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CARNOSINE, A THERAPEUTIC POTENTIAL FOR CRITICAL LIMB  
ISCHEMIC

By

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B.S., Kwame Nkrumah University of Science and Technology, 2008

M.S., University of Louisville, 2015

A Dissertation

Submitted to the Faculty of the  
School of Medicine of the University of Louisville  
In Partial Fulfillment of the Requirements  
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Doctor of Philosophy in Biochemistry and Molecular Biology

Department of Biochemistry and Molecular Genetics

University of Louisville

Louisville, Kentucky

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A Dissertation Approved on

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## DEDICATIONS

This work is dedicated to my dad of blessed memory who encouraged me to pursue a career in the sciences and to my beloved mom without whose care and tireless support I wouldn't have made it this far. I also appreciate the support of my siblings who have been there for me through it all. Lastly but not least to my supportive and loving husband, thank you for your care, sacrifice and understanding.

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## ABSTRACT

### CARNOSINE, A THERAPEUTIC POTENTIAL FOR CRITICAL LIMB ISCHEMIA

Adjoa Agyemang Boakye

July 27<sup>th</sup>, 2016

Critical Limb ischemia (CLI) is a serious manifestation of peripheral artery disease. Advanced CLI patients are poor candidates for vascular surgeries. Numerous studies have shown that hypoxia inducible factor 1 $\alpha$  (HIF1- $\alpha$ ) plays an important role in recovery from murine hindlimb ischemia (HLI). HIF1- $\alpha$  is regulated by oxygen dependent prolyl hydroxylases (PHDs). Previous studies have shown that inhibition of PHDs by metal quenchers and viral delivery of HIF1- $\alpha$  improves blood flow to the ischemic limb. However, clinical trials with these therapies are largely negative partly because they do not address the underlying chronic oxidative stress. Based on recent observations that endogenous histidyl dipeptides such as carnosine can chelate metals and quench reactive carbonyls, we hypothesize that supplementation of carnosine can promote revascularization to enhance wound healing through aldehyde quenching and metal chelation. C57BL/6 mice were subjected to hindlimb ischemia (HLI) surgery by ligating the femoral artery and vein and supplemented with carnosine (1g/L) for 21 days. Laser Doppler analysis showed that blood flow in carnosine treated mice was significantly increased (31 $\pm$ 2%) compared with the non-treated mice (20 $\pm$ 2%) after 14 and 21 days

(carnosine  $50\pm6\%$  vs non-treated  $28\pm4\%$ ;  $p<0.05$ ) of recovery from surgery. Vascular density measured by microfil-perfusion-microCT was significantly enhanced by carnosine treatment compared with non-treated HLI mice. Similarly, muscle regeneration and isolectin staining was significantly increased compared to the non-treated mice. Mobilization of endothelial progenitor cells (Flk<sup>+</sup>/Sca<sup>+</sup>) and VEGF expression in the ischemic limb was significantly increased by carnosine supplementation. Also, 4-HNE protein adduct levels were decreased in ischemic limbs of carnosine treated mice. Levels of carnosine in the ischemic muscle were increased approximately 2 fold compared with non-treated HLI mice. Pre-treatment of C2C12 cells with carnosine and its analogue methyl carbinine, that lacks the ability to quench metals, resulted in increased nuclear levels of HIF1- $\alpha$  and VEGF secretion in hypoxic carnosine treated cells compared with methyl carbinine treated hypoxic cells. Collectively, our results demonstrate that carnosine treatment improves blood flow by increasing HIF1- $\alpha$  stabilization and endothelial progenitor cell mobilization thus, can be used as a safe therapeutic intervention for CLI patients



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## CHAPTER I

### GENERAL INTRODUCTION

#### **Epidemiology of Peripheral Artery Disease**

Recent epidemiological data estimates that non-communicable chronic diseases are the leading cause of morbidity and mortality worldwide [1]. This burden is expected to rise within the next decades because of an increase in exposure to risk factor for these diseases [2]. Therefore efforts have to be put in place to identify risk factors as well as cost-effective treatment and preventive strategies to combat this burden. One best example of this chronic disease condition that is often underdiagnosed is peripheral arterial disease.

Peripheral arterial disease (PAD) refers to narrowing or complete atherosclerotic occlusion of one or more upper or lower extremity artery [3]. Although this definition covers arterial disorders other than the coronary vasculature, most reports and guidelines limit it to occlusive diseases in branches of the lower aorta [4]. It's important to note that non-atherosclerotic PAD such as fibromuscular dysplasia and vasculitis, although rare, also exist [5]. Atherosclerotic PAD is estimated to affect > 200 million people worldwide and approximately 8 million people in the United States [2, 6]. Even though PAD represents a distinct disease state it is also a predisposing factor for coronary artery and cerebrovascular disease [7, 8]. PAD is also the leading cause of morbidity due to the associated

decline in limb function as well as limb loss in certain individuals [9]. Recently, the Institute of Medicine has listed PAD as a high research priority area to reduce mortality and morbidity associated with PAD [10].

The classical symptom for PAD is intermittent claudication which is leg pain resulting from walking or exercise that is relieved at rest [7]. This pain occurs because during walking or exercise metabolic demands of the limbs are not met due to the narrowing of blood vessel thus, limiting the capacity of the circulatory system [2, 11]. This deficit in blood supply exerts the same discomfort as angina for coronary heart disease resulting in calf muscle cramping and fatigue [12]. Most patients with PAD may not exhibit any symptoms of intermittent claudication because they do not subject themselves to exertional exercise, or the atherosclerotic plaque is not sufficient to elicit any hemodynamic burden. Current estimates show that only 10-20% of patients presents with intermittent claudication and the remainder are either asymptomatic or present with symptoms that are atypical [13, 14]. This high prevalence of asymptomatic leg ischemia and exertional pain other than intermittent claudication may explain why PAD is often underdiagnosed. Progressive unabated atherosclerotic burden in PAD results in critical limb ischemia (CLI). CLI is the most severe form of PAD which manifests as ischemic rest pain, gangrene, necrosis and ulcers. CLI is associated with high rates for limb amputations and death [15].

Due to the limitations associated with using intermittent claudication as a diagnostic tool, ankle brachial index (ABI) is routinely used in clinical practice as a diagnostic tool. ABI refers to ratio of systolic pressure at the ankle to that in the

arm. An ABI value of  $<0.9$  is defined as PAD while values between 0.9 and 1 represents borderline values [7]. Values  $> 1$  are considered normal however, in patients with calcified noncompressible lower extremity arteriopathy, ABI values  $> 1.4$  have been reported [16]. Although ABI is highly specific it has a low sensitivity [17]. This is partly due to the fact that ABI only becomes abnormal with significant hemodynamic lesions and diseases of less severity might not be detected [18]. Imaging techniques such as Doppler ultrasound and angiography are employed as diagnostic tools but each of these imaging techniques has their limitations of being either invasive or not providing accurate measurements of the atherosclerotic burden [19]. The lack of good diagnostic tools and clearly defined symptoms explains why for some patients the first manifestation of PAD is critical limb ischemia.

The risk factors for PAD are same as traditional risk factors for cardiovascular disease such as cigarette smoking, diabetes mellitus, hypertension, dyslipidemia and ageing [19-21]. Amongst these risk factors smoking is considered to be the strongest predictor for PAD. Studies have shown that smoking plays a prominent role in PAD compared to other atherosclerotic diseases and cessation of smoking reduces the risk of PAD associated morbidity and mortality [7, 22]. In addition to smoking, ageing is also a potent risk factor for PAD. Recent studies suggest that up to 15% of the adult population (i.e.  $> 45$  years) have PAD [6, 23] and the incidence of age driven PAD is likely to increase [2]. Diabetes mellitus is another important risk factor for PAD. Studies with human diabetics have shown that 1% increase in glycosylated hemoglobin increases the incidence of PAD by



approximately 28% [24]. Diabetes also affects the anatomic distribution of disease leading to a bad prognosis in these individuals [25, 26]. Dyslipidemia and hypertension, though risk factors, contribute less to the pathogenesis of PAD as compared to other vascular events such as myocardial infarction and ischemic stroke [23]. Family history of PAD has also been shown to be a predisposing factor. This is partly due to inheritability of risk factors such as dyslipidemia and diabetes mellitus [18]. Further research into the genetics of PAD may reveal other candidate genes which may act as prognostic markers for PAD. Other risk factors such as obesity, homocysteinemia, C-reactive protein, race and ethnicity have not been studied in details but there are evidences to suggest that they contribute to PAD development [7].

### **Pathophysiology of peripheral artery disease**

The pathophysiology of atherosclerotic PAD is complex involving chemokines, growth factors, cytokines, signaling molecules and different cell types [9, 27-29]. Atherosclerosis, the underlying cause of PAD, refers to buildup of fatty plaques in blood vessels that limits blood flow to the target organs. Exposure of vascular cells to lipoproteins triggers a series of reactions involving endothelial cells, vascular smooth muscle cells and the innate immune system. A cascade of events results in foam cell formation, cell death, calcification and arterial remodeling, that progressively leads to narrowing of the blood vessel [30, 31]. Imaging results from PAD patients show narrowing of lower aorta, femoral, iliac, popliteal and tibial arteries [12, 16]. This narrowing causes intermittent claudication in some individual, but majority of PAD patients are usually asymptomatic [23, 32]. The

hemodynamic changes associated with these occlusion are diagnosed as a reduction in ankle brachial index and is dependent on the location of the occlusion and existence of blood vessels other than the occluded vessel linking the preoccluded and postoccluded sites [9]. Ultimately, supply of nutrients and oxygen to the metabolically active skeletal muscle tissue distal to the site of occlusion is hampered resulting in tissue ischemia.

Prolonged ischemic insult results in skeletal muscle dysfunction that ultimately leads to tissue death and necrosis due to accumulation of toxic metabolites, decrease in intracellular pH, shift to anaerobic metabolism, and ultimately a reduction in cellular energy charge [33]. Although restoring blood flow to the ischemic limb is essential to salvage and restore limb metabolic activity, reperfusion is also detrimental to the muscle [34-36]. The exact mechanisms involved in reperfusion injury are not clear, however, several studies have shown that restoration of molecular oxygen exacerbates reperfusion injury. Studies by Korthuis et al. showed that reperfusion with normal blood causes microvascular dysfunction and myocyte necrosis, however, this effect was attenuated when the skeletal muscle was perfused with anoxic blood [37, 38]. Similarly, studies showing that antioxidant enzymes such as superoxide dismutase and glutathione peroxidase or their mimetics and free radical scavengers exhibit protective effects in hindlimb ischemia and clearly demonstrate a causal role of oxygen derived reactive metabolites in the pathogenesis of reperfusion injury [39-41].

PAD patients experience several bouts of ischemia and reperfusion. During walking or exercise, metabolic demands of muscle exceed oxygen supply due to

arterial narrowing mimicking ischemia and during resting periods, reperfusion occurs in the ischemic limbs. Unlike the heart or brain, atherothrombotic events in the muscle do not cause acute conditions like myocardial infarction or stroke and the mechanism underlying these tissue specific responses to ischemia are not clear. Repeated bouts of ischemia and reperfusion results in ion pump dysregulation, mitochondrial dysfunction, ROS generation, inflammation and apoptosis. These processes if unrestricted result in damage to the muscle fibers [9, 23, 32].

Ischemia reperfusion is followed by the reparative phase that involves stimulation of pathways to ensure repair of damaged tissue and restore organ function [42]. During this stage ROS production occurs in limited amounts which serve as signaling molecules. Minute production of ROS activate transcription of growth factors such as vascular endothelial growth factor (VEGF) that results in revascularization and tissue regeneration [43, 44]. Bone marrow derived mononuclear cells also play an important role in this reparative process [45-48]. However, these adaptive responses are hindered in patients with PAD. Although, most of the pathways elicited in the reparative phase are aimed to restore organ function, chronic activation of these reparative processes contribute to organ failure due to fibrosis [42]. Thus, the symptoms can progress from intermittent claudication to rest pain to chronic non-healing ulcers and culminate in gangrene and eventual amputation.

## **Ischemic Phase Injury**

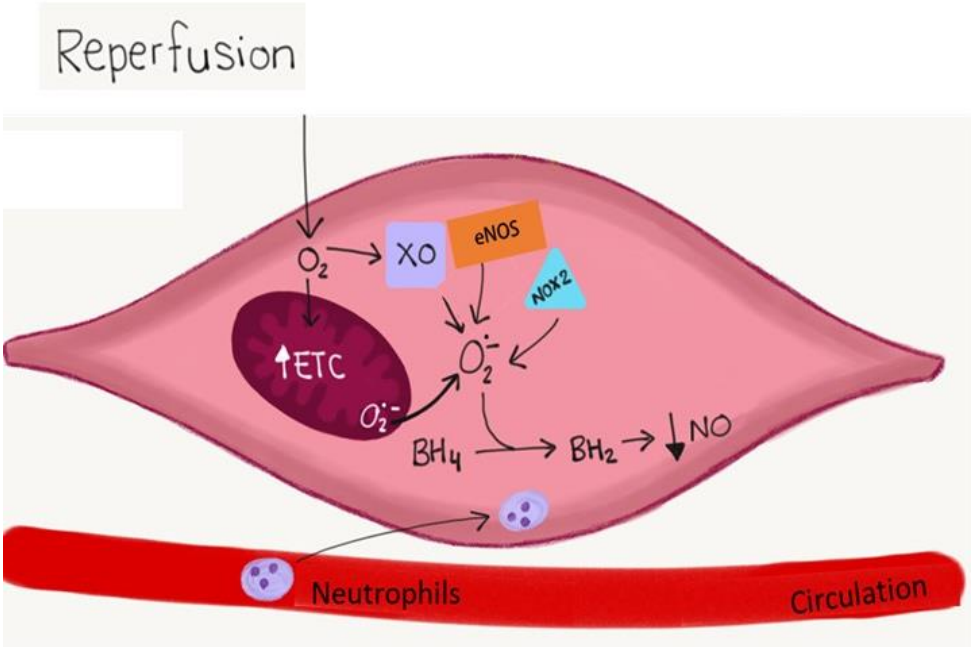
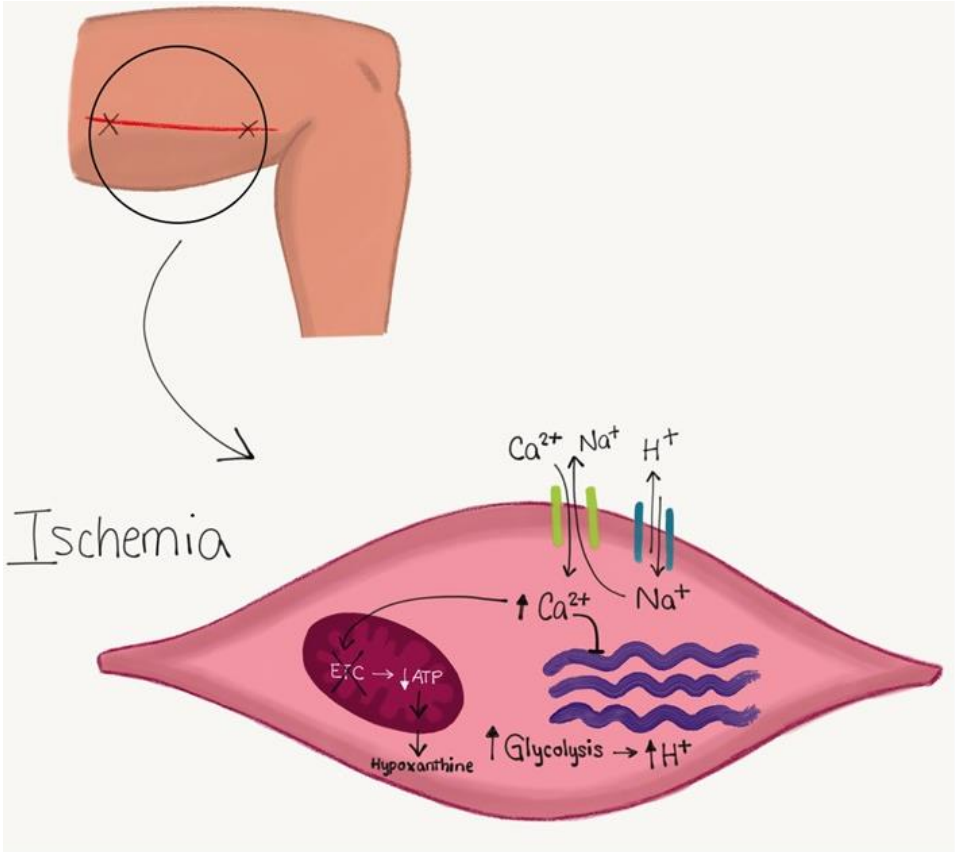
Ischemic insult to a tissue results in tissue hypoxia due to limited or inadequate supply of oxygen. The absence of oxygen prevents aerobic metabolism through the mitochondria resulting in the reliance on anaerobic glycolysis for energy generation [49]. Due to the low efficiency of glycolysis in utilization of cellular energy sources, the decrease in mitochondrial metabolism depletes ATP levels and decreases cellular pH due to accumulation of H<sup>+</sup> ions. Additionally, fatty acid catabolism is decreased due to depleted oxygen levels which results in accumulation of harmful lipids [50, 51]. The absence of an effective circulatory system not only limits the availability of oxygen but also decreases the extrusion of toxic metabolites from the cell. To restore pH to its normal levels, the cell increases extrusion of H<sup>+</sup> leading to increased intracellular transport of Na<sup>+</sup> through Na<sup>+</sup>/H<sup>+</sup> exchanger. Because ATP production is decreased during ischemia, ATP regulated ion pumps such as Na<sup>+</sup>/ K<sup>+</sup> ATPase, that maintains low intracellular Na<sup>+</sup>, is inhibited resulting in increased intracellular Na<sup>+</sup> [42, 52]. The increase in intracellular Na<sup>+</sup> decreases the concentration gradient across the membrane which increases the outward movement of Na<sup>+</sup> through the Na<sup>+</sup>/ Ca<sup>2+</sup> exchanger thereby reversing the function of this exchanger in resting membranes and increasing intracellular calcium (**Fig. 1**) [42].

Intracellular calcium levels are tightly regulated by the Na<sup>+</sup>/ Ca<sup>2+</sup> exchanger and Ca<sup>2+</sup> ATPase which regulates transport across the plasma membrane. Likewise, the ryanodine receptors and ATP- dependent SERCA which functions to regulate sarcoplasmic reticulum calcium transport can affect intracellular Ca<sup>2+</sup> levels [53,

54]. During ischemia  $\text{Na}^+/\text{Ca}^{2+}$  exchanger activity is increased, intracellular calcium levels are elevated and extracellular transport through the membrane bound  $\text{Ca}^{2+}$  ATPase or transport to the sarcoplasmic reticulum through SERCA are both decreased due to depleted ATP levels [55]. This phenomenon of increased intracellular calcium concentration during ischemia is referred to as calcium overload. With the increase in intracellular uptake and diminished uptake into the calcium storing organelle (sarcoplasmic reticulum), mitochondrial transport of  $\text{Ca}^{2+}$  is increased which limits oxidative phosphorylation. The increase in intracellular  $\text{Ca}^{2+}$  also activates  $\text{Ca}^{2+}$  dependent proteases, phosphatases and phospholipases such as protease calpains which degrade cytoskeletal and endoplasmic reticulum proteins leading to cell death [56]. Furthermore, phospholipase activation leads to membrane phospholipid degradation and accumulation of free fatty acids such as arachidonic acid, a prostaglandin inducer. Since membrane phospholipids are important in maintaining membrane structure their degradation disrupts membrane integrity and potentiates the loss of ion pumps and membrane bound receptors [57].

In addition to the above mentioned pathway, ischemia also activates pathways which do not contribute directly to the ischemic phase injury but lay the foundation for reperfusion injury e.g., conformational changes in oxidant producing enzymes, such as xanthine oxidase is induced by the increase in proton generation in ischemia [58, 59].

Figure 1



**Figure 1: Mechanisms contributing to ischemia/reperfusion induced tissue**

**injury**: Ischemia resulting from arterial occlusion leads to increased anaerobic respiration. The ensuing acidosis and reduced ATP levels results in increased intracellular  $Ca^{2+}$ . ATP hydrolysis also leads to the accumulation of hypoxanthine, a substrate of xanthine oxidase (XO). Dysfunctioning of electron transport chain (ETC) and activation of enzymes capable of producing ROS are also initiated. During reperfusion, the return of oxygen leads to exuberant ROS production. ROS could result from xanthine oxidase (XO) which produces superoxide anion as a by-product of hypoxanthine catabolism. NAPDH oxidase (Nox2), and mitochondria represent other sources of ROS during reperfusion. ROS generation results in eNOS uncoupling due to decreased intracellular BH4 levels. This leads to decrease levels of antiahesive NO and subsequent invasion of phagocytic cells.

## **Reperfusion Injury**

During reperfusion, return of molecular oxygen to the ischemic tissues aggravates tissue injury initiated by the initial ischemic insults. Because reperfusion phase of the disease is amendable to treatment it represents a valuable step to study. Although, research over the past decades has improved our understanding of the molecular processes involved in this injury, the exact mechanisms are not completely understood. Numerous studies have shown that ROS generation during reperfusion is the pivotal casual factor in reperfusion injury [33, 42] . ROS are documented to initiate pathways that lead to leukocyte infiltration, inflammation and mitochondrial permeability pore transition [49, 60, 61].

ROS can reacts with lipids to generate highly reactive lipid peroxidation product such as acrolein and 4hydroxynonenal (4HNE) [62]. ROS can also modify functional groups of proteins and can induce damage to DNA [60, 61]. Although extensive research has been conducted to examine the role of ROS regulators e.g., enzymes, mimetics, antioxidants and free radical scavengers as therapeutic interventions in ischemic related diseases such as PAD, the molecular mechanisms underlying reperfusion injury is not fully understood.

### *Sources of reactive oxygen species during reperfusion*

Oxygen is like a two-edged sword that increases our efficiency in utilization of energy deriving substrate but also results in the generation of ROS that cause tissue damage. Under physiological conditions ROS are generated by enzymes such as xanthine oxidase, NADPH oxidase and the electron transport chain [33].



Xanthine oxidase(XO) is a key enzyme of purine catabolism pathway which can exist in two forms; an NAD<sup>+</sup>-dependent dehydrogenase or oxygen dependent oxidase form [58]. During ischemia the dehydrogenase isoform is converted to the oxidase isoform through either proteolysis of a small portion of the enzyme or oxidation of a thiol residue in the enzyme [58, 63, 64]. Moreover, ischemia results in increased degradation of ATP to adenosine, which is deaminated into hypoxanthine, a substrate of xanthine oxidase [33, 65]. The increase in substrate availability primes the enzyme so that once molecular oxygen is provided during reperfusion there is an increase in its catalytic activity resulting in superoxide generation. Studies have shown that xanthine oxidase activity is increased during reperfusion [66]. Additionally, inhibition of xanthine oxidase by inhibitors such as allopurinol prevents reperfusion injury thus establishing a role for xanthine oxidase in reperfusion-induced ROS production [66].

During reperfusion injury NADPH Oxidase contributes to superoxide anion (O<sub>2</sub><sup>-</sup>) generation. Different isoforms of this enzyme are expressed on leukocytes, endothelial cells and mitochondria [67, 68]. All isoforms of this enzyme catalyze the formation of O<sub>2</sub><sup>-</sup> using NADPH as substrate [68]. Unlike the neutrophilic isoform which is activated during infection or tissue damage the vascular isoform, Nox 4, produces low amount of O<sub>2</sub><sup>-</sup> to maintain normal vascular tone through endothelial nitric oxide synthase (eNOS) activation [69]. However, under ischemic conditions even the vascular isoform increases the generation of O<sub>2</sub><sup>-</sup> which is augmented by superoxide production from infiltrating granulocytes [49, 68, 70, 71].

Under physiological conditions oxygen is reduced to water by the electron transport chain however, approximately 2% of molecular oxygen is partially reduced which generates  $O_2^-$  [42, 72]. Mitochondria contributes to 95% of ROS generated under physiological conditions [42]. ROS in the mitochondria are generated primarily from electron leaks in complexes I and III and this phenomenon is increased during reperfusion [42, 73, 74]. This reperfusion associated exuberant ROS production can result due to redox sensitive iron-sulfurs of the mitochondria complexes undergoing oxidation/reduction reactions [75], nitration of complexes I and III and disruption of cardiolipin-respiratory chain super assemblies in complexes I – III [76-78] culminating in loss of activity of these complexes.

