This study also revealed that L-citrulline can be transported by a system similar to alanine-serine-cysteine system (ASC), with an apparent substrate concentration at half the maximal velocity (*Km*) of 4.10 ± 0.86 mM and a maximal rate of metabolism (*Vmax*) of 18.7 ± 1.66 mmol/g wet weight tissue/30 min for its transport. Although the amount of amino acids transported through the mucosal membrane mainly depends on the concentration of the amino acids themselves, the absorption of L-citrulline at enterocytes is not affected by its intake (Romero et al., 2006). The proximal convoluted tubules are the main site of L-citrulline absorption (Romero et al., 2006). System B° is the main transporter for neutral amino acid with Na^+ ions in the renal membrane (Romero et al., 2006). Because of that L-citrulline is transported by this Na^+-dependent transporter system (Romero et al., 2006). In addition, L-citrulline transported into endothelial cells by the neutral amino acid system N transporter 1 (SN1) to provide the cells with L-arginine, which is in turn converted to NO and L-citrulline (Simon et al., 2003). Also, this study revealed that L-citrulline transport markedly is inhibited by substrates selective for amino-acid transport systems N such as glutamine, histidine, and asparagine. Smooth muscle cells transport L-citrulline slowly, unlike L-arginine, with an apparent *K*m=1.6±0.2 mM and *V*max=5.9±0.6 pmol μg−1 protein min−1 through Na^+-independent system L or partially Na^+-dependent system N. This transport depends on the amount of of circulating competitor amino acids (Wileman, Mann, Pearson, & Baydoun, 2003).

1.4.3. L-citrulline is a Better Source for L-arginine

In the intestinal mucosa, dietary arginine can be catabolized to citrulline and the synthesized citrulline is exported to the kidneys for arginine resynthesis (Figure 1.5). In this case, arginine can be protected to be taken by the liver and metabolized (Bahri et al., 2013). Therefore, the proper supply of L-arginine for the whole body is L-arginine-L-citrulline homeostasis (Romero et al., 2006). As L-citrulline is not metabolized in the liver, about 80% of L-citrulline produced by enterocytes reaches the systemic circulation as L-arginine (Brosnan & Brosnan, 2004). Several studies confirmed that L-citrulline supplementation elevates L-arginine plasma levels more than L-arginine does. Therefore, L-citrulline is recognized to be a potent endogenous precursor of L-arginine (Brosnan & Brosnan, 2004; Curis et al., 2005; Moinard et al., 2007; Romero et al., 2006; Wu et al., 2009). Its superior effect for therapeutic purposes could be attributed to its ability to inhibit arginase activity, unlike L-arginine supplementation, which enhances arginase expression and activity (S. N. Kaore & Kaore, 2016). For instance, Persson et al reveal that the effect of L-citrulline, but not L-arginine, in prevention of glomerular hyper-filtration and proteinuria resulted from diabetes mellitus in rats (Persson, Fasching, Teerlink, Hansell, & Palm, 2014). Moreover, L-citrulline can prevent us from unwanted effects such as excessive ureagenesis caused by arginine (Romero et al., 2006). Another reason of interest with regards to L-citrulline over L-arginine as a method to increase circulating L-arginine, is that L-citrulline is more easily handled and it is tasteless, odourless, and nonhygroscopic, while L-arginine is extremely bitter and highly water absorbent (Suzuki, Morita, Kobayashi, & Kamimura, 2016).

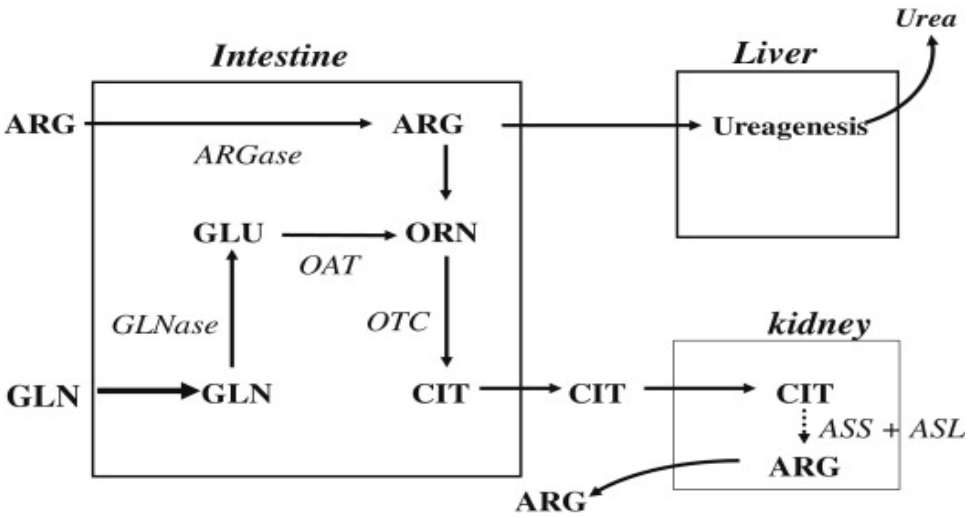

Figure 1.5: L-arginine-L-citrulline Homeostasis (Bahri et al., 2013)
Interorgan metabolism of citrulline and related amino acids. GLNase, Glutaminase; OAT, Ornithine aminotransferase; OCT, Ornithine carbamoytransferase; ARGase, Arginase; ARG, Arginine; CIT, Citrulline; ORN, Ornithine; GLN, Glutamine; GLU, Glutamate; ASS, Argininosuccinate synthetase; ASL, Argininosuccinate lyase.

1.5. Clinical Use and Safety of L-citrulline Supplementation

According to several studies, L-citrulline supplementation is used in therapeutic conditions and situations due to it being a potent precursor of L-arginine, which in turn boosts NO production in most cells (Sureda et al., 2009; Suzuki et al., 2016; Waugh, Daeschner, Files, McConnell, & Strandjord, 2001). As illustrated by many studies, cardiovascular diseases, as well as diabetes, hypertension, atherosclerosis, hyperhomocysteinemia have shown deficiencies in L-arginine supply (Romero et al., 2006).

L-citrulline might mediate vasoprotection (Sureda et al., 2009), which was demonstrated in a phase 2 study of sickle cell disease (Waugh et al., 2001). It has been shown that there was a reduction in the number of leukocytes and neutrophils in circulation (Moinard et al., 2007), and inhibition of cell adhesion and activation of leukocytes, and suppression of endothelial damage (Sureda et al., 2009) with L-citrulline treatment. Sureda A, et al. showed that NO-mediated major

changes in markers of oxidative stress and high concentrations of neutrophil nitrite. However, effects of L-citrulline on immunity not only contributed to NO synthesis, but also could be due to polyamines derived from L-arginine and ornithine, and its anti-oxidant properties (Papadia et al., 2018). Recently, L-citrulline has been used in children with Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes (MELAS) syndrome instead of L-arginine, because it increases NO production, arginine flux, plasma arginine, and citrulline flux more than L-arginine does. In addition, it has been revealed that L-citrulline supplementation significantly decreased plasma lactate compared with L-arginine (Papadia et al., 2018). Also, L-citrulline has important impact on patients with hypertension, as Figueroa et al reveal that L-citrulline increased wave reflection time contributes to the reduction in aortic systolic blood pressure response to cold pressor test (Figueroa, Trivino, Sanchez-Gonzalez, & Vicil, 2010) while watermelon supplementation reduced arterial stiffness and aortic systolic blood pressure in hypertensive obese postmenopausal women (Figueroa, Wong, Hooshmand, & Sanchez-Gonzalez, 2013). L-citrulline reversed the defects in the renal citrulline–arginine pathway or arginine reabsorption, which causing spontaneously hypertensive rats (Shilpa N. Kaore, Amane, & Kaore, 2013). 3 g/day L-citrulline supplementation has been demonstrated to improve left ventricular ejection fraction and right ventricular ejection fraction at rest and stress (Balderas-Munoz K, 2012; Orozco-Gutiérrez JJ, 2010), as well as decreasing systolic pulmonary artery pressure in heart failure patients (Shilpa N. Kaore et al., 2013). El-Kirsh et al indicated the beneficial effects of L-citrulline on hypercholesterolemic rats (El-Kirsh, Abd El-Wahab, & Abd-Ellah Sayed, 2011). According to several studies, watermelon juice mitigated metabolic syndrome in Zucker diabetic fatty rats by reducing fat accretion, lowering blood glucose concentration and free fatty acids, and improving acetylcholine-induced vascular relaxation. All

these effects have a positive impact on patients with type 2 diabetes (T2D) (Wu et al., 2007), as well as preventing or ameliorating cardiovascular complications of diabetes (Hoang, Padgham, & Meininger, 2013; Romero et al., 2008). L-citrulline increased ASL protein expression and prevented lung injury as well as pulmonary hypertension induced by hyperoxia in newborn rats (Ali et al., 2012; Sopi et al., 2012; Vadivel et al., 2010). L-citrulline showed an important role in the prevention of kidney failure, as it improved intrarenal oxygenation and kidney function, prevented renal oxidative stress and severe functional and morphological renal deterioration, protected against rhabdomyolysis-induced acute renal failure and glycerol-induced acute renal failure in rats (Liu et al., 2013). Also, Persson et al. found that L-citrulline administration prevented diabetes mellitus-induced increases in glomerular filtration rate and proteinuria (Persson et al., 2014). Improvement of sperm quality in male fertility administered L-citrulline for one month, without any significant adverse effects (Stanislavov R, 2014). Experimental studies reveal the challenging effect of L-citrulline to reduce the toxicity of anticancer agent which showed an attractive therapeutic effect for ASS-deficient tumours (Agrawal V, 2012 ; Mauldin et al., 2012). Another crucial role regarding L-citrulline is that maintaining protein homeostasis and improving muscle mass related to malnutrition as proved by many studies (Shilpa N. Kaore et al., 2013; Sylwia Osowska et al., 2008). Studies in humans have shown that L-citrulline may act as a performance enhancer, as it reduced muscle fatigue via promoting aerobic energy production in exercising muscle (Bendahan et al., 2002). It has also been shown to increase performance during high-intensity anaerobic exercise (Pérez-Guisado & Jakeman, 2010). Similarly, studies in rats suggest that L-citrulline enhances gastrocnemius muscle performance, as L-citrulline-malate supplementation reduced both the phosphocreatine and oxidative cost of contraction following electrically induced transcutaneous stimulation (Benoît

Giannesini et al., 2011). A recent study conducted by Villareal, et al revealed that L-citrulline supplementation increases exercise performance in mice due to upregulation of PGC-1α expression in skeletal muscle (Villareal, Matsukawa, & Isoda, 2018).

L-citrulline, unlike L-arginine, is considered to be orally safe and well tolerated even in high doses (i.e., >10 g in 1 bolus) (Romero et al., 2006). The bioavailability of L-citrulline is not limited by the intestinal absorption even at high dose, while intestinal absorption of L-arginine is rapidly saturated and caused osmotic diarrhea at high loads (J. K. Collins et al., 2007; Moinard et al., 2007; Romero et al., 2006). Although according to Collins et al, different loading doses of 2 g, 5 g, 10 g, or 15 g produced no adverse effects in healthy subjects (J. K. Collins et al., 2007), L-citrulline shortened the time required for completion of myocardial depolarization and repolarization (decreases QT intervals) in healthy subjects (Kameda et al., 2011). This risk on cardiovascular needs to be clarified by further studies (S. N. Kaore & Kaore, 2016).

1.6. Mitochondria

Mitochondria are energy-producing organelles, in which cellular respiration and energy (ATP) production take place, and they contain their own genome (Diaz & Moraes, 2008). In addition to their role in cell metabolism by energy production, they are considered a major site for production of reactive oxygen species (ROS) and a key player in apoptosis and calcium homeostasis (Sanchis-Gomar F, 2014). Oxidative phosphorylation is a series of reactions that utilize the energy derived from NADH and $FADH_2$ electron carriers to produce more ATP (Szablewski, 2011). A complex of proteins that are embedded in the inner membrane of the mitochondria use the stored energy from NADH and $FADH_2$ mediated electron transfer to pump protons into the membrane space (Szablewski, 2011). An electrical and chemical gradient of

protons results from this process (Szablewski, 2011). The reaction of producing ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi) was driven from the use of the ATP synthase (ATPase) to the proton gradient (Szablewski, 2011). These proteins (called cytochromes) that are embedded in the inner mitochondria membrane, as well as the ATPase are the constituents of the electron transport chain (Szablewski, 2011)(Figure 1.6). Four complexes I, II, III, IV that are essential in the electron transportation. Ubiquinone (within the membrane) carries the electrons between complexes I and III, whereas cytochrome c (in the membrane space) carries the electrons between complexes III and IV (Szablewski, 2011). Hydrogen atoms, released from the entrance of the reduced NAD (as NADH) and reduced FAD (as $FADH_2$) to the chain at complex I and complex II respectively (Szablewski, 2011). Complexes I, III, and IV (except complex II) used this energy to pump protons actively across the membrane into the intermembrane space. In complex IV, final end-product (water) formed from the combining of the electrons with protons and oxygen (Szablewski, 2011). As oxygen acts as the final electron acceptor, aerobic respiration is impossible to take place without it, and only anaerobic respiration can occur (Szablewski, 2011). A chemiosmotic gradient across the membrane is created from the reservoir of proton ions that formed due to the stored energy (Szablewski, 2011). By ATP synthase, the protons move back into the matrix down this chemiosmotic gradient across the membrane. The enzyme ATPase makes ADP and Pi to be bound together to form ATP (Szablewski, 2011). Moreover, the electron transport chain forms three molecules of ATP, and one molecule of H_2O, if a donor of protons and electrons is NADH, while it forms two molecules of ATP and one molecule of H_2O, in the case of $FADH_2$ (Szablewski, 2011).

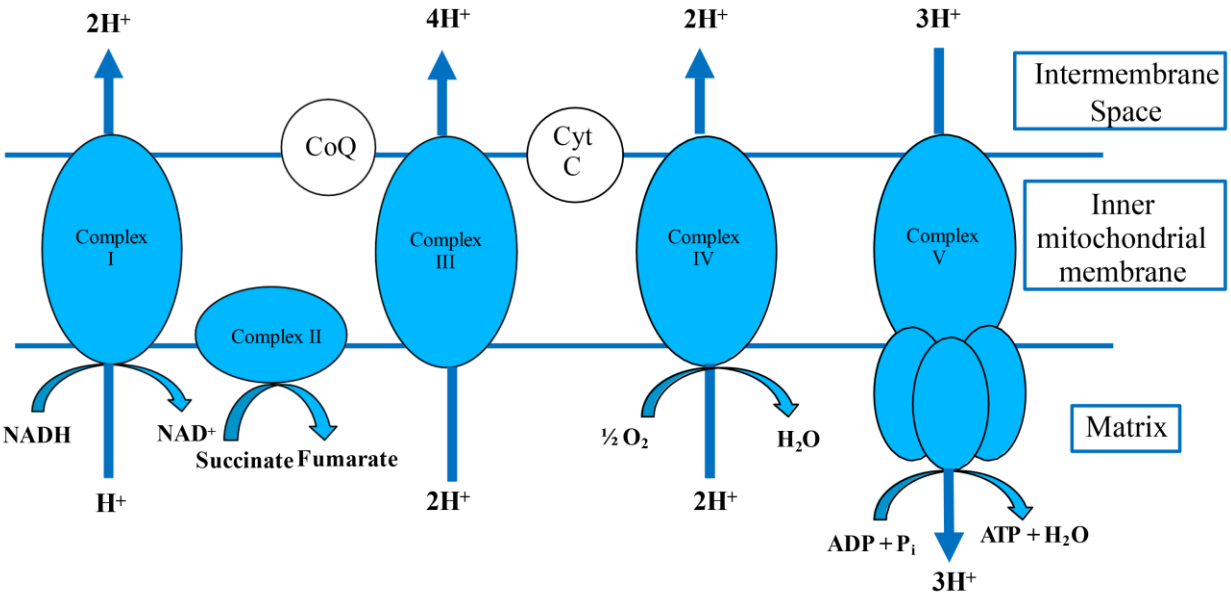

Figure 1.6: Electron Transport Chain

Enzyme complexes of electron transport proteins; the electrons are shuttled between these proteins which is used to pump protons (H^+) to the space between the inner and the outer membrane. This creates a gradient that is used to finally produce ATP.

1.6.1. Mitochondrial Biogenesis

Mitochondrial biogenesis requires the coordination of several mechanisms and the participation of various stimuli, in order to control the number of mitochondria and maintain optimal mitochondrial function (Diaz & Moraes, 2008). These mechanisms include nuclear-mitochondrial communication, mitochondrial protein expression and import, mitochondrial DNA (mtDNA) gene expression, assembly of multi-subunit enzyme complexes, and regulation of mitochondrial fission and fusion. Stimuli for biogenesis include calcium, transcription factors and transcriptional coactivators (Diaz & Moraes, 2008). Any defect in mitochondrial biogenesis can lead to a diseased state. For example, mutations in mtDNA can cause defects in mitochondrial biogenesis and in multiple respiratory chain (Diaz & Moraes, 2008).

1.6.1.1. Mitochondrial Fusion and Fission

Fusion and fission are two processes that are involved in mitochondrial biogenesis (Diaz & Moraes, 2008)(Figure 1.7). They are considered as a normal function of mitochondrial dynamics, to maintain their proper morphologies and cellular functions (Diaz & Moraes, 2008). These events play critical roles during development, cell division and apoptosis (Diaz & Moraes, 2008). Many studies suggest the relation between fusion and fission and the distribution of mitochondrial DNA within the cell, as well as their inheritance into daughter cells (Diaz & Moraes, 2008). One study shows that loss of mitochondrial DNA and respiratory function due to fusion defects, which were reversible in mammalian cells (C. A. Chen H, Chan DC, 2005). Mitofusins (Mfn1 and Mfn2) and optic atrophy 1 (OPA1) have been identified in mammalian species as proteins that are involved in mitochondrial fusion (Hoppins, Lackner, & Nunnari, 2007). Mutations in these two genes responsible for mitochondrial fusion, Mfn2 and OPA1, cause defects in peripheral and retinal ganglia neurons, which eventually lead to two neurodegenerative diseases: Charcot-Marie-Tooth 2A and Autosomal Dominant Optic Atrophy respectively (Alexander et al., 2000; Delettre et al., 2000; Züchner et al., 2004). Another study showed that cerebellum Mfn2 knockout mice leads to neuronal degeneration, respiratory function deficiency, and abnormal mitochondrial distribution (M. J. Chen H, Chan DC, 2007). Regarding mitochondrial fission, dynamin-related protein (Drp1) and human mitochondrial fission 1 (hFis1) are proteins that involved in the fission process (Diaz & Moraes, 2008). It seems that endophilin B, belonging to a family of self-assembling proteins shown to remodel membranes in endocytosis (Karbowski, Jeong, & Youle, 2004) and by sumoylation of Drp1, which protects the protein from degradation (Harder Z, 2004) are the most likely regulators for mitochondrial fission. A study demonstrates that Drp1 overexpression causes fragmentation of the mitochondrial network and protects against

Ca2+-mediated apoptosis (Szabadkai G, 2004). Therefore, Calcium signalling in cells is the most crucial regulator for mitochondrial fusion and fission processes (Diaz & Moraes, 2008).