In addition to the leaks in the electron transport chain,  $O_2^-$  is generated by monoamine oxidase, growth factor adapter Shc, and mitochondrial Nox 4 [42, 49]. These enzymes are activated through ischemia mediated increased availability of substrate, changes in enzyme conformation or subcellular localization [42]. Furthermore, mitochondrial generated  $O_2^-$  can leak into cytosol, activate mitochondrial anion channel and result in mitochondrial permeability transition [79].

In addition to these ROS generating systems endothelial nitric oxide synthase (eNOS) uncoupling also generates ROS during reperfusion injury. eNOS catalyzes the reaction that leads to the production of vasodilatory and antiadhesive compound, nitric oxide, and requires tetrahydrobiopterin (BH4) as a cofactor. BH4 transfers electrons from oxygen to arginine leading to formation of L-citrulline and

NO. During reperfusion  $O_2^-$  oxidizes BH4 [80, 81] which diminishes BH4 shuttling capacity leading to eNOS uncoupling and additional  $O_2^-$  production. BH4 levels are reduced during ischemia/reperfusion (I/R) injury and treatment with BH4 protects from I/R injury [80].  $O_2^-$  can also react with NO and generate highly reactive nitrosative product [49] Thus, increased ROS generation trigger more ROS production thereby increasing the oxidative stress burden. **Fig. 1** provides a summary of ROS sources during reperfusion.

#### *Mechanisms of oxidant-mediated cellular injury*

ROS can attack the entire array of biomolecules found in the body causing damage to these biomolecules [82]. These damaging effects can occur due to ROS induce peroxidation of membrane lipid, protein crosslinking or degradation, and DNA hydroxylation [49]. These direct effects of ROS could explain some of the phenotype observed during reperfusion. For example peroxidation of membrane lipids affects membrane fluidity and barrier functions which then leads to electrolyte accumulation, cell volume dysregulation, cellular edema and eventual lysis of myocytes [33]. Lipid peroxidation of enzymes can lead to loss of function, modification of enzyme function or even target the enzyme for degradation. For instance oxidative inactivation of the Krebs cycle enzyme aconitase results in the production of hydroxyl radicals by the enzyme thus, enhancing the oxidative stress already present [83]. In addition, second messenger generation could occur due to peroxidation which then disrupt cellular signaling pathways and activates cell death pathways in some instances [84]. ROS can increase the frequency of mutations in DNA and cause changes in transcript sequences accounting for

aberrant protein synthesis. These changes in protein synthesis activate the unfolded protein response leading to endoplasmic reticulum stress and cytotoxic pathway activation [85]. ROS also appear to play a role in the increased microvascular permeability associated with reperfusion of ischemic skeletal muscle. This notion is supported by the observation that administration of free radical scavengers attenuates reperfusion induced microvascular permeability [38, 86].

Mitochondrial ROS generation can lead to mitochondrial permeability transition thereby allowing leakage of mitochondrial contents into the cytosol. For instance leakage of cytochrome c into the cytoplasm can activate proapoptotic signaling pathways [42, 49]. Mitochondrial DNA is particularly susceptible to ROS induced damages due to the lack of protection by histones, unavailability of adequate DNA repair machineries, and proximity to ROS producing sites [42]. Infiltration of activated neutrophils also generate ROS such as hypochlorous acid and superoxide in addition to secretion of hydrolytic factors which results in further tissue injury [33].

### *Inflammation*

Inflammation refers to a complex pathologic response involving an intricate and highly dynamic sequence of events that occurs in affected blood vessels and adjacent tissues in response to an injury or infection. Both infection and injury have been shown to elicit a similar immune response. In the early 1990, Matzinger proposed the danger theory to explain an immune response that encompasses both the response to infection and injury. The danger theory states that an immune

response is triggered by danger signals released by the body's own cells and that the presence of non-self is not sufficient to elicit the immune response [87]. Under stressed conditions such as ischemia, the immune response can be triggered by modification of intra-cellular components e.g. it has been shown that during ischemia calcium overload leads to calcium pyrophosphate complex formation. Also, uric acid accumulates due to xanthine oxidase activation. Both calcium pyrophosphate complex and uric acid have the abilities to bind and activate the inflammasome [88, 89]. Inflammasomes are intracellular protein complexes that mediate the production of cytokines such as TNF- $\alpha$ , activate inflammatory signaling pathways such as NF- $\kappa$ B, as well as activate proapoptotic caspases [88]. These cytokines attract neutrophils to the site of injury.

A large body of evidence suggests that ROS is involved in leukocyte recruitment. Endothelial cells or isolated blood vessels that were exposed to H<sub>2</sub>O<sub>2</sub> became more adhesive to neutrophils [90, 91] which is, in part, mediated by depleted NO levels [92, 93]. Additionally, chemoattractant molecules such as platelet activating factor and leukocyte B4 receptor are upregulated by ROS [33]. Furthermore, ROS also activates complement system that increases neutrophil recruitment [33]. Direct causal relationship between ROS production and neutrophil infiltration has been established by using antioxidant enzymes or ROS scavengers which diminishes neutrophil infiltration in both cultured cells and animal studies [33]. Neutrophils cause injury by releasing enzymes such as collagenase and elastase which degrade the basement membrane of capillaries [33]. They also secrete oxidants such as hypochlorous acid that increases protease levels which

exacerbate inflammation by activating cytokine production [94, 95]. The direct evidence showing that neutrophils are causal in the inflammation process is provided by studies showing that microvascular dysfunction is attenuated in animals that are rendered leukopenic [96, 97]

#### *Neutrophil recruitment to ischemic tissues*

Leukocyte recruitment to the ischemic tissue is a complex series of event that involves a number of adhesive molecules [96, 98]. Using gain and loss of function studies several mediators on both the endothelial cell and granulocytes have been shown to play pivotal role in these processes. For instance neutralizing antibody against common  $\beta$ - subunit (CD18) on neutrophils or P-selectin on activated endothelial cell prevents neutrophil adhesion and migration [99]. Additionally, adhesive molecules such as ICAM- 1 are also important in mediating interactions with CD18 which provide an avenue for direct transfer of neutrophilic content to the muscle [100]. CD18/ICAM-1 adherence reactions contribute to microvascular barrier disruption, which allows transmigration of neutrophils to the skeletal muscle [101, 102]. This observed upregulation in the levels of pro-adhesive molecules occurs in the presence of decreased NO levels, which accentuates the activated adhesive endothelial phenotype. Studies that disrupted the adhesive interactions between neutrophil and endothelium have demonstrated a decline in neutrophil-dependent cell injury. Likewise, establishment of these interactions not only allows the phagocytic cells to migrate to sites of inflammation but also appears to facilitate the destructive potential of the granulocytes [103, 104]. Neutrophil-endothelium interactions are therefore essential for immigration of activated leukocytes. In

addition to these interactions, modifications are induced in the cytoskeletal structure of the endothelium which modulates the transmigration process [105]. This close proximity of neutrophils to endothelial cells is required for the production of leukocyte dependent injury since adherent neutrophils have an increased oxidant release capacity than suspended neutrophils [33].

#### *Capillary no-reflow phenomenon*

Reperfusion does not always lead to restoration of tissue perfusion but could lead to a condition referred to as capillary no-reflow. Capillary no-reflow is defined as a suboptimal tissue perfusion without any evidence of vessel obstruction due to atherothrombosis [49]. Although poorly understood, studies show a direct correlation between leukocyte numbers and the percent of no-reflow capillaries. Depletion of neutrophils also abolishes no-reflow in reperfused tissues [49, 102, 106] implying that neutrophils and ROS are involved in capillary no reflow.

This phenomenon of capillary no-reflow is initiated as a result of ROS and granulocyte mediated proinflammatory milieu generation and ion-pump dysfunction. The proinflammatory stimuli lead to modification of adhesive molecules on both neutrophils and endothelial cells. Leukocytes then roll on the endothelial surface, get tethered and eventually egress into the underlying tissue. This phenomenon results in endothelial barrier dysfunction allowing enhanced vascular protein leakage. The ensuing increased transcapillary fluid filtration results in edema and increased interstitial pressure. Edema exerts physical compression on the vessel especially for tissues such as muscles, which cannot readily expand due to the presence of a restrictive fascial sheath, resulting in the

capillary no-reflow phenomenon [33, 49]. Altered ion pump function is partly responsible for edema formation and reducing edema by treatment with a hyperosmotic saline dextran solution prevents endothelial cell swelling [107]

### **Reparative Phase**

This response phase to ischemic insult involves activation of processes that seek to restore tissue perfusion and heal the damage tissue. Three key processes are involved in the revascularization responses namely vasculogenesis, angiogenesis and arteriogenesis (**Fig.2**). Although these processes are distinct in some aspects they do share overlapping mediators such as growth factors and cytokines. Furthermore, one process could influence the subsequent process i.e. vasculogenesis could lead to angiogenesis. Tissue regeneration and repair is a sequel of revascularization that involves growth factors, tissue resident stem cells and bone marrow derived stem cells

#### *Angiogenesis*

Angiogenesis is a well-studied process that involves proliferation, sprouting and migration of endothelial cells to form new thin walled capillaries [108]. The initial trigger for angiogenesis is local ischemia. In 1988 Goldberg and colleagues while examining the expression of erythropoietin gene observed that hypoxia, cobalt chloride or nickel chloride increased mRNA expression of erythropoietin through a common pathway [109]. They hypothesized that this common pathway involved a ligand dependent conformational change in a heme containing protein. Later Semenza *et al.* identified a cis-acting enhancer element in erythropoietin gene and



a multimeric transcriptional regulatory complex which binds directly to a 50-base pair hypoxia-inducible enhancer element in the 3' region of erythropoietin gene. These transcription factors were referred as hypoxia inducible factor (HIF) [110, 111].

The HIF family transcription factors are now known to include three constitutively expressed  $\beta$ -isoforms, whose regulations are oxygen independent, and at least three oxygen-dependent  $\alpha$ -isoforms [112]. Of the three  $\alpha$ -isoforms, HIF1- $\alpha$  is the most ubiquitously expressed whereas HIF2- $\alpha$  and HIF3- $\alpha$  are more restricted to specific tissues [113]. All three isoforms have been shown to interact with the  $\beta$ -isoforms and activate target gene transcription [114, 115]. However, an alternately spliced transcript of HIF3- $\alpha$ , inhibitory Per-ARNT-Sim (PAS) domain protein, appears to be a negative regulator of transcription [116]. Due to the ubiquitous nature of HIF1- $\alpha$ , it is the most widely studied isoform.

HIF1- $\alpha$  is a basic helix-loop helix (bHLH), Per-ARNT-Sim (PAS) domain containing protein. It contains an oxygen-dependent degradation and transactivation domains. The basic helix-loop helix domain is essential for its DNA binding activity whereas the PAS domain is involved in heterodimerization with HIF- $\beta$ . The oxygen dependent degradation and transactivation domains regulate HIF1- $\alpha$  stability and coactivator binding respectively [113, 117].

Initially it was thought that the putative HIF1- $\alpha$  regulator was a heme iron containing protein, but further studies established that stabilization of HIF1- $\alpha$  is mediated by non heme containing enzymes identified as prolyl hydroxylases (PHDs). PHDs are iron-containing dioxygenases that require  $\alpha$ -ketoglutarate and

oxygen for their activity [118]. The discovery of PHDs and HIF1- $\alpha$  helped us better understand the role of molecular oxygen in the molecular mechanisms that are activated during hypoxia. In the presence of molecular oxygen HIF1- $\alpha$  is targeted for proteasomal degradation through PHD mediated hydroxylation of two essential proline residues in the oxygen dependent degradation domain. The reaction requires  $\text{Fe}^{2+}$ ,  $\alpha$ -ketoglutarate and oxygen and involves splitting molecular oxygen into two. One oxygen is used as a substrate for hydroxylation and the other used for oxidation of  $\alpha$ -ketoglutarate to succinate [119]. Hydroxylation on the proline residues act as a specificity determinant allowing for recruitment of the E3 ubiquitin ligase, von Hippel-Lindau tumor suppressor (pVHL), thereby targeting HIF1- $\alpha$  for proteasomal degradation. Each of the hydroxylation sites can independently interact with VHL potentially contributing to rapid degradation of HIF1- $\alpha$  [116]. However, under hypoxic conditions when oxygen becomes limiting PHD activity is inhibited thereby preventing proline residue hydroxylation on HIF1- $\alpha$  [118]. Furthermore,  $\text{Fe}^{2+}$  in the catalytic site of the enzyme is loosely bound to 2-histidine-1- carboxylate coordinate motifs which makes  $\text{Fe}^{2+}$  susceptible to displacement [113]. Therefore, metal chelators such as deferoxamine can induce HIF1- $\alpha$  stabilization even in the presence of molecular oxygen [120, 121].

In addition to this modification, hydroxylation of an asparagine residue in the transactivation domain by asparagine hydroxylase prevents interactions with coactivators [116]. Other posttranslational modifications such as acetylation and phosphorylation have been shown to occur in HIF1- $\alpha$ . Acetylation of a conserved lysine residue in the oxygen degradative domain has been shown to be important

in HIF1- $\alpha$ -pVHL interactions and stability. Also, phosphorylation by MAPK is reported not to affect stability or transactivation but rather increase HIF1- $\alpha$  transcriptional activity [113, 122, 123]. Other less common modifications are SUMOylation which activates and represses transactivation and cysteine residue S-nitrosation which increases transactivation [124-127]. Cytokines, ROS and growth factors could also activate HIF-1 $\alpha$  in a hypoxia independent manner [113, 117]. However, studies have shown that prolyl hydroxylation is the most important regulator of HIF1-  $\alpha$  stability.

Once proline residue hydroxylation is inhibited under hypoxic conditions, HIF1- $\alpha$  translocate into the nucleus and interacts with its coactivators. This transcriptional complex is formed on genes that contain the cis-acting element that was initially identified in the erythropoietin gene. Using promoter analysis on genes that are modified by hypoxia this consensus sequence, referred to as hypoxia response element (HRE), has been shown to be present in hundreds of genes involved in erythropoiesis, Fe<sup>2+</sup> metabolism, angiogenesis, glycolysis, immunity, cell proliferation, and matrix and barrier regulation [113]. The ability of this single factor to regulate these diverse genes involved in almost all the adaptations to hypoxia makes its regulation a viable target in the treatment of ischemic pathologies.

The genes involved in angiogenesis that are regulated by HIF1- $\alpha$ , include epidermal growth factor, platelet-derived growth factor, angiopoietin, stromal-derived growth factor and vascular endothelial growth factor (VEGF) which is the most potent endothelial specific mitogen [108, 128].

VEGF family of growth factors consists of seven family members named VEGFA - F and PIGF with VEGF-A being the prototype. With the exception of VEGF-F and PIGF all five VEGF family members exist in mammalian cells [129]. These are a family of secreted proteins each containing a cysteine-knot structure with eight invariant cysteine-residues that are involved in inter and intra molecular disulfide bond formation [130]. The angiogenic effects of secreted VEGF are propagated by binding with the VEGF tyrosine kinase receptors. Three VEGF tyrosine kinase receptors designated VEGFR1-3 have been identified. Each receptor has a single membrane-spanning region, seven immunoglobulin-like domains in the extracellular region and a conserved tyrosine kinase sequence interrupted by a kinase insert domain [130]. VEGFR2 appears to be the most important receptor in VEGF-mediated proliferation of endothelial cells [131]. On the other hand VEGFR1 is likely involved in negative regulation of VEGFR2 mitogenic effects [132]. VEGFR3 is usually limited to lymphatic endothelial cells and seems to regulate proliferation and migration of these cells. In terms of its activation it differs from the other two VEGFRs because it undergoes proteolytic cleavage in the extracellular domain into two disulfide linked polypeptides [129].

Binding of VEGF to VEGFR2 results in autophosphorylation of several tyrosine residues in the kinase-insert domain and C-terminal domain. Each of these autophosphorylation site mediates specific interactions between receptors and downstream signal transducers [129]. In human's tyrosine1175 phosphorylation creates a docking site for phospholipase C- $\gamma$ 1 (PLC $\gamma$ 1), growth factor receptor-bound protein 2 and SHC. PLC $\gamma$ 1 docking leads to activation of protein kinase C

through diacylglycerol generation from phosphatidylinositol 4, 5-bisphosphate. Diacylglycerol is known to activate MAPK cascade which promotes cell survival and proliferation. This pathway also lead to  $Ca^{2+}$  signaling, prostaglandin production and increased vascular permeability [133]. Another signaling pathway which can be activated is the PI3K signaling cascade. Docking of PI3K to phosphotyrosine leads to activation of protein kinase B (PKB)/AKT leading to phosphorylation of proapoptotic caspase 9 and BAD which inhibits proapoptotic factors and increase proliferation and survival. PKB/AKT pathway also increase eNOS activity through interaction with HSP90 and leads to increased vascular permeability [129, 133]. Modifications in focal adhesion proteins are also induced through SRC and SHB docking. This causes actin reorganization facilitating endothelial and smooth muscle cell migration which are necessary for angiogenesis. The immune/ inflammatory pathways are also activated by VEGF signaling through the JAK/STAT pathway [133]. Receptor activation also leads to production of platelet- activating factor by endothelial cells which stimulate their mitosis and migration [129]. Chemoattractant molecule production is also induced by receptor signaling which allows for monocyte recruitments [134]. By regulating factors involved in every step of the angiogenic process i.e. vascular cell permeability, migration, proliferation and sprouting, VEGF acts as a potent angiogenic factor. Proliferation of the vascular cells has been observed as early as 24 hour after the induction of hindlimb ischemia in murine models and peaks at days 3 and 7 [135]. VEGF also works synergistically with other growth factors which enhance angiogenic response [136]. The increase in endothelial cell mitosis

also corresponds to morphological changes in smooth muscle cells which represents a shift from contractile to proliferative phenotype such as appearance of rough endoplasmic reticulum and many free ribosomes [137].

### *Vasculogenesis*

Asahara and colleagues showed for the first time in 1997 that the cells isolated from the blood can form new vessels thus challenging the dogma that postnatal vascularization is operated by proliferation of pre-existing endothelial cells. They showed that a subset of CD34+ (hematopoietic stem cell) population of cells in peripheral blood upon culture express endothelial cell markers such as CD31 and these cells could be incorporated into newly formed blood vessels following murine hindlimb ischemia [45]. This new cell population was named endothelial progenitor cells (EPCs). Further studies demonstrated the existence of more than one endothelial cell progeny [138]. Since then other cell populations such as myeloid cells, side populations and tissue resident stem cells have been shown to differentiate or transdifferentiate into endothelial cells [139]. Due to the heterogeneity of these cell populations, especially in humans, no specific marker has been identified to describe putative EPC however, a panel of markers is consistently used as a surrogate marker for cells displaying vascular regenerative properties [139]. EPCs were originally defined as cells that were positive for the hematopoietic cell marker, CD34 and the endothelial cell marker, VEGFR2, but CD34 is not exclusive to hematopoietic stem cells. Low expression of CD34 is observed on matured endothelial cells. The more immature hematopoietic stem cell marker, CD133 is commonly used however, most studies use all three markers

(VEGF,CD34,CD133) [140, 141]. The stem cell antigen-1 (Sca-1), a common marker used to enrich hematopoietic stem cells in rodent tissues, can also be used in conjunction with VEGFR2 to identify EPCs [142].

Following the discovery of these cells, numerous studies using different models of ischemia were done to test the role of EPCs in promoting neovascularization, and to the best of our knowledge all these studies have shown protective effects. The incorporation of EPCs into new vessels is dependent on ischemia because low incorporation rates are observed basally [143]. Moreover, under non ischemic conditions basal levels of circulatory EPCs are maintained while a pool of the cells are stored in the bone marrow microenvironment referred to as the stem cell niche. These cells can then egress out of the niche following ischemia [128, 144]. Cardiovascular risk factors such as diabetes and smoking are associated with decreased levels of circulatory EPCs whereas cardio protective factors such as exercise and drugs that reduce the levels of blood low-density lipoprotein (e.g. statins) increase the circulating levels [128, 139, 145]. These observations suggest a direct effect of EPCs on cardiovascular health and an ischemia related mechanism that increases the transmigration of these cells to sites of ischemia. A complex coordinated response involving cytokines, chemokines and proteases are required to enable mobilization from the bone marrow [144, 145].

Based on the observations that VEGF is involved in embryonic blood vessel growth and angiogenesis Asahara et al. tested the hypothesis that VEGF is also involved in post-natal vasculogenesis [146]. They showed that treatment with VEGF increases EPC mobilization and this effect was abolished by using neutralizing

antibodies against VEGF [147]. VEGF binds the VEGF receptor which leads to activation of PI3K/AKT pathway and increased NO production. NO directly activates matrix metalloproteinase 9 that cleaves membrane kit ligand into soluble kit ligand. C-kit expressed on the surface of EPC is a receptor for soluble kit ligand. The interaction between C-kit and its ligand allows EPC to egress from the bone marrow [128].

Stromal-derived factor-1 (SDF-1), a stromal protein which interacts predominantly with the chemokine receptor CXCR4, is also involved in EPC mobilization. EPCs express CXCR4 and interaction between CXCR4<sup>+</sup> EPC and SDF-1 maintains EPCs in the bone marrow niche [148]. This interaction suggests that EPCs could respond to increased circulatory SDF-1 level and vice versa. Because SDF-1 is a transcriptional target of HIF-1 $\alpha$ , SDF-1 levels are increased in both the target tissue and circulation during hypoxia [117]. In addition to hypoxic tissues, matured endothelial cells, pericytes, platelet and EPCs can express SDF-1 to induce EPC chemotaxis [149, 150]. Both matrix metalloproteinase mediated disruption of SDF-1 and EPC interactions within the stem cell niche as well as elevation of SDF-1 within the circulation are required for mobilization. Also, the extent of EPC mobilization is directly proportional to blood SDF-1 levels suggesting that a concentration gradient created by SDF-1 act as a chemoattractant for EPC [151]. Both SDF1 and VEGF activate the nitric oxide synthesis pathway and a synergistic role of both factors has been observed to be involved in EPC mobilization even though deletion of each individual factor exerts substantial effect on this process [139].



Since EPCs represent a rare population of bone marrow cells, chemoattraction is of utmost importance in their mobilization and recruitment to injured blood vessels thus, SDF1 and VEGF are potent regulators of EPC mobilization. Trials with human patients have shown that the migratory capacity of EPCs or bone marrow cells towards VEGF or SDF1 respectively enhances the functional improvements of patients after stem cell therapy [152]. In addition to these factors, studies have implicated other factors such as granulocyte-colony stimulating factor, monocyte-chemoattractant protein-1, erythropoietin, angiopoietin-1, lipid derived product such as sphingosine-1-phosphate and more recently estrogen [128, 139, 144, 146] to increase EPC mobilization. Once in the peripheral blood, EPC homing to sites of ischemia occurs through adhesion and transmigration. The activity and expression of matrix degrading cysteine protease, cathepsin G, is required for tissue invasion by EPCs [144].

### *Arteriogenesis*

Due to the importance of molecular oxygen in regulating aerobic metabolic, aerobes have evolved to have alternate pathways for blood to reach the same volume of tissue. These set of blood vessels supplying the same volume of tissue are referred to as collateral anastomoses. The process of remodeling already existing collateral anastomoses into larger conductance artery is referred to as arteriogenesis (**Fig 2**). The initial driving force for arteriogenesis is physical, however, bone marrow derived cells and tissue derived factors play additional role in this remodeling process [135, 153]. Arteriogenesis is essential because an

increase in capillary numbers without a corresponding increase in the number of large conductance arteries is not sufficient to ensure tissue perfusion.

Blood vessels regress if they are not constantly perfused and expand on chronic perfusion. When the perfusion rates are high, they develop thicker vessel walls and become highly conductant. Our circulatory system has evolved to have these backup routes for blood flow which are not normally perfused or perfused suboptimally under normal conditions known as anastomoses [28]. These blood vessels serve as backup system so that during ischemia when the principle artery is blocked, blood flow is not completely shut due to the availability of these conduit vessels. The success of arteriogenic process is primarily dependent on the availability of a collateral network linking preocclusive and postocclusive circulation [135].