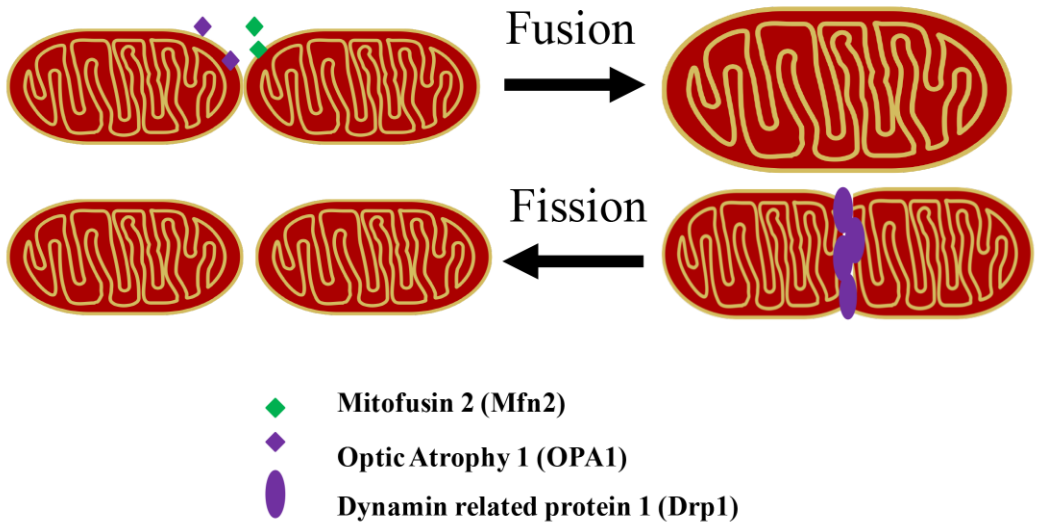

Figure 1.7: Mitochondrial Fusion and Fission; adapted from (Milone & Benarroch, 2012)

1.6.1.2. Transcription Factors and Coactivators that Regulate Mitochondrial DNA Synthesis

Human mitochondrial DNA (mtDNA) is a double-stranded circular molecule of 16,569 bp encoding 2 ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs) and 13 proteins that form part of the multi subunits complexes of the oxidative phosphorylation system (7 subunits of complex I, 1 subunit of complex III, 3 subunits of complex IV and 2 subunits of complex V) (Diaz & Moraes, 2008)as shown in Figure 1.8. mtDNA can separate into two strands, a heavy (H-strand) and a light (L-strand) by alkaline gradient centrifugation experiments due to their differential content of guanosine and cytidine (Anderson et al., 1981). The H-strand encodes for the 2 rRNAs, 12 of the polypeptides and 14 of the tRNAs whereas the L-strand encodes for only

one of the polypeptides (ND6) and 8 tRNAs (Anderson et al., 1981). The maintenance of hundreds to thousands of copies of mtDNA in one cell is crucial for normal function of the respiratory chain that is responsible for aerobic ATP production (Kanki et al., 2004).

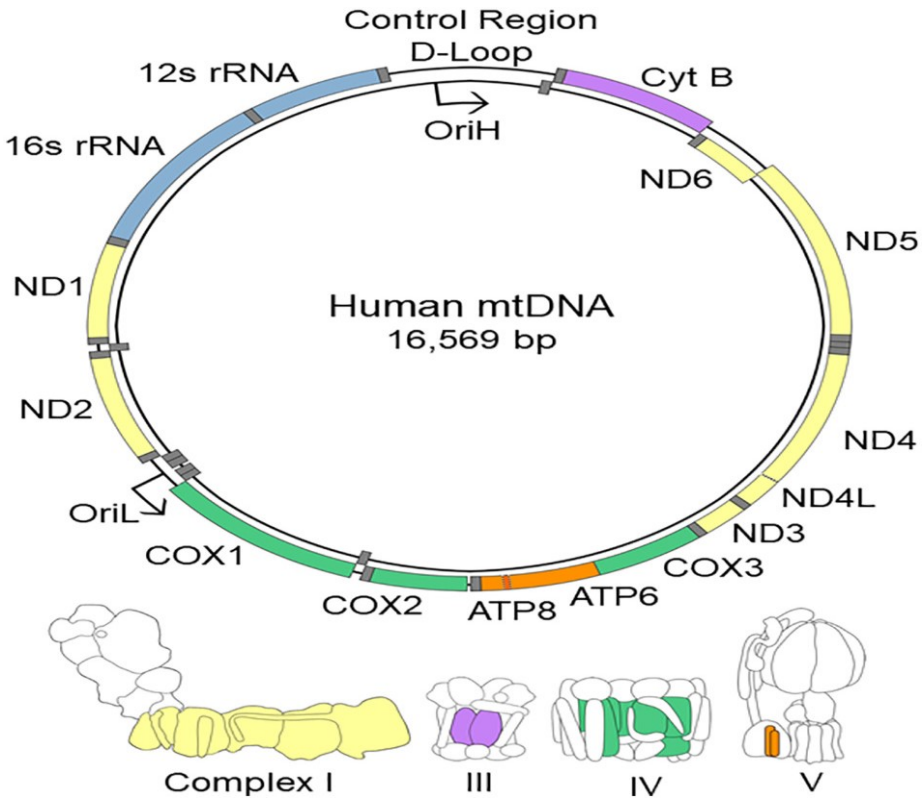

Figure 1.8: Structure of the Human Mitochondrial DNA (Chocron, Munkácsy, & Pickering, 2019)

Transcription factors and transcriptional coactivators play an essential role in the regulation of mtDNA transcription and replication, as well as the expression of mitochondrial proteins encoded in the nuclear genome participating in oxidative phosphorylation and their import (Diaz & Moraes, 2008). Transcriptional coactivators of the peroxisome proliferator-activated receptor γ -coactivator-1 (PGC-1) family, PGC-1α, PGC-1β and PRC (PGC-1 related coactivator) (Scarpulla, 2002) works in concert with the nuclear respiratory factor 1 and 2 (*Nrf1*

and *Nrf2*) (Joseph V. Virbasius, 1993) and the estrogen-related receptor (ERRα), which are considered as the most prevalent transcription factors activating promoters of mitochondrial genes (Diaz & Moraes, 2008) as illustrated in Figure 1.9. In addition, this family of coactivators stimulates not only mitochondrial biogenesis but also regulates several metabolic pathways such as cellular respiration, thermogenesis and hepatic glucose metabolism (Diaz & Moraes, 2008). Whereas PGC-1α is mainly involved in the regulation of gluconeogenesis, PGC-1β regulates β - oxidation of fatty acids (Lin et al., 2003; Ling et al., 2004). Srivastava and Moraes demonstrate an improvement of respiration in cells with mtDNA mutations associated with overexpression of PGC-1α and PGC–1β (Srivastava, Barrett, & Moraes, 2007). Lin et al showed mitochondrial proliferation in skeletal muscle and a switch in fiber type composition from the more prominent type II (glycolytic) to type I (oxidative) in transgenic mice overexpressing PGC-1α (Lin et al., 2002). Another study suggests the involvement of PGC-1α to increase mitochondrial mass in skeletal muscle due to endurance exercise (Diaz & Moraes, 2008). Mitochondrial transcription factor A (*Tfam*), another transcription factor for mtDNA synthesis, enhances mtDNA transcription in a promoter-specific fashion in the presence of mitochondrial RNA polymerase and mitochondrial transcription factor B (*Tfb1m* or *Tfb2m*). Research confirms its involvement in maintaining the integrity of mDNA (Kanki et al., 2004). Regarding *Tfb2m*, a study showed a decrease in *Tfb2m* level caused mitochondrial impairment, eliciting a complex retrograde response including a shift to glycolysis (Adán et al., 2008).

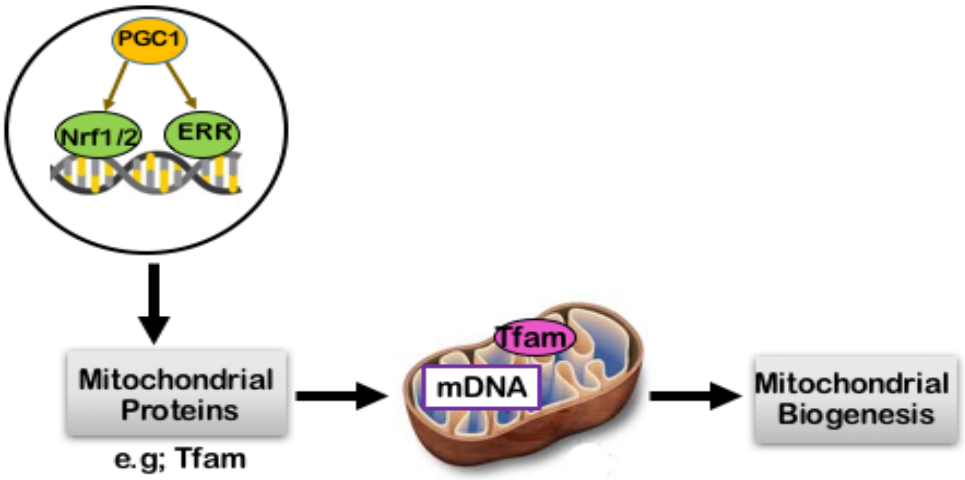

Figure 1.9: Role of PGC-1α in Regulation of Mitochondrial Biogenesis; adapted from (Dillon, Rebelo, & Moraes, 2012)

PGC-1 coactivators regulate mitochondrial biogenesis by activating transcription factors such as NRF-1/2 and ERRα, thereby increasing the expression of nuclear DNA-encoded mitochondrial proteins. These mitochondrial proteins are then imported into the mitochondria. One such protein is Tfam, which when upregulated, leads to increased mtDNA replication and increased expression of mtDNA-encoded proteins. These processes initiate an increase in mitochondrial biogenesis.

1.6.1.3. L-citrulline as a Supplement to Improve Performance and Mitochondrial Function

Regular exercise and physical activity decrease the risk of development of obesity, T2D, hypertension, and cardiovascular diseases, as well as increase life expectancy about 8-10 years (Chen et al., 2015; Safdar et al., 2011). Several studies have revealed that one of many important benefits imparted by exercise is the improvement of skeletal muscle function. These improvements were performed by enhancing mitochondrial function (Dubé et al., 2008; Safdar et al., 2011; Villareal et al., 2018; Yan, Okutsu, Akhtar, & Lira, 2011). PGC-1α has a crucial role in regulation of mitochondrial biogenesis, which in turn positively impact mitochondrial function (Diaz & Moraes, 2008). Muscle-specific overexpression of PGC-1α increases mitochondrial biogenesis

and capillary density in skeletal muscle, and in turn improves maximal oxygen uptake (VO_2max) and exercise capacity (Tadaishi et al., 2011). Studies in humans have shown L-citrulline may act as a performance enhancer, as it reduced muscle fatigue in 18 men performing finger flexions at 1.5 second intervals lighting a 6 kg weight. It was postulated that this benefit was due to augmented aerobic energy production in exercising muscle, as ^{31}P magnetic resonance spectroscopy (^{31}P MRS) studies revealed a 34% increase in oxidative ATP production and 20% increase in the rate of phosphocreatine recovery within the exercising muscle (Bendahan et al., 2002). It has also been shown to increase performance during high-intensity anaerobic exercise, as L-citrulline reduced muscle soreness and repetition number during flat barbell bench presses in 41 male volunteers (Pérez-Guisado & Jakeman, 2010). Similarly, studies in rats suggest that L-citrulline enhances gastrocnemius muscle performance, as L-citrulline malate supplementation reduced both the phosphocreatine and oxidative cost of contraction following electrically induced transcutaneous stimulation (B. Giannesini et al., 2011). In addition, a recent study conducted with Villareal, et al revealed that L-citrulline supplementation increases exercise performance in mice due to upregulation of PGC-1α expression in skeletal muscle (Villareal et al., 2018).

1.7. Obesity and T2D

Although the World Health Organization (WHO) considers obesity as a disease since 1948, it did not consider it as a public health problem until 1997 (James, 2009). Obesity is defined as an abnormal or excessive accumulation of body fat that presents a risk to health (Organization, 2018). Obesity often results from increased calorie intake and reduced levels of physical activity, though genetics can also be implicated (Kylie Conroy, 2014; "Nutraceuticals and health : review of human evidence,"). Body mass index (a person's weight (in kilograms) divided by the square

of his or her height (in metres)) is the most widely used method for classifying being overweight or obese. A person with a BMI of 30 or more is generally classified as obese, while a person with a BMI equal to or more than 25 is classified as overweight (Organization, 2018). Throughout the world, the prevalence rate of overweight and obesity has increased over the past decade (Kylie Conroy, 2014); and it is considered a major risk factor for many chronic diseases, including diabetes, cardiovascular diseases and cancer (Organization, 2018). Regardless of its effect on peoples' overall social and economic well-being, obesity is recognized as a direct cause of 1 in 10 premature deaths among Canadian adults aged 20 to 64 (O. Canada, 2015). According to population surveillance studies in Canada, there was a significant increase in the prevalence of obesity over the past three decades (O. Canada, 2015). In 2014, the Canadian Community Health Survey revealed that over 5 million adults have obesity meanwhile 30% or more than one in three adults is obese according to the 2015 Canadian Health Measures Survey (O. Canada, 2015). In particular, there is general consensus that a well-characterized consequence of obesity is T2D. In 2016, it has been reported that 7.0% of Canadians aged 12 and older (roughly 2.1 million people) was diagnosed with diabetes (S. Canada, 2017). Overweight or obese Canadians aged 18 and older were more likely to diagnosed with diabetes than normal weight (S. Canada, 2017). According to the 2016 Canadian statistics, 13.2% of obese Canadians, 6.6% of overweight Canadians, and 3.6% of those classified as having a normal weight were diagnosed with diabetes (S. Canada, 2017).

1.7.1. Glucose Homeostasis in Metabolic Health

Glucose homeostasis is mainly controlled by two opposing hormones, insulin and glucagon, which may act on the liver, fat, and skeletal muscle primarily (Szablewski, 2011). In

the fasting state, glucose output from the liver determines plasma glucose levels, whereas majority of glucose is taken up by the skeletal muscle in case of the fed state (Saltiel, 2016). Glucose transporter proteins (GLUT) facilitate glucose entry into the cell. Table 1.7 shows the five identified subtypes that differ in characteristics and their distributions (Wilcox, 2005). By these proteins, different cell types can utilize the glucose according to their specific functions (Wilcox, 2005). Most brain cells have GLUT1 as the principal transporter protein that can move glucose intracellularly despite the low glucose and insulin levels seen during the fasting state. However, GLUT4, the major glucose transporter protein in adipose cells and muscle cells, requires insulin for its action and has a much higher Km for glucose (Wilcox, 2005). Muscle accounts for about 60-70% of insulin stimulated whole body glucose uptake while adipose tissue accounts for about 10% of whole-body insulin mediated uptake (Smith, 2002). In the fed state, insulin is secreted to promote glucose uptake into the muscle via GLUT4 and glycogen synthesis via activation of glycogen synthase. In addition, energy can be produced anaerobically via glycolysis. In the case of adipose tissue, insulin stimulates glucose transportation to the adipocyte through GLUT4 and promotes lipogenesis while suppressing lipolysis (Wilcox, 2005). Both muscle and adipose tissue do not rely on glucose as a major fuel source for energy during the fasted state, when insulin levels are low (Wilcox, 2005). In muscle cells insulin deficiency can promote protein catabolism, releasing amino acids for gluconeogenesis. In addition, adipocytes liberate free fatty acids into the circulation for direct utilization by other organs such as heart, muscle and liver (Wilcox, 2005). In the liver, free fatty acids are converted to ketone bodies, which are considered as an alternative energy substrate for the brain during starvation (Wilcox, 2005).

Glucose is an essential metabolic substrate for energy production (Szablewski, 2011). Hence, once it enters the cells, it is either converted to glycogen or oxidized by various catabolic pathways to produce ATP (Szablewski, 2011). Glycolysis is the first pathway in the complete oxidation of glucose in the cytoplasm of the cell. In this process one molecule of glucose is catabolized to two molecules of pyruvate, two molecules of ATP and two molecules of NADH (Szablewski, 2011). In aerobic glycolysis the resultant pyruvate is transported into the mitochondria so that it can be oxidized into acetyl-CoA via pyruvate dehydrogenase, the rate-limiting enzyme of glucose oxidation (Szablewski, 2011). Further series of reactions occurs through oxidizing the acetyl-CoA within the Krebs Cycle (also known as the citric acid cycle) (Szablewski, 2011). In addition, the Krebs cycle produces NADH and $FADH_2$ reducing equivalents, which donate their electrons to the complexes of the electron transport chain to drive ATP production via oxidative phosphorylation as described in 1.6 (Szablewski, 2011).

However, in the absence of oxygen anaerobic glycolysis proceeds instead (Szablewski, 2011). Glycolytic-derived pyruvate is reduced to lactate via lactate dehydrogenase (Szablewski, 2011). This reaction happens in heavily exercising muscle where oxygen supply is not enough for aerobic metabolism. The accumulated lactate is removed via transporters and delivered to other tissues where it can be used as an energy source (Szablewski, 2011).

Table 1.7: Glucose Transporter Proteins; adapted from (Wilcox, 2005)

ISOFORM	Tissue Distribution	Affinity for Glucose	Km
GLUT1	Brain microvessels Red blood cells Placenta Kidney All tissues	High	1 mmol/L
GLUT2	Liver Kidney β cell Small intestine	Low	15-20 mmol/L
GLUT3	Brain neurons Placenta Foetal muscle All tissues	High	<1 mmol/L
GLUT4	Muscle cells Fat cells Heart	Medium	2.5-5 mmol/L
GLUT5	Small intestine Testes	Medium	6 mmol/L

Isoforms of glucose transporter protein, their distribution and affinity in the tissues; Km, substrate concentration at half the maximal velocity.

1.7.2. Insulin Signaling and Glucose Metabolism

Insulin is a hormone that is normally secreted by the islet beta cells of the pancreas, which acts to regulate blood glucose levels (Figure 1.10) (Wilcox, 2005). It binds to insulin receptors to mediate its action. The insulin receptor consists of a heterotetramer, 2 α extracellular glycoprotein subunits and 2 β intracellular glycoprotein subunits linked by disulphide bonds (Kido, Nakae, & Accili, 2001). As long as insulin binds to the extracellular α subunit, it results in a conformational change that allows ATP to bind to the intracellular component of the β subunit (Wilcox, 2005). ATP binding activates tyrosine kinase through the phosphorylation of the β subunit. Tyrosine kinase phosphorylates intracellular substrate proteins called insulin responsive substrates (IRS) (Wilcox, 2005). These IRS proteins are responsible for mediating further cellular actions of insulin by binding other signaling molecules (Kido et al., 2001). There are four IRS proteins;

IRS-1, IRS-2, IRS-3, IRS-4. IRS-1 and 2 distribute and overlap in many tissues. IRS-1 is the major IRS in skeletal muscle, IRS-2 is the main IRS in liver (Kido et al., 2001), IRS-3 is found only in adipose tissue, β cells and liver and IRS-4 in thymus, brain and kidney (Burks & White, 2001; Withers & White, 2000). Phosphorylated IRS proteins bind to many proteins, some of them with enzymatic activity such as src-homology-2 domain proteins (SH2), and others that lack enzymatic activity such as adaptor protein Grb2. The src-homology-2 domain proteins (SH2) include important enzymes such as phosphatidylinositol 3-kinase (PI 3-kinase). PI 3-kinase phosphorylates and activates serine and threonine kinases such as Akt/protein kinase B (PKB), protein kinase C (PKC) and phosphatidylinositol dependent protein kinases1& 2 (PIPD 1&2) to mediate insulin's metabolic effects. These effects include the translocation of glucose transporter proteins, glycogen, lipid and protein synthesis, anti-lipolysis and the control of hepatic gluconeogenesis (Wilcox, 2005).