Upon occlusion of an artery, the pressure in the distal part of occlusion drops while that in the proximal site is enhanced [153]. However, if preexisting anastomoses exist between these two sites the steep pressure gradient generated allows for increase flow through these collaterals [135]. Increased flow through the less perfused artery increases the fluid shear stress due to the viscous drag that blood flow exerts on the endothelium [28]. However, this represents a relatively weak force and cannot fully account for the total morphological changes occurring in arteriogenesis, suggesting additional biological or physical factors are involved in increasing the blood flow [135]. Studies have shown that endothelial cells which are in direct contact with the blood are the first to sense this force and transduce the mechanical stimuli into intracellular biochemical response resulting in changes

in gene expression and vascular remodeling [135, 137, 154]. Using genetic techniques over 40 genes have been found to have shear-stress response element [155-157]. Through the activation of these genes, fluid shear stress is converted into intracellular biochemical signals mediated by an array of biomolecules including receptors, ion channel, and cytoskeletal proteins. Studies have shown that an increase in fluid shear stress increases bradykinin B2 GPCRs activity [158]. Fluid shear stress can also induces cytoskeletal rearrangement, activate integrins, and increase calcium influx through the P2X4 purinoreceptors signaling all of which leads to endothelial cell activation [153, 159]. The transcription factor NF- $\kappa$ B activated during the inflammatory phase also mediates some of these transcriptional processes involved in arteriogenic gene expressions [160]. Collectively, these changes results in alterations in gene transcription and signal transduction [135]. Hence, the quiescent collateral endothelium is converted into a highly activated layer supporting infiltration and adhesion of leukocytes. Perhaps, the greatest modulators of the arteriogenic responses are monocyte/macrophages. These monocyte/macrophages have been shown to adhere to the luminal side of growing collaterals after coronary or femoral artery ligation [161, 162]. Using different methods to control circulating monocyte numbers after arterial ligation studies have shown that the number of circulating monocytes correspond to the extent of arteriogenesis [162, 163]. Specifically the inflammatory subsets of monocytes are responsible for arteriogenesis [47]. That circulating monocytes play a role suggests that there are signals that allow these circulating cells to be recruited to the site of injury. Monocyte chemoattractant

protein 1 (MCP-1) is a chemokine whose expression is increased by fluid shear stress. Upregulation of MCP-1 leads to monocyte/macrophage adhesion to the endothelium and subsequent accumulation in the perivascular space [161]. Evidence for MCP-1 and its receptor CCR2 involvement in this recruitment is provided by the observations that either knockout of MCP-1 or CCR2 attenuates arteriogenesis through diminished monocyte recruitment [163, 164]. The migration of monocytes is a complex process because the cells have to traverse through the endothelial barriers. Because monocytes generate high levels of matrix metalloproteinases and plasminogen activator, they are able to pass through the barrier by matrix degradation [165].

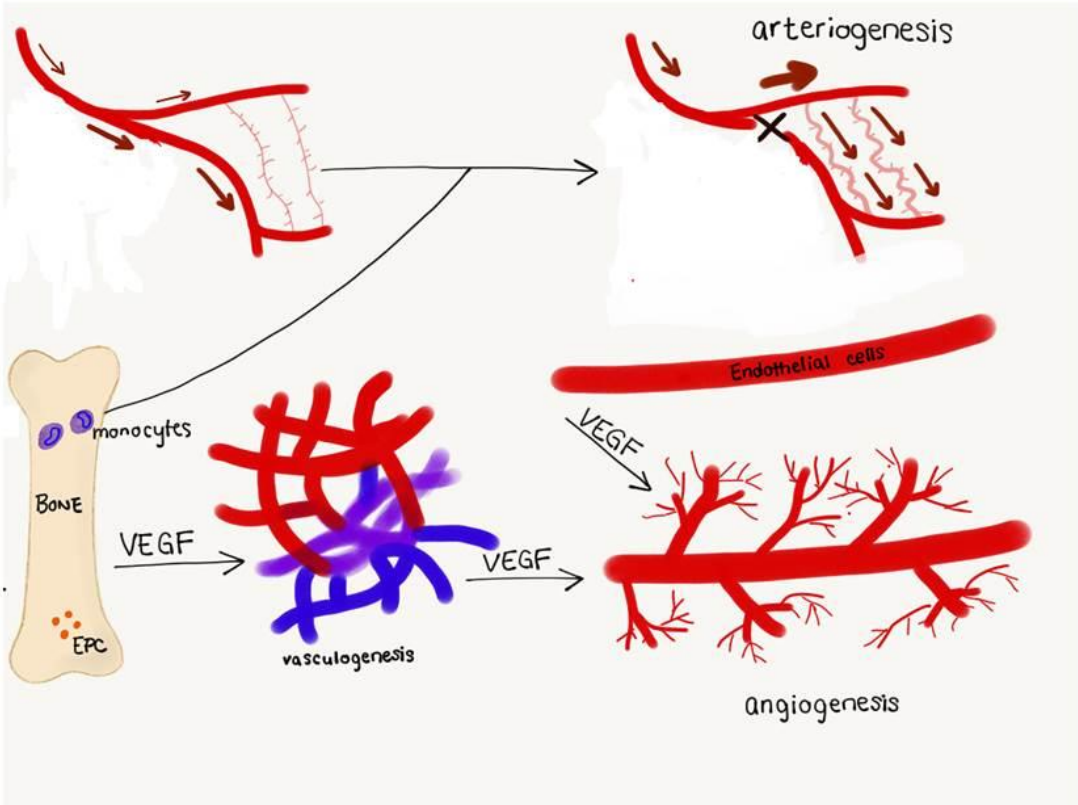
In addition to these cells another leukocyte population that has been shown to play a role in the arteriogenic process are the CD4<sup>+</sup>T lymphocytes. Using CD4<sup>+</sup> T lymphocyte knockout mice Stabile and colleagues showed a decreased collateral network formation in these mice after hindlimb ischemia [48]. This decrease in collaterals was attributed to diminished monocyte/macrophage recruitment in the knockout mice. Furthermore, CD8<sup>+</sup> T lymphocytes and mast cells are also known to play a role in the arteriogenic process [135, 166].

Although the role of different cells in arteriogenesis has been elucidated the exact contribution and the signaling pathways that are activated by each of these cell type is currently unknown. However, the recruited monocytes/macrophages have been reported to secrete growth factors such as VEGF and FGF, increase vasodilatory NO production, and secretes MMPs that participate in extracellular matrix and skeletal muscle digestion to create additional space for growing

collaterals [153]. This vascular remodeling process is not limited to endothelial cells but proliferation and remodeling of vascular smooth muscle cells have also been observed. This proliferative capacity of the smooth muscle cells is mediated by matrix metalloproteinase degradation of the basement membrane [153].

Figure 2

Reparative phase



**Figure 2: Mechanisms contributing to tissue revascularization after an ischemic event:** Ischemia induced VEGF production leads to mobilization of endothelial progenitor cells (EPC) from the bone marrow into the circulation. The circulating progenitor cells then migrate to sites of ischemic injury and form blood island (vasculogenesis). Through further VEGF stimulation these island differentiate to form new blood vessel (angiogenesis). Also, existing endothelial cells can migrate and proliferate through VEGF activity to form new capillary network (angiogenesis). In the process of arteriogenesis, the paracrine action of monocytes leads to remodeling of preexisting anastomoses into large conductance arteries

## **Treatment strategies for PAD**

Risk factor modification is important for managing PAD. Smoking cessation is recommended for PAD patients because smoking is the single most important modifiable risk factor. Smoking cessation improves functional performance, reduces risk for amputation and increases survival rates in PAD patients [7, 22, 167]. Diabetes mellitus is associated with vascular abnormalities such as endothelial dysfunction and intensive glycemic control has been demonstrated to reduce microvascular complication. Hence, comorbid individuals are encouraged to exercise good glycemic control [168, 169]. Supervised exercise programs are the most effective noninvasive intervention for improving pain free walking [170]. Intermittent claudication results from inadequate oxygen supply to the limb. Exercise has been shown to improve oxygen delivery through angiogenesis and increase endothelial dependent vasodilation. It can also increase mitochondrial biogenesis, reduce inflammation and ROS generation; which play key roles in the pathophysiology of PAD [171]. These beneficial effects of exercise have been shown to be responsible for its effectiveness in preventing intermittent claudication.

Pharmacologically two drugs, cilostazol and pentoxifylline, are approved for treatment of PAD. Cilostazol is a quinolone derivative which inhibits type III phosphodiesterase, and pentoxifylline is a methylxanthine derivative. Cilostazol induces vasodilation, increase angiogenesis through peroxisome proliferator-activated receptor- $\gamma$  and cAMP pathways and suppresses apoptosis of endothelial cells [172, 173]. Pentoxifylline, on the other hand, prevents platelet aggregation, has anti-inflammatory properties and acts as a hemorrheologic agent [174].



All the above treatment options are important in PAD however, chronic ischemia and reperfusion in these PAD patients overtime culminate in critical limb ischemia and irreversible tissue loss. CLI has an annual incidence of 500-1000/million people and is present in 12% of the adult US population. The mortality rate is 25% within a year of diagnosis. Morbidity is high in these individuals with about 30% requiring amputation in the first year of diagnosis [15].

In patients with advanced PAD and critical limb ischemia, risk factor management and treatment with pharmacological agent is not sufficient to prevent limb loss. These patients require surgical or endovascular interventions however, majority of these patients are not amendable to these treatment options [175]. For instance patients with poorly controlled diabetes mellitus have lower vessel patency rates and also present with increased rate of adverse limb events when subjected to endovascular techniques [170]. Furthermore, in some patients the operated artery can be re-narrowed after the initial surgical procedure leading to multiple revascularization surgeries [12]. Likewise some patients may not be good candidates for either percutaneous or surgical treatment due to the anatomic distribution of the occluded arteries being so severe to permit relieve of pain or the presence of comorbid conditions such as end-stage renal disease [25, 176]. The later patient population represent the “no-option patient group” accounting for 30% of CLI patients [15]. Currently, no effective treatment is available for these individuals with amputation being the only hope for pain alleviation. In these patients the chronic ischemia burden exceeds the capacity of the peri-ischemic vessels to diffuse oxygen and nutrient to the organ as well as endogenous

reparative mechanisms [177]. Recent focus of therapeutic intervention to the no-option patients has been delivery of exogenous cellular and molecular agents to promote revascularization. Studies using animal models have shown that administration of HIF1- $\alpha$  and growth factors such as VEGF and fibroblast growth factors (FGF) can enhance vasculogenesis, angiogenesis and arteriogenesis in ischemic limbs, attenuate tissue injury, increase muscle regeneration and improve limb function [117, 178-181]. These preclinical studies provided basis for clinical trials to deliver these exogenous agents to PAD/CLI patients. Several clinical trials have been conducted with these agents to elucidate their efficacy in patient populations. A phase III clinical trial testing the efficacy of FGF in no-option CLI patients showed no change in time to amputation or death between treated and placebo groups [182, 183]. Similarly, trials using VEGF or HIF1- $\alpha$  showed no change in peak walking time or maximal treadmill time respectively [184, 185]. These trials with single agents have largely been negative or resulted in no measureable difference between groups despite promising preclinical, phase I and in some cases phase II clinical trials [182]. Like growth factors, many animal studies have reported a role for bone marrow derived and peripheral blood mononuclear cells in recovery from HLI [45, 47, 48]. Several cell types such as peripheral blood or bone marrow mononuclear cells or isolated cell population from blood or bone marrow using selective markers have been injected and tested for their efficacy. Over 50 clinical trials have been conducted and some are still testing these cell therapies [175, 177]. Modest level of efficacy have been observed in these studies with regard to end-points such as ankle brachial index, pain-free

walking, tissue perfusion etc. Most of these clinical trials with these cells did not yield encouraging results which could be due to the limitations in identifying the correct dose, mode and frequency of administration of these agents [175]. It is worth noting that significant improvement has been made over the past decade in fine-tuning these factors. Also, the patient population in the clinical setting are individuals with diverse cardiovascular risk factors which have been associated with decreased circulating progenitor cell number and function. A number of studies have reported impaired mobilization or increased senescence of endogenous EPCs in hypertension, hypercholesterolemia, and diabetes mellitus [186-188]. The potential effect of these risk factors on the function and activity of exogenously administered progenitor cells was demonstrated by a study showing that diabetic impairment in angiogenesis could be reversed if progenitor cells were administered together with agents to promote synergism with local trophic pathways [189]. This suggests that agents that can target multiple deleterious pathways involved in PAD progression could be important treatment options for PAD patients.

### **Project objective**

Impaired tissue perfusion secondary to atherosclerosis is the underlying cause of several adverse chronic conditions such as PAD [7]. Unabated ischemia resulting from this atherosclerotic burden results in tissue necrosis and loss in these patients [190]. Treatment options for these patients include use of pharmacological agents such as cilostazol and antiplatelet therapy in early stage disease patients to relieve pain. Endovascular or bypass surgery can be employed in advanced patients [12,

16, 170]. Use of angiogenic agents such as VEGF, FGF and hepatocyte growth factor have also been tested [175]. More recently autologous bone marrow or peripheral blood cells have been tested as treatment option for these individuals [177]. Although these treatments are currently available, their efficacy have been marginal in part because they do not account for the complex interplay of factors such as ROS and chronic inflammation that underline the disease condition. Therefore, new therapies are needed that could target these multiple underlying causes such as oxidative stress and chronic inflammation.

Revascularization after ischemia is tightly regulated by the transcription factor HIF1- $\alpha$ . Activation of HIF1- $\alpha$  leads to the expression of angiogenic genes such as VEGF which is essential for EPC mobilization (vasculogenesis) as well as sprouting and migration of endothelial cells to form new thin walled vessels (angiogenesis). HIF1- $\alpha$  can also regulate the arteriogenic response through its upregulation of monocyte-chemoattractant protein-1 expression thus, HIF1- $\alpha$  is a viable target for ischemic diseases [117]. HIF1- $\alpha$  is negatively regulated by prolyl hydroxylases (PHDs) which are Fe<sup>2+</sup> requiring enzymes. Therefore, strategies to inhibit PHD using either pharmacological agents or Fe<sup>2+</sup> chelators have emerged as possible treatment options for these individuals. Animal studies using PHD inhibitors have shown protection in HLI through HIF1- $\alpha$  stabilization [60, 120, 180]. However, the use of these chelators and inhibitors could be toxic and HIF1- $\alpha$  gene therapy for CLI patients is largely negative [185].

Recent observations have shown increased levels of reactive carbonyls in ischemic muscles of PAD patients [191, 192]. In addition to promoting tissue injury

directly through reaction with biomolecules these molecules affect the response involved in revascularization. At high concentrations, lipid peroxidation products such as 4-hydroxynonenal are reported to inhibit VEGF production [193]. Similarly acrolein, a highly reactive lipid peroxidation product, has been reported to inhibit EPC mobilization [142]. Moreover, high levels of oxidative stress has been shown to induce senescence of stem/progenitor cells [177, 194]. These observations suggest that oxidant stress may contribute in part to the defective revascularization process observed in PAD patients and may suggest why the administration of proangiogenic agents in the clinical setting was not effective. Thus, removal of lipid-peroxidation products in ischemia could be another strategy to increase the revascularization potential and attenuate tissue injury.

Carnosine is an endogenous dipeptide, comprising histidine and the non-proteinogenic amino acid  $\beta$ -alanine, which is synthesized by an ATP grasp domain containing enzyme carnosine synthase (ATPGD1) [195]. Carnosine is present in high concentration in skeletal muscle, heart and brain. It is a food constituent that is highly abundant in chicken and beef. Studies have shown that carnosine is a potent chelator of divalent metals such as  $\text{Fe}^{2+}$  and  $\text{Cu}^{2+}$  [196, 197]. Given the observation that  $\text{Fe}^{2+}$  chelators such as deferoxamine can increase HIF1- $\alpha$  levels and improve recovery from HLI [198] we rationalized that carnosine can increase revascularization in HLI through HIF1- $\alpha$  stabilization. Furthermore, our lab and others have shown that carnosine can form conjugates with lipid peroxidation products thereby limiting their reactivity [199]. Given the extensive evidence that supports a role of lipid peroxidation product in tissue injury associated with

ischemia and recent evidence linking their levels to diminished progenitor cell levels and angiogenesis [142, 192], we postulated that quenching of reactive aldehydes by carnosine could be beneficial in HLI.

In this series of investigations, we hypothesized that carnosine can promote post-ischemic angiogenic and arteriogenic responses through metal chelation and aldehyde quenching. To address this hypothesis we tested the effect of oral carnosine supplementation on revascularization after mouse HLI. We used Laser Doppler imaging technique and microfil perfusion coupled with micro-CT analysis to determine tissue perfusion. The effect of changes in tissue perfusion on muscle integrity and lipid peroxidation product levels were analyzed. We examined the role of carnosine on ischemia-induced EPC and immune cell mobilization and recruitment. We used cell culture models to analyze the molecular pathways that are regulated by carnosine. Results from this study shows that carnosine improves blood flow in the ischemic limb of the HLI mice and provides the platform to start clinical trials in PAD patients and determine whether supplementation with carnosine can improve blood flow/ABI in these patients.

## CHAPTER II

### ORAL CARNOSINE SUPPLEMENTATION PROMOTES REVASCULARIZATION IN A MURINE HINDLIMB ISCHEMIA MODEL

#### **Introduction**

Carnosine, ( $\beta$ -alanyl-L-histidine), was discovered in the late 19<sup>th</sup> century by the Russian chemist, V. Gulewitch as a non-protein nitrogen-containing compound in meat; hence the name carnosine (from the Latin word *carnis* meaning meat/flesh) [200]. Currently, about 10 variants of carnosine have been identified which are classified into the histidyl dipeptide family [200]. All vertebrates' tissue analyzed have one or more derivatives of histidyl dipeptides with mammals usually possessing carnosine and one methylated isoform i.e. either anserine or balenine, except for humans, that have carnosine only [201]. Carnosine is highly abundant in skeletal muscle and other excitable tissues such as heart and brain. The skeletal muscle concentration in humans is about 20-30mmol/kg dry weight hence carnosine is one of the top ten metabolites in the skeletal muscle [202]. Such high concentrations in muscle suggest a critical role of this dipeptide in skeletal muscle function thus, creating the need to understand its metabolism and function.

Carnosine is synthesized by the enzyme carnosine synthase (ATPGD1). Recent work by Drozak, et al. characterized this enzyme as an ATP-grasp domain containing protein [195]. The enzymatic reaction involves peptide bond formation

between  $\beta$ -alanine and histidine (**Fig. 3**), with  $\beta$ -alanine being the rate limiting substrate [203].  $\beta$ -alanine is synthesized from catabolism of uracil, decarboxylation of aspartic acid and oxidation of 3-aminopropanal [204-206]. In addition to these endogenous synthesis,  $\beta$ -alanine can also be taken as a supplement to increase tissue carnosine levels [203]. Intracellular transport of  $\beta$ -alanine is achieved through TAUT and PAT-1 transporters [207].

The presence of a  $\beta$ -amino acid in carnosine makes it resistant to tissue peptidases. Hydrolysis of carnosine into constituent amino acid is catalyzed by a specific peptidase referred to as carnosinase. Carnosinase exists in two isoforms that are present in the serum and cytosol respectively [201, 208]. The activity and expression of these degradative enzymes vary across species with humans having a higher serum carnosinase activity and expression than rodents [209, 210]. The cytosolic isoform of the enzymes is highly abundant in liver, kidney and skeletal muscle of humans and rodents [200, 207]. Regardless of the tissue distribution cytosolic isoform has a lower activity compared with serum isoform [201].

Carnosine is transported across the plasma through the activity of a family of proton coupled transporters (PHT1, PHT2, PEPT2, and PEPT1) [211-213]. PEPT1 is highly expressed in small intestines and plays an essential role in the absorption of ingested carnosine into the bloodstream [213]. PEPT2 on the other hand is expressed on the plasma membrane of several tissues such as kidney and skeletal muscle. Studies with the PEPT2 KO mice have shown that PEPT2 is involved in the extrusion of carnosine from the skeletal muscle and import of carnosine to the kidney and brain [214]. However, studies by Everaert et al.



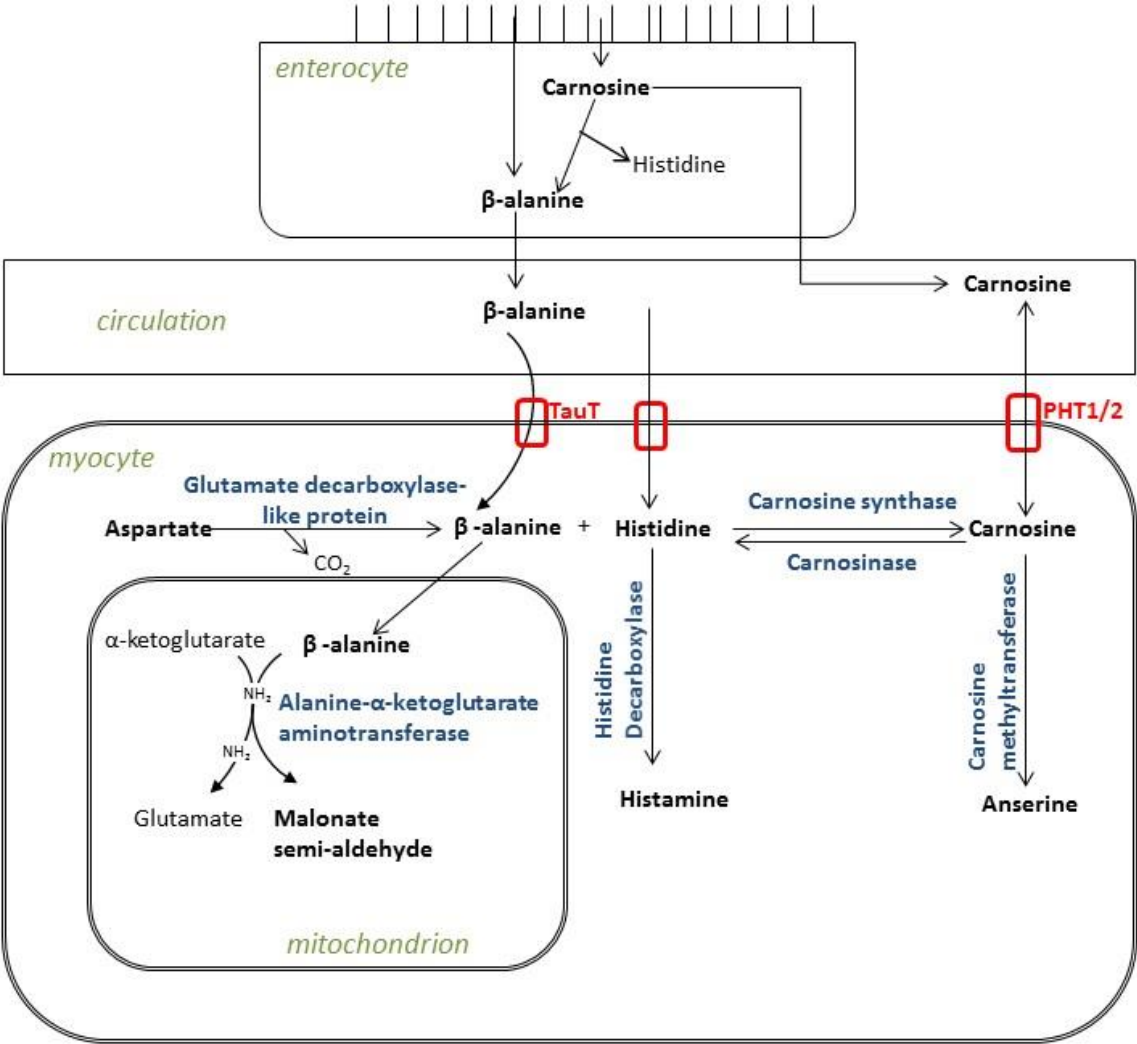
showed that the relative expression of PEPT2 transporters in muscle is low and PHT1 is the key transporter for carnosine in the skeletal muscle [207]. Because these transporters are non-specific it is difficult to elucidate their contribution in regulating intracellular carnosine levels. Although  $\beta$ -alanine is considered the rate limiting substrate, histidine is also known to affect carnosine levels [203, 215]. Decarboxylation of histidine to histamine by histidine decarboxylase can affect intracellular carnosine concentration [207]. Also,  $\beta$ -alanine can be transaminated by  $\beta$ -alanine-2-oxoglutarate transaminase and  $\beta$ -alanine-pyruvate transaminase. Inhibition of these transaminase enzymes increase carnosine levels in skeletal muscle and cardiac tissue [216]. Carnosine can be methylated to its analogues anserine or ophidine, decarboxylated to carcinine or acetylated to acetyl carnosine however, these modifications are species-specific [201, 217]. **Fig. 3** provides a summary of factors regulating carnosine homeostasis in rodent muscle.

Carnosine has the ability to buffer intracellular pH, because the imidazole ring of histidine, has a pKa close to physiological pH that imparts buffering capacity to histidyl dipeptide. During intensive exercise lactic acid is accumulated which is decomposed to lactate and proton that leads to decreased pH thus limiting muscle function [217]. A direct role of carnosine in regulating proton levels was described by Severin et al. who showed that treatment with carnosine prevents acidification induced by lactic acid without affecting lactate accumulation thus, preserving excitation-contraction-coupling in isolated frog muscle [217, 218]. Carnosine also affects intracellular calcium regulation presumably through modulation of sarcoplasmic reticulum calcium release and increases the sensitivity of contractile

proteins to calcium [219]. Hence, both pH buffering and calcium handling properties of carnosine are essential in regulating muscle contraction. In addition to pH buffering carnosine is known to exhibit antioxidant properties, [220] quench highly reactive carbonyl species [199, 221] and chelate divalent metals such as  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{Zn}^{2+}$  [196, 197]. Carnosine also regulates neutrophil function, exhibits anti-aging and anti-inflammatory functions, regulates enzymes such as phosphorylase a, modulates gene transcription and possibly translation through regulation of eukaryotic initiation factor 4E protein [222-224].

Based on its well-characterized role in scavenging ROS, calcium regulation, metal chelation and aldehyde quenching, we sought to elucidate a role for carnosine in recovery from hindlimb ischemia (HLI). In these studies we used a well-characterized murine model of HLI to determine the therapeutic potential of carnosine in HLI.

Figure 3



**Figure 3: Schematic overview of factors regulating muscle carnosine homeostasis in mice:** Ingested carnosine can be absorbed as the intact molecule or degraded into constituent amino acids within the intestinal lumen. Carnosine can be transported to the myocyte through the activity of PHT1/2 transporters. Intramuscular carnosine can be degraded, methylated or stored. On the other hand intramuscular  $\beta$ -alanine transport is through TauT. In the muscle it can be used for carnosine synthesis or transaminated for energy generation. Histidine, although not the limiting substrate, can be decarboxylated to histamine

## **Experimental Procedure**

**Animals and reagents:** Male C57BL6J mice (16-18 weeks old) were obtained from Jackson Laboratory (Bar harbor, ME). Mice had access to chow *ad libitum* and were placed on a 12hr light/dark cycle. All protocols and procedures were approved by the Institutional Animal Care and Use Committee of the University of Louisville. Carnosine, hemoxilin and eosin (H&E) dyes were obtained from Sigma Aldrich, Microfil dye from Flow Tech Inc and isolectin antibody from Molecular Probes.

**Hindlimb Ischemia Surgery (HLI):** The murine hindlimb ischemia (HLI) model used in this study involved ligation of the femoral artery and vein. The mice were anaesthetized using 1-3% isoflurane in 100% oxygen at a flow rate of 1L/min. Once fully anaesthetized, depilatory cream was applied to the right hindlimb to remove excess hair and expose the skin. Betadine and 70% alcohol were applied consecutively then a 5mm incision was made on the thigh within the femoral triangle. The inguinal fat pad was pulled aside to expose the underlying neurovascular bundle. To obtain a good view of the vasculature, the membranous femoral sheath was removed followed by ligation of the femoral artery and vein at two points which were 2mm apart. The intervening blood vessel was excised and the skin closed with two discontinuous sutures. Mice were allowed to recover from the surgery. Sham operated mice were treated as described above except that the vessels were not ligated. Regarding carnosine supplementation two different protocols were used. In the first study mice were pre-treated with carnosine at a concentration of 1g/L in their drinking water for 7 days and then subjected to either

sham or HLI surgery. This study group will be referred to as pre-treated mice. The second protocol involved carnosine treatment after HLI surgery. Mice were subjected to HLI and sham surgeries and immediately supplemented with carnosine (1g/L) in drinking water throughout the recovery process. This latter group will be referred to as post-treated study.

**Laser Doppler Perfusion Imaging (LDPI):** Post ischemia tissue perfusion was monitored using Peri Scan PIM II laser Doppler device (Perimed). These analyses were carried out on whole limb for the pre-treated group on post-operative day 14 and on the feet for the post-treated group on post-operative days 7, 14 and 21. Mice were anesthetized as described above, placed in the supine position (for whole limb imaging) or prone position (for feet imaging) and body temperature maintained throughout the imaging by placing them on a heating pad set at 37°C. Blood flow in the limbs was displayed as laser frequency changes using different color pixels. Images were analyzed with both NIH image J and moorLDI V6 PC analysis software. The ratio of the flow obtained for the ligated (ischemic) and contralateral limb was reported as the percentage of tissue perfusion recovered.

**Vascular Casting and Micro- Computed Tomography (microCT) analysis:**

After 21 days of recovery from HLI, the post-surgery treated mice were intraperitoneally injected with 100 µl of 100 unit/ml heparin to prevent blood coagulation and sedated with pentobarbital. When the mice were fully sedated an incision was made in the thoracic cavity to expose the heart. A blunted 25 gauge needle was cannulated through the left ventricles. The mice were systemically perfused at a constant rate of 5ml/min with the vasodilation solution (100 µM

adenosine, 10  $\mu$ M sodium nitroprusside and 0.05 wt. /vol bovine serum albumin). Undiluted microfil vascular casting agent (Flow Tech Inc, MA) was mixed with 10% volume of curing agent and perfused systemically until a good filling of the vasculature was obtained. The casting was allowed to polymerize at room temperature and the entire hindlimb limb deskinning. To obtain whole mount images of these limbs the deskinning limbs were placed in increasing concentration of glycerol (40%, 60%, 80% then 100%) successively for 24 hours at each concentration. Whole mount images were obtained from these samples by imaging with a digital CCD camera (Nikon). For microCT analyses the samples were further processed by fixing in 10% neutral buffered saline for 18-20 hours and decalcified with Cal EX II for 48 h. Samples were stored in 70% ethanol until imaging. MicroCT imaging was carried out on a MicroCAT (Siemens) using following parameters; 80kVp, 200  $\mu$ A and 2 x 2 binning to obtain a pixel resolution of 1024 x 1024 with 34  $\mu$ M voxel sizes. Following the setup of appropriate thresholds for soft tissue elimination, blood vessel volumes were quantified using Analyze software. Similar to LDPI the ratio of blood vessel volumes for the ischemic and contralateral limbs was used to assess the extent of arterial filling.

**Histology**: Histological analyses were conducted on formalin fixed tissues for the hematoxylin and eosin (H&E) staining and OCT-frozen sections for isolectin staining. For H&E staining, following euthanasia the hamstring muscles were excised, formalin fixed and paraffin embedded. Cross sections of muscles were made, mounted on slides and stained with hematoxylin and eosin. The muscle cross sections were analyzed for muscle regeneration and necrosis. For isolectin

staining, hamstring and gastrocnemius muscles were harvested after euthanasia, placed in cryomolds and covered fully with OCT compound. The molds were rapidly placed in cold 2-methyl butane to freeze quickly and prevent ice crystal formation. Cryosections were prepared and incubated with isolectin antibody (Molecular probes) which is already conjugated with a fluorophore. For nuclear staining the slides were counterstained with 4',6-Diamidino-2-Phenylindole Dilactate (DAPI). Samples were imaged using Nikon Eclipse Ti microscope and analyzed using NIS-Element AR analysis software (Nikon).

**Metabolic phenotyping:** Limb function/physical activity of the HLI mice was assessed using metabolic chambers (TSE phenomaster system; Bad Homberg Germany). We determined mice movement (both ambulatory and fine movements) as a measure of limb function. In addition to these parameters we also examined their respiratory exchange ratio (RER), oxygen consumption, and whole body energy expenditure.

**Complete Blood Count Analysis:** Following pentobarbital euthanasia, blood samples were collected by cardiac puncture. The blood samples were placed on ice for 30 mins. The samples were analyzed on Abbott cell-dyn system to obtain a complete hematological profile of the mice.

**Data analysis and statistics:** All the experimental results are represented as mean  $\pm$  SEM. For studies involving only two groups the unpaired student T- test was used for analysis whereas for studies involving groups that are greater than two one-way ANOVA was used with Bonferroni corrections. All statistical analysis



was done with GraphPad analysis software. A p value < 0.05 was considered significant.

## **Results**

### **Limb perfusion is enhanced in the ischemic limb of carnosine treated mice:**

To test the prophylactic potential of carnosine, mice were pre-treated with or without carnosine at a concentration of 1g/L in drinking water for 7 days and subjected to HLI surgery. Carnosine treatment was continued during the recovery process. Our results show that tissue perfusion was significantly increased in the carnosine pre-treated ( $63 \pm 6\%$ ) compared to the non-treated ( $19 \pm 3\%$ ) HLI mice (**Fig. 4**). Based on these results we investigated the therapeutic potential of carnosine in promoting wound healing responses. Wild type (WT) C57/BL6 mice (18 week old) were subjected to HLI and sham surgeries. After surgeries, mice were treated with or without carnosine (1g/L) in drinking water. Recovery of blood flow was monitored by LDPI over a period of 21 days. No difference was observed between the non-treated and carnosine treated groups after 7 days of recovery however, 14 days after the surgery carnosine treated mice showed a significantly higher tissue perfusion ( $31.2 \pm 2\%$ ) compared to non-treated mice ( $20.2 \pm 1.42\%$ ). This increased tissue perfusion persisted after 21 days of recovery in carnosine treated mice ( $49 \pm 6\%$ ) compared to ( $28 \pm 4\%$ ) non-treated HLI mice (**Fig 5**). Collectively these results show that carnosine pre-treatment or post-treatment augments wound healing responses to increase blood flow in the ischemic limb.

**Carnosine supplementation augments post ischemic arteriogenesis:** Three processes namely arteriogenesis, angiogenesis and vasculogenesis have been

shown to account for revascularization in post-natal life. To better understand which of these processes are responsible for promoting carnosine-mediated revascularization we performed Microfil casting coupled with microCT analysis to determine the vascular density and filling in the post-treated HLI mice following 21 days of recovery. Microfil casted images showed an increase in surface vasculature in carnosine treated compared to the non-treated mice (**Fig 6A**). MicroCT analysis of microfil–casted samples provides a detailed view of the entire mouse hindlimb vasculature thereby allowing for the visualization of an extensive tortuous network of arteriogenic collaterals and capillary network. These analyses showed that vascular density in the carnosine treated HLI mice was significantly enhanced compared to non-treated HLI mice (**Fig 6B and C**) suggesting that supplementation of carnosine increases arteriogenesis in the ischemic limb.

**Carnosine increases muscle regeneration and neovascularization:** To determine if the increase in blood flow induced by carnosine affects the morphological changes induced by ischemia, we performed histopathological analysis using Hematoxylin and Eosin (H&E) staining. Histological assessment of the hamstring muscle close to the site of ligation showed an increase in muscle regeneration after 7 days of recovery in the ischemic muscles of non-treated mice characterized by centrally located nuclei and regular polygonal shaped myofibers. Notably, this effect was enhanced with carnosine treatment (**Fig 7**). Furthermore, staining of skeletal muscle with endothelial cell marker isolectin B4 showed that carnosine treatment following HLI significantly increased capillary density

compared to the non-treated HLI mice (**Fig 8**) suggesting that angiogenesis is involved in carnosine-mediated revascularization.

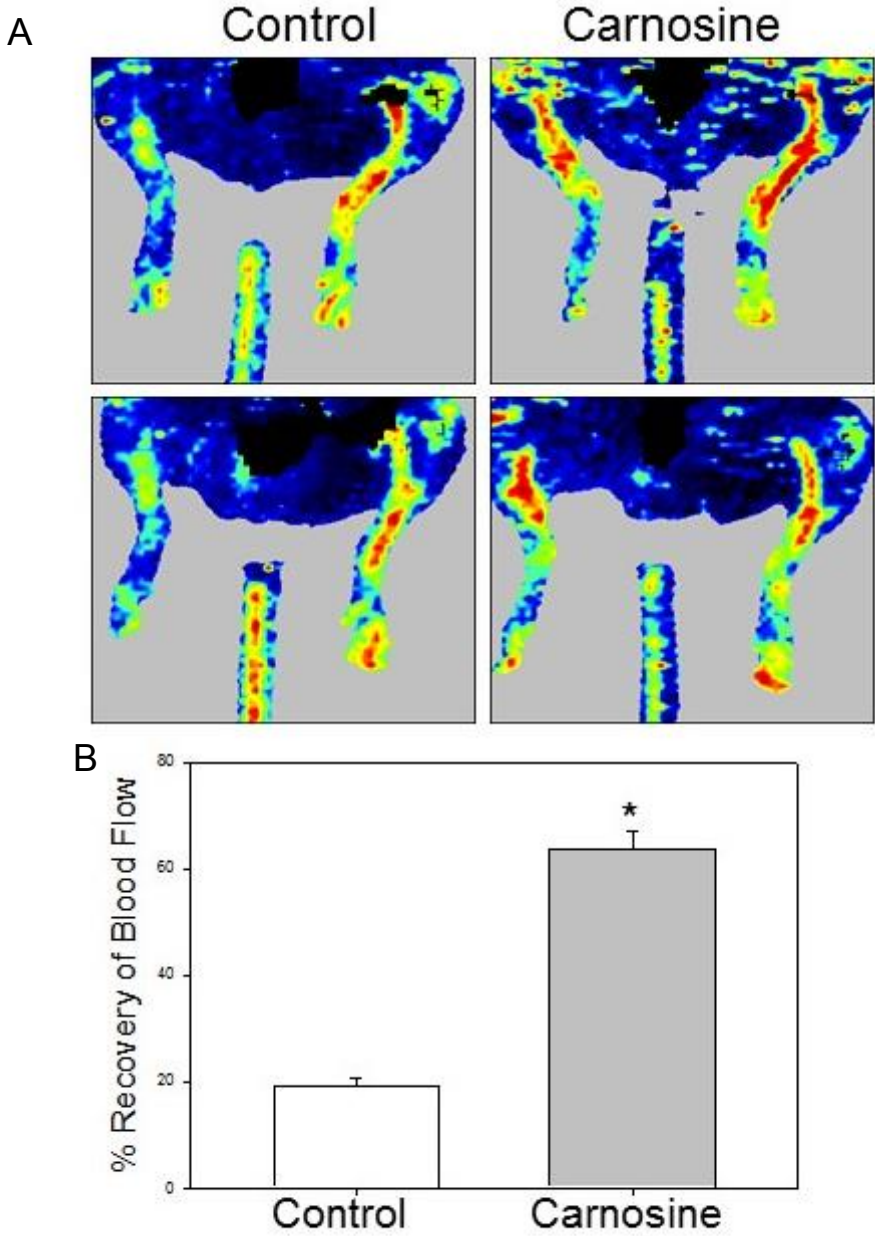
**Carnosine supplementation improves limb function in the HLI mice:**

Peripheral arterial disease (PAD) is considered to be one of the leading causes of morbidity in adults [7]. To test whether carnosine can be used as a therapeutic intervention to increase physical activity in PAD patients, we measured the ambulatory and fine movements of HLI mice in metabolic cages after 14 days of recovery from surgery. Our results show that there is no change in fine movements between the non-treated and carnosine treated HLI mice (**Fig 9C**). However, a significant increase in ambulatory movement was observed in the carnosine treated compared to the non-treated mice (**Fig 9B**). We observed a trend towards an increase in total movement with carnosine treatment although this change did not achieve statistical significance (**Fig 9A**). Further, we observed that  $VO_2$  was significantly increased in carnosine treated ( $4050.9 \pm 160.03$ ) compared to non-treated ( $3278.6 \pm 166.02$ ) mice. Similarly,  $VCO_2$  was increased to  $3977 \pm 133.7$  in carnosine-treated compared to  $3278.6 \pm 166.02$  in non-treated HLI mice (**Fig 9 D and E**). No change in respiratory exchange ratio (RER) was observed. Taken together, these results demonstrate that increase in blood flow by carnosine treatment is concomitantly followed by restored limb function.

**Complete Blood Count (CBC) analysis:** To evaluate the overall health of the mice following ischemia and carnosine treatment we performed CBC analysis after 3 days of recovery from HLI. We observed a trend towards a decrease in white blood cells, neutrophils, monocytes, eosinophils and platelets with ischemia.

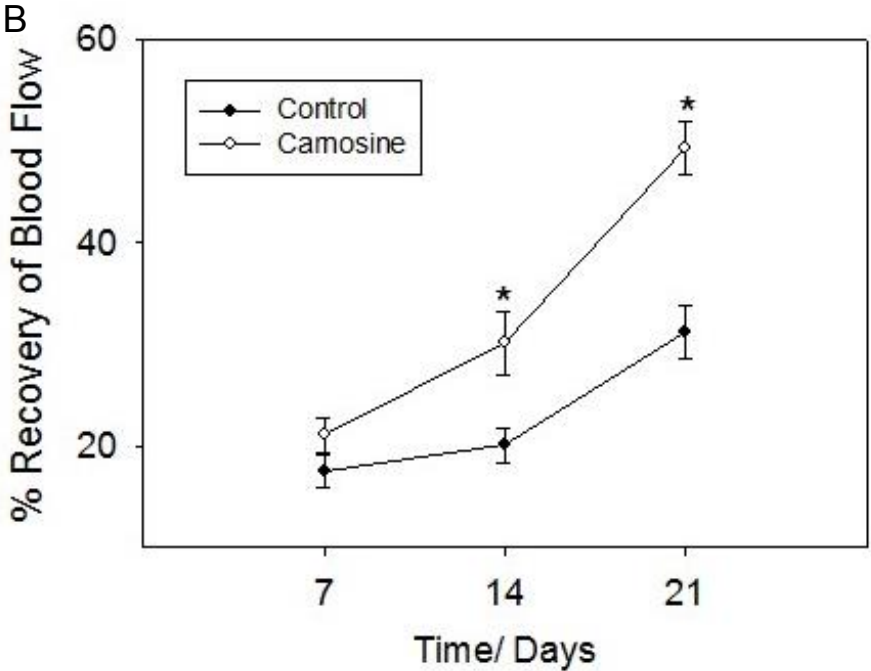
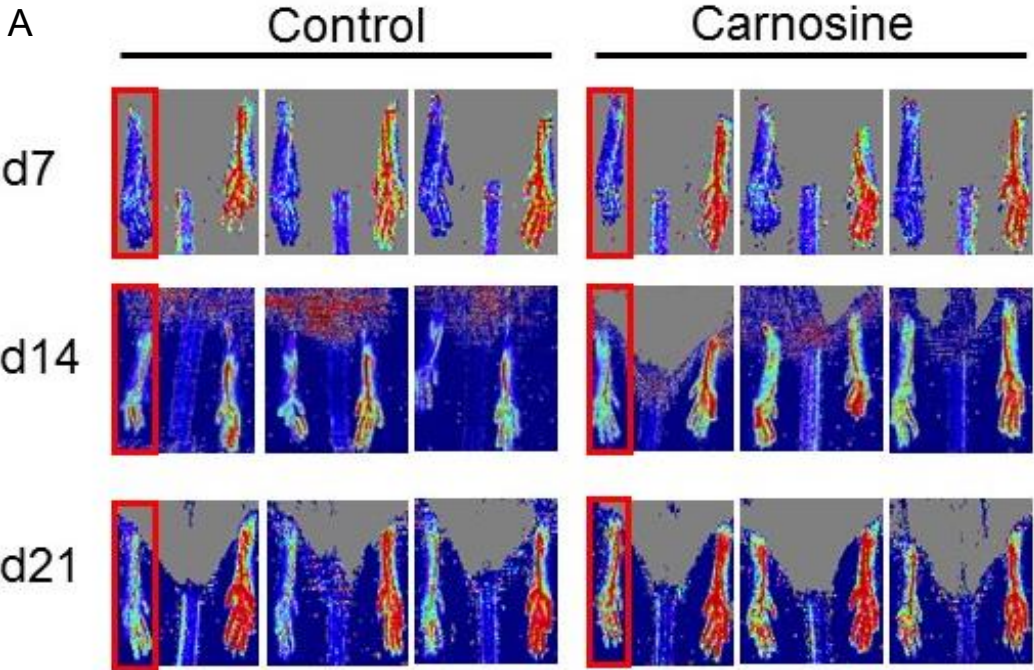
These levels were restored to that in the sham with carnosine treatment after ischemia (**Fig 10 A-C, E, J**). Lymphocyte, red blood cells, hemoglobin and hematocrit levels however remained unchanged between the groups (**Fig 10 D, F, H, I**).

Figure 4



**Figure 4: Carnosine pre-treatment improves tissue perfusion.** WT C57/BL6 mice (17 week old) were pre-treated with/without carnosine for 7 days and subjected to HLI. **(A)** Doppler images of control and carnosine treated mice following 14 days of recovery from HLI. Red represents normal perfusion, blue, a marked reduction in tissue perfusion and yellow and green represent a trend towards restoration of normal perfusion **(B)** Quantification of the Doppler images. Values are represented as a mean for n=5 mice in each group. \*P<0.01 vs non-treated mice.

Figure 5



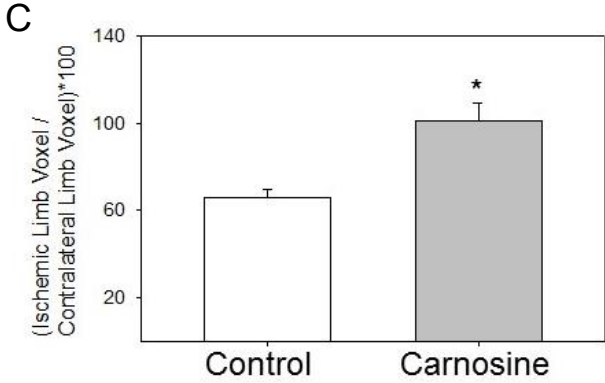
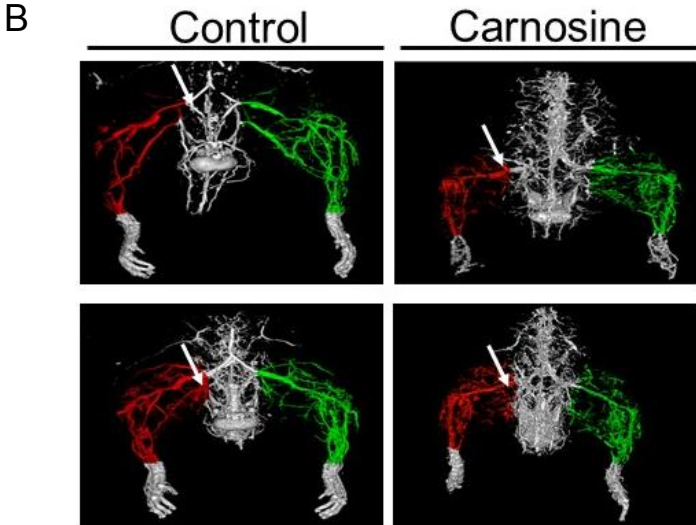
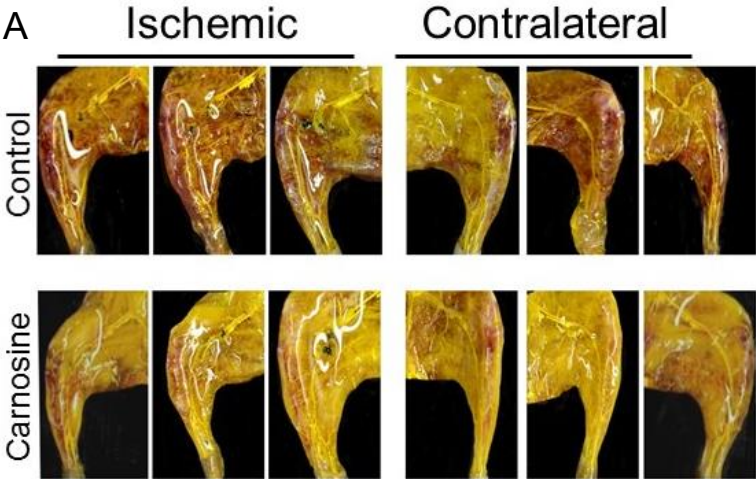
**Figure 5: Post-surgery carnosine treatment improves tissue perfusion: (A)**

Representative laser Doppler perfusion images monitoring foot perfusions on post-operative days 7, 14 and 21 in control and carnosine treated WT C57/BL6 mice.