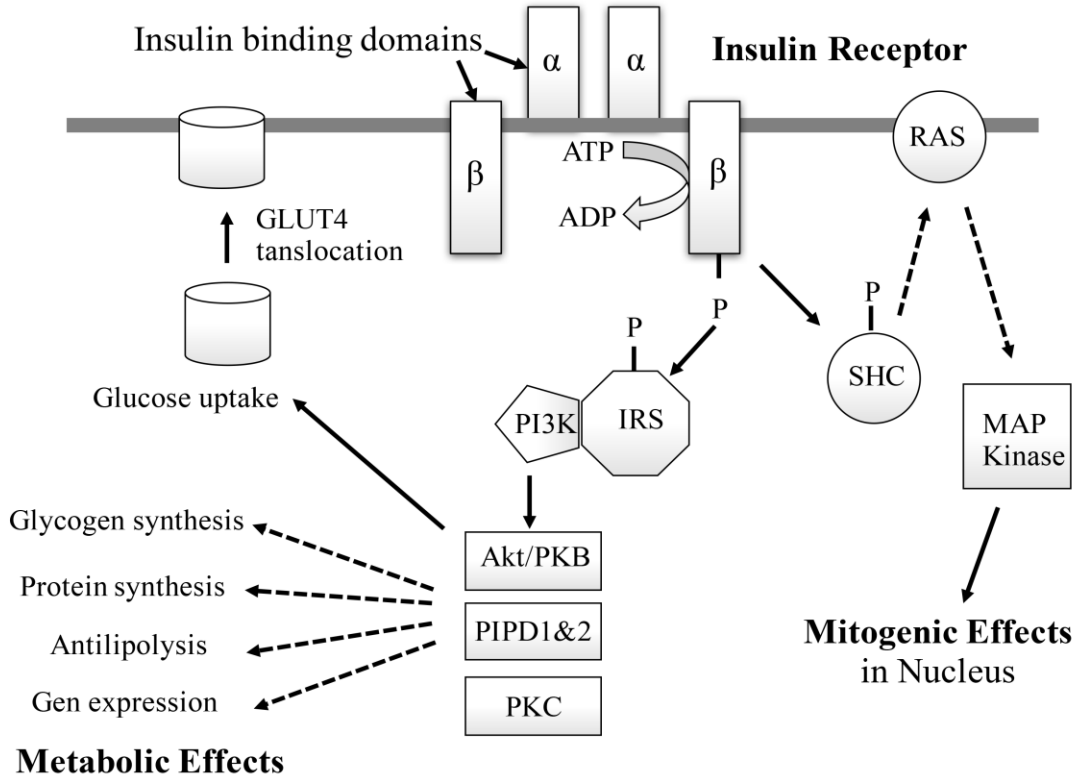

Figure 1.10: Insulin Signaling Pathway; adapted from (Wilcox, 2005)
The insulin action is mediated by the activation of multiple signaling pathways. The pathways are started by binding ATP the intracellular component of the β subunit, and then activating tyrosine kinase, which phosphorylate IRS proteins. The IRS/PI 3-K pathway leads to the generation of PIP_3 and the consequent activation of PIP_3-dependent kinases. IRS, Insulin responsive substrates; PI3K, Phosphatidylinositol 3-kinase; PKB, Protein kinase B; PKC, Protein kinase C; PIPD 1&2, phosphatidylinositol dependent protein kinases1& 2; MAPK, mitogen-activated protein kinase.

1.7.3. Proposed Mechanisms of Insulin Resistance due to Obesity

Insulin resistance is a hallmark feature of obesity. Insulin resistance occurs when insulin cannot suppress hepatic glucose output and promote peripheral glucose disposal (Ye, 2007), whereas hyperinsulinemia means that plasma insulin levels are elevated (Ye, 2013). As insulin resistance and hyperinsulinemia are common features of obesity, there is still confusion about their relationship in the pathology of T2D (Ye, 2007). It is often thought that hyperinsulinemia results from insulin resistance, as pancreatic beta-cells try to compensate insulin resistance by secreting more insulin to stimulate glucose disposal. However, recent studies suggest that a high

level of insulin that results from lipotoxicity may directly cause insulin resistance (Ye, 2013). Both views are illustrated in Figure 1.11 and 1.12, and despite which occur first, insulin resistance or hyperinsulinemia, T2D is often the end result.

Figure 1.11: Compensation Hypothesis

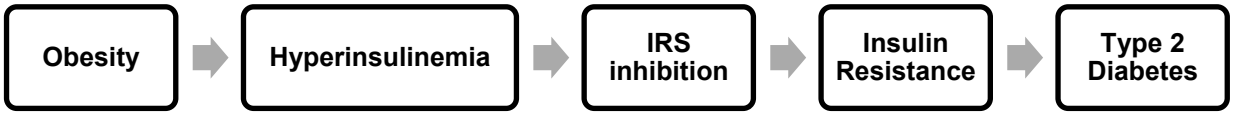

Figure 1.12: Insulin Hypothesis

1.7.4. Diet-induced Obesity and Insulin Resistance Model

An imbalance of food intake, basal metabolism, and energy expenditure result in obesity, followed by chronic complications such as diabetes and cardiovascular disease, which contributes to an increasing health care burden and decreasing life expectancy (Flier, 2004; C.-Y. Wang & Liao, 2012). In order to understand pathophysiological changes and adaptation that happen through decades of obesity, the mouse model of diet-induced obesity has become one of the most important tools (C.-Y. Wang & Liao, 2012). The main contributor to the increasing obesity prevalence in humans is increasing availability of the high-fat/energy dense foods in modern society over the past two decades (Wisse, Kim, & Schwartz, 2007). Thus, there have been many

studies characterizing the responses of animals exposed to high-fat diets in the last 20-30 years (Zhang et al., 2010). Because the metabolic abnormalities of C57BL/6J mouse closely parallel that of the human obesity progression pattern, the C57BL/6J mouse model is better characterized than some other models (S. Collins, Martin, Surwit, & Robidoux, 2004; C.-Y. Wang & Liao, 2012). In this model, mice are often supplied with a 60% kcal from lard fat diet compared with lean mice controls, who receive 10% kcal from lard diet (C.-Y. Wang & Liao, 2012).

1.7.5. L-citrulline Metabolism in Obesity

The pioneering work of Felig et al. shows an increase in plasma levels of valine, leucine, isoleucine, tyrosine, and phenylalanine from 20 plasma amino acids measured in obese subjects (Felig, Marliss, & Cahill, 1969). Thus, recent metabolomics studies report significant alterations in plasma amino acid levels, particularly elevations of branched-chain amino acids (BCAA) (Fiehn et al., 2010; She et al., 2007; Vannini P, 1982; T. J. Wang et al., 2011; Wijekoon, Skinner, Brosnan, & Brosnan, 2004) and aromatic amino acids (AAA) (Fiehn et al., 2010; T. J. Wang et al., 2011) in obese and diabetic subjects. Although, findings in human studies are so far less consistent, recent research in mice suggests that obesity is also associated with increased plasma citrulline levels (Sailer et al., 2013). This study shows that there was an increase only in plasma citrulline and ornithine levels in mice with diet-induced obesity while mice treated with streptozotocin displayed not only elevation in citrulline and ornithine levels but also increases in plasma concentrations of BCAAs and AAAs. Verdam et al report that plasma citrulline levels increased in obese subjects with hyperglycemia (Jourdan et al., 2015; Verdam et al., 2011). As obesity development is associated with increased plasma citrulline concentrations, this could contribute to the metabolic syndrome (Sailer et al., 2013).

1.8. Thesis Rationale, Hypotheses and Objectives

1.8.1. Rationale

Nutraceuticals are defined as products isolated or purified from food that are generally sold in medical forms. The demand for these products is increasing in the past decades, because of their nutritional value, physiological benefits, potential health benefits, as well as protective effects against chronic diseases.

Our nutraceutical L-citrulline is an organic α-amino acid found naturally in high quantities in watermelons, onions, and garlic (Kayleen St. John, 2014). Furthermore, L-citrulline is synthesized almost exclusively in the intestine and requires arginine and glutamine for its biosynthesis in the Urea Cycle (Bahri et al., 2013). It serves as a by-product of nitric oxide synthase, which converts L-arginine into NO and L-citrulline. It can also serve as a precursor for L-arginine biosynthesis, and thus has been used as a supplement to increase circulating arginine concentrations in situations of arginine deficiency (Papadia et al., 2018; Waugh et al., 2001). Studies in humans have shown that L-citrulline may enhance performance as it reduced muscle fatigue via promoting aerobic energy production in exercising muscle (Bendahan et al., 2002). It has also been shown to increase performance during high-intensity anaerobic exercise (Pérez-Guisado & Jakeman, 2010). Similarly, studies in rats suggest that L-citrulline enhances gastrocnemius muscle performance, as L-citrulline-malate supplementation reduced both the phosphocreatine and oxidative cost of contraction following electrically induced transcutaneous stimulation (Benoît Giannesini et al., 2011). A recent study conducted by Villareal *et al*. revealed that L-citrulline supplementation increases exercise performance in mice due to upregulation of PGC-1α expression in skeletal muscle (Villareal et al., 2018). Based on the aforementioned studies, it is possible that L-citrulline's potential beneficial actions on exercise performance

involve increases in mitochondrial biogenesis and in turn mitochondrial function. Of importance, a number of studies have demonstrated that increasing mitochondrial function protects against obesity-induced impairments in glucose homeostasis (Dubé et al., 2008; Gupte, Bomhoff, Swerdlow, & Geiger, 2009; Seth et al., 2007). Despite increases in skeletal muscle mitochondrial function frequently being associated with improved glucose homeostasis, it remains enigmatic as to whether L-citrulline supplementation for the purposes of enhancing performance, has secondary actions on glycemia.

1.8.2. Hypotheses

- L-citrulline supplementation will improve exercise capacity and glucose homeostasis in insulin resistant, obese mice.
- L-citrulline supplementation will improve insulin sensitivity and insulin signaling in skeletal muscle.
- L-citrulline supplementation will reverse fat-diet-induced impairment in the factors and coactivators that regulate mitochondrial DNA synthesis, and in turn increase mitochondrial biogenesis.
- L-citrulline supplementation mediates its in-vivo actions via direct effects on skeletal muscle.

1.8.3. Objectives

- To investigate whether L-citrulline supplementation influences mitochondrial biogenesis, exercise performance, and glucose homeostasis during experimental obesity.
- To investigate the effect of different doses of L-citrulline on transcription factors and coactivators that regulate mitochondrial biogenesis and insulin signaling in C2C12 cells.
- To determine the specific signaling pathways transduced in the skeletal muscle following

treatment with L-citrulline.

- To determine whether L-citrulline supplementation mediated in vivo actions due to direct actions on the muscle or due to boosting L-arginine levels.

Chapter 2: Materials and Methods

2. Materials and Methods

2.1. Chemicals and Reagents

L-citrulline, L-arginine ammonium persulfate (APS) protease and phosphatase inhibitors sodium chloride (NaCl) and methanol were purchased from Sigma-Aldrich (Oakville, ON, Canada). Beta-mercaptoethanol was acquired from Sigma Life Science product (Japan). Glucose was purchased from EMD Millipore (Etobicoke, ON, Canada), and insulin (Novolin) (biosynthetic human insulin) was purchased from Novo Nordisk (Mississauga, ON, Canada). The insulin ELISA assay kit was obtained from Alpo Diagnostics (Salem, New Hampshire, USA). Plasma collecting EDTA coated microfuge tubes were obtained from Sarstedt (Nümbrecht, Germeny). Pierce™ ECL western blotting substrates were purchased from Thermo-Fisher scientific (Burlington, ON, Canada). Trans-Blot membranes (pure nitrocellulose) were purchased from BioRad Laboratories (Mississauga, ON, Cnada). Monoclonal and polyclonal primary (Akt, P-Akt, GSK3β, P- GSK3β, and Hsp90 antibodies) and secondary antibodies (anti-rabbit and anti-mouse antibodies) were obtained from Cell Signaling Technology (Danvers, Massachusetts, USA), except Hsp90 which was obtained from BD biosciences (San Jose, CA, USA). Tris, HCl, dithiothreitol (DTT) and cDNA kits were purchased from Invitrogen (Invitrogen, Carlsbad,), EDTA from Caledon (Gergetown, ON, Canada), glycerol from Fluka (Flemington, New Jersey, USA). Glycine, sodium dodecyl sulfate (SDS) were obtained from Fisher Bioreagent (USA). Tween 20 was obtained from TM of Corda International PLC (USA). Tetramethylethylenediamine (TEMED) made in China. Unifilter P81 96-well filterplates were obtained from Whatman (Florham Park, New Jersey). Triazol reagent and TAQ man Primers (*Ppargc1a, Nrf1, Tfam, Tfbm2, Ppia*) were purchased from Thermo Fisher Scientific, and designed SYBR green Primers (*Ppargc1a, Nrf1, Tfam, Ppia*) were obtained from Integrated DNA Technology (IDT).

2.2. Methods

2.2.1. Animals

All animals received care according to the guidelines of the Canadian Council on Animal Care and all experimental procedures performed in mice were approved by the University of Alberta Health Sciences Animal Welfare Committee. 10-week-old C57BL/6J (Jackson Laboratory) males fed either a low-fat diet (LFD, 10% kcal from lard, Research Diets D12450J) or a high-fat diet (HFD, 60% kcal from lard, Research Diets D12492), while receiving drinking water supplemented with either vehicle or L-citrulline (0.6 g/L) for 15 weeks. As the mouse's average drinking water is ~ 5 mL/day (Bachmanov, Reed, Beauchamp, & Tordoff, 2002), the daily L-citrulline dose is 100 mg/kg, which is consistent with several previous studies (Bendahan et al., 2002; Pérez-Guisado & Jakeman, 2010; Schwedhelm et al., 2008). All experimental groups were subjected to several physiological assessments throughout 15 weeks, and upon study completion all animals were euthanized via intraperitoneal (IP) injection of sodium pentobarbital (12 mg) following an overnight fast and at 15 min post-administration of saline or insulin. Tissues (e.g. gastrocnemius muscle, liver) were extracted and immediately snap frozen in liquid N_2 using Wollenberger tongs precooled to the temperature of liquid N_2, and stored at -80^{o}C.

2.2.2. In-vivo Experiments

2.2.2.1 Glucose Tolerance Test

Intraperitoneal glucose tolerance testing was performed in over-night fasted mice with free access to their water by administering 2 g/kg glucose. Blood glucose was measured at 0, 15, 30, 60, 90, 120 min post glucose injection via tail bleeding by using Contour Next blood glucose monitoring system (Bayer) as previously described (Ussher et al., 2010). In addition, blood

samples were collected in tubes containing the anticoagulant agent (EDTA) at 0 and 30 min post-glucose administration, in order to measure plasma insulin levels as described later.

2.2.2.2. Insulin Tolerance Test

Intraperitoneal insulin tolerance testing was assessed in mice following a 6 hr fast with free access to a drinking water, using an insulin dose of 0.7 U/kg (Novolin (biosynthetic human insulin), Novo Nordisk). Then blood glucose was measured at 0, 15, 30, 60, 90, 120 min after insulin administration as described in 2.2.2.1. As the possibility of hypoglycemia could happen in mice undergoing this procedure, a syringe of glucose (50% glucose in water) was always ready in case of emergency. Mice that became hypoglycemic during the insulin tolerance test (blood glucose < 2.0 mM) were injected with glucose to prevent death and the animal was excluded from data analysis.

2.2.2.3 Exercise Tolerance Test

The test was conducted by running mice on calibrated, motor-driven treadmill (Columbus Instruments) at a starting speed of 3 m/min for 2 min, followed by increasing speeds of 4 m/min for 2 min, 5 m/min for 2 min, 6 m/min for 6 min, 8 m/min for 10 min, 10 m/min for 3 min, 12 m/min for 3 min,14 m/min for 3 min, 16 m/min for 3 min, 18 m/min for 3 min, 20 m/min until exhaustion. The time was recorded at exhaustion point; when mice would no longer run for more than 5 sec at a time, and remained on the shock grid even when touched with a gloved hand to attempt assisting the animal back onto the treadmill. For data collection, the first 6 min is not included, as it is considered an acclimatization period for mice to become familiar with the treadmill.

2.2.2.4. Indirect Calorimetry

Oxymax comprehensive lab animal monitoring system (Columbus Instruments) was used to assess in-vivo energy metabolism via indirect calorimetry. The mice were singly housed in a metabolic cage with free access to water and food for 48 hr. The first 24 hr were used to allow mouse acclimatization to their new housing environment, whereas the final 24 hr period was utilized for data analysis. This method provides measurement of total metabolism, oxygen consumption (VO_2) and the carbon dioxide production (VCO_2), respiratory exchange ratios (RER), heat production, activity, as well as food and water intake. By using the following equation, both the VO_2 and the VCO_2 can be calculated taking the differences between the input and output oxygen flow, and the output and input carbon dioxide flow respectively. The unit for these values is litter per minute (LPM) (Even, Mokhtarian, & Pele, 1994).

$$VO_2 = (FinO_2 * Vin) - (FoutO_2 * Vout)$$

Where:

$FinO_2$ and $FoutO_2$ are oxygen fraction at input and output

Vin and Vout are the input and output ventilation rate

$$VCO_2 = (FoutCO_2 * Vout) - (FinCO_2 * Vin)$$

Where:

$FinCO_2$ and $FoutCO_2$ are carbon dioxide fraction at input and output

Vin and Vout are the input and output ventilation rate

Since the VO_2 and the VCO_2 were calculated, the RER was calculated as the ratio between VO_2 and VCO_2. As RER is a ratio, it does not have a unit.

$$RER = VCO_2 / VO_2$$

By the following equation, heat can be calculated through the determined caloric value (CV) based on the observed RER and the observed VO_2.

$$Heat = CV * VO_2$$

Where:

$$CV = 3.815 + 1.232 * RER$$

2.2.2.5. Magnetic Resonance Imaging (MRI) Body Composition Analysis

An EchoMRI-4in1/700 body composition analyzer was utilized to deliver precise body composition measurements of fat, lean, free water, and total water masses in fully conscious animals via specialized Nuclear Magnetic Resonance (NMR) Relaxometry-based technology. EchoMRI™ analyzers are not only exceedingly easy to operate, but also precise and accurate measurements carried out. Mice were inserted in a special tube without restriction or anesthesia. After placement of the tube in the analyzer, the scanning takes ~2 min for each mouse with the results extracted into Microsoft Excel for subsequent analysis.