Region of interest is indicated by the red boxes **(B)** Quantification of foot perfusion rates expressed as percentage of blood flow in the ischemic limb to the contralateral limb. n= 5-9. \*p<0.01 vs control mice

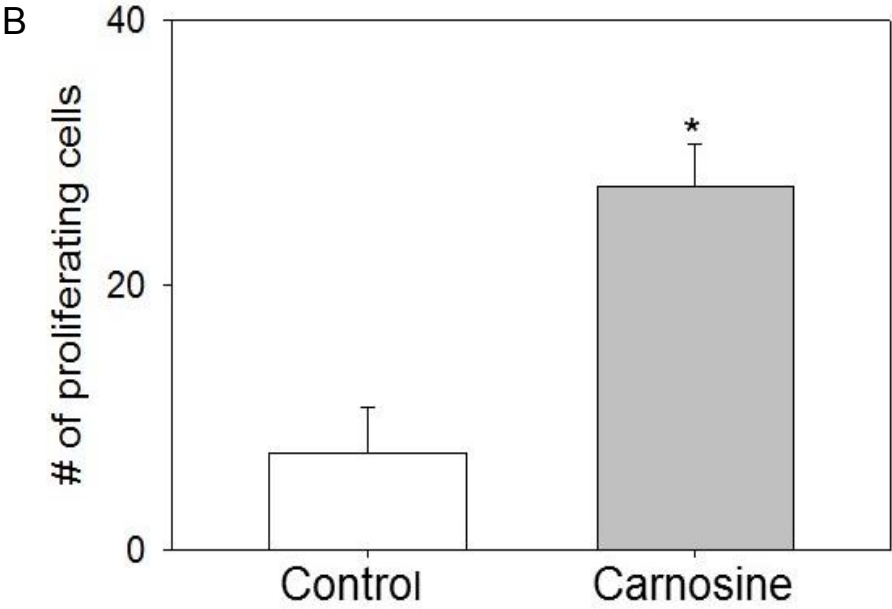
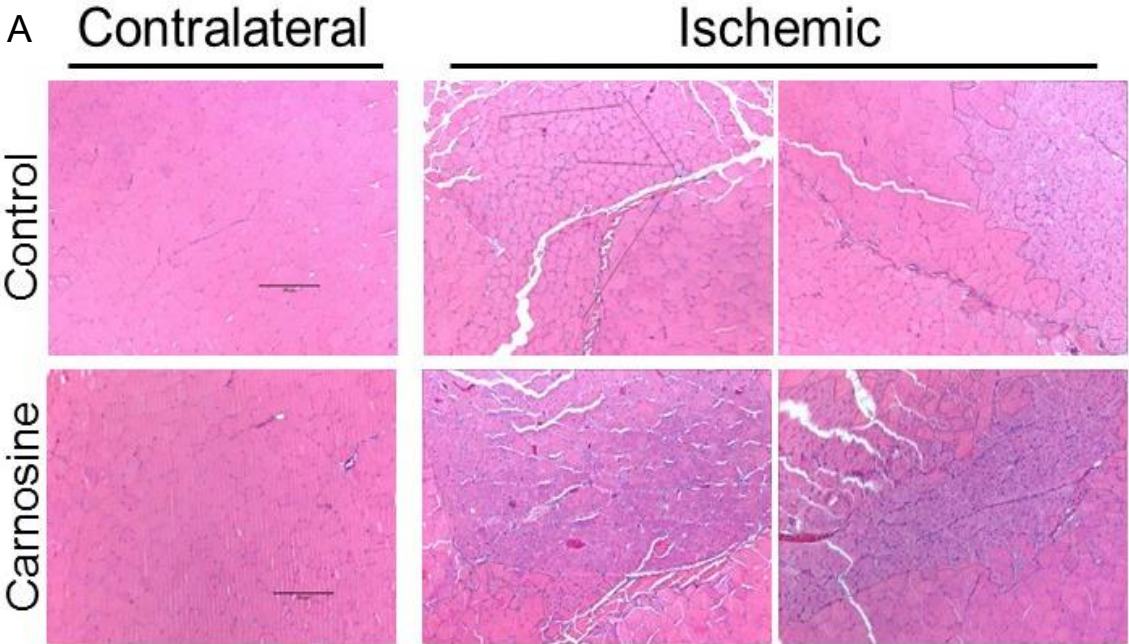


Figure 6



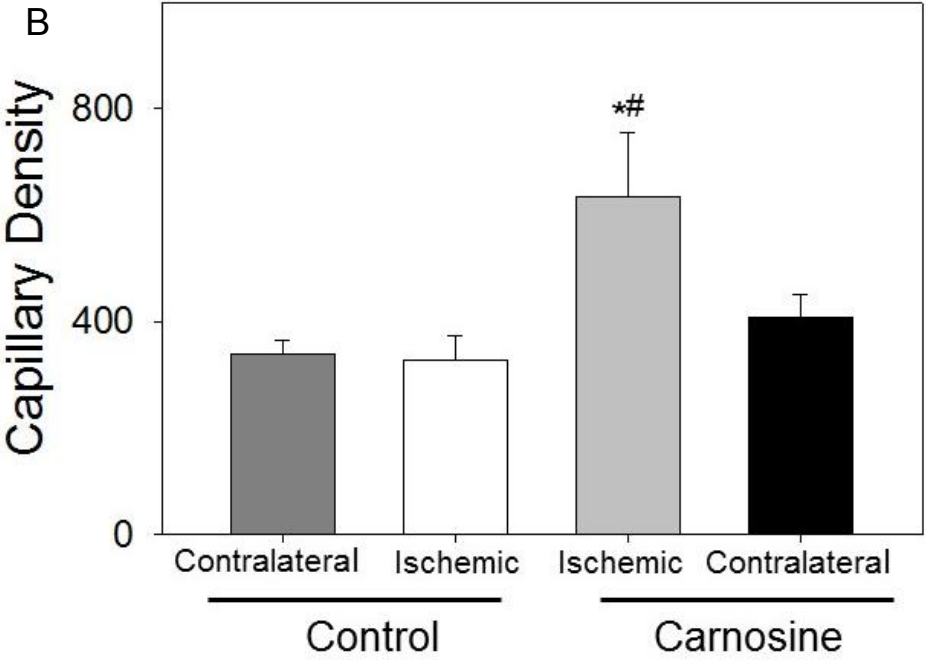
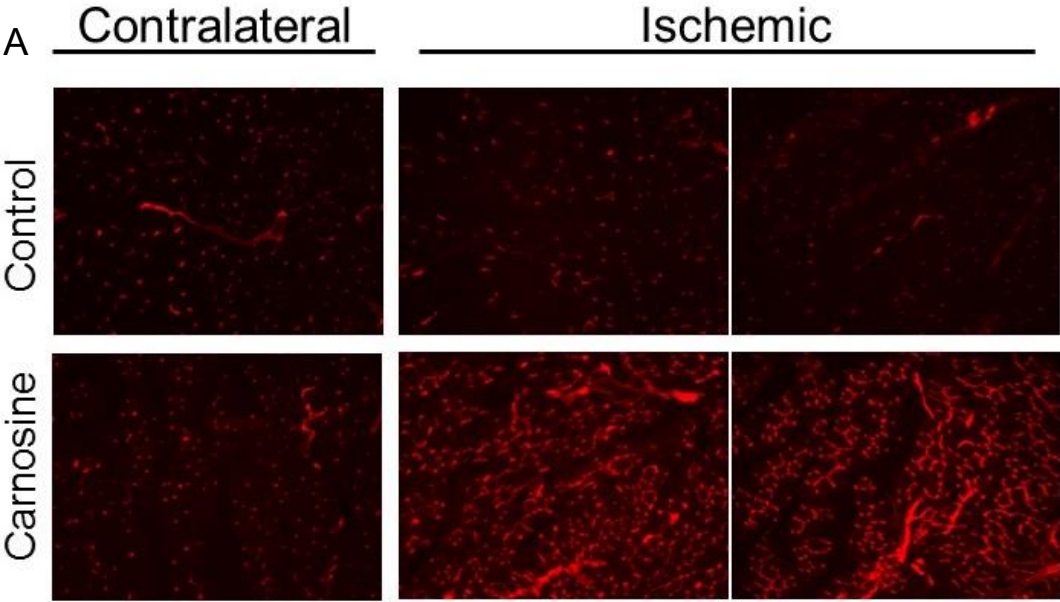
**Figure 6: Whole mount images and microcomputed tomography (micro-CT) angiograms demonstrating that carnosine treatment increases arteriogenesis.** WT C57/BL6 mice subjected to HLI were allowed to recover for 21 days with or without carnosine supplementation. **(A)** Representative whole mount images of carnosine treated and non-treated glycerol clarified microfil perfused limbs. Results shows increased microfil filling (increase yellow coloration) in ischemic limbs of carnosine-treated compared with non-treated mice. **(B)** Representative images of micro-CT scans demonstrating increased vascular growth in carnosine treated compared with non-treated mice. Arrows indicate proximal ligation sites. **(C)** Quantification, represented as a percentage of vascular density of ligated limb to contralateral limb. n=10 mice in each group. \* $p < 0.01$  vs control mice.

Figure 7



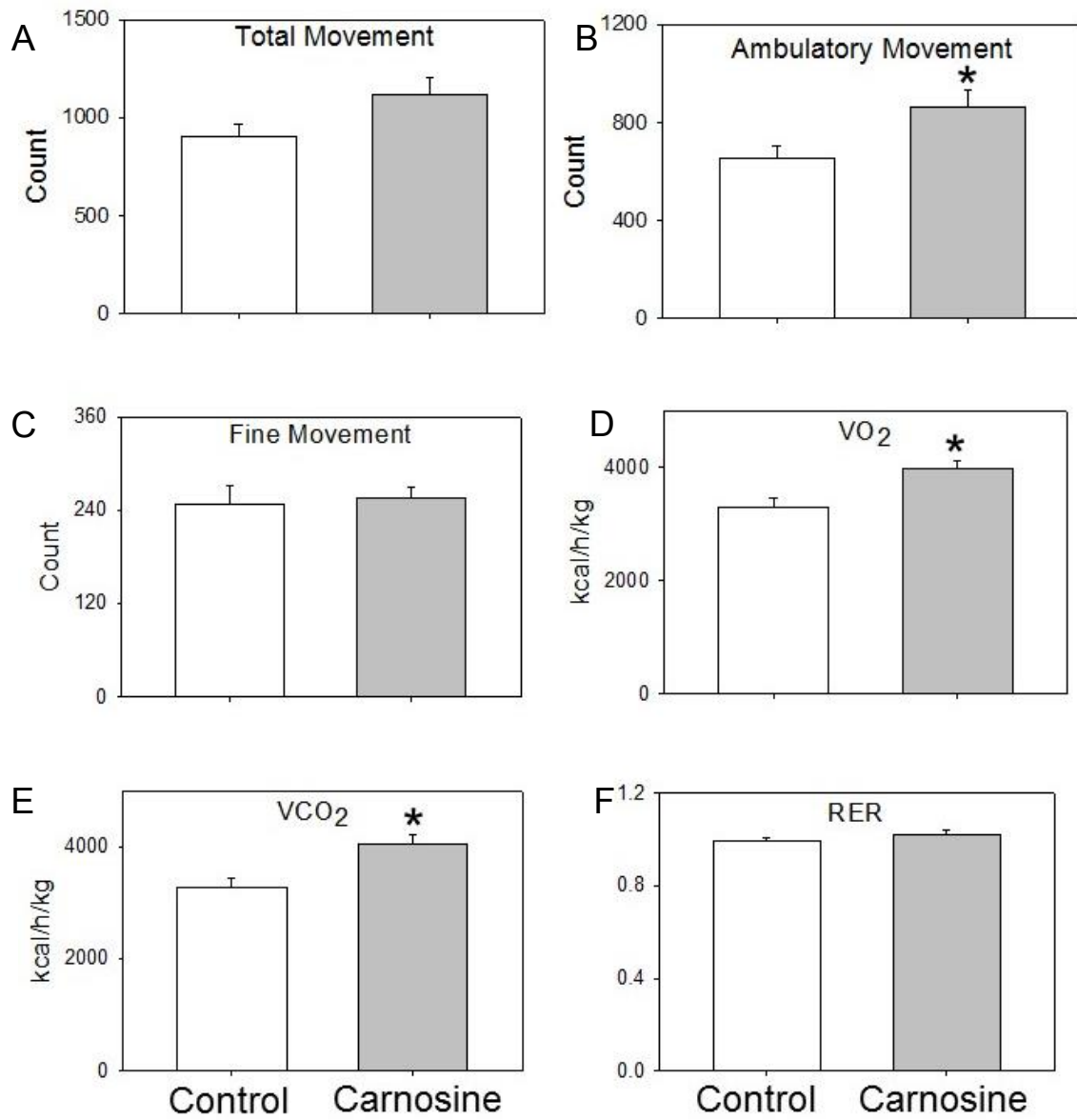
**Figure 7: Carnosine treatment increases muscle regeneration and attenuates ischemic tissue injury:** H&E staining of paraffin embedded sections of the hamstring muscle captured under bright field conditions with 10X objective. **(A)** Representative images for non-treated and carnosine treated mice after 7 days of recovery from HLI surgery. Contralateral limbs are included for comparison. **(B)** Data expressed as mean  $\pm$  SEM for the number of regenerated myocyte (myocytes with centrally located nuclei) from 4-5 fields per section (4-5 sections/mouse). n=5 for each treatment. \* $p < 0.01$  compared to non- treated HLI mice.

Figure 8



**Figure 8: Neovascularization is increased with carnosine treatment: (A)**  
Isolectin B4 staining of ischemic and contralateral hamstring muscles of non-treated and carnosine-treated HLI mice following 21 days of recovery **(B)**  
Quantitative analysis of capillary density. n=5 for each treatment. \* $p < 0.05$  vs non-treated ischemic limb. #  $p < 0.05$  vs non-treated contralateral limb.

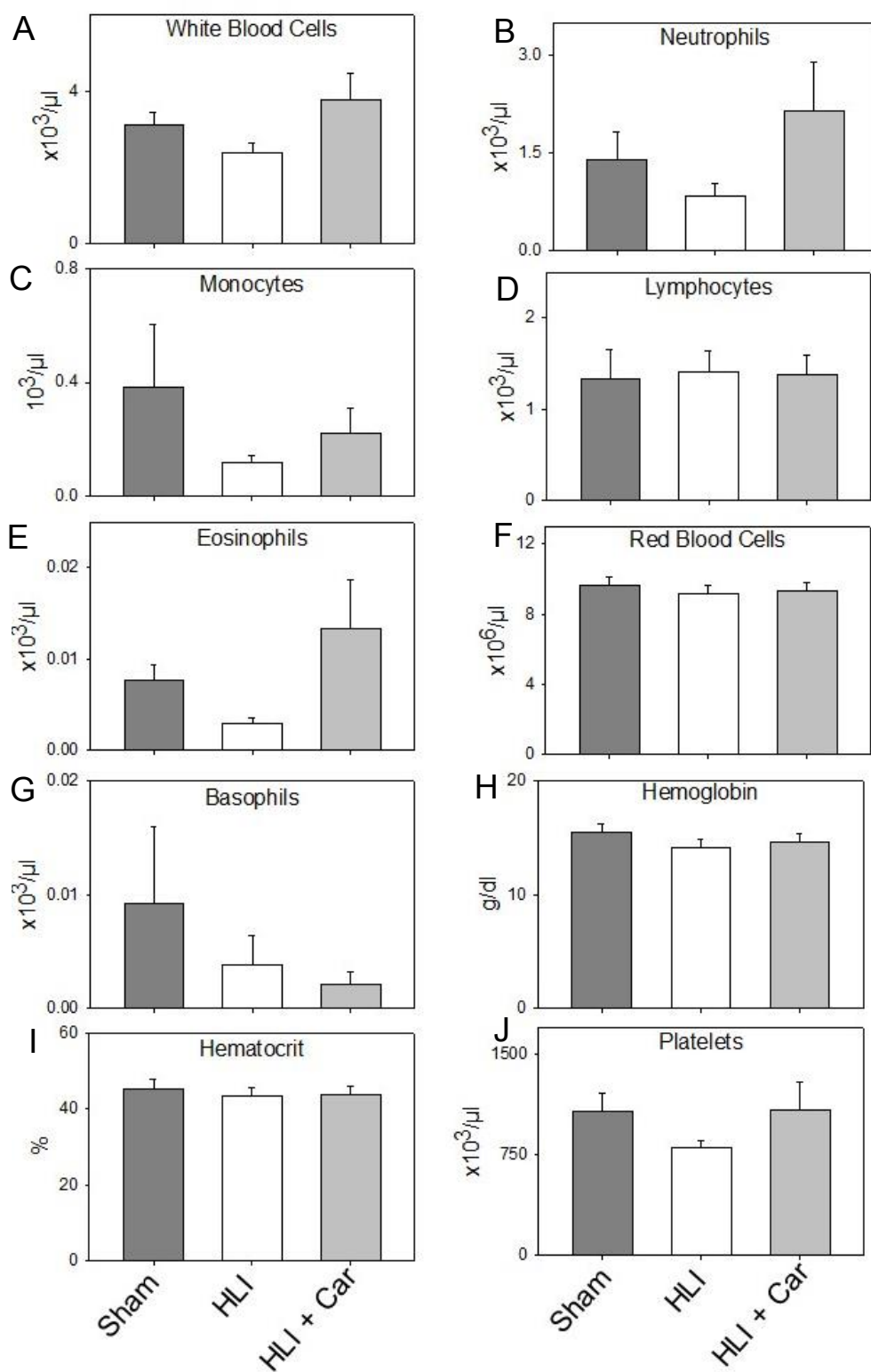
Figure 9



**Figure 9: Physical activity and metabolic rate is increased in carnosine treated mice.** Measurements of **(A)** total movement, **(B)** ambulatory movement and **(C)** fine movement of carnosine treated and non-treated mice after 14 days of recovery from HLI were used to assess the physical activity. Metabolic state assessment was done by examining, **(D)**  $VO_2$ , **(E)**  $VCO_2$  and **(F)** respiratory exchange ratios (RER) in the treated and non-treated HLI mice. Data are presented as mean  $\pm$  SEM of n=5 mice in each group. \* $p < 0.05$  vs non-treated mice (control).



Figure 10



**Figure 10: Blood cells populations and properties are not affected by carnosine treatment.** Complete Blood Count of (A) white blood cells and the subpopulations of white blood cells i.e. neutrophils, monocytes, lymphocytes, eosinophils (B-E,G) in peripheral blood were determined along with (F) Red blood cells, (H) hemoglobin, (I) hematocrit, (J) platelets levels. All measurements were taken following 3 days of recovery from surgery. No changes were observed in any of these blood cell populations.

## **Discussion**

The results from this study show that carnosine increases blood flow in the ischemic limb by promoting post-ischemic angiogenic and arteriogenic response. Our results demonstrate that carnosine supplementation increases arterial volume/diameter, capillary density and muscle regeneration in the HLI mice compared with non-treated HLI mice. Furthermore, limb function was significantly improved in the carnosine treated HLI mice.

A major goal of this study was to evaluate the therapeutic potential of carnosine in critical limb ischemia. This is particularly important because of the high rates of mortality and morbidity in these individuals [15]. The model of murine HLI employed in this study is important in evaluating changes induced in critical limb ischemia and monitor efficacy of possible candidate drugs [175]. Ligation of femoral artery is reflective of the human disease in which occlusion of the femoral artery is frequently observed [12, 225].

Laser Doppler Perfusion Imaging (LDPI) provides noninvasive recording of microvascular tissue perfusion allowing for longitudinal assessment of blood flow after occlusion [226]. Our results show that pre-treatment of carnosine in mice significantly increased tissue perfusion in the HLI mice compared to non-treated mice. Furthermore, our results showing that carnosine supplementation improves blood flow after HLI surgery suggests that carnosine can be used as therapeutic intervention for PAD patients. We did not observe any toxic effects within the doses that were used in this study although symptoms of paresthesia are observed in humans with single doses higher than 800 mg [227].

One of the limitations of LDPI is the limited depth of penetration thus, only surface vascular perfusion can be monitored. The femoral artery is fairly superficial to be monitored by LDPI, however, the femoral artery serves as a conduit for other vessels and its occlusion disrupts flow to the entire limb. Therefore, it is important to monitor developing collateral networks and arterioles deep within the muscle to evaluate the extent of damage and recovery. Microfil-perfusion coupled with microCT imaging, offers a more holistic assessment for evaluating vascular remodeling. This silicon-based radio-opaque agent once injected through the left ventricles form a 3-D cast of the vasculature. The mineral phase of bone, due to its radio-opaque nature, can interfere with micro-CT analysis. Therefore, formic acid decalcification is used to remove the mineral phase of bone without affecting the already casted vasculature [228]. With additional threshold settings to eliminate soft tissue extensive network of tortuous arterioles can be observed, which a hallmark of arteriogenesis is. Our results show an increase in the appearance of tortuous vessels with carnosine treatment. We also observed a significant increase in the arteriolar filling with carnosine treatment by using Analyze software. This technique cannot adequately quantify capillary density due to its low resolution and detection limits. Therefore, it is primarily used to quantify the extent of arteriogenesis thus, suggesting that carnosine treatment increases arteriogenesis in HLI mice.

Angiogenesis is an important contributor to post-ischemic revascularization. Due to the limitation of microfil-perfusion in measuring capillary density, we used isolectin staining to determine the role of carnosine in angiogenesis. Isolectin

staining was significantly more in carnosine treated ischemic hamstring muscle following 3 weeks of recovery compared to non-treated mice. Interestingly, no difference was observed between the contralateral limb and ischemic limbs of non-treated mice. Collectively, our results demonstrated that carnosine treatment increases both angiogenesis and arteriogenesis in HLI mice.

Excessive myofiber damage has been observed in the gastrocnemius muscle of peripheral artery disease patients [192]. We evaluated the effect of carnosine on ischemia induced tissue injury. After 7 days of recovery from ischemia we observed an increase in the number of regenerated muscle in the ischemic muscle of carnosine treated compared with non-treated mice. This effect was sustained at 14 days of recovery and tissue injury significantly reduced at this time point (**data not shown**). To further determine whether the recovery of tissue perfusion and restoration of muscle structure improves limb function we determined physical activity by monitoring their movement in a metabolic cage. These results showed that physical activity of carnosine treated mice was significantly improved compared to non-treated mice. Although, metabolic cage analysis provided sufficient evidence for limb function, treadmill exercise can be employed in future to evaluate stress-induced influences in limb function.

In conclusion, despite the great utility of the murine HLI model some limitations exist. Firstly, in PAD the underlying cause is atherosclerosis while in this model obstruction is achieved surgically. Also, the clinical patients present with several other cardiovascular risk factors such as diabetes which is associated with microvascular dysfunction. Future investigations examining the role of carnosine

in the presence of one or more of these risk factors may be important to further evaluate the use of carnosine for the patient population.

In summary our results provides the first evidence that carnosine improves wound healing after HLI. Our results show that carnosine can regulate arteriogenesis, angiogenesis and attenuate tissue injury. These results demonstrate the potential therapeutic utility of carnosine. However, further studies are needed to evaluate the optimal dose, frequency of dose and best mode of administration for the PAD patients.

## CHAPTER III

### CARNOSINE IMPROVES REVASCULARIZATION BY AUGMENTING THE POST-ISCHEMIC ANGIOGENIC RESPONSE

#### **Introduction**

Peripheral artery disease refers to atherosclerotic narrowing or blockage of arteries that supply blood to the limb. Risk factors include smoking, diabetes, ageing, hypertension, dyslipidemia and obesity and modification of these risk factors represent a viable option in the management of PAD [7, 170].