2.2.3. Ex-vivo Experiments

After animal euthanasia, blood samples were collected from our mice using cardiac puncture technique into tubes containing the anticoagulant agent EDTA. To separate the plasma, the blood samples were centrifuged at 3000 rpm for 10 min and the plasma was collected and stored at -80°C. In addition, the following tissues (heart, soleus and gastrocnemius muscle, liver and fat) were collected from each mouse and immediately frozen in liquid nitrogen. Frozen soleus and gastrocnemius tissues were powdered in a motor and pestle cooled to the temperature of liquid nitrogen, followed by storage of all frozen samples in -80°C freezers for further analysis.

2.2.3.1. Western Blot analysis

A portion of frozen soleus and gastrocnemius powder (20 mg) was weighed and homogenized in buffer containing 50 mM Tris HCl (pH 8 at 4°C), 1 mM EDTA, 10% (wt/vol) glycerol, 0.02% (wt/vol) Brij-35, 1 mM DTT, and protease and phosphatase inhibitors by using homogenizer machine for 30 Sec. After leaving the homogenate for 10 min in ice, centrifuge it at 10,000 x g for 20 min and store the resulting supernatant in -80°C freezers for immunoblotting. Bradford protein assay was used to measure protein concentration of homogenate. Tri/glycine SDS-PAGE (10% sodium dodecyl sulfate polyacrylamide gel electrophoresis) method was used for separating proteins. The separated proteins were transferred onto a 0.45 μm nitrocellulose membrane via wet-transfer system. Nonspecific binding sites on the membrane were blocked with 5% milk in 0.5% Tween20-tris buffered saline (TBST) for 1 hr at room temperature. Membranes were probed with either anti-Akt (Cell Signaling Technology, 1/1000 dilution), anti-phosphoSerine 473 Akt (Cell Signaling Technology, 1/1000 dilution), anti-GSK3β (Cell Signaling Technology, 1/1000 dilution), anti-phosphoSerine 9 GSK3β (Cell Signaling Technology, 1/1000 dilution), anti-Hsp90 (BD biosciences, 1/1000 dilution) antibodies in 3% bovine serum albumin (BSA) in TBST over-night at 4°C. Then, membranes were washed with TBST three times for 10 min and probed with anti-rabbit (Cell Signaling Technology, 1/2000 dilution) secondary antibody in 5% fat-free milk. However, in case of Hsp90 measurement, the membranes were probed with anti-mouse (Cell Signaling Technology, 1/2000 dilution) secondary antibody. Enhanced chemiluminescence reagent (Thermo-Fisher scientific) was utilized to detect immunoblots via Image Quant LAS 4000 mini while Image J software was used for quantification.

2.2.3.2. Real-Time PCR analysis

RNA extraction and cDNA preparation has to be carried out prior to performing real-time PCR.

2.2.3.2.1. TRiazol RNA Isolation

TRiazol reagent (500 µl) was added for a portion of frozen soleus and gastrocnemius powder, and homogenized by using a mechanical tissue homogenizer. Samples were frozen immediately in dry ice. Once all samples were homogenized and frozen on dry ice, they were stored at -80ºC until next day, following which they were thawed for process of TRizol extraction of RNA. Once thawed, samples were left at room temperature for 5 min, 0.1 mL chloroform was added and mixed vigorously, using a vortex machine. After leaving samples at room temperature for 10-15 min, samples were centrifuged for 15 min at 12,000 x*g* at 4ºC. Then supernatant was transferred into new autoclaved 1.5 mL Eppendorf tubes, 0.25 mL ice cold isopropanol was added, and the samples sat for 5-10 min at room temperature. Samples were centrifuged again for 10 minutes at 12,000 x*g* at 4°C to pellet the RNA. Isopropanol was either poured out into a waste container or sucked up via vacuum, without disturbing the resulting pellet. For washing the RNA pellet, 1 mL of 75% ice-cold ethanol was added and centrifuged for 5 minutes at 7,500 xg at 4°C. This step was repeated to ensure optimal washing. After removing last wash, tubes were left open to let the RNA pellet air-dry for 20 min. Then, the pellet was resuspended in PCR grade (RNAse/DNAse free) water. In case the pellet did not dissolve well, low heat was applied for 5 min. Finally, a NanoDrop 2000 spectrophotometer was used to quantify RNA concentration in each sample.

2.2.3.2.2 SuperScript III cDNA Synthesis

The resulting RNA concentration was used to synthesize the first-strand cDNA. First, a mixture of RNase free water and 10x DNAse buffer was added to 1-5 µg of RNA of each sample to a total volume of 10 µl. Then, ice-cold mixture of EDTA and random primers was added to each sample, and kept at 70°C for 12 min. After leaving the samples in ice for 2min, the following reaction was performed by adding a mixture of (5x buffer, DTT, dNTP mix) to each sample at 25°C for 5 min. The final reaction involved addition of the SuperScript III (Invitrogen, Carlsbad, CA) reverse transcriptase to each tube, and keeping in 70°C for 75 min.

2.2.3.2.3. Real-time PCR Quantification and Data analysis

A real-time PCR machine (Bio-Rad Laboratories Inc.) was used for quantitative analysis of targeted mRNA expression in 96 well optical reaction plates. Reaction mixture contains yellow water and primer was added to a blend of total cDNA plus blue water mixture in the plate. After that, the plate was covered with PCR seals, and centrifuged at 22 °C for 2 min. The plate was placed in PCR machine for running. PCR primers are shown in Table 2.1 and 2.2. The real-time PCR data was measured by relative gene expression, which was analyzed via $2^{-\Delta\Delta Ct}$ method (where CT is threshold cycle). This method was described in Applied Biosystems and as described by Livak and Schmittgen (Livak & Schmittgen, 2001). The reference peptidyl-prolyl isomerase gene (*Ppia*, also known as cyclophilin), housekeeping internal control gene, was used to normalize all gene expression.

Table 2.1: TAQ man Primers (Thermo Fisher Scientific)

Gene	TAQ man Primer
Ppargc1a	Mm01208835-m1
Nrf1	Mm01135606-ml
Tfam	Mm0044785-ml
Tfbm2	Mm01620397-sl
Ppia	Mm02342430-g1

Table 2.2: Designed SYBR Green Primers (Integrated DNA Technology (IDT))

Gene	Forward Primer	Reverse Primer
Ppargc1a	5'- TAT GGA GTG ACA TAG AGT GTG CT 3'	5'-CCA CTT CAA TCC ACC CAG AAAG -3'
Nrf1	5'- AGC ACG GAG TGA CCC AAA C-3'	5'-TGT ACG TGG CTA CAT GGA CCT -3'
Tfam	5'-GGA ATG TGG AGC GTG CTA AAA3'	5'-GCT GGA AAA ACA CTT CGG AATA-3'
Ppia	5'-GCT GGA CCA AAC ACA AAC G3'	5'-ATG CCT TCT TTC ACC TTC CC-3'

2.2.3.3. Enzyme-Link Immunosorbent Assay for Insulin Measurement

Blood samples were collected in specific tubes coated with anticoagulant agent (EDTA) at 0 and 30 min post-glucose administration during intraperitoneal glucose tolerance, following by centrifuging at 3000 rpm for 10 min to collect plasma samples in order to measure plasma insulin levels via Enzyme-linked Immunosorbent Assay Kit (Alpco Diagnostics) as previously described (Rami Al Batran, 2018). 96 well plate and all the reagents were brought to room temperature before use. 5 µL of each sample was added to each well, followed by 75 µL of enzyme conjugate and provided film was used to seal the plate. Then, the plate was incubated in an orbital microplate shaker at 700-900 rpm for 2 hr at room temperature. Following, a working straight wash buffer was used 6 times for washing the plate. In order to start the reaction, 100 µL of substrate was added to each well and the plate placed again in the microplate shaker. After 30 min, 100 µL of stop

solution was added and the plate was gently shaken to stop the reaction. The absorbance of the plate was measured at 450 nm wavelength, in order to determine plasma insulin levels “ng/mL”. To be noted, the measurement should be done within 30 min after adding the stop solution, and all the air-bubbles were removed prior to measurement.

2.2.3.4 Mitochondrial Respiration Levels Assessment

Mitochondrial oxygen consumption was assessed in freshly saponin-permeabilized gastrocnemius muscle using a Clark oxygen electrode connected to an Oxygraph Plus recorder (Hansatech Instruments Ltd., Norfolk, England) as previously described (Darwesh, Jamieson, Wang, Samokhvalov, & Seubert, 2018; Kuznetsov et al., 2008). Saponin-permeabilized muscle was loaded in a chamber, contained 2 ml of the respiration medium (EGTA 0.5 mM, $MgCl_2.6H_2O$ 3 mM, taurine 20 mM, KH_2PO_4 10 mM, HEPES 20 mM, BSA 0.1%, potassium-lactobionate 60 mM, mannitol 110 mM, DTT 0.3mM, pH 7.1, adjusted with 5 N KOH) at 30°C. Saline, 30 μM L-citrulline, 1 mM L-citrulline, 100 μM L-arginine or 200 μM L-arginine was added to the chamber too. To determine basal respiration, complex I substrates glutamate (10 mM) and malate (5 mM) were added and oxygen consumption was recorded (State 4 respiration). Then, 1mM ADP was added and ADP-stimulated respiration rate was determined (State 3 respiration). Respiration rate was represented as nmol O_2 consumed per mg protein per minute. The respiratory control ratio (RCR) was calculated as the ratio between ADP stimulated respiration rates (State 3 respiration) and basal (State 4 respiration).

2.2.4. In-vitro Experiments

2.2.4.1. Cell Culture

C2C12 myoblasts (American Type Culture Collection) were cultured in six-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin. Cells were incubated in a water-jacketed CO2 incubator maintained at 37°C with 95% O2 and 5% CO2 (vol/vol). Upon confluence, cells were differentiated into myotubes via DMEM containing 2% horse Serum and 1% (vol/vol) penicillin-streptomycin for 5 days.

2.2.4.2. C2C12 myotubes for Western Bolting

After 5 days of differentiation, C2C12 myotubes were treated with either glucose free DMEM containing 30, 300 μM, 1 mM L-citrulline, 100, 200 μM L-arginine or saline for 2 hr. Treated cells were homogenized in buffer containing 50 mM Tris·HCl (pH 8 at 4°C), 1 mM EDTA, 10% (wt/vol) glycerol, 0.02% (wt/vol) Brij-35, 1 mM DTT, and protease and phosphatase inhibitors, after treated with 0.1 nM and 1nM insulin for 30 min. The cells were scraped off and collected into 1.5 ml Eppendorf tubes. After sonication for 2 min, the cells were kept on ice for 30 min. the tubes were centrifuged at maximum speed (14,000 x*g*) for 10 min, and the supernatant was collected in 1.5 tubes Eppendorf and either stored in -80ºC freezers for future use or thawed for process of protein estimation by Bradford assay. After that, the samples were subjected to western blot protocols as described in 2.2.3.1.

2.2.4.3. C2C12 myotubes for Real-Time PCR

Another set of differentiated C2C12 myotubes were treated with the same concentration of L-citrulline and L-arginine for 24 hr, and harvest pellet cell for RNA isolation. Starting with adding 100 μl of trypsin to each well and leaving the plate at 37°C incubator for 5 min, then 1 ml of complete media (10% FBS DMEM media) was added in each well. Then, the cells were collected

in 1.5 ml tubes and centrifuged at maximum speed for 20 sec. The cell pellet was obtained by aspirating the supernatant from the tubes. 1 mL of PBS was added to cell pellet and slowly pipette up and down to not generate bubbles in order to resuspend the pellet for 30 sec to 1 min. The tubes were centrifuged again at maximum speed for 20 sec and the supernatant was removed. The resultant cell pellet, either was stored in –80°C freezers or 500 µL TRiazol reagent was added for RNA extraction, followed by cDNA preparation and Real-Time PCR Quantification as prescribed in 2.2.3.2.

2.2.5. Statistical Analysis

All values are presented as means ± mean of standard error (SEM) using the statistical program Graph Pad Prism version 6.0c. The Significant differences were determined by the use of an unpaired two-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc analysis, as we have two parameters (Treatment, Diet). The differences were considered significant when P-value < 0.05.

Chapter 3: Results

3. Results

3.1. L-citrulline supplementation improves obesity-induced glucose intolerance but reveals a trend to worse insulin sensitivity

C57BL/6J mice were fed either a LFD or a HFD and supplemented with either vehicle control (VC) or L-citrulline (100 mg/Kg) in their drinking water for 15 weeks, which had no effect on body weight (Table 3.1) and food intake in both lean and obese mice (Figure 3.2A). Assessment of glucose tolerance following 11 weeks of L-citrulline supplementation revealed an improvement in glucose tolerance only in obese mice (Figure 3.1A/B), which may be attributed to changes in circulating insulin levels, as the increase in insulin at 30-min versus 0-min of the GTT was showed a trend to increase in both lean and obese L-citrulline supplemented mice (Figure 3.1C).

Table 3.1: Effect of L-citrulline supplementation on body weight and body composition in lean and obese mice

	Body Weight			Lean mass	Fat mass	% Fat mass
	Week 1	Week 7	Week 15			
LFD Control	24.666±0.486	28.233±0.643	29.183±0.595	22.575±1.325	3.27±0.6	11.659±2.515
LFD L-citrulline	23.9±0.75	27.316±1.108	28.35±1.127	22.586±0.843	3.84±0.026	13.283±0.464
HFD Control	25.483±0.749	45.6±1.386*	50.616±0.578*	24.997±0.666	19.1±0.835*	40.586±0.693*
HFD L-citrulline	26.5±0.573	41.64±3.017*	48.98±2.028*	25.4±0.728	15.9±0.943#	35.828±1.145#

The body weight and body composition were measured via magnetic resonance imaging in lean and obese mice treated with L-citrulline (n = 3-8). Values represent mean ± SE. $^{*}P<0.05$, indicates a significant difference from LFD Control. $^{\#}P<0.05$, indicates a significant difference HFD Control. LFD = low fat diet, HFD = high fat diet.

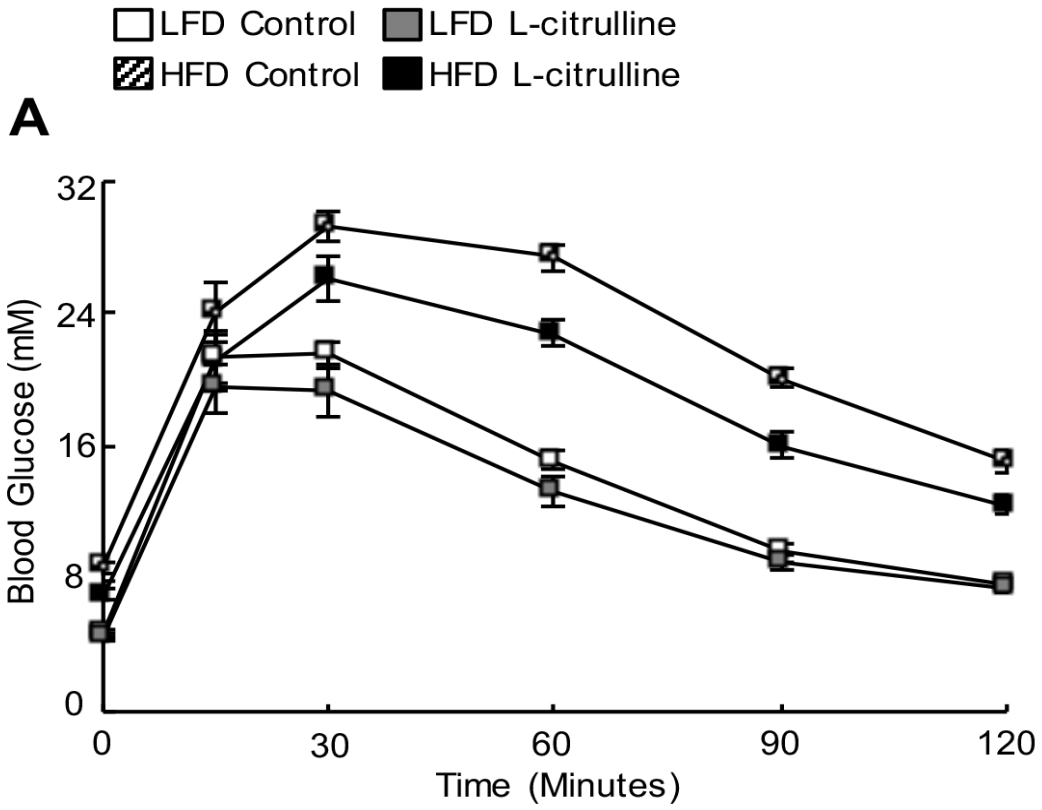

Figure 3.1: L-citrulline supplementation improves glucose homeostasis in obese mice.

(A) Glucose tolerance in lean and obese mice treated with either VC or L-citrulline (n = 10-15). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis.

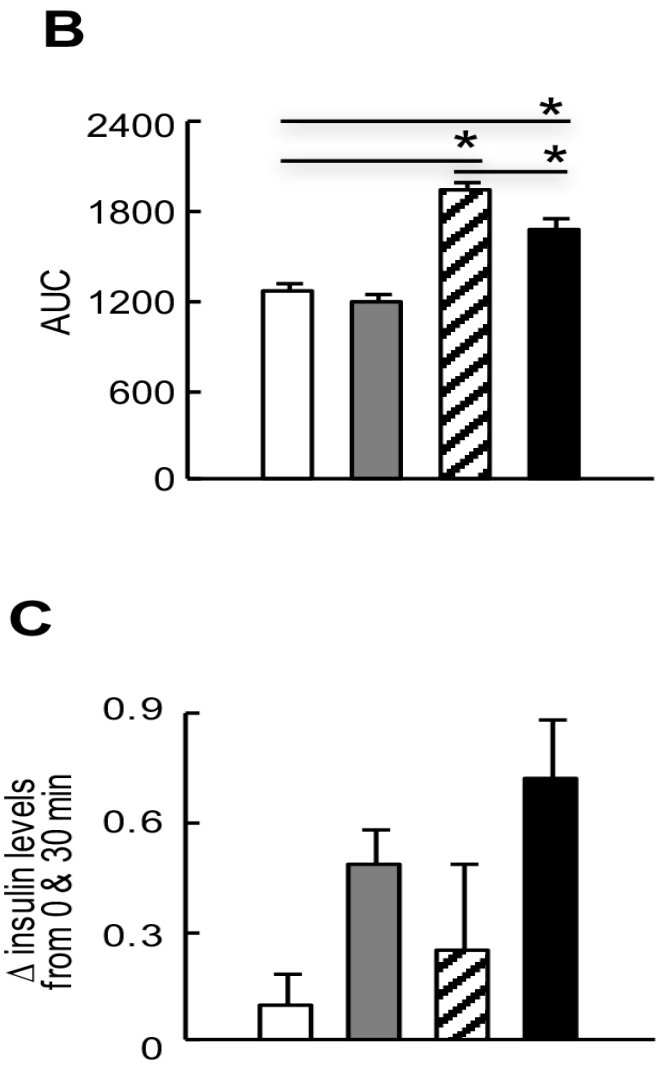

Figure 3.1: L-citrulline supplementation improves glucose homeostasis in obese mice.

(B) Area under the curve during the glucose tolerance test (n = 10-15). (C) Plasma insulin levels during the glucose tolerance test at 0 and 30 min post-glucose administration (n = 5-11). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *$P < 0.05$.

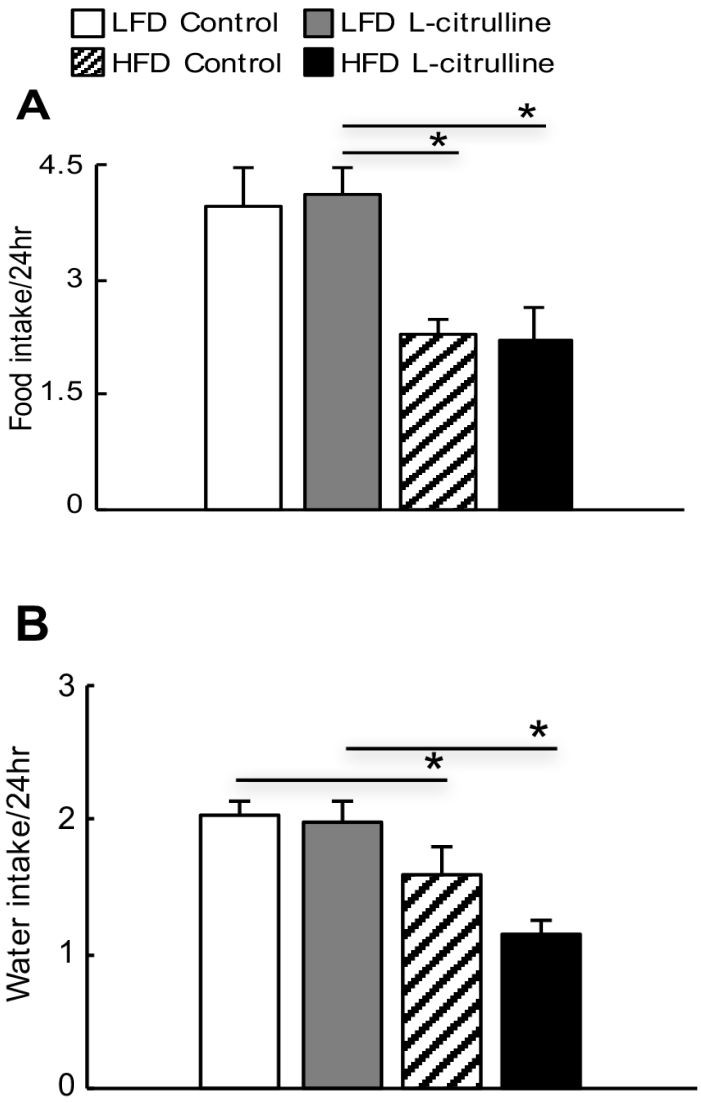

Figure 3.2: Food and water intake for lean and obese mice treated with either VC or L-citrulline. (A) Twenty-four-hour food intake for lean and obese mice treated with either vehicle or L-citrulline (n = 4-7). (B) Twenty-four-hour water intake for lean and obese mice treated with either VC or L-citrulline (n = 4-7). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *$P < 0.05$.

Conversely, L-citrulline supplementation revealed a trend to worse insulin tolerance in obese mice (Figure 3.3A/B). This worsening was associated with a trend to impairment in insulin signaling, as insulin-stimulated Akt phosphorylation in gastrocnemius but not soleus muscles was nonexistent when compared to VC supplemented mice, though no differences in insulin-stimulated glycogen synthase kinase 3β (GSK3β) phosphorylation were observed (Figure 3.3C/D). Of interest, these adverse actions of L-citrulline on skeletal muscle insulin tolerance/signaling may be indirectly mediated, since direct treatment of differentiated C2C12 myotubes with pharmacological concentrations of L-citrulline (2 hr and followed by 30 min insulin treatment), did not worsen insulin-stimulated Akt or GSK3β phosphorylation (Figure 3.4A/B). In addition, our observations are not due to boosting L-arginine concentrations, as direct treatment of differentiated C2C12 myotubes with pharmacological concentrations of L-arginine (2 hr and followed by 30 min insulin treatment), did not worsen insulin-stimulated Akt or GSK3β phosphorylation (Figure 3.5A/B).

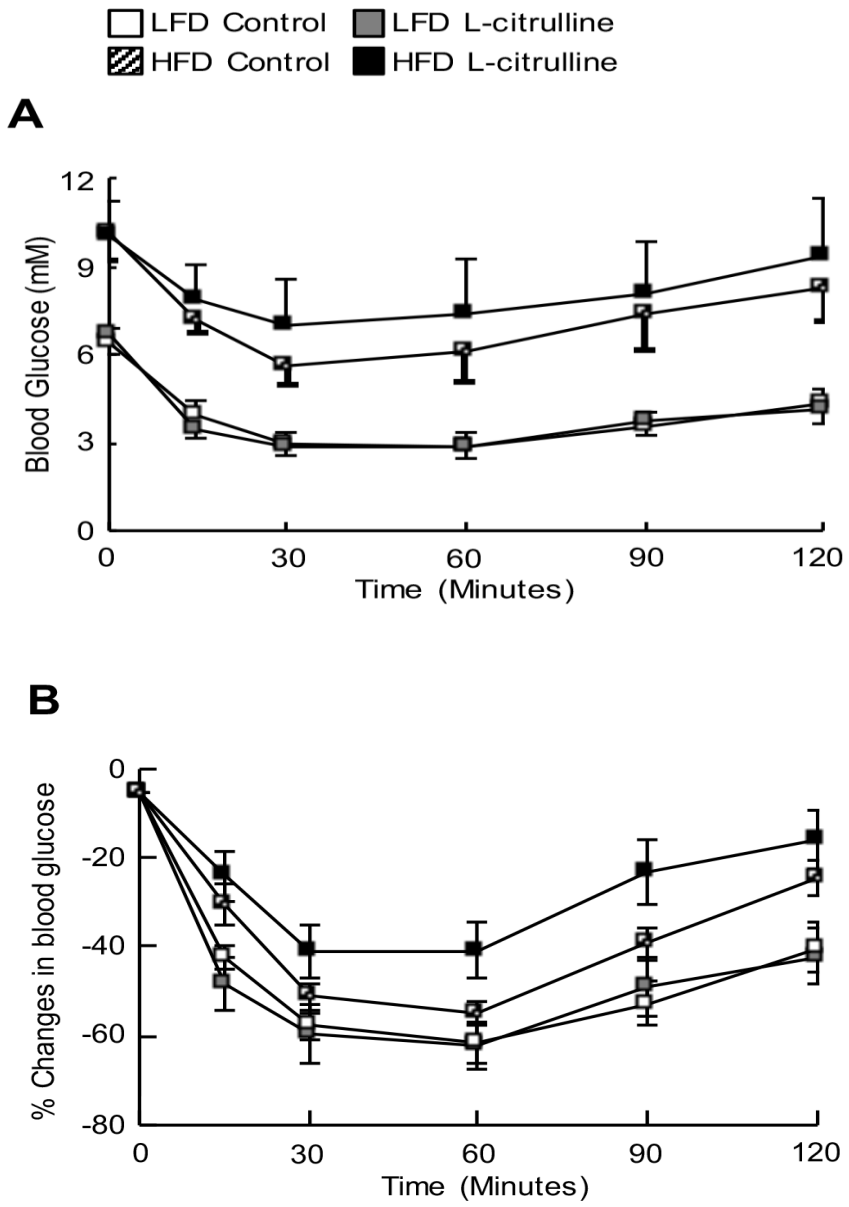

Figure 3.3: L-citrulline supplementation reveals a trend to worse insulin tolerance test in obese mice.

(A) Insulin tolerance in lean and obese mice treated with either VC or L-citrulline (n = 7-17). (B) Normalized glucose level during insulin tolerance test (n = 7-17). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis.

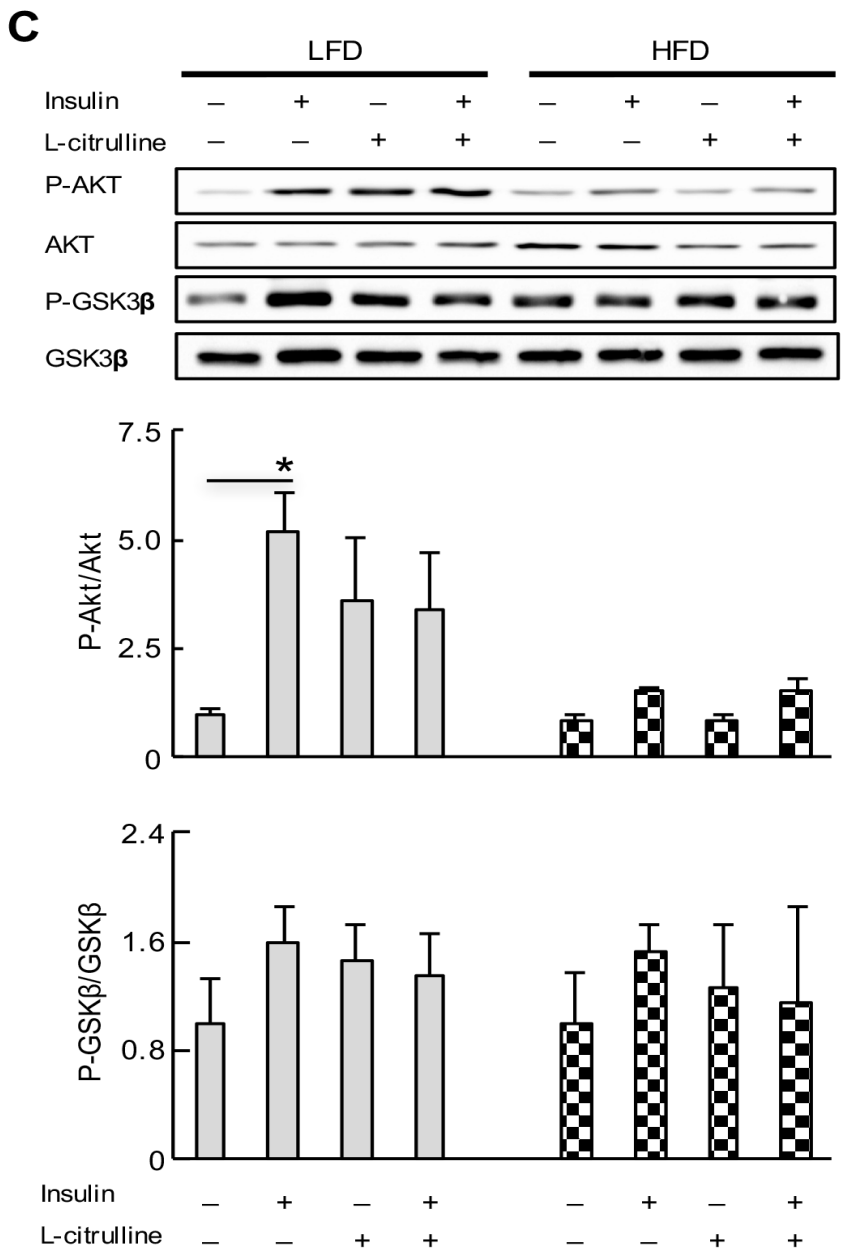

Figure 3.3: L-citrulline supplementation reveals a trend to worse insulin tolerance test in obese mice.

(C) Insulin signaling (Akt and GSK3β phosphorylation) in soleus from both lean and obese mice treated with either VC or L-citrulline (n = 4). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *$P < 0.05$.

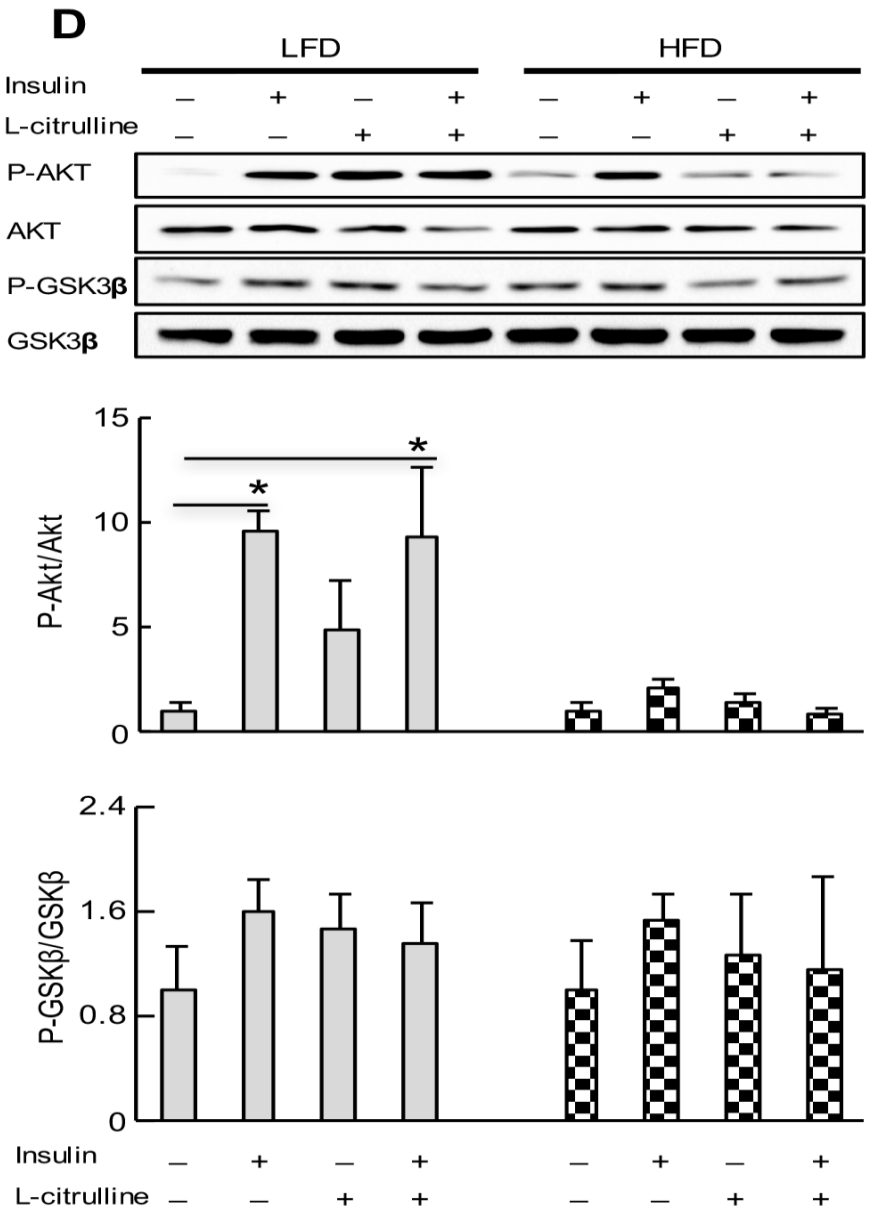

Figure 3.3: L-citrulline supplementation reveals a trend to worse insulin tolerance test in obese mice.

(D) Insulin signaling (Akt and GSK3β phosphorylation) in gastrocnemius from both lean and obese mice treated with either VC or L-citrulline (n = 4). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *$P < 0.05$.

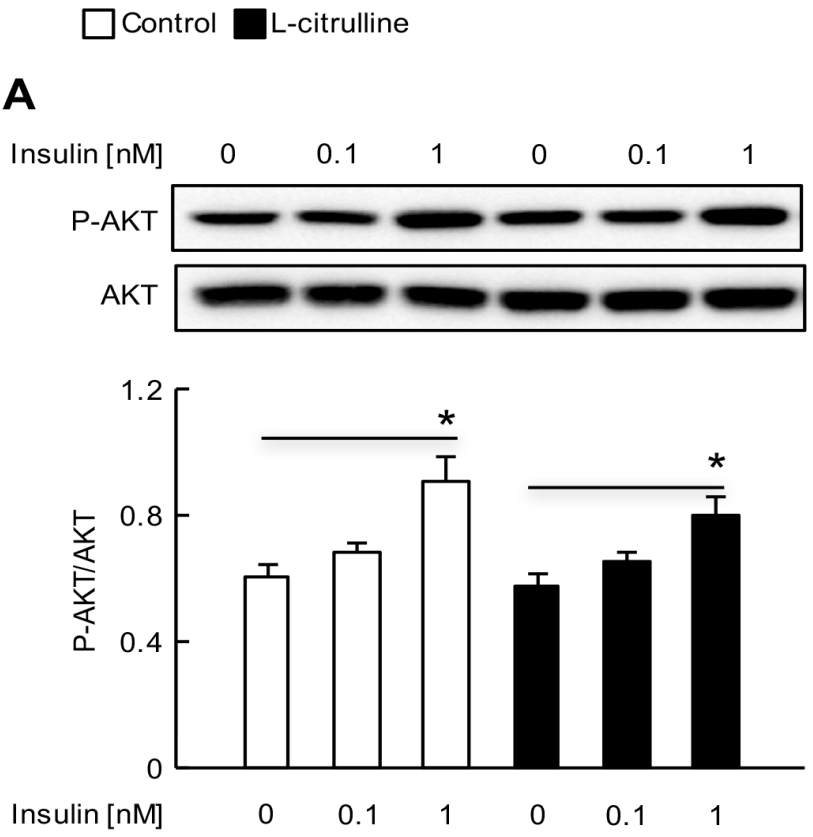

Figure 3.4: L-citrulline supplementation does not affect insulin signaling in C2C12 myotube cell line.

(A) Insulin signaling (Akt phosphorylation) in C2C12 cells treated with either VC or L-citrulline and then stimulated with insulin for 30 min. (n = 8). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *$P <$ 0.05.

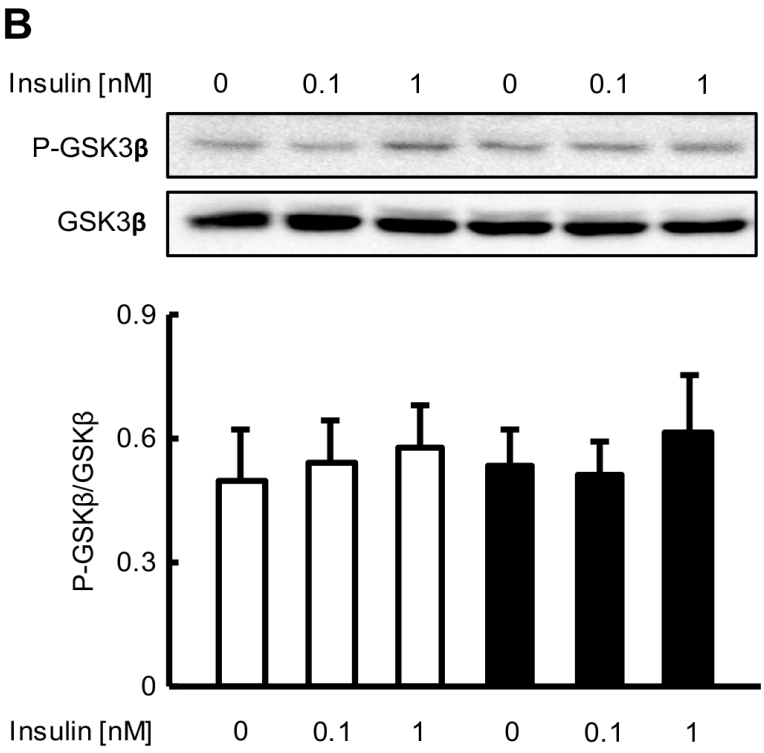

Figure 3.4: L-citrulline supplementation does not affect insulin signaling in C2C12 myotube cell line.