Carnosine levels have been shown to be negatively associated with some of these risk factors while its supplementation is essential in modifying these risk factors. For instance in apolipoprotein E null mice, which are highly susceptible for developing atherosclerosis, carnosine was effective in preventing the development of atherosclerosis [229]. A decrease in carnosine levels have been observed in Type II diabetic humans [230]. In animal models of obesity, improved insulin sensitivity and reduced cardiovascular risk factors such as dyslipidemia and hypertension have been demonstrated with carnosine treatment [231-233]. Ageing in humans is also associated with a decrease in muscle carnosine content [234]. Interestingly exercise, an important non-invasive option for improving intermittent claudication and rest pain is associated with an increase in muscle carnosine

levels [235, 236]. These observations suggest a role of carnosine in vascular health and therefore a viable candidate to investigate.

In diverse models of ischemia carnosine has been observed to play a protective role. Intraperitoneal administration of carnosine at a concentration of 100mg/kg attenuates ischemic injury induced by cerebral ischemia [237]. Similarly, carnosine treatment promotes recovery of cardiac function in isolated heart model of ischemia/ reperfusion [199, 238]. Likewise, intraperitoneal administration of carnosine protected hearts from Adriamycin-induced cardiotoxicity and restored cardiac function after ischemia [239]. Similar effects have been observed in hepatic, renal and testicular models of ischemia with carnosine treatment [240-244].

The protective effects of carnosine are mostly attributed to quenching of reactive aldehydes by carnosine [245]. This property of carnosine is particularly important because under ischemic conditions generation of reactive aldehydes is increased and pathways that remove these aldehyde are overwhelmed thus, carnosine supplementation is essential to compensate for this deficit [33]. Studies with cerebral model of ischemia have shown that carnosine supplementation imparts neuroprotection by improving mitochondrial function and diminishing autophagy [246]. Inhibitory effects of carnosine on mast cell degranulation and histamine release was reported in in-vitro models of ischemia [247]. Vasodilatory effects and regulation of myocyte contractility through modulation of calcium homeostasis are likely to contribute to anti-ischemic effects of carnosine.

Although the anti-ischemic effects of carnosine are known, its regulation on the vascular adaptations to ischemia has not been studied. The mouse hindlimb ischemia



represents a good model to study vascular adaptations to ischemia and explore the effects of therapeutic candidates on this process. In this model the role of various players such as inflammatory cells, cytokines and progenitor/stem cells can be monitored in a temporal fashion to determine their possible causal role in the recovery process. In this series of investigations we examined the effect of carnosine supplementation on post-ischemic angiogenic/vasculogenic response. Using flow cytometric analysis we evaluated ischemia induced mobilization of endothelial progenitor and immune cells to establish a role for carnosine in the regulation of these factors. We also determined whether carbonyl quenching by carnosine can be a potential contributor to the vascular and tissue adaptations in HLI mice.

### **Experimental Procedure**

**Material:** Tyrosine–Histidine, anserine (Bachem), protease inhibitor cocktail, bovine serum albumin, nonafluoropentanoic acid and heptafluorobutyric acid (Sigma-Aldrich), fibrous tissue RNA isolation kit (Qiagen), VEGF (Santa-Cruz), anti-carnosine (Abcam), anti-4-hydroxynonenal (Cosmo Bio), avian virus reverse transcriptase, deoxy nucleotide triphosphate mix (Promega), primers (IDT), SYBR green (VWR), Pierce ECL plus (Thermo Fischer).

### **Liquid chromatography / Mass spectrometry (LC/MS/MS) analysis of histidyl**

**dipeptides:** Hamstring muscles were homogenized in a lysis buffer; phosphate buffered saline, protease inhibitor cocktail and 200 $\mu$ M Tyr-His (internal standard; IS). The homogenates were sonicated and centrifuged at 16000 g. Following centrifugation, the supernatant was de-proteinated with 70% perchloric acid and centrifuged again. The resulting supernatant was neutralized with 750 mM

ammonium hydrochloride and diluted 1:1 with the mobile phase (90% water: 10% acetonitrile and 0.1% heptafluorobutyric acid). Samples were injected onto HP 1100 LC and separated on a Polar RP column. Histidyl dipeptides (carnosine and anserine) were detected by using Micromass Quattro LC/MS/MS system [216]. The data for histidyl dipeptides were acquired by monitoring the following transitions 226.95→110.22 (carnosine); 241.1→109.2 (anserine); 319→ 110.22 (Tyr-His). The peptides were quantified using the peak area ratio of histidyl-dipeptide and internal standard. MassLynx mass spectrometry software from Waters was used for the analysis.

**qRT-PCR analysis:** Following euthanasia hamstring muscle of mice were harvested and pulverized. Total RNA was obtained using Qiagen Fibrous Tissue RNA isolation kit. RNA quantification and quality were obtained by taking ratio of the absorbance at 260nm to 280nm using Nanodrop 1000A spectrophotometer (Thermo). To synthesize cDNA the isolated RNA and oligo dT primer mixture were heated at 70°C for 5 mins to allow the primers anneal with mRNA templates. dNTP's and reverse transcriptase (avian virus reverse transcriptase from Promega) enzymes were added to the mixture and the resulting mixture was incubated at 42°C for 1 h followed by heating at 94°C for 5 min to denature the enzyme. Relative gene expression was analyzed by using cDNA, SYBR green and a pair of forward and reverse primers. The cycling conditions include; 5 min at 95°C, 45 cycles of 10 seconds at 95 °C, 20 sec at 60 °C and 25 sec at 72 °C . Ct values were obtained in triplicates. Hypoxanthine- guanine phosphoribosyl transferase 1 (HPRT1) was used as internal control gene. Measurements were made on Prism 7900 HT (Applied Biosystem). Fold changes in

mRNA expression between the groups were calculated according to the comparative  $C_t$  method that involves comparing the  $C_t$  values of the gene of interest and the internal control.

**Flow cytometry:** To identify the role of leukocytes and endothelial progenitor cells (EPC's) in carnosine mediated recovery from HLI, flow cytometry analysis of peripheral blood, bone marrow and skeletal muscle from HLI and sham operated mice were carried out on BD LSR II flow cytometer. Mice were allowed to recover for 3 days. Following euthanasia blood was collected by cardiac puncture, and the femur, tibia and muscles (hamstring and gastrocnemius) were dissected. Blood samples were lysed, blocked by Fc and washed with 1% bovine serum albumin in PBS. The samples were stained for leukocyte markers using a cocktail of the following antibodies, CCR2, CD11b, F4/80, Gr-1, Ly6C, 7/4, CD19, CD4 and CD8. Samples were stained with antibody cocktail for 30 mins, washed with BSA-PBS. For EPC's identification samples were treated in the same way except stained with Flk-1<sup>+</sup> and Sca1<sup>+</sup> antibodies.

For bone marrow analysis, femur and tibia from both the ischemic and contralateral limbs were flushed with Hank's balanced salt solution (HBSS) to obtain the bone marrow. The bone marrow was separated by ficoll and the buffy coat was washed with 1% bovine serum albumin in PBS. Samples were blocked by Fc and stained for EPC and leukocyte markers respectively and analyzed by LSR.

To determine the leukocyte infiltration, the hamstring and gastrocnemius muscle of ischemic and contralateral limbs were minced in HBSS and digested in 0.2% Type II

collagenase at 37°C for 1h. The digested tissues were passed through a 50 micron cell strained, washed with 1% BSA in PBS, Fc blocked and stained for leukocytes.

**Western blot analysis:** Tissue lysates were prepared in RIPA buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% NP-40). Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride (PVDF) membrane and blocked with 5% milk in Tris buffered saline (TBS) with 1%-3% Tween 20. The blots were incubated in primary antibodies at 4°C for overnight followed by secondary antibody incubation at room temperature for 1h. Horseradish peroxidase (HRP) conjugated secondary antibodies were used in all analysis. Membranes were developed using HRP substrate (ECL plus form Pierce) and scanned with Typhoon bioimager (GE healthcare)

**LC/MS/MS analysis of carnosine-aldehyde conjugates:** To evaluate the extrusion of carnosine-aldehyde conjugates in urine, mice were placed on 3% glucose and 0.125% saccharin solution (1g /L). Urine was collected at different time intervals (1, 2, 3 and 12 h) after the supplementation to determine baseline conjugates levels. Post-surgery changes in urinary conjugates were determined by collecting urine 1, 2, 3, and 12 h after the HLI surgery. The urine samples were centrifuged at 16000 g for 10 min to remove debris and spiked with 30 µl of 1mM Tyr-His (internal standard) per 500 µL of urine. The urine samples were deproteinated by centrifuged at 16000 g for 30 mins in a 3000 Da cut off filter. The filtrates were separated on HP 1100 LC connected to a Polar RP column using 5mM nonafluoropentanoic acid (NFPA) as the mobile phase. Detection of the carnosine aldehyde conjugates and their reduced

forms was done on a Micromass Quattro *LC/MS/MS* system [199]. The ratio of peak intensity of conjugates and the internal standard was used for quantification. MassLynx mass spectrometry software was used for analysis.

## **Results**

**Carnosine transport to the ischemic limb is increased with carnosine supplementation:** Based on the observations that limb perfusion and function are increased with carnosine treatment, we next analyzed carnosine levels in the skeletal muscle of carnosine treated and non-treated HLI as well as sham-operated mice. *LC/MS/MS* analysis of the hamstring muscle showed that carnosine levels were significantly increased in the carnosine treated HLI compared to the sham and non-treated HLI mice (**Fig. 11A**). Surprisingly, no changes in carnosine levels were observed in the carnosine treated sham mice suggesting that carnosine transport is increased in the ischemic limb only (**Fig 11A**). To determine the factors responsible for increase in intra- muscular carnosine levels, we determined the expression of enzymes and transporters involved in maintaining homeostasis of endogenous carnosine levels. We determined the expression of transporters (PHT1, PEPT1, PEPT2, TauT), and enzymes (ATPGD1 and CNDP2). No differences were observed in the mRNA levels of ATPGD1 and CNDP2 suggesting that there is no change in the synthesis and degradation of carnosine by HLI surgery and carnosine treatment. However, the expression of carnosine and dipeptide transporters PEPT2 and PHT1 were significantly increased in carnosine-treated HLI compared with non- treated HLI mice suggesting that the increased expression of transporters may be responsible for the increased carnosine levels in the ischemic tissue (**Fig. 11B**).

**Carnosine treatment increases EPC's mobilization:** Numerous studies have shown that EPCs are mobilized from bone marrow to the site of ischemic injury to increase post-natal vasculogenesis and angiogenesis [128]. Because, we observed an increase in capillary density in the ischemic muscle of carnosine treated mice, we tested whether carnosine improves angiogenesis by increasing EPCs mobilization from the bone marrow to the site of injury. We determined EPCs levels in the peripheral blood and bone marrow using stem/progenitor cell marker Sca-1 and the endothelial cell marker Flk-1 (VEGFR2). Our results show that following 3 days of recovery from HLI there was no change in the circulating EPCs levels between the HLI and sham treated animals, however, a significant increase in EPCs mobilization was observed in the carnosine treated HLI mice (**Fig. 12E**). No change in EPCs mobilization was observed in the sham operated carnosine treated mice (**Fig. 12E**). No change was observed in the bone marrow EPCs following 3 days of recovery from HLI (**Fig. 12 F**). However, after 7 days of recovery EPC levels were significantly increased in the peripheral blood of HLI compared with sham operated mice. This increase was further enhanced in the carnosine treated mice (**Fig. 12 G**). Levels of EPC in the bone marrow of both non-treated and carnosine treated HLI mice were decreased compared to sham operated mice (**Fig. 12H**).

**Carnosine treatment does not affect the inflammatory response induced by ischemia:** In addition to EPC's, bone marrow derived mononuclear cells have been shown to regulate neovascularization and collateral growth. We therefore examined whether the levels of macrophages (CD11b<sup>+</sup>, F4/80<sup>+</sup>), monocytes (CD11b<sup>+</sup>, F4/80<sup>-</sup>), inflammatory monocyte subset (Ly6Chi, 7/4hi), resident monocyte subset (Ly6Clo,

7/4lo) B cells (CD19<sup>+</sup>), CD4<sup>+</sup> and CD8<sup>+</sup> lymphocyte levels were changed in the blood, bone marrow and skeletal muscle (NB. lymphocytes were evaluated in blood samples only and macrophages in the muscle samples only). The gating strategies are summarized in **(Fig.13)**. All analyses were done after post-ischemia (3 days). Our results show that the levels of bone marrow inflammatory monocytes were increased in the HLI carnosine treated and non- treated mice **(Fig. 14E)**, but no change in the total and resident monocyte populations **(Fig. 14 D, F)** were observed. In the peripheral blood no change was observed in the monocytic populations with HLI or carnosine treatment **(Fig. 14 A-C)**. Analysis of the skeletal muscle showed that monocytes and macrophages levels were increased by HLI however, carnosine supplementation had no additive effect on these cells **(Fig. 15A-D)**. In addition to flow cytometry results, qRT-PCR analysis of the monocyte chemo-attractant molecule (MCP-1/CCL2) and its receptor CCR2 showed MCP-1 and CCR2 expression were increased by HLI however, carnosine treatment had no additive effect **(Fig. 15 E-F)**. We also examined the mRNA levels of cytokines as well as cytokine regulated effector molecules which affect the inflammatory response such as VCAM, Tie2, TNF $\alpha$ , Angiopoietin, and IL-6. None of these factors were changed by HLI or carnosine treatment **(Fig. 15G)**. Collectively, these results demonstrate that carnosine treatment does not affect immune cell populations and inflammatory process induced by ischemia.

**Carnosine treatment increases VEGF levels in ischemic muscle:** Based on our observations that EPC mobilization is increased by carnosine supplementation and previous studies showing that vascular endothelial growth factor (VEGF) regulates

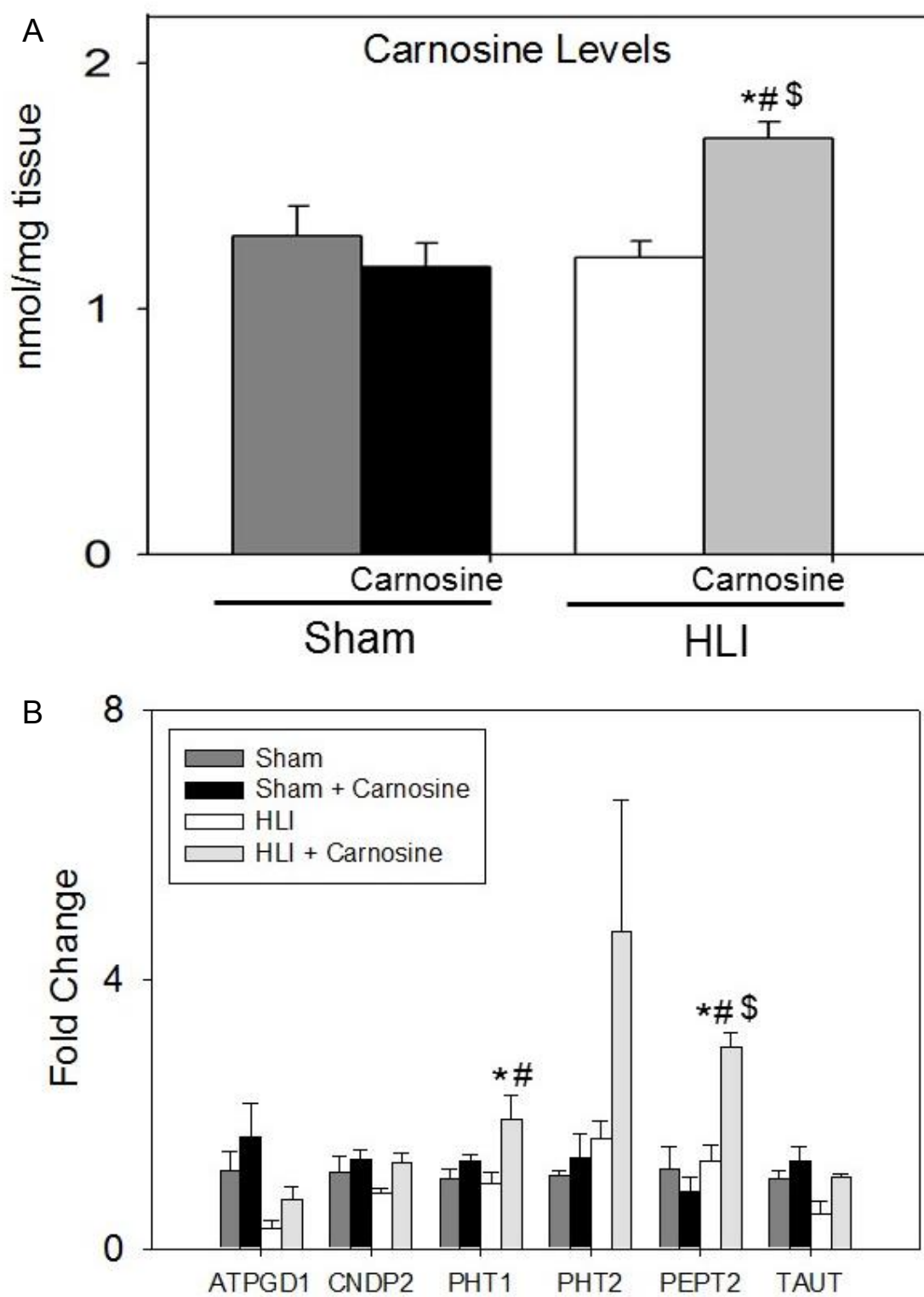
EPC's mobilization we determined VEGF levels in the ischemic muscle of the non-treated and treated mice. VEGF mRNA (**Fig. 16A**) and protein expression (**Fig 16. C, D**) were significantly increased in the carnosine treated mice compared to the non-treated HLI mice suggesting that increase in VEGF levels may be involved in increasing EPC mobilization. No change in VEGFR2 expression was observed. These results suggest that the carnosine treatment increases VEGF levels in the skeletal muscle which concomitantly increases EPC mobilization.

**Carnosine treatment increases extrusion of reactive aldehyde and protein carnosinylation:** Several studies have shown that generation of reactive aldehydes is increased in the ischemic muscle of HLI mice and PAD patients [191, 192]. Studies from our lab and others have shown that carnosine has the ability to react with aldehydes and can also bind with the protein-aldehyde adducts to generate carnosinylated protein adduct [199]. We therefore determined whether carnosine supplementation alone can increase the urinary extrusion of carnosine-reactive aldehydes conjugates. *LC/MS/MS* analysis showed that carnosine treatment increased the extrusion of carnosine-propanal (carnosine-acrolein) conjugates compared with non-treated mice (**Fig. 17 A**). The extrusion of carnosine-propanal conjugates was significantly increased after HLI surgery compared to the baseline. Notably, the extrusion of carnosine-propanal conjugates was significantly increased by carnosine treatment in HLI mice (**Fig 17 A**). Western blot analysis of the ischemic and contralateral tissues showed that generation of 4-HNE protein adduct was increased in the ischemic tissue and carnosine treatment decreased the generation of these adducts (**Fig 18 A, C**). Similarly, generation of carnosinylated protein



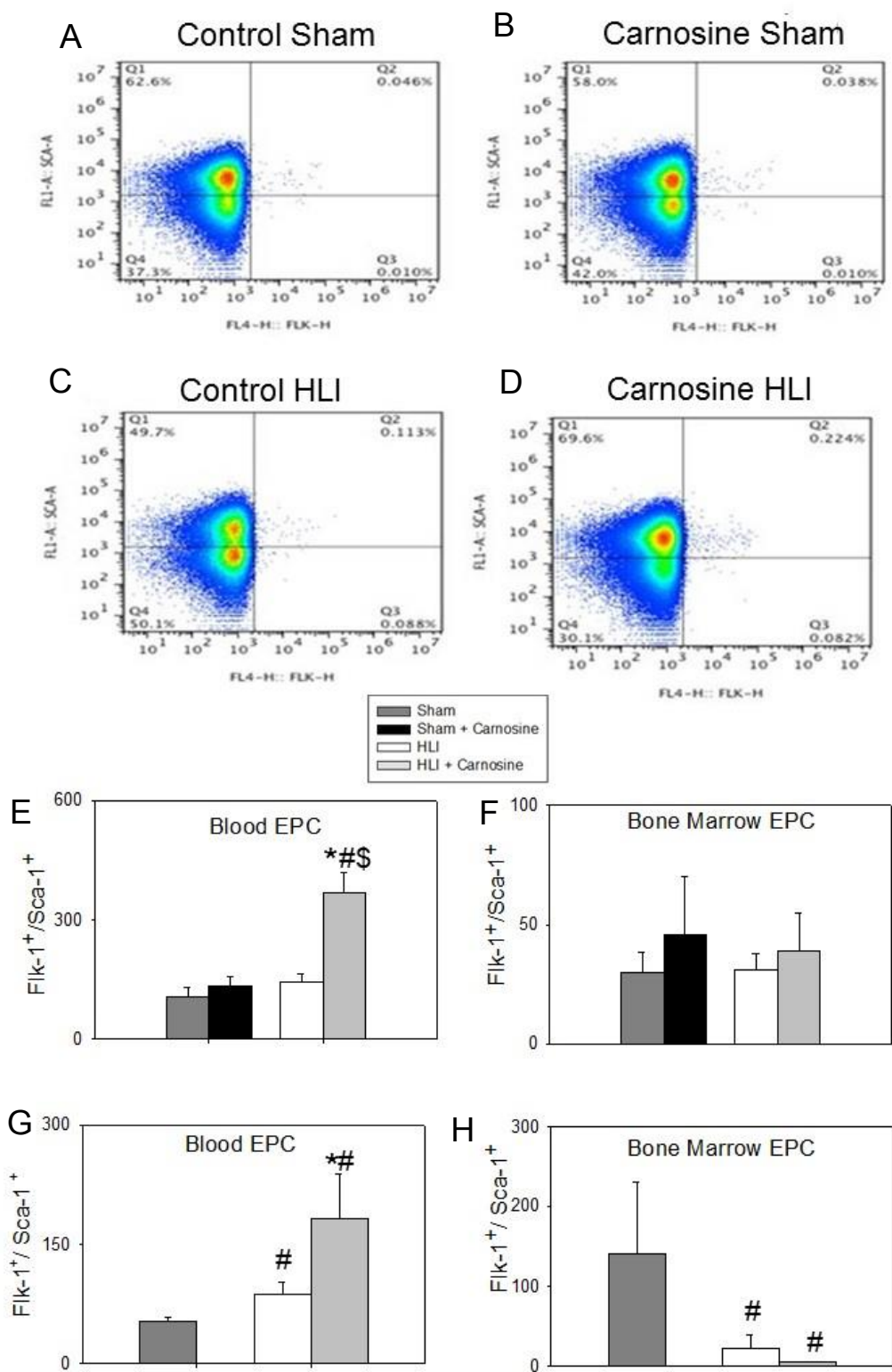
adducts, which prevents the propagation of the free radical chain reaction initiated by these reactive aldehydes, was increased in the ischemic muscle of non-treated HLI mice and supplementation of carnosine enhanced carnosinylated protein adduct generation (**Fig 18 B, D**). These results suggest that aldehyde quenching by carnosine could also contribute to wound healing responses mediated by carnosine.

Figure 11



**Figure 11: Oral administration of carnosine increases carnosine levels in the ischemic muscle. (A)** *LC/MS/MS* analysis of carnosine in the hamstring muscle of non-treated and carnosine-treated HLI mice. Treated and non-treated sham operated mice were used for comparison. **(B)** qRT-PCR analysis of the different enzymes and transporters that regulate carnosine levels. \* $p < 0.05$  non-treated HLI mice, #  $p < 0.05$  vs non-treated sham and \$ $p < 0.05$  vs carnosine-treated sham. n=5

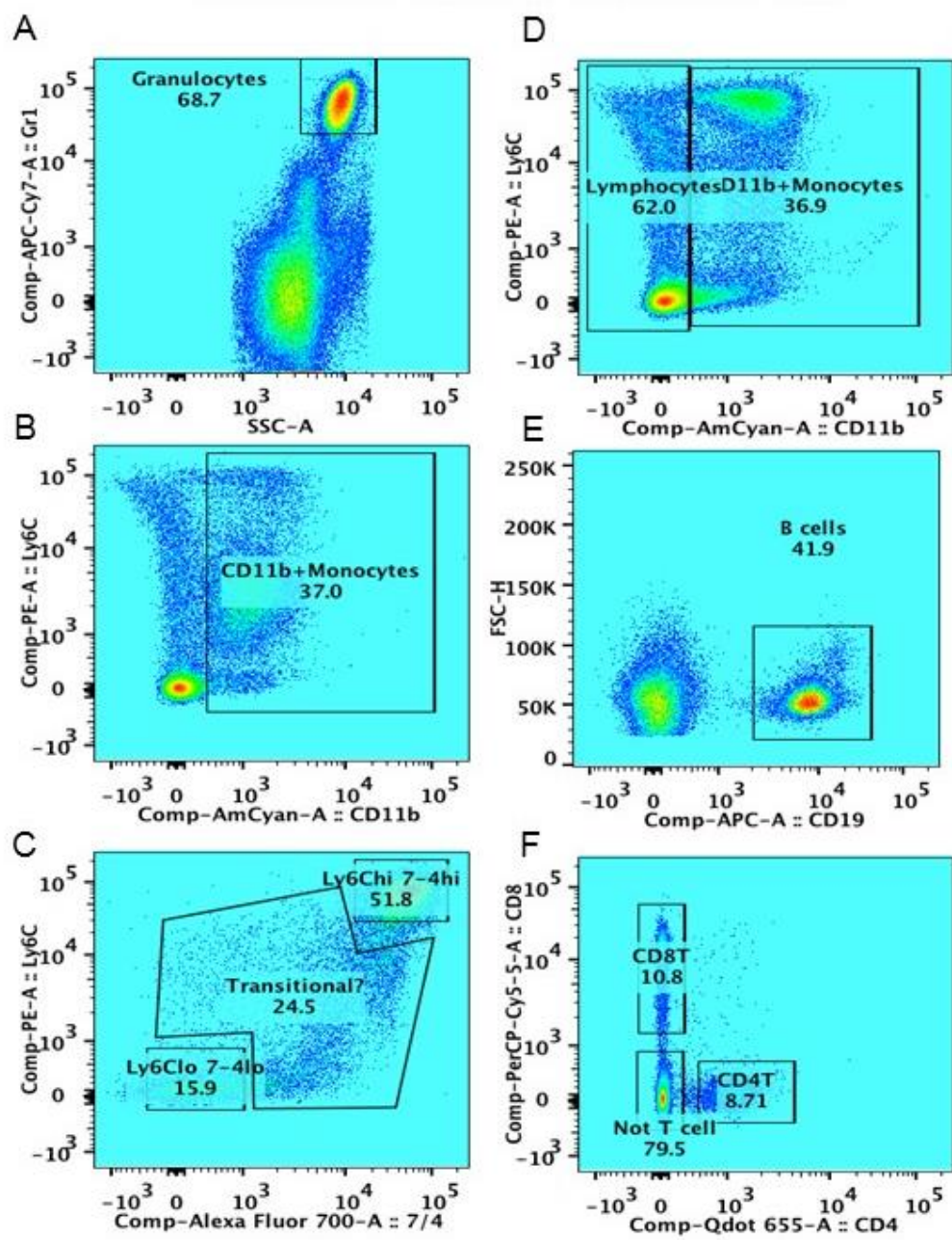
Figure 12



**Figure 12: Carnosine increases EPCs mobilization.** EPCs were identified as a subpopulation of the live lymphocytic cells that were double positive for Flk-1 and Sca-1. Representative dot blot of Flk-1<sup>+</sup>/Sca-1<sup>+</sup> cells in peripheral blood after 3 days of **(A)** Sham **(B)** Sham + carnosine **(C)** HLI and **(D)** HLI + carnosine treatment. Flk<sup>+</sup>/Sca<sup>+</sup> cells in **(E)** blood and **(F)** bone marrow after 3 days of recovery. **(G and H)** represents EPC levels in blood and bone marrow respectively after 7 days of recovery. \*p< 0.05 non-treated HLI mice, # p < 0.05 vs non-treated sham and \$p<0.05 vs carnosine-treated sham. n= 5-10 for each group.

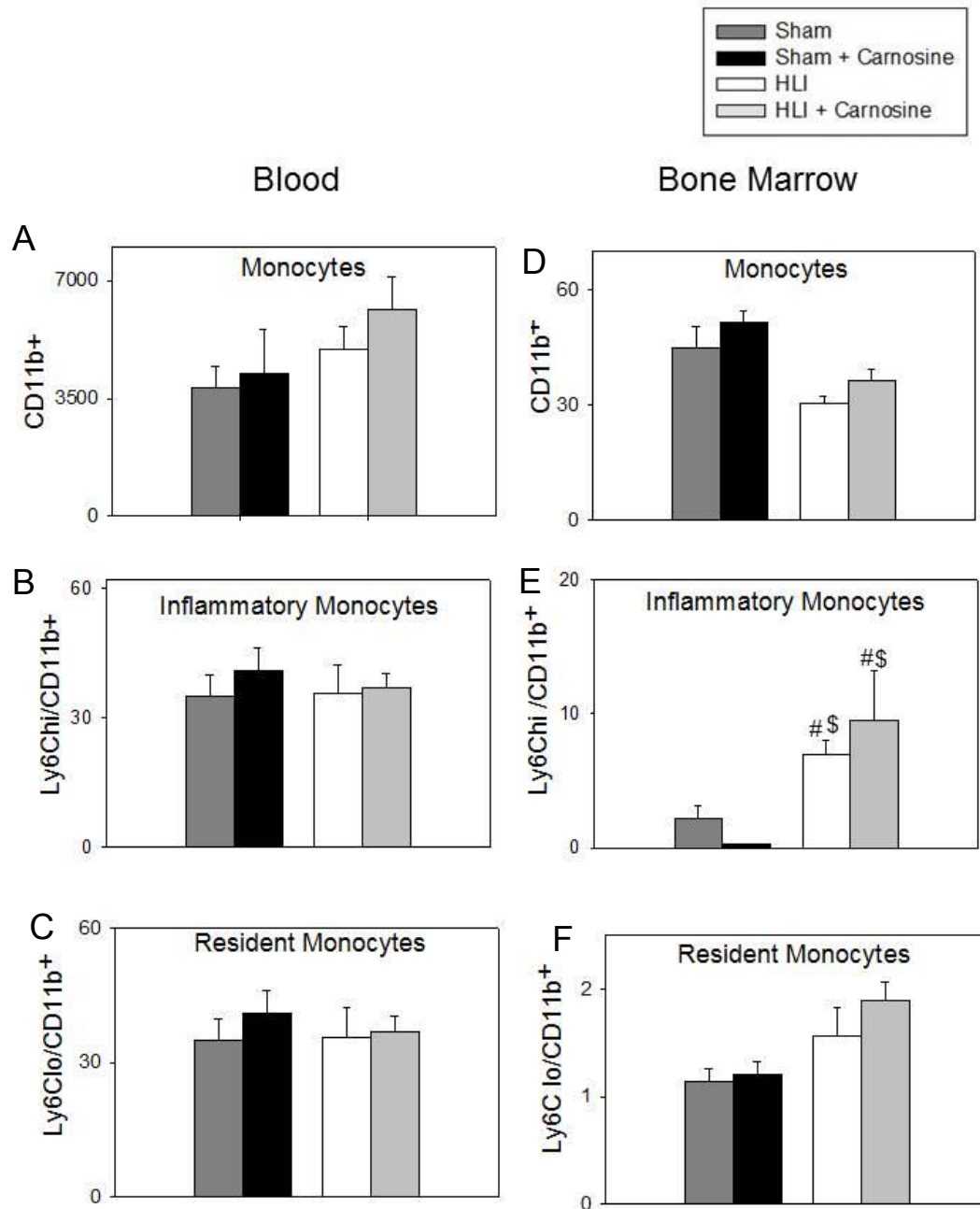
Figure 13

Flow Cytometry Gating Strategy for Immune Cells



**Figure 13: Gating strategy for evaluating immune cell regulation after HLI:** To evaluate the effect of carnosine treatment and ischemia on the immune cell populations we employed the following gating strategy **(A)** Cells identified as Gr-1<sup>+</sup> were defined as granulocytes and therefore excluded from further analysis. **(B)** Monocytes were identified by using CD11b<sup>+</sup> and Ly6C<sup>+</sup> antibodies. **(C)** Inflammatory subset of monocytes was identified as high for both Ly6C and 7/4 (hereby referred to as Ly6C<sup>hi</sup>). The resident monocytes are low for both Ly6C and 7/4 (referred to as Ly6C<sup>lo</sup>). To identify the lymphocytic subpopulations we **(D)** classified the cells that were neither monocyte nor granulocyte gate as lymphocytes. **(E)** B-lymphocytes were identified as the subset of the lymphocytes that are positive for CD19. **(F)** The remaining cells were identified as being CD4<sup>+</sup> or CD8<sup>+</sup>.

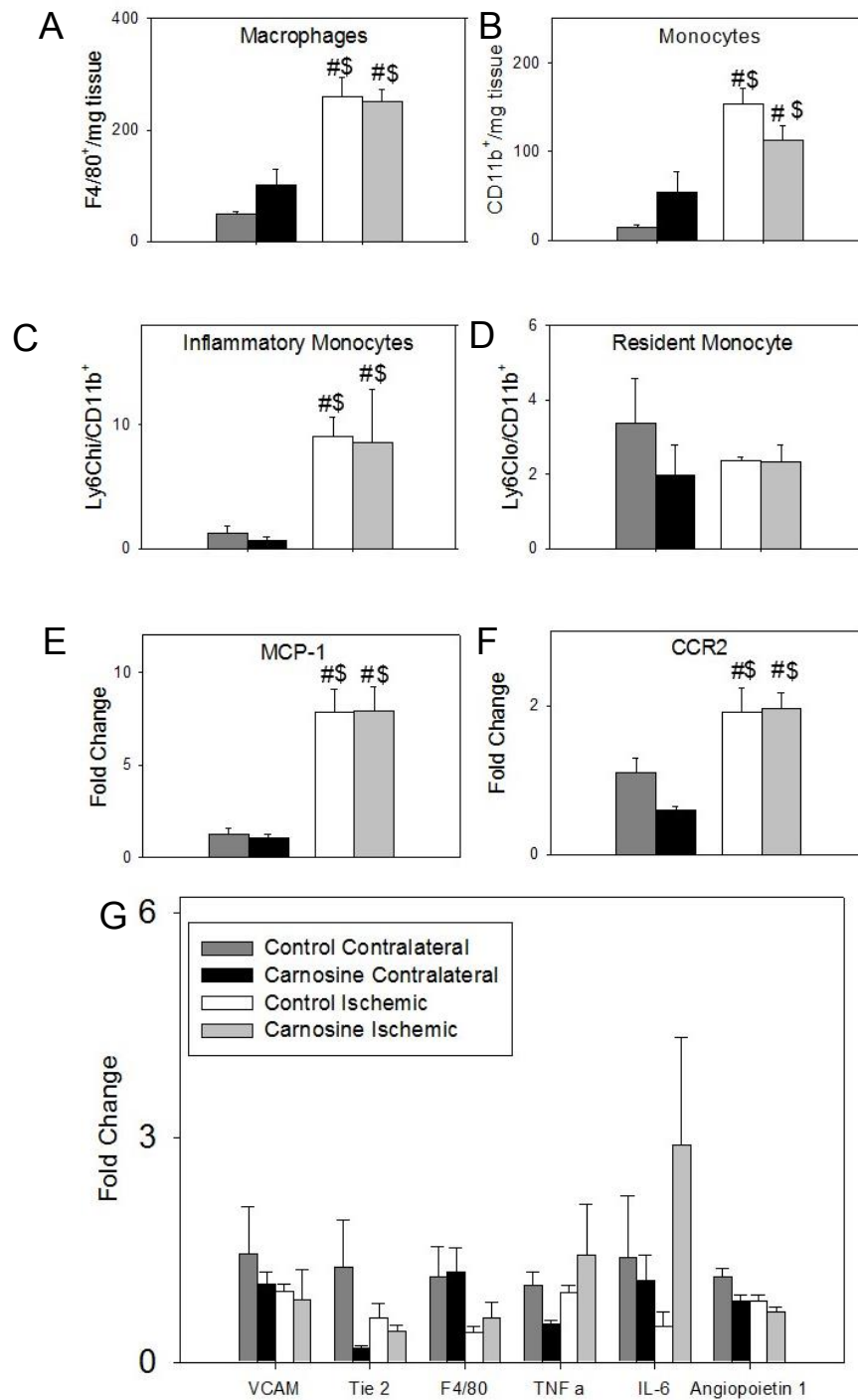
Figure 14





**Figure 14: HLI increased inflammatory monocyte population in the bone marrow.** Flow cytometry analysis of (A-C) blood monocyte subpopulation and (D-F) bone marrow monocyte subpopulations following 3 days of recovery from HLI. Inflammatory and resident monocytes results are expressed as percentage of total monocytes that were either Ly6C<sup>hi</sup> or Ly6C<sup>lo</sup>. Data is presented mean±SEM, #p<0.05 vs non-treated sham and \$p<0.05 vs carnosine-treated sham, n=6-10 in each group.

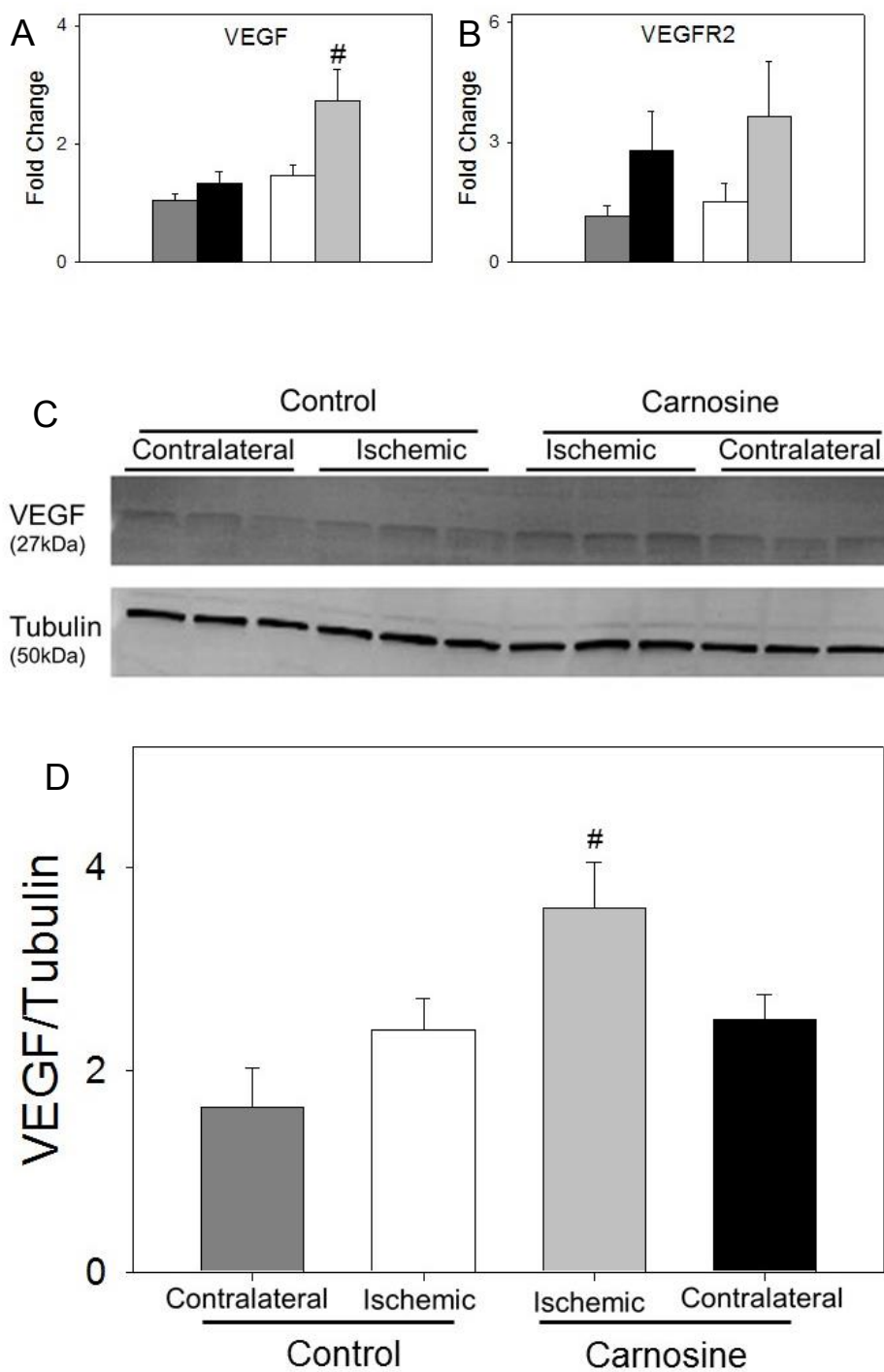
Figure 15



**Figure 15: HLI increases the inflammatory response in the skeletal muscle:**

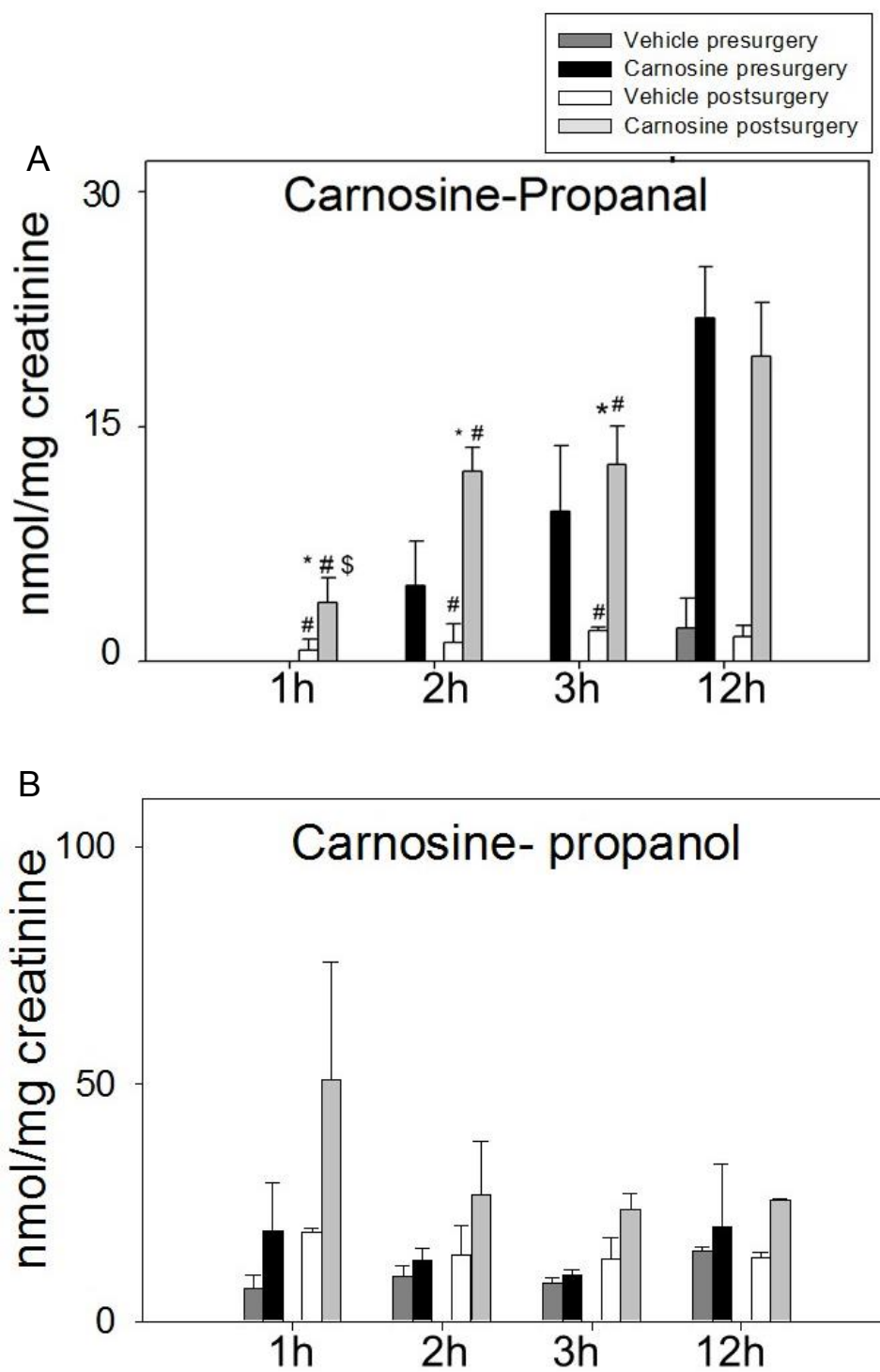
Flow cytometry analysis of the skeletal muscle showing recruitment of (A) macrophages (B) monocytes (C) inflammatory monocytes and (D) resident monocyte in the ischemic muscles. (E, F) qRT-PCR analysis for the expression of the monocyte-chemoattractant protein -1 (MCP-1) and its receptor CCR2. (G) Represents qRT-PCR analysis of various inflammatory cytokines in the hamstring muscle. Data is presented as mean  $\pm$  SEM, #p<0.05 vs non-treated contralateral limb and \$p<0.05 vs carnosine-treated contralateral limb, n=5 in each group.

Figure 16



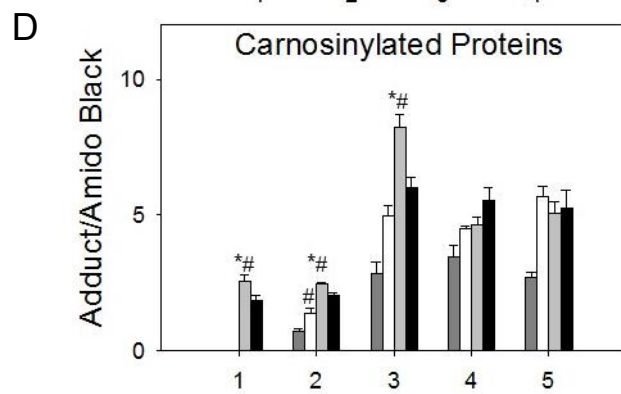
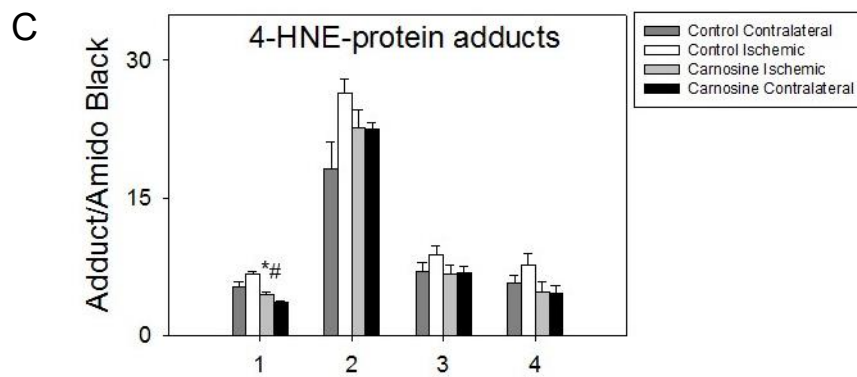
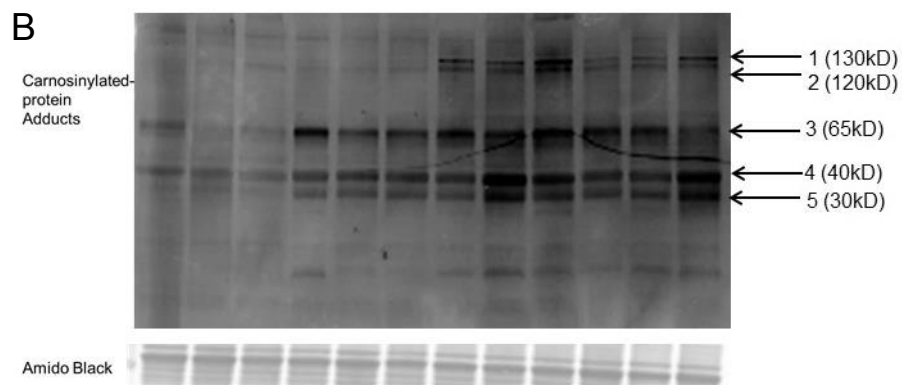
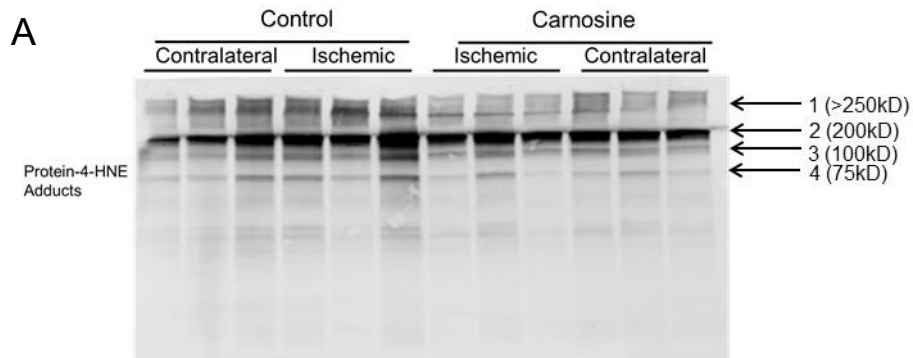
**Figure 16: Carnosine treatment increases VEGF expression in the ischemic muscle. (A)** VEGF mRNA and **(B)** VEGFR2 mRNA expression in the ischemic and contralateral muscles after 3 days of ischemia **(C)** VEGF protein levels in contralateral and ischemic muscle of carnosine treated and control mice. **(D)** Data is presented as mean  $\pm$  SEM. VEGF expression normalized to tubulin. #  $p < 0.05$  vs non-treated contralateral limb.  $n=5$  mice in each group.