(B) Insulin signaling (GSK3β phosphorylation) in C2C12 cells treated with either VC or L-citrulline and then stimulated with insulin for 30 min. (n = 8). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis.

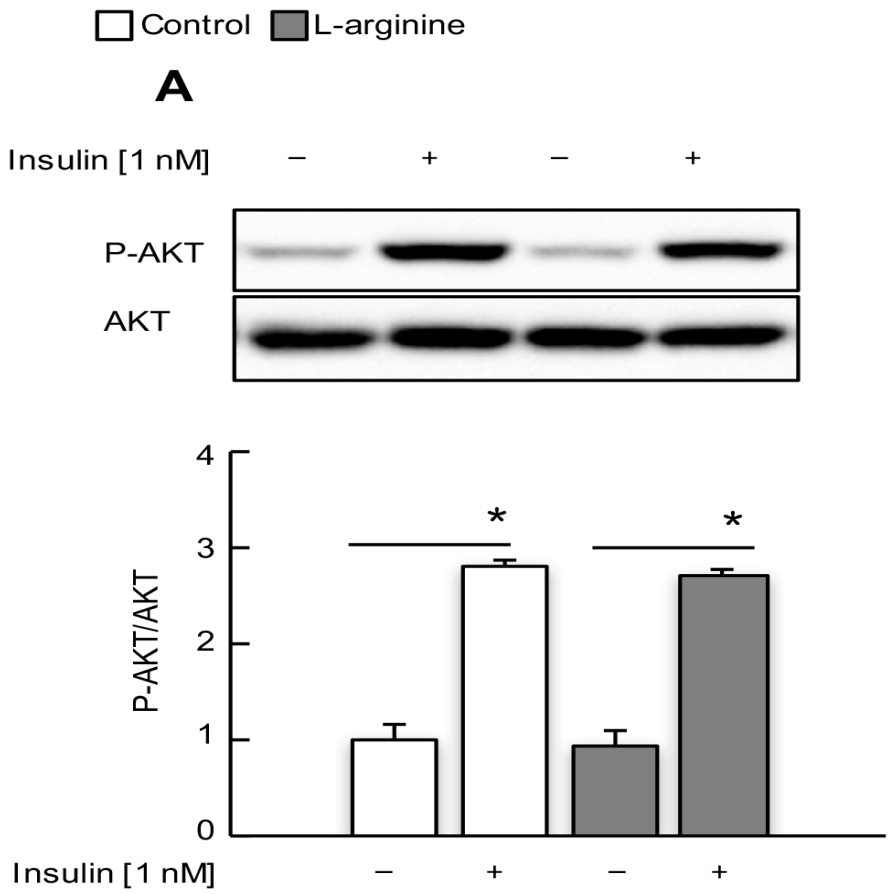

Figure 3.5: L-arginine supplementation does not affect insulin signaling in C2C12 myotube cell line.

(A) Insulin signaling (Akt phosphorylation) in C2C12 cells treated with either VC or L-arginine and then stimulated with insulin for 30 min. (n = 6). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *P < 0.05.

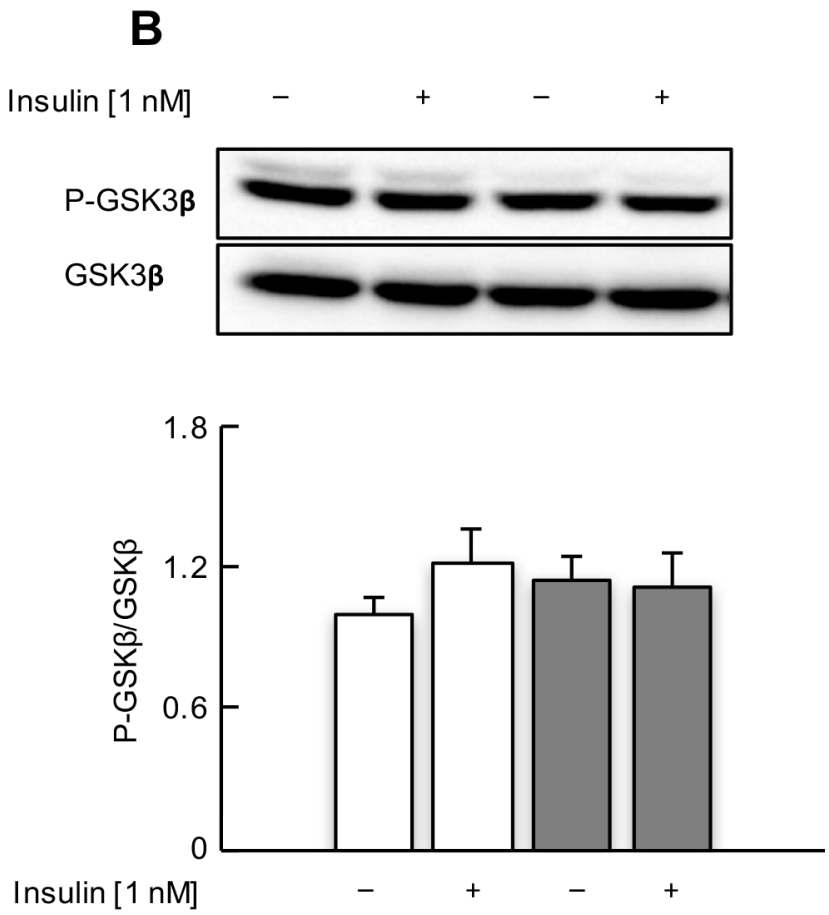

Figure 3.5: L-arginine supplementation does not affect insulin signaling in C2C12 myotube cell line.

(B) Insulin signaling (GSK3β phosphorylation) in C2C12 cells treated with either VC or L-arginine and then stimulated with insulin for 30 min. (n = 6). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis.

3.2. L-citrulline supplementation increases exercise capacity in both lean and obese mice

To evaluate the effect of L-citrulline supplementation on exercise performance, both lean and obese mice supplemented with L-citrulline for 8 weeks were run on a forced exercise treadmill at gradually increasing speeds until exhaustion. Consistent with previous studies, experimental obesity impaired exercise capacity in mice (Fukushima et al., 2014; Ussher et al., 2010), while our findings demonstrated that L-citrulline has salutary actions on exercise performance, as we observed significant increases in the time and distance that both lean and obese mice were able to run on the forced exercise treadmill (Figure 3.6A/B).

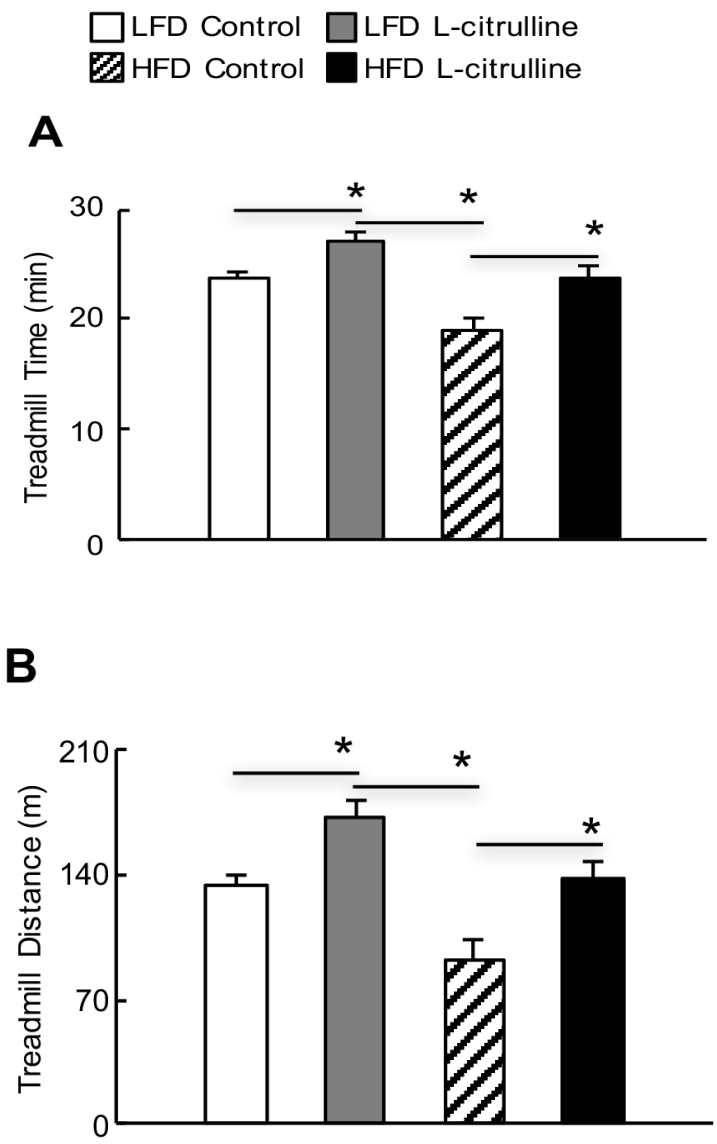

Figure 3.6: L-citrulline supplementation increases an aerobic exercise capacity in both lean and obese mice.

(A) Treadmill time in lean and obese mice treated with either VC or L-citrulline (n = 5-6). (B) Treadmill distance in lean and obese mice treated with either VC or L-citrulline (n = 10-11). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *$P < 0.05$.

3.3. L-citrulline supplementation increases whole-body consumption rates but does not impact substrate preference in obese mice

We next assessed *in vivo* energy metabolism in lean and obese mice following 10 weeks of L-citrulline supplementation via indirect calorimetry, which revealed significant increases in whole-body oxygen consumption rates in obese mice during the initial hours of the dark cycle (Figure 3.7A/B). This increase was associated with a trend to increased ambulatory activity in obese mice (Figure 3.8A/B), but was not associated with changes in substrate preference, as respiratory exchange ratios were similar in both lean and obese mice supplemented with L-citrulline (Figure 3.9A/B). To further support our observations that L-citrulline supplementation does not modify substrate preference, and to assess whether L-citrulline has direct actions on muscle mitochondria that enhance respiration, we quantified respiratory control ratios in permeabilized fibres from gastrocnemius muscles of lean mice. Direct treatment of permeabilized fibres with pharmacological concentrations of L-citrulline or L-arginine had no impact on mitochondrial respiration rates or respiratory control ratios (Figure 3.7C).

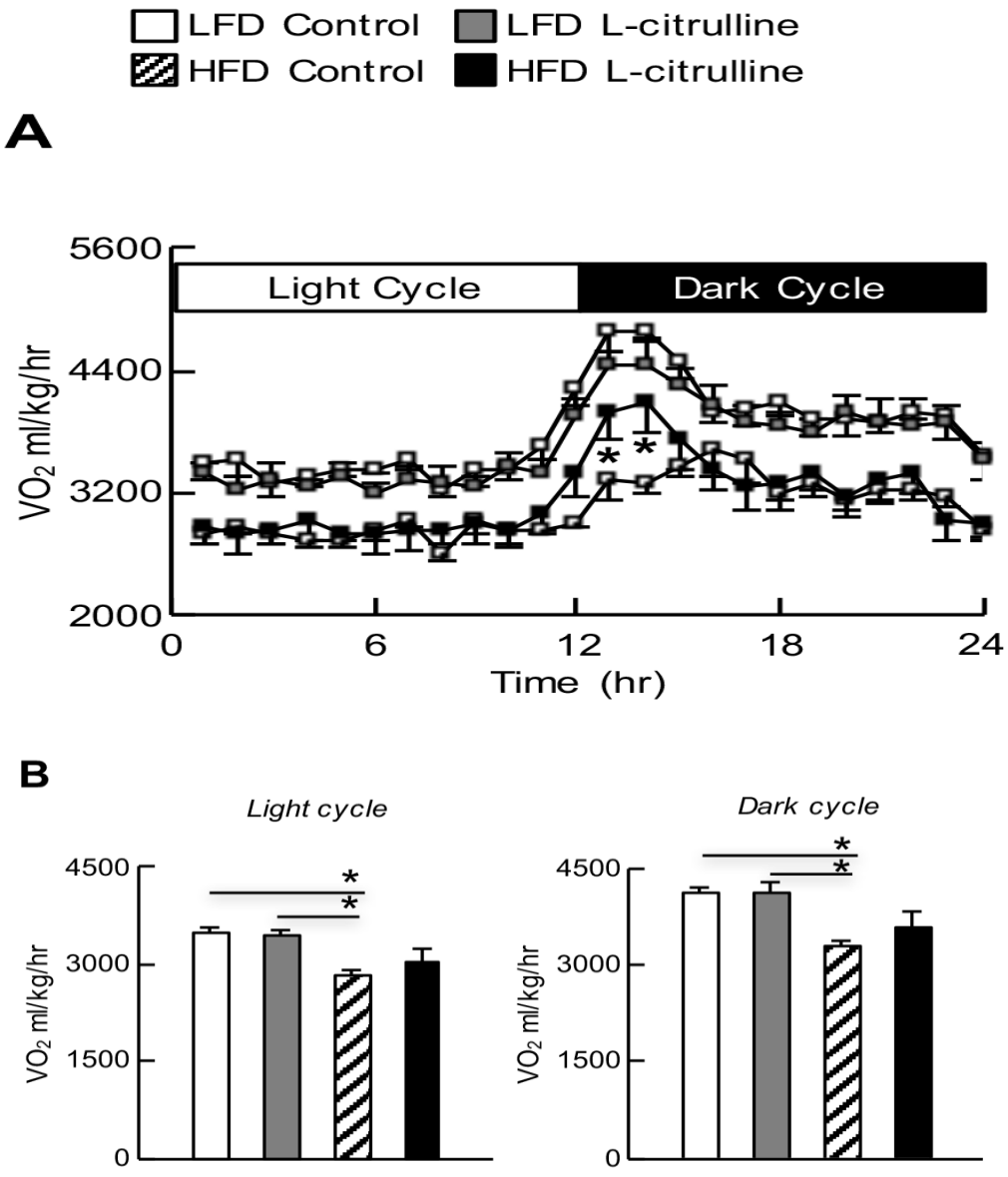

Figure 3.7: L-citrulline supplementation improves whole-body oxygen consumption rates in obese mice.

(A) Twenty-four-hour (B) Light and Dark cycle whole-body oxygen consumption rates in lean and obese mice treated with either VC or L-citrulline (n = 4-7). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *$P < 0.05$.

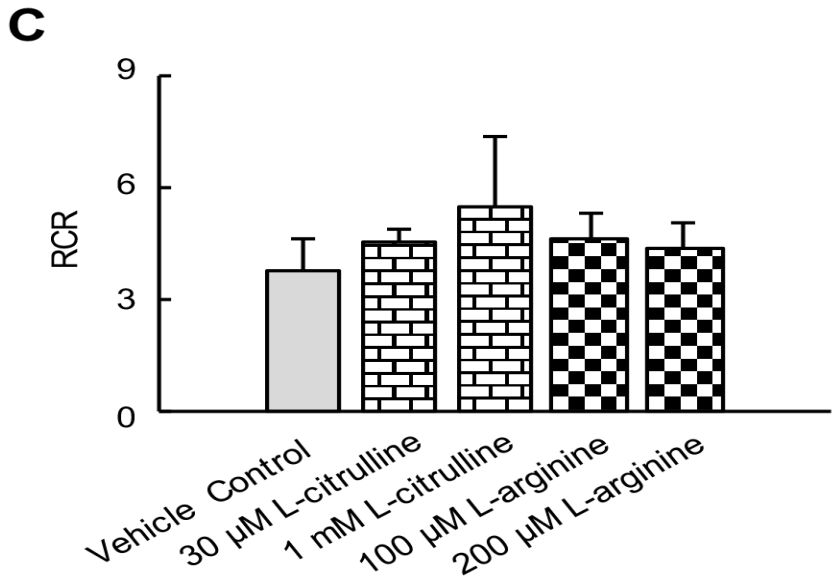

Figure 3.7: L-citrulline supplementation improves whole-body oxygen consumption rates in obese mice.

(C) Respiratory control ratio (RCR) of direct treatment of perimeablized muscles with VC, L-citrulline or L-arginine (n = 5-6). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis.

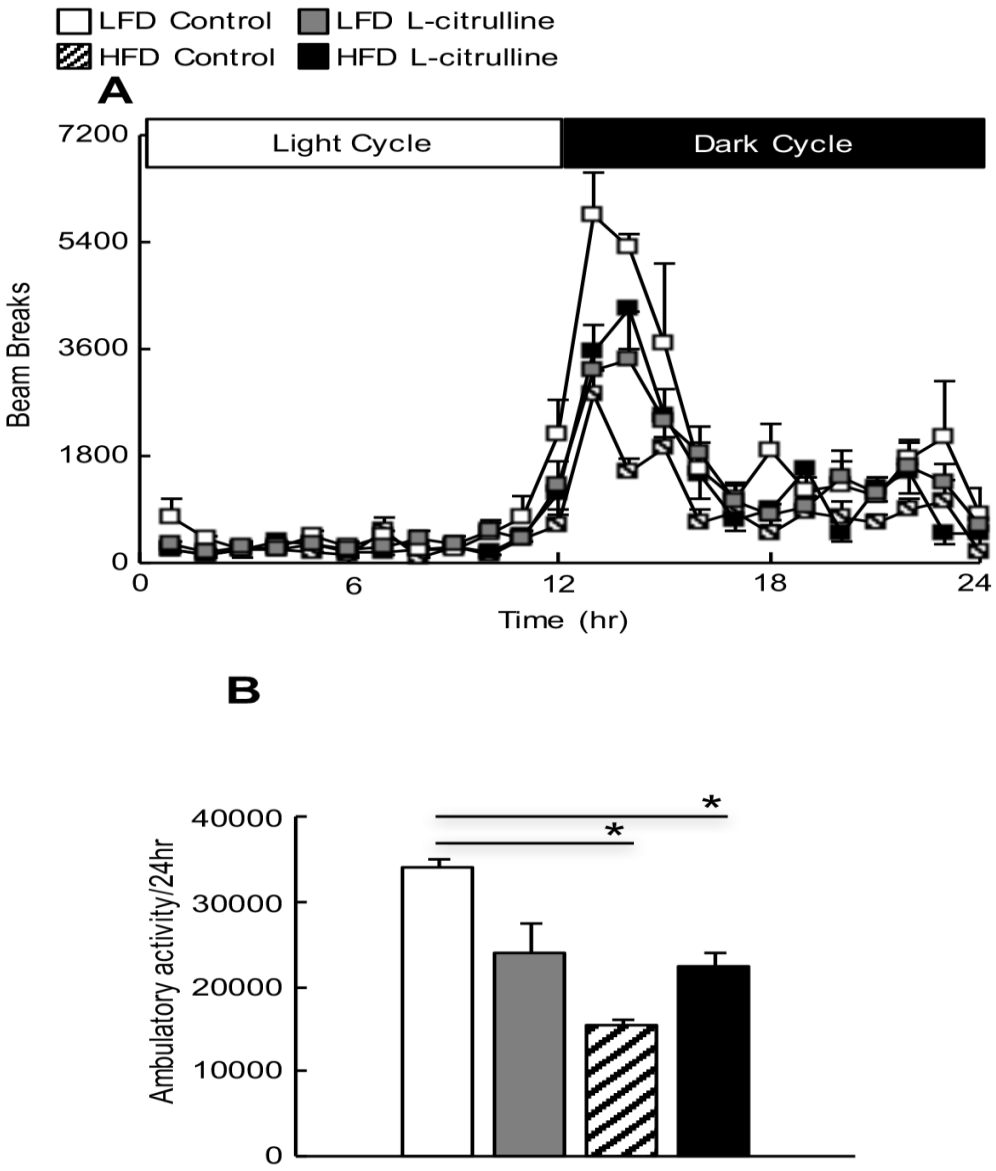

Figure 3.8: L-citrulline supplementation shows a trend to increased ambulatory activity and increases heat production in obese mice.

(A) & (B) Twenty-four-hour ambulatory activity for lean and obese mice treated with either VC or L-citrulline (n = 4-7). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *$P < 0.05$.

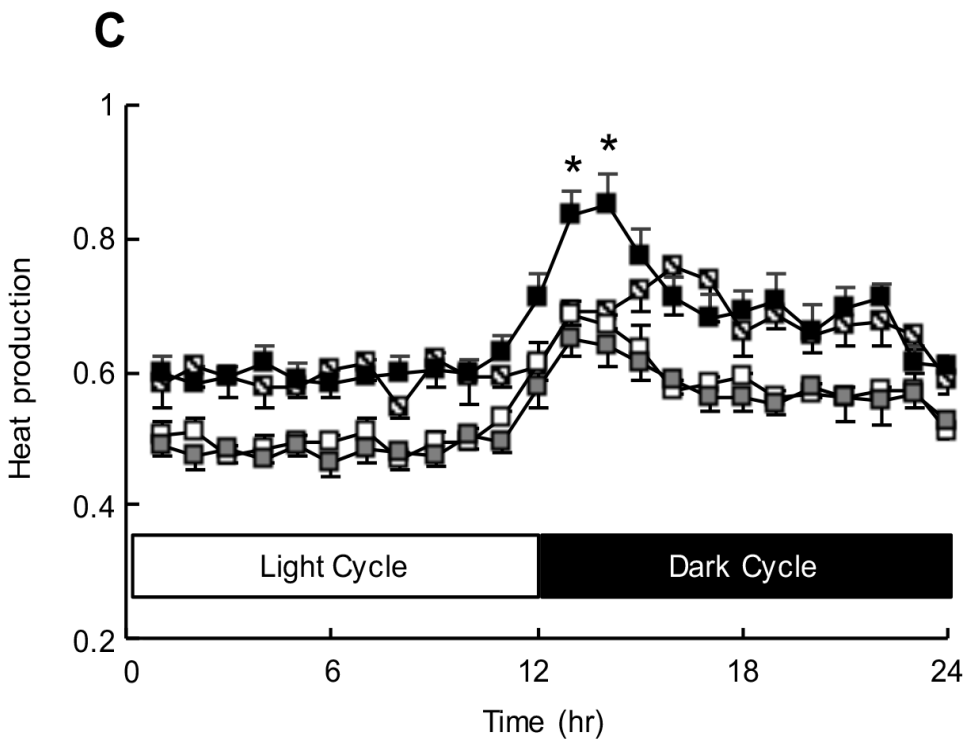

Figure 3.8: L-citrulline supplementation shows a trend to increased ambulatory activity and increases heat production in obese mice.

(C) Twenty-four-hour heat production for lean and obese mice treated with either VC or L-citrulline (n = 4-7). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *$P < 0.05$.

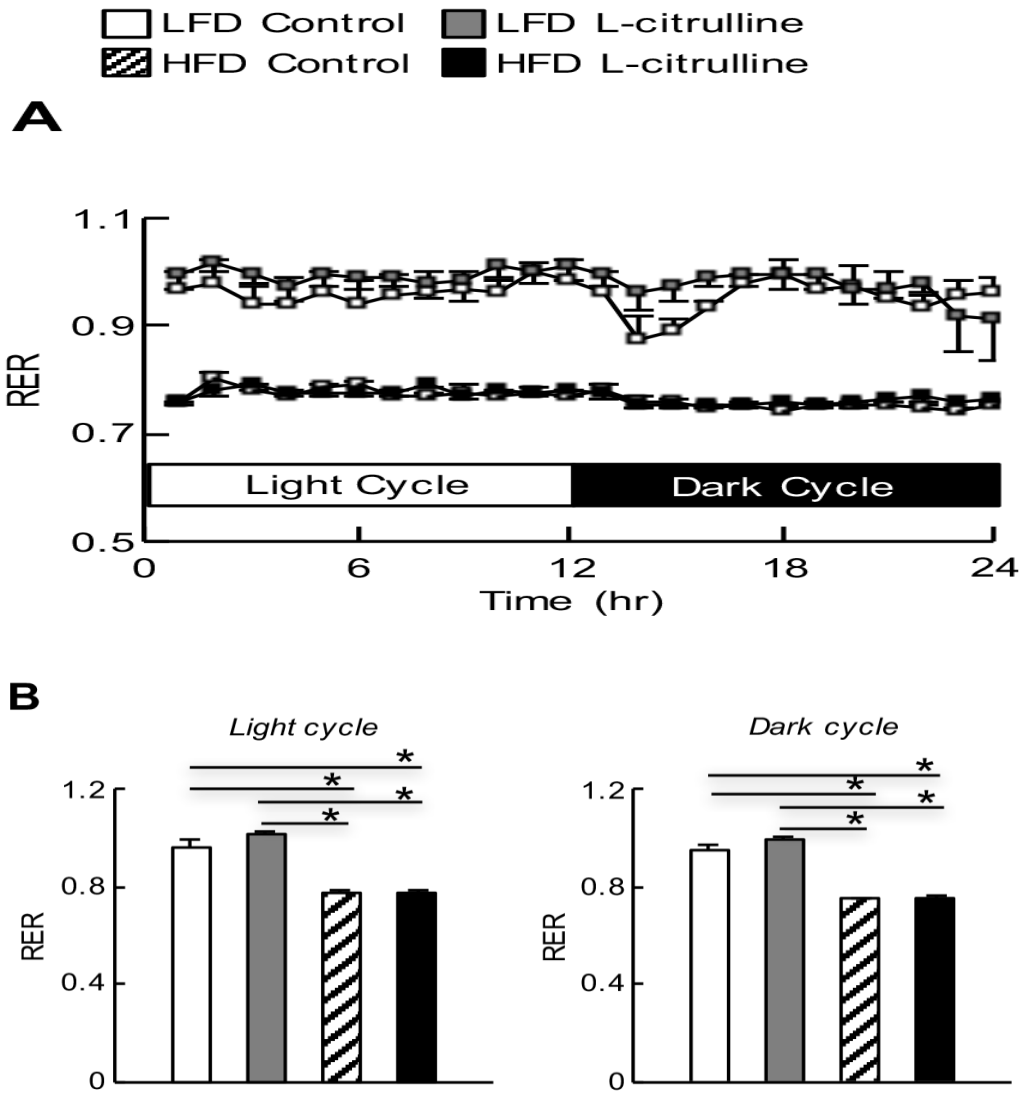

Figure 3.9: L-citrulline supplementation does not affect substrate preference in lean and obese mice.

(A) Twenty-four-hour (B) Light and Dark cycle respiratory exchange ratio in lean and obese mice treated with either VC or L-citrulline (n = 4-7). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *$P < 0.05$.

3.4. L-citrulline supplementation reverses obesity-induced impairments in the expression of key regulators of mitochondrial function/biogenesis

Because L-citrulline has been shown to improve muscle performance and aerobic energy metabolism in preclinical and clinical studies (Bendahan et al., 2002; Benoît Giannesini et al., 2011; Pérez-Guisado & Jakeman, 2010; Villareal et al., 2018), we quantified mRNA expression for a number of factors associated with the regulation of mitochondrial function and/or biogenesis (Diaz & Moraes, 2008; Dillon et al., 2012; Handschin & Spiegelman, 2008). Experimental obesity resulted in significant reductions or trends to reductions in the mRNA expression of peroxisome proliferator activated receptor gamma coactivator-1 α (*Ppargc1a*), nuclear respiratory factor 1 (*Nrf1*), mitochondrial transcription factor A (*Tfam*), and mitochondrial transcription factor B2 (*Tfbm2*) in gastrocnemius muscles, the majority of which were prevented via supplementation with L-citrulline (Figure 3.10A). Conversely L-citrulline supplementation had no effect on the expression of *Ppargc1a*, *Nrf1*, *Tfam*, or *Tfbm2* in soleus muscles, and experimental obesity had minimal impact on the expression of these genes when compared to their lean counterparts (Figure 3.10B). To determine whether the actions of L-citrulline on gastrocnemius muscle mRNA expression profiles were due to direct actions on the muscle, we treated differentiated C2C12 myotubes with increasing concentrations of L-citrulline. Similar to our *in-vitro* observations assessing insulin signaling, direct treatment of C2C12 myotubes with L-citrulline had negligible influence on the mRNA expression of *Ppargc1a*, *Nrf1*, *Tfam*, or *Tfbm2* versus their saline treated counterparts (Figure 3.10C). Moreover, to determine whether these *in-vivo* improvements were due to increases in L-arginine levels, we treated differentiated C2C12 myotubes with physiological and pharmacological concentration of L-arginine. Our results showed no changes in mRNA expression of all these genes (Figure 3.11A).

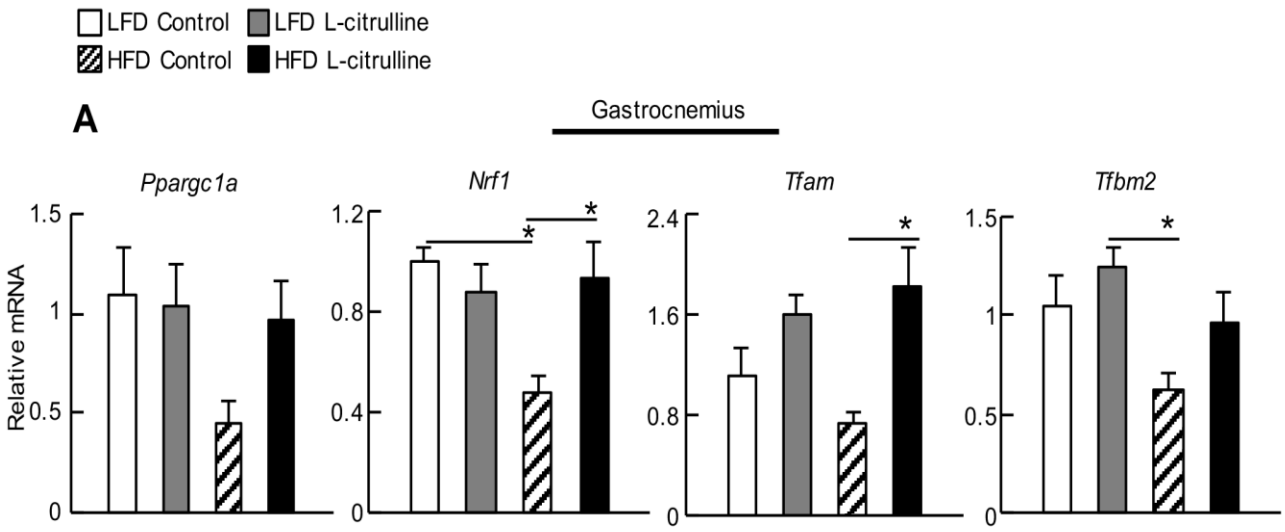

Figure 3.10: mRNA expression of factors that regulate mitochondrial biogenesis/function in mice and C2C12 myotubes treated with L-citrulline.

(A) mRNA expression of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargca*), mRNA expression of Nuclear respiratory factor 1 (*Nrf1*), mRNA expression of Mitochondrial transcription factor A (*Tfam*), and mRNA expression of Mitochondrial transcription factor b2 (*Tfbm2*) in gastrocnemius of lean and obese mice treated with either VC or L-citrulline (n = 5-6). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *$P < 0.05$.

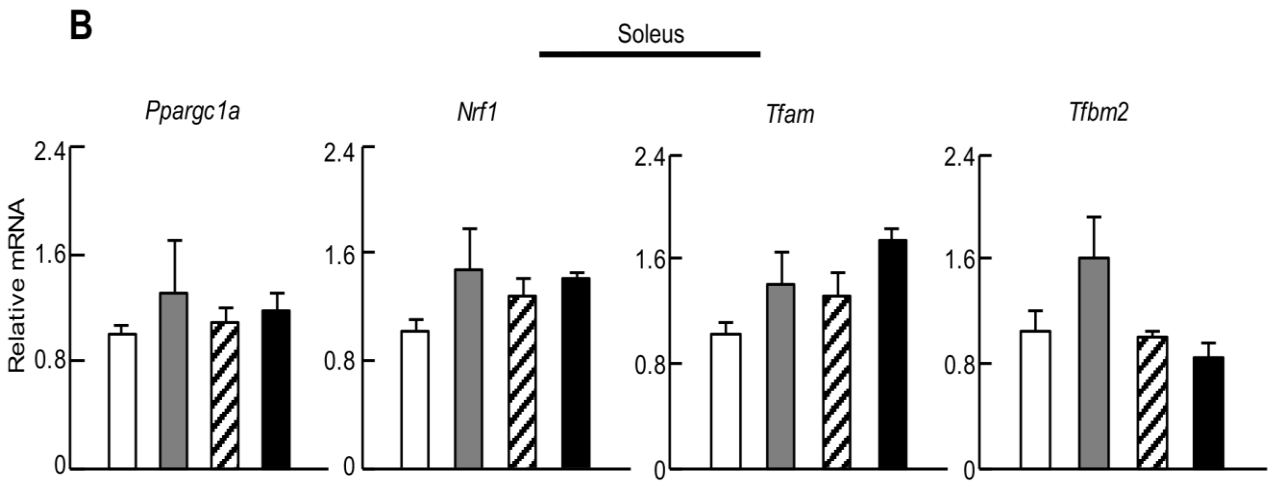

Figure 3.10: mRNA expression of factors that regulate mitochondrial biogenesis /function in mice and C2C12 myotubes treated with L-citrulline.

(B) mRNA expression of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargca*), mRNA expression of Nuclear respiratory factor 1 (*Nrf1*), mRNA expression of Mitochondrial transcription factor A (*Tfam*), and mRNA expression of Mitochondrial transcription factor b2 (*Tfbm2*) in soleus of lean and obese mice treated with either VC or L-citrulline (n = 5-6). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis.

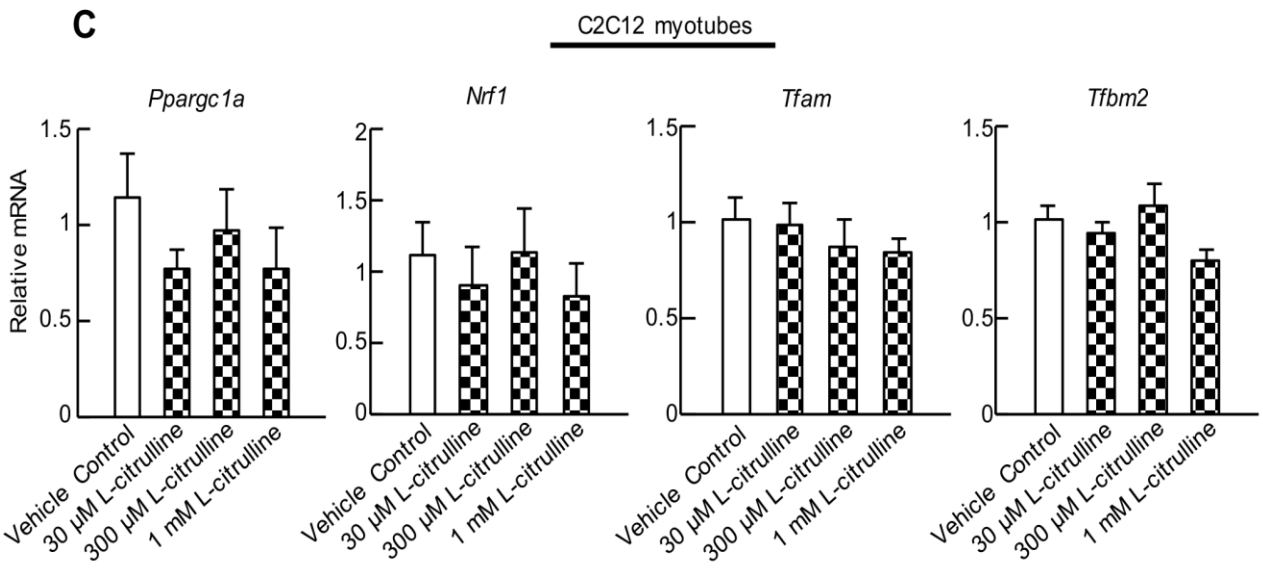

Figure 3.10: mRNA expression of factors that regulate mitochondrial biogenesis/function in mice and C2C12 myotubes treated with L-citrulline.

(C) mRNA expression of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargca*), mRNA expression of Nuclear respiratory factor 1 (*Nrf1*), mRNA expression of Mitochondrial transcription factor A (*Tfam*), and mRNA expression of Mitochondrial transcription factor b2 (*Tfbm2*) in C2C12 myotubes cell line treated with either VC or L-citrulline for 24 hours (n = 4-6). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis.

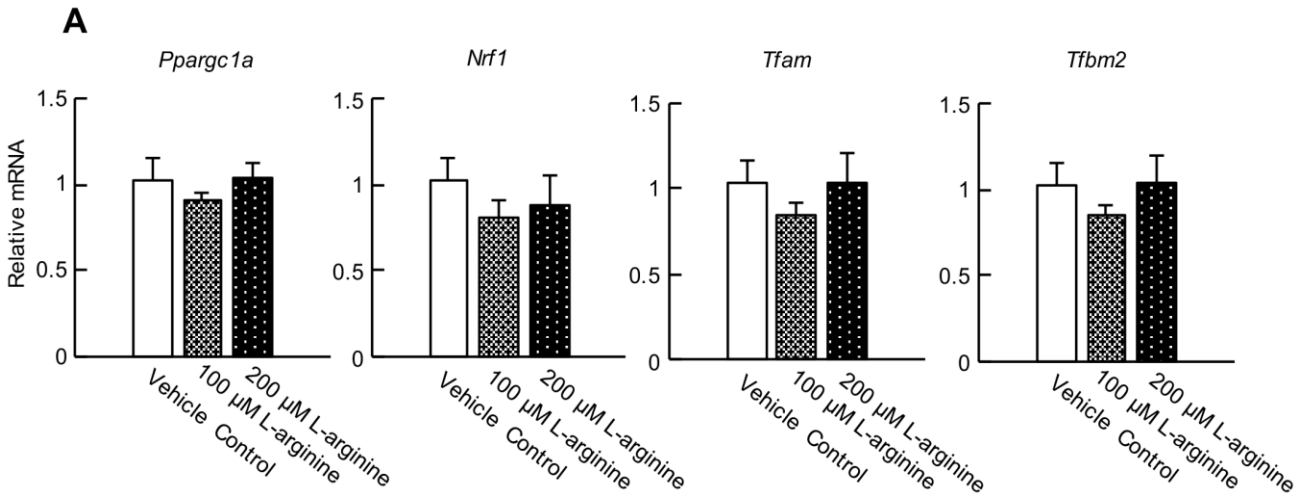

Figure 3.11: mRNA expression of factors that regulate mitochondrial biogenesis/function in C2C12 myotubes treated with L-arginine.

(A) mRNA expression of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargca*), mRNA expression of Nuclear respiratory factor 1 (*Nrf1*), mRNA expression of Mitochondrial transcription factor A (*Tfam*), and mRNA expression of Mitochondrial transcription factor b2 (*Tfbm2*) in C2C12 myotubes cell line treated with either VC or L-arginine for 24 hours (n = 4). Values represent means ± SEM. Differences were determined using a two-way ANOVA, followed by a Bonferroni post-hoc analysis.

Chapter 4: Discussion

4. Discussion

The current study is the first to examine the effect of L-citrulline supplementation on obesity-induced insulin resistance, which is relevant as recent research reported an increase in plasma L-citrulline levels in obese individuals (Verdam et al., 2011). Moreover, as previous studies used L-citrulline in combination with malate, which is one of intermediates of the Krebs cycle, it is essential to examine the effect of L-citrulline alone. Our results support previous findings with L-citrulline supplementation, as we reveal that L-citrulline supplementation improves exercise performance, as both lean and obese mice supplemented with L-citrulline in the drinking water were able to run 28% and 47% longer on a forced exercise treadmill respectively. In addition, novel actions of L-citrulline supplementation on obesity-induced insulin resistance and dysglycemia, where L-citrulline improves glucose homeostasis and this was associated with an increase in whole-body oxygen consumption rates. Moreover, it reverses obesity-induced impairments in the expression of key regulators of mitochondrial function/biogenesis. Surprisingly, L-citrulline supplementation revealed a trend to worse insulin tolerance and insulin signaling in obese mice. Such observations should be taken into consideration for individuals choosing to consume L-citrulline as a nutraceutical supplement in attempts to improve their exercise performance.

The increase we observed in exercise performance in both lean and obese mice supplemented with L-citrulline was expected, as previous studies in humans have reported similar observations as L-citrulline/malate reduced muscle fatigue in 18 men performing finger flexions at 1.5 second intervals lighting a 6 kg weight (Bendahan et al., 2002). It was postulated that this benefit was due to augmented aerobic energy production in exercising muscle, as ^{31}P magnetic

resonance spectroscopy (^{31}P MRS) studies revealed a 34% increase in oxidative ATP production and 20% increase in the rate of phosphocreatine recovery within the exercising muscle. Likewise, citrulline/malate co-supplementation also reduced muscle soreness and repetition number during flat barbell bench presses in 41 male volunteers (Pérez-Guisado & Jakeman, 2010). In preclinical studies, citrulline/malate supplementation improves muscle efficiency in electrically stimulated gastrocnemius muscles from anesthetized Wistar male rats, as seen by decreases in both the phosphocreatine and oxidative costs of contraction during ^{31}P MRS studies (Benoît Giannesini et al., 2011). Conversely, a single dose of L-citrulline failed to improve maximum number of repetitions over 5 sets, time to exhaustion, and maximal oxygen consumption during chest press exercise in 22 volunteer athletes (11 male/11 female) (Cutrufello, Gadomski, & Zavorsky, 2015). Reason's for the discrepancy between these studies remains unknown. However, a possible explanation could stem from the fact that the latter study utilized L-citrulline supplementation alone versus citrulline/malate co-supplementation or used it as a single dose, though Takeda and colleagues have shown that L-citrulline supplementation improves time to exhaustion and reduces circulating lactate levels during swimming exercise in ICR mice (Takeda, Machida, Kohara, Omi, & Takemasa, 2011).

The inclusion of malate as a co-supplement is thought to account for the potential improvement in aerobic energy metabolism, since malate is a key intermediate of the Krebs Cycle (Gibala, Young, Taegtmeyer, & Taegtmeyer, 2000). Our results support the findings of Takeda and colleagues, however, since L-citrulline supplementation alone was sufficient to improve aerobic capacity on a forced exercise treadmill. Moreover, it has been reported that mitochondrial dysfunction results from obesity-induced insulin resistance (Watt et al., 2006). It could be our

obese, insulin resistant mice have impaired mitochondrial function, as we observed reduced expressions of some transcription factors in high-fat diet mice and in whole-body oxygen consumption rates. The RER ratios for both obese mice treated with saline or L-citrulline are close to 0.7, which indicates these mice are using fat as their primary source of energy, and there are no impairments in muscle fatty acid oxidative capacity. However, we observed increases in whole-body oxygen consumption rates only in L-citrulline supplemented obese mice, suggesting that mitochondrial function was improved via L-citrulline supplementation. Because PGC-1α is a key regulator of mitochondrial biogenesis/function and aerobic energy metabolism in skeletal muscle (Chan & Arany, 2014; Dillon et al., 2012), we explored whether L-citrulline supplementation enhances PGC-1α expression, though no differences were observed in both gastrocnemius and soleus muscles from lean mice when compared to their vehicle control-supplemented counterparts. Such observations contrast studies in lean mice supplemented for 15-days with L-citrulline, as Villareal and colleagues demonstrated L-citrulline mediated increases in swimming exercise performance, which was associated with an upregulation of PGC-1α expression in gastrocnemius muscles (Villareal et al., 2018). The incompatibility of our observations with those of Villareal and colleagues could be due to the fact that our mice were not frequently exercising and we simply assessed performance during a single aerobic exercise challenge. In addition, our daily dose of L-citrulline supplementation was significantly lower, which could also explain why we did not observe increased gastrocnemius muscle *Ppargc1a* expression. Although L-citrulline did not have direct actions in lean mice resulting in increased *Ppargc1a* expression, L-citrulline supplementation reversed the impairment of experimental high-fat diet-induced obesity on gastrocnemius but not soleus muscle *Ppargc1a*, *Nrf1*, *Tfam*, or *Tfbm2* mRNA expression. Because these genes all represent key regulators of mitochondrial biogenesis/function and subsequent

energy metabolism (Dillon et al., 2012; Handschin & Spiegelman, 2008), preventing obesity's actions on this mRNA expression profile in skeletal muscle may explain the prevention of obesity mediated reductions in whole-body oxygen consumption rates we observed. Reason's for why obesity does not decrease the expression of these genes in soleus muscle is not clear, but could be due to the fact that as a much more oxidative red muscle, the soleus has adaptive mechanisms in place to offset the detrimental actions of obesity on oxidative gene expression. It should be noted that the L-citrulline mediated increases we observed in whole-body oxygen consumption rates were relatively mild despite being statistically significant, and were only seen during the initial hours upon the transition to the dark cycle of the mouse. As skeletal muscle accounts for ~20-30% of resting energy expenditure (Zurlo, Larson, Bogardus, & Ravussin, 1990), we do posit that L-citrulline's actions on skeletal muscle, in particular white skeletal muscle (e.g. gastrocnemius), contribute to the increase in whole-body oxygen consumption rates we observed in obese mice.

We initially surmised that our *in-vivo* observations in mice were due to direct actions on skeletal muscle, as studies have shown that L-citrulline acts directly on skeletal muscle myocytes *in-vitro*, where it can enhance muscle protein synthesis and prevent wasting (Ham et al., 2015; Plénier et al., 2017). However, differentiated C2C12 myotubes treated with L-citrulline demonstrated no changes in *Ppargc1a*, *Nrf1*, *Tfam*, or *Tfbm2* mRNA expression, suggesting that perhaps indirect actions account for the skeletal muscle phenotype we observed in obese mice supplemented with L-citrulline. L-citrulline supplementation has been proposed as a novel approach to increase circulating arginine concentrations since arginine can be recycled from citrulline, while L-arginine is a precursor for nitric oxide (NO) synthesis, and NO has been shown in numerous studies to improve mitochondrial function (Baldelli, Lettieri Barbato, Tatulli,

Aquilano, & Ciriolo, 2014). We therefore assessed whether increases in L-arginine could account for our *in-vivo* observations following L-citrulline supplementation of obese mice, but treatment with pharmacological levels of L-arginine also had no effect on *Ppargc1a*, *Nrf1*, *Tfam*, or *Tfbm2* mRNA expression. It will be important for future studies to elucidate the indirect mechanisms by which L-citrulline supplementation prevents obesity-mediated impairments in the expression of genes regulating mitochondrial function in white muscle, and whether this contributes to improvements in aerobic exercise performance. In contrary, according to Villareal et al, L-citrulline supplementation increases expression of PGC-1α in C2C12 myotubes (Villareal et al., 2018). In fact, C2C12 myotubes are normal cells, therefore, it is important for future studies to treat insulin resistant muscle with either L-citrulline or L-arginine to confirm our findings.

It is valuable to measure mitochondrial respiration rates as a marker of mitochondrial function (Schuh, Jackson, Khairallah, Ward, & Spangenburg, 2012). As expected, direct treatment of muscles with L-citrulline did not produce any changes in mitochondrial oxygen consumption. This finding supports that the improvement in whole body consumption was not due to the direct effect of L-citrulline on muscle. Moreover, it was not due to boosting L-arginine, as a direct treatment of gastrocnemius muscle with L-arginine did not increase mitochondrial respiration rates. Therefore, we assume that another indirect mechanism could account for these L-citrulline-mediated effects. To confirm L-citrulline supplementation and its effect on mitochondrial respiration, it is crucial to repeat this experiment on muscle isolated from control and L-citrulline supplemented obese and lean mice.

These improvements in whole-body oxygen consumption were associated with a trend to increased ambulatory activity and an increase in heat production in treated obese mice during the initial hours upon the transition to the dark cycle. This suggests that L-citrulline supplementation shows a trend to improvement in impaired activity due to diet-induced obesity.

Importantly, it has been suggested in numerous studies that interventions leading to increased mitochondrial function can protect against obesity-induced dysglycemia. For instance, a study showed that receptor-interacting protein 140 (RIP140) deletion enhances mitochondrial oxidative energy metabolism and improves insulin sensitivity in both obese and aged mice (Seth et al., 2007). Heat therapy (20 min at 41ºC) in obese rats improves mitochondrial function and skeletal muscle insulin sensitivity (Gupte et al., 2009). In addition, exercise training in older obese adults increases mitochondrial function and type 1 muscle fibre content, which is associated with marked improvements in insulin sensitivity (Dubé et al., 2008). Our results support the notion of mitigating the obesity effects by enhancing mitochondrial biogenesis/function, as we observed that obese mice supplemented with L-citrulline had a significant decrease in blood glucose levels in response to a glucose tolerance test. Thus a novel finding of L-citrulline supplementation is dependent on feeding state in reduction of blood glucose as these improvements were associated with a trend to increased circulating insulin levels. But, we did not observe any difference in blood glucose levels in the fasting state, in contrast with Wu et al observation, as they showed a decrease in glucose serum concentrations in Zucker Diabetic Fatty Rats after 6 hr fasting (Wu et al., 2007). Reason behind this incompatibility could be a high dose of watermelon juice used comparing with our dose. Moreover, another study in humans was consistent with our study, as it also reported no difference in blood glucose levels in the resting state. However, it showed no changes in plasma

insulin levels after high-intensity exercise, suggesting a reduction in nitric oxide-mediated pancreatic insulin secretion or increased insulin clearance due to L-citrulline administration (Hickner et al., 2006). Therefore, it will be important for future studies to determine whether L-citrulline has direct actions on the islet β-cell that could account for our findings. In addition, there may be liver effects, as liver also controls carbohydrate metabolism and therefore the balance between the muscle and liver regulation of glucose uptake and output, respectively, may contribute to overall blood glucose homeostasis in our study (Szablewski, 2011). Therefore, further studies are essential to assess role of the liver in these improvements by *in-vivo* and *in-vitro* studies. We also assessed whether L-citrulline may be improve glucose homeostasis in obese mice via improving muscle insulin sensitivity, but to our surprise, L-citrulline supplementation showed a trend to worse insulin tolerance in obese mice. This worsening was associated with a trend to impairment in insulin stimulated Akt phosphorylation in gastrocnemius but not soleus muscle, which was also unexpected since gastrocnemius muscle was where we observed prevention of the obesity-induced decline in *Ppargc1a*, *Nrf1*, *Tfam*, or *Tfbm2* mRNA expression. It is worth noting that despite numerous studies postulating that enhancing mitochondrial function can improve insulin sensitivity and glycemic control, others have suggested that increasing oxidative metabolism in the absence of elevated energy demand can actually overload mitochondria and impair insulin sensitivity and overall glucose homeostasis (Koves et al., 2008; Muoio & Neufer, 2012). As several studies suggest improvement in insulin sensitivity due to exercise (Dubé et al., 2008), it could be that insulin sensitivity improved if our supplemented mice were frequently exercising. Although the mechanism by which L-citrulline supplementation may demonstrate a trend to impairment in muscle insulin signaling/sensitivity remains unknown, it may involve mTORC1 signaling, as L-citrulline has been shown to activate mTORC1 and its downstream target

S6K (Plénier et al., 2017). Moreover, it has been reported that amino acid infusion increases activation of S6K1 and deactivation of IRS-1 via inhibitory phosphorylation on multiple serine residues, which prevents Akt activation and induces insulin resistance (Tremblay et al., 2005). Similar to what we observed with our mRNA expression profiles, L-citrulline's action on muscle insulin signaling/sensitivity is likely indirectly mediated and not the result of increasing arginine concentrations, as we did not observe impaired insulin-stimulated Akt phosphorylation in differentiated C2C12 myotubes treated with either L-citrulline or L-arginine. Also, we cannot exclude the fact C2C12 cells are immortalized, and it is important to repeat our experiments on insulin resistant muscle cells or primary human muscle cells.

Our results thus showed that there was no difference in body weight, but fat mass significantly decreased in obese mice treated with Lcitrulline; however, our limitation was that we did not measure fat mice prior to supplementation. This reduction was independent of food intake, as there was no difference in food consumption between control and L-citrulline supplemented obese mice. It is essential to investigate the mechanism of this beneficial effect of L-citrulline, which whether attributed to NO-mediated action or other mechanism, as regulation of the arginine–NO pathway could result in reduction in unfavorable fat mass in animals (Jobgen, Fried, Fu, Meininger, & Wu, 2006). Moreover, we do not know whether whole-body oxygen consumption rates were lower in control obese mice because of increased fat mass, as we did not normalize whole-body oxygen consumption to lean mass of our mice.

Another thing that should be taken in our consideration is that water intake through 24 hr for obese mice supplemented with L-citrulline was less than vehicle control group during the

metabolic cages measurement. This inconsistency may be due to the fact that mice are likely under stress in a small, single cage without environment enrichment, as we observed that both lean and obese mice consumed less water than average drinking water in a C57 mouse, which is ~ 5 mL (Bachmanov et al., 2002). This will suggest that it is important in future studies to measure the level of L-citrulline and analyze its correlation with all parameters measured in our study.

Our study was also limited by its focus on the muscle as the only target of L-citrulline action. Thus, it is recommended to determine whether L-citrulline may also affect mitochondrial biogenesis/function in heart, liver, islet β-cells and adipose tissue, as mitochondrial function has also been reported to play a critical role in these tissues (Vernochet et al., 2012; Weksler-Zangen et al., 2013). As we observed that obese mice supplemented with L-citrulline had a significant decrease in blood glucose levels in response to a glucose tolerance test, these improvements were associated with a trend to increase in circulating insulin levels. Therefore, it will be important for future studies to determine whether L-citrulline has direct actions on the islet β-cell that could account for our findings. Furthermore, assessment of respiration in permeabilized muscle fibres to verify whether mitochondrial oxygen consumption increased in lean and obese mice treated with L-citrulline.

Taken together, our study supports findings from previous studies implicating the nutraceutical L-citrulline as an aerobic performance enhancer, with new findings demonstrating that these actions are preserved in obesity, and that L-citrulline also attenuates obesity-induced dysglycemia, as well as obesity induced mitochondrial dysfunction. Nevertheless, the trend to a deterioration in insulin sensitivity following L-citrulline supplementation in obese mice suggests

that its broad use as a potential nutraceutical should be minimized, particularly in obese subjects. Further verification in preclinical studies and in clinical trials to reveal the beneficial effects and the possible adverse effects with long term L-citrulline intake both in healthy and obese individuals is required.

Future directions:

As our supplement L-citrulline reverses obesity-induced dysglycemia, as well as obesity induced mitochondrial dysfunction, and these benefits are not due to the direct action or due to boosting L-arginine levels, it is important to determine the mechanism of action. Therefore, it will be important for future studies to determine whether L-citrulline has direct actions on the islet β-cell that could account for our findings of a trend to increased circulating insulin levels. Moreover, it will be important for future studies to elucidate the indirect mechanisms by which L-citrulline supplementation prevents obesity-mediated impairments in the expression of genes regulating mitochondrial biogenesis/function in white muscle, and whether this contributes to improvements in aerobic exercise performance. In contrary, according to Villareal et al, L-citrulline supplementation increases expression of PGC-1α in C2C12 myotubes (Villareal et al., 2018). Our study reveals that a direct treatment of gastrocnemius muscle with L-citrulline or L-arginine did not increase mitochondrial respiration rates. Hence, we assume that another indirect mechanism could be behind L-citrulline's effect on mitochondrial biogenesis/function. To confirm whether L-citrulline supplementation increases mitochondrial function/respiration, it is crucial to repeat this experiment on permeabilized fibres isolated from lean and obese mice supplemented with either saline or L-citrulline. In addition, L-citrulline supplementation showed a trend to worse insulin tolerance in obese mice, and this worsening was associated with a trend to impairment in insulin

stimulated Akt phosphorylation in gastrocnemius. Although the mechanism by which L-citrulline supplementation demonstrates a trend to impairment in muscle insulin signaling/sensitivity remains unknown, it may involve mTORC1 signaling, as L-citrulline has been shown to activate mTORC1 and its downstream target S6K (Plénier et al., 2017). Moreover, it has been reported that amino acid infusion increases activation of S6K1 and deactivation of IRS-1 via inhibitory phosphorylation on multiple serine residues, which prevents Akt activation and induces insulin resistance (Tremblay et al., 2005). However, L-citrulline's action on muscle insulin signaling/sensitivity is likely indirectly mediated and not the result of increasing arginine concentrations, as we did not observe impaired insulin-stimulated Akt phosphorylation in differentiated C2C12 myotubes treated with either L-citrulline or L-arginine. Therefore, to determine the mechanism by which L-citrulline shows trend to deterioration in insulin sensitivity, measuring mTORC1 and its downstream target S6K, as well as IRS-1 activities could be useful.

A number of studies utilizing endothelial NOS-/- mice (eNOS-/- mice) have shown that eNOS regulates mitochondrial function (Cook et al., 2004; Duplain et al., 2001; Le Gouill et al., 2007; Ojaimi et al., 2005). Conclusions from these studies implicate NO formation as the key element of the eNOS reaction mediating these changes in mitochondrial function. However, as the other by-product of eNOS enzymatic activity is L-citrulline formation, it is important to determine the relative importance of L-citrulline versus NO formation in eNOS-mediated alterations in mitochondrial function and oxidative phosphorylation. Therefore, lean and obese eNOS-/- mice and their wild-type (WT) littermates will either be supplemented with vehicle control, L-citrulline (100 mg/kg in the drinking water), or S-nitrosoglutathione (GSNO, 2 mg/kg intraperitoneal injection. As GSNO acts as an exogenous NO donor, such studies will allow us to elucidate

whether L-citrulline and/or NO are more critical for eNOS-induced alterations in mitochondrial function. Then, all our experiments should be repeated in this model.

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