Figure 17



**Figure 17: Quenching of reactive aldehydes is increased with carnosine treatment.** *LC/MS/MS analysis* of pre- and post-surgery urinary metabolite levels (A) carnosine-propanal and (B) carnosine-propanol. \* $p < 0.05$  non-treated HLI mice, #  $p < 0.05$  vs non-treated sham and \$ $p < 0.05$  vs carnosine-treated sham.  $n=4$ .

Figure 18





**Figure 18: Carnosine treatment decreases 4-HNE protein adducts formation and increases protein-carnosinylation.** (A) Western blots images of 4-HNE protein adducts and (B) carnosinylated proteins adducts in ischemic and contralateral muscle following 21 days of recovery from HLI. Blots were developed using anti-HNE and anti-carnosine antibodies (C) Represents the analysis of 4 HNE protein adduct and (D) carnosinylated protein adducts, data is presented as mean  $\pm$  SEM #  $p < 0.01$  vs contralateral and \* $p < 0.01$  vs HLI n=5 mice in each group.

## Discussion

The results from this study show that carnosine treatment results in increased circulating EPC. This suggests a role for neovascularization in carnosine mediated revascularization. Cytokine analysis showed that monocyte chemoattractant protein-1 and its receptor CCR2 were increased by HLI however, no additive effect was observed with carnosine treatment. Angiogenic growth factor VEGF was significantly increased in carnosine treated HLI mice suggesting a possible VEGF dependent mechanism involved in EPC mobilization. We observed no changes in carnosine transporter and enzyme expression with ischemia although carnosine supplementation under ischemic conditions increased PHT1 transporter expression. 4-HNE protein adducts were significantly depleted and the extrusion of carnosine-propanal conjugates was increased in carnosine treated HLI mice.

In our investigation we found that after 3 days of recovery from HLI surgery levels of circulating EPCs in carnosine pre-treated mice was significantly increased compared to non-treated mice whereas EPC levels were significantly mobilized after 7 days in the carnosine post-treated mice. These results suggest that carnosine enhances the adaptive response to ischemia which is essential because, a timely robust revascularization response after an ischemic insult is necessary to prevent further damage to the tissue. Analysis of bone marrow EPC levels from ischemic limb showed diminished levels in both treated and non-treated tissue after 7 days of recovery suggesting that the observed circulatory EPC levels may be coming from the bone marrow. However, it is possible that hypoxia could affect EPC survival in bone marrow niche. Future experiments using labelled bone marrow may be

essential to delineate these pathways. These studies will be critical to define the effect of carnosine on mobilization, survival, proliferation and homing of progenitor cells. Surprisingly, carnosine treatment did not increase circulating EPC levels in the sham operated mice suggesting an ischemia mediated regulatory effect on carnosine metabolism.

Ischemic injury activates the innate immune response leading to upregulation of cytokines and chemokines in the injured tissue. Monocyte chemoattractant protein (MCP-1/CCL2) that binds to the receptor C-C chemokine receptor type 2 (CCR2) is a potent chemokine for monocytes/macrophages. These monocytes/macrophages contribute to the initiation and resolution of inflammation and revascularization. Message levels of MCP-1 and CCR2 were increased in both carnosine treated and non-treated HLI mice. Recent data has shown that the inflammatory subset of monocytes (Ly6C<sup>hi</sup>) is involved in the recovery process. We examined monocyte levels in bone marrow, peripheral blood and the skeletal muscle. Our results show ischemia induced increases in the levels of inflammatory monocytes in both bone marrow and adductor muscle however, carnosine treatment exerted no additional effects. Other cytokines such as VCAM-1, TNF- $\alpha$ , IL-6 and angiopoietin-1 which are known to play critical roles in inflammation were not affected by carnosine treatment. Inflammation is like a double edge sword that can enhance revascularization but excessive inflammation can increase oxidative stress, myocyte apoptosis and tissue fibrosis [248]. Hence, that carnosine did not obliterate or augment the inflammatory response may be essential to ensure reparative processes without initiating detrimental effects of inflammation.

Based on our results that EPCs levels are increased in carnosine treated HLI mice, we next investigated carnosine metabolism in the HLI mice. No differences were observed in the carnosine levels and transporters between the sham and the HLI mice. However, levels of carnosine were significantly increased in carnosine treated HLI mice. Similarly, expression of carnosine transporters PHT1 and PEPT2 was increased in ischemic muscle of carnosine treated mice. Because these transporters are pH sensitive our results suggest that the change in pH by ischemia and the increase in carnosine levels may increase the transporter expression.

Several studies have demonstrated that VEGF is a potent inducer of EPC mobilization [147]. To determine whether the increase in EPC mobilization in carnosine treated mice is due to increased VEGF generation we next determined mRNA and protein levels of VEGF in ischemic hamstring muscle. Our results show that VEGF mRNA and protein levels were significantly increased in the carnosine treated mice compared to the non- treated HLI mice suggesting that the increase in muscle carnosine by carnosine supplementation may be involved in regulating VEGF levels. VEGF is known to regulate endothelial cell sprouting and new vessel formation, thus the increase in VEGF levels by carnosine supplementation may be involved in increasing the capillary density through a combination of angiogenesis and vasculogenesis.

Numerous studies have shown that generation of reactive carbonyls is increased in the gastrocnemius of PAD patients and murine models of HLI [192]. Since carnosine quenches reactive carbonyl we analyzed the levels of HNE adducts in the HLI mice. Our results shown that levels of 4-HNE protein adducts were increased with ischemia

and the generation of these adducts was significantly reduced in the carnosine treated HLI mice. Previous studies from our lab had shown that conjugates of carnosine-propanal forms adduct with cardiac proteins and accumulation of these carnosinylated proteins adducts is increased in ischemic aldose reductase-null hearts [199]. We therefore explored further whether carnosinylated proteins are increased by carnosine supplementation. Our results show that levels of carnosinylated protein adducts was significantly increased in the ischemic limb of the carnosine treated mice suggesting that the capping of aldehyde protein adducts by carnosine may prevent generation of high molecular weight adducts. Furthermore, we also observed that the extrusion of carnosine-acrolein conjugates was significantly increased by carnosine supplementation suggesting that the increased extrusion of reactive aldehydes by carnosine may attenuate ischemic injury by diminishing carbonyl stress.

Collectively, we demonstrate that carnosine supplementation increases EPC mobilization, VEGF expression and aldehyde quenching in the HLI mice

## CHAPTER IV

### MECHANISMS UNDERLYING CARNOSINE MEDIATED REVASCULARIZATION.

#### **Introduction**

Timely reperfusion is critical in attenuating tissue injury from critical limb ischemia, myocardial infarction or cerebrovascular ischemia. Vasculogenesis, angiogenesis and collateral artery growth account for revascularization after vascular damage to ensure tissue reperfusion. Our results show that carnosine level is increased in mice that were supplemented with carnosine after HLI surgery. This increase was associated with increased neovascularization, collateral artery growth, reduced muscle damage and improved limb function. Further we observed that mobilization of EPC is increased by carnosine supplementation.

Previous studies have shown that levels of circulating stem/progenitor cells depend on their ability to proliferate in bone marrow niche and be mobilized under cellular stress such as ischemia. Once mobilized they can home to sites of endothelial cell damage and incorporate into newly formed blood vessels [128]. Therefore, we examined key processes that can trigger mobilization of these progenitor cells from the bone marrow. These processes are hypoxia induced (1) Increase in nuclear HIF1- $\alpha$  levels (2) VEGF transcription and (3) VEGF secretion into the extracellular space.

HIF1- $\alpha$  is a central mediator of revascularization. Its stabilization and nuclear translocation leads to expression of genes such as VEGF which is essential for mobilization of EPCs as well as sprouting, migration and proliferation of endothelial cells to form new thin walled vessels [113]. These effects of VEGF are primarily mediated through its receptor VEGFR2. This receptor tyrosine kinase once activated through VEGF binding can activate diverse growth stimulatory and migratory pathways through docking of various downstream mediators [146]. HIF1- $\alpha$  can also promote immune response which is important for arteriogenesis. Hence, HIF1- $\alpha$  regulation is a viable target for developing anti-ischemic therapeutics.

Under normoxic conditions it is targeted for proteasomal degradation through the activity of prolyl hydroxylases (PHDs) which require divalent iron for their activity. Studies have shown that inhibition of PHDs by divalent iron chelators promote recovery from HLI through HIF1- $\alpha$  stabilization [198]. Based on our observation that EPC mobilization is increased by carnosine treatment and that carnosine has the ability to chelate divalent metals we investigated whether carnosine can stabilize HIF1- $\alpha$  and increase VEGF expression.

To test whether carnosine can increase HIF1- $\alpha$  stabilization, we used murine myoblast cell line C2C12 cell and determined the effect of oxygen and nutrient deprivation on HIF1- $\alpha$  nuclear levels, VEGF secretion and transcription. We investigated the effect of carnosine and its non-hydrolysable isoform octyl-D-carnosine on HIF1- $\alpha$  nuclear levels and VEGF secretion. To determine if chelation of Fe<sup>2+</sup> by carnosine was essential for increasing nuclear HIF1- $\alpha$  levels, we used the carnosine analogue, methyl carbinine, which cannot chelate Fe<sup>2+</sup>. Collectively, our

results demonstrate that metal chelating property of carnosine increases the activity of revascularization mediators.

## **Experimental Procedure**

**Materials:** HIF 1- $\alpha$  (Santa-Cruz), octyl-D-carnosine and methyl carnosine (custom made).

**Fe<sup>2+</sup> chelation by carnosine and its analogs:** This was carried out based on the principle that free Fe<sup>2+</sup> can form a polymaltose complex with ferrozine and that this complex can be quantified by looking at the absorbance at 560nm. In the presence of a chelator however, some of the Fe<sup>2+</sup> will be chelated thereby allowing for only the left over Fe<sup>2+</sup> to complex with ferrozine. Therefore, a decrease in absorbance at 560nm signifies chelation by the compound being investigated. The experimental procedure is described below. Firstly, 7.5 $\mu$ l of 2mM FeCl<sub>2</sub> solution was added to 277.5 $\mu$ L of varying concentration of the dipeptides for 3 minutes (dipeptide solutions were prepared in water supplemented with 10% dimethylformamide). The reaction was inhibited by adding 15 $\mu$ L of 5mM ferrozine solution and incubating the mixture for 10mins at RT. Absorbance at 560nm was monitored using a spectrophotometer. The well characterized divalent metal chelator EDTA was used as a positive control in these experiments.

**In vitro hypoxia studies:** Murine myoblast cells (C2C12) were used in these studies. The cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 0.1% penicillin streptomycin till they were 70-80% confluent. They were then allowed to differentiate by replacing the FBS with horse serum. First



intracellular uptake of the various peptides (carnosine, methyl carbinine, and octyl-D-carnosine) was obtained using *LC/MS/MS* (described above for carnosine determination). To mimic ischemia in these cells, they were subjected to nutrient deprivation by replacing the differentiation media with a solution containing 117mM NaCl, 0.9mM CaCl<sub>2</sub>, 12mM KCl, 0.49mM MgCl<sub>2</sub>, 4mM HEPES, 20mM sodium lactate and 5.6mM L-glucose. Also oxygen deprivation was achieved by placing cells in a sealed humidified chamber (Billups- Rothenberg Inc) and replacing the oxygen with 95%N<sub>2</sub> and 5%CO<sub>2</sub>. The cells were harvested at indicated times after hypoxia into the appropriate buffer for either qRT-PCR or Western blot analysis. Hypoxia studies were carried out for non-treated, carnosine, octyl carnosine and methyl carbinine treated cells.

## Results

**Fe<sup>2+</sup> chelation by different histidyl dipeptide:** Based on our results that carnosine augments wound healing response and literature showing carnosine has the ability to chelate divalent metals, we next sort to delineate the contribution of metal chelation to wound healing. Carnosine forms stable complex with transition metals such as Cu<sup>2+</sup> [249]. To determine whether carnosine can chelate Fe<sup>2+</sup> which is essential for PHD activity we determined Fe<sup>2+</sup> chelation property using the protocol established by Canabady et al. [197]. Our results show that carnosine exhibits metal chelation to the same extent as EDTA (**Fig. 19D**). We also synthesized methyl carbinine, a carnosine analogue that lacks the carboxyl group required for metal chelation and has a methyl group on the imidazole nitrogen (**Fig. 19A**). Our results show that methyl carbinine had little or no Fe<sup>2+</sup> chelation property. Anserine, a naturally occurring carnosine

analogue also showed reduced metal chelation capacity (**Fig. 19D**). Glycine-glycine was used as a dipeptide control in this experiment. N.B. peptide structures are shown in **Fig 19 A-C**.

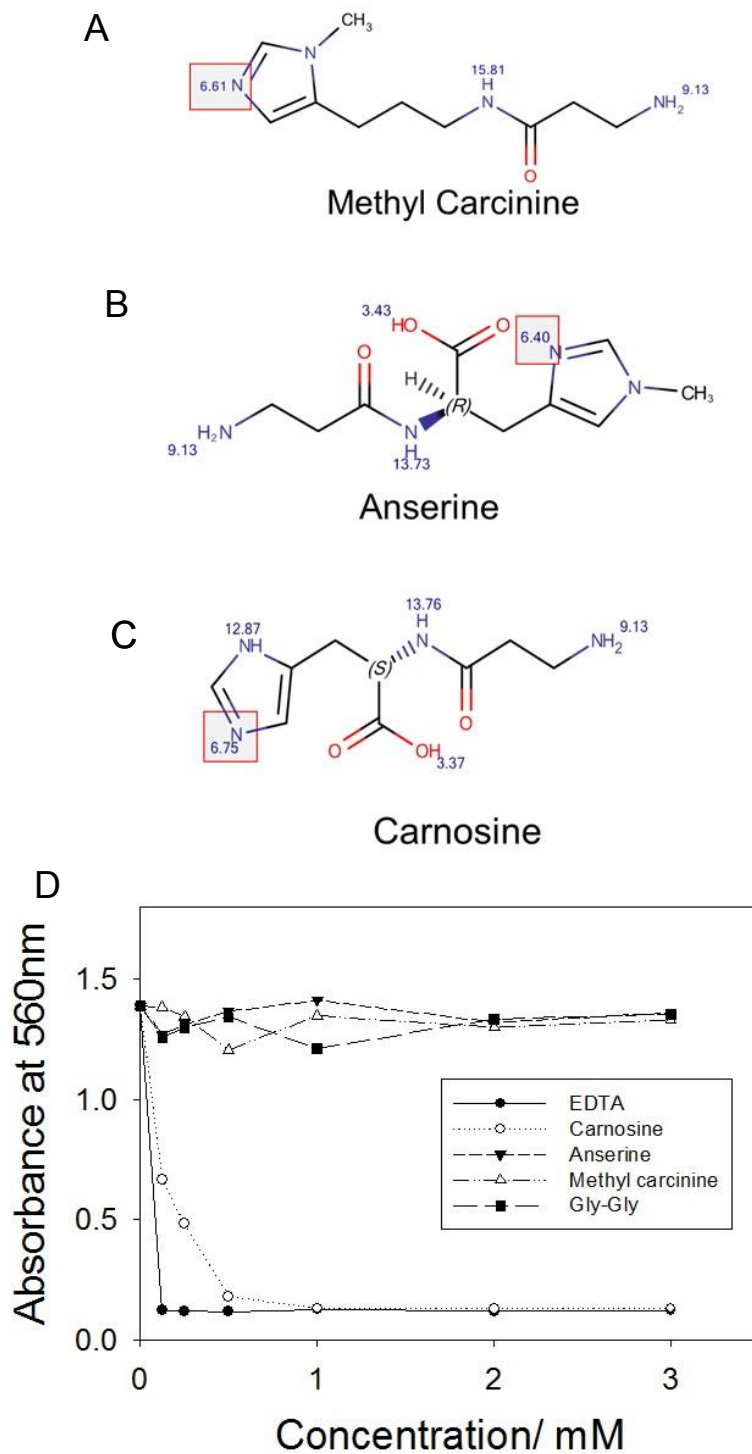
**Uptake of histidyl dipeptides by C2C12 (murine myoblast cells):** To determine whether the histidyl dipeptides are taken up by these cells, we incubated C2C12 cells with carnosine(1mM), methyl carbinine(1mM) and octyl D-carnosine (100 $\mu$ M) respectively at indicated times (**Fig. 20 A, B**). Increased intracellular concentrations were observed for all three peptides. Also, octyl-D-carnosine treatment resulted it's de-esterification to carnosine (**Fig. 20 A, B**).

**Carnosine treatment increases HIF1-  $\alpha$  nuclear levels and VEGF secretion in hypoxic C2C12 cells:** To determine the contribution of the myocytes to the adaptive process, we first examined time dependent changes in VEGF secretion with hypoxia in C2C12 cells. We observed that VEGF levels were increased in conditioned media with increasing times of hypoxia (**Fig 21 A**). To determine the effect of carnosine administration on HIF1- $\alpha$  stabilization and signaling, we pretreated the murine myoblasts C2C12 cells with 1mM carnosine for 12-15 h and subjected the cells to different times of hypoxia. HIF-1  $\alpha$  nuclear level was increased after 1h of hypoxia and this increase in nuclear HIF1- $\alpha$  was significantly enhanced by carnosine treatment (**Fig. 21B, C**). VEGF secretion in the conditioned media was also increased by carnosine treatment compared to non-treated hypoxic cells (**Fig. 21 D**). These results clearly suggest that carnosine increases HIF1- $\alpha$  nuclear translocation and VEGF levels.

**Octyl-D-carnosine increases HIF1- $\alpha$  nuclear levels and VEGF secretion:** To determine whether the whole molecule of carnosine or its constituent amino acids increase HIF1- $\alpha$ , we treated hypoxic C2C12 with the non-hydrolysable isoform of carnosine, ODC (100 $\mu$ M). VEGF secretion and mRNA expression were increased by ODC treatment compared with non-treated hypoxic cells (**Fig 22C, D**). Similarly, nuclear levels of HIF1- $\alpha$  was also increased by ODC treatment (**Fig 22A, B**). These observations suggest that the effect of carnosine on the angiogenic response is due to the whole molecule and not due to constituent amino acids.

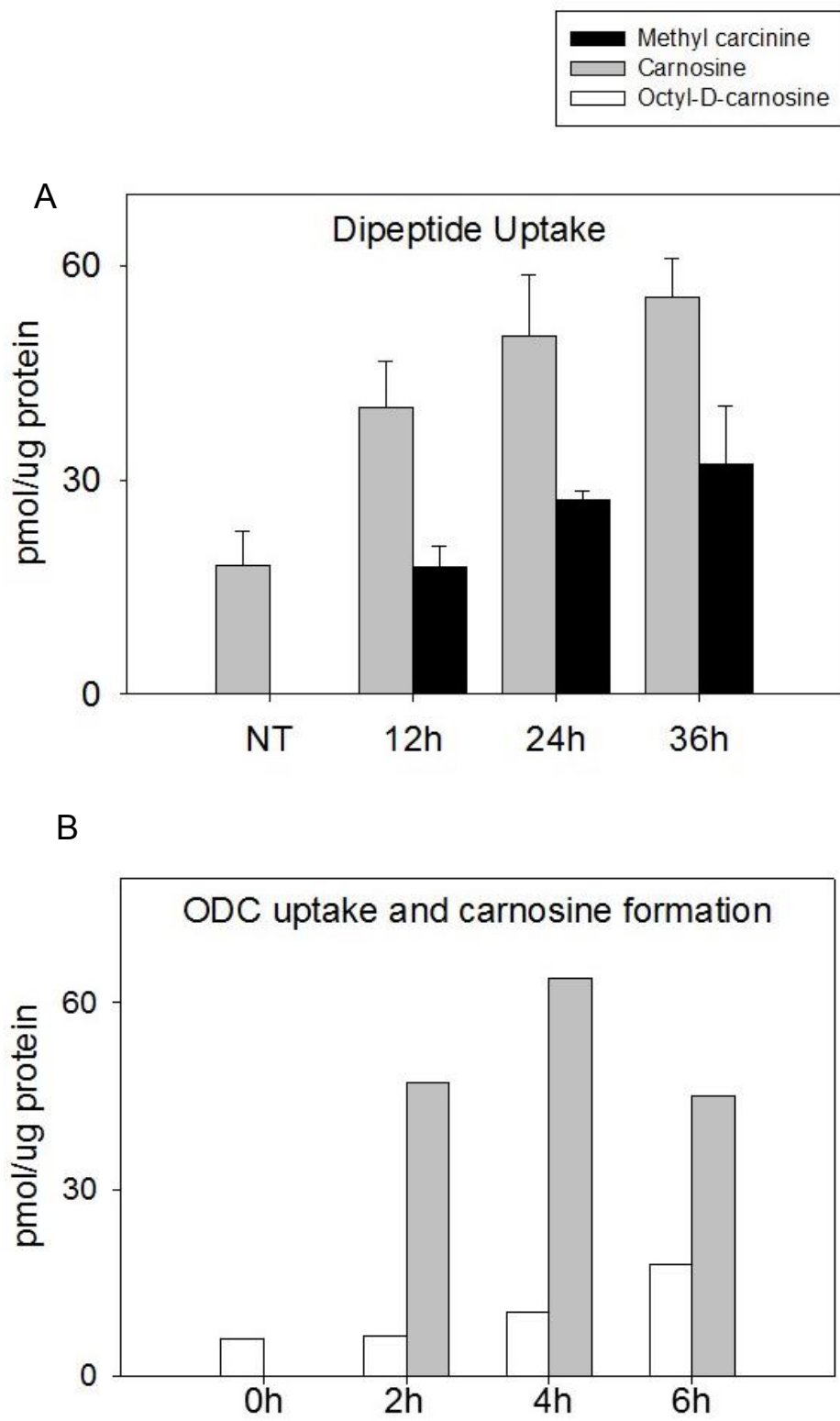
**Metal chelation by carnosine contributes to VEGF secretion and HIF1- $\alpha$  nuclear content:** We tested the hypothesis that the increase in VEGF secretion and HIF1- $\alpha$  nuclear levels by carnosine treatment is due to Fe<sup>2+</sup> chelation by carnosine. To test our hypothesis we pretreated C2C12 cells with methyl carbinine which lacks the ability to chelate metals (**Fig. 19D**). Our results show that carnosine treatment increases HIF1- $\alpha$  nuclear content whereas for cells that were treated with methyl carbinine and subjected to hypoxia there was no change in HIF1- $\alpha$  levels compared to non-treated cells. (**Fig. 23A, B**). Similarly, VEGF secretion was hindered with methyl carbinine treatment (**Fig. 23C**). Taken together these results suggest metal chelation of carnosine promotes angiogenic response

Figure 19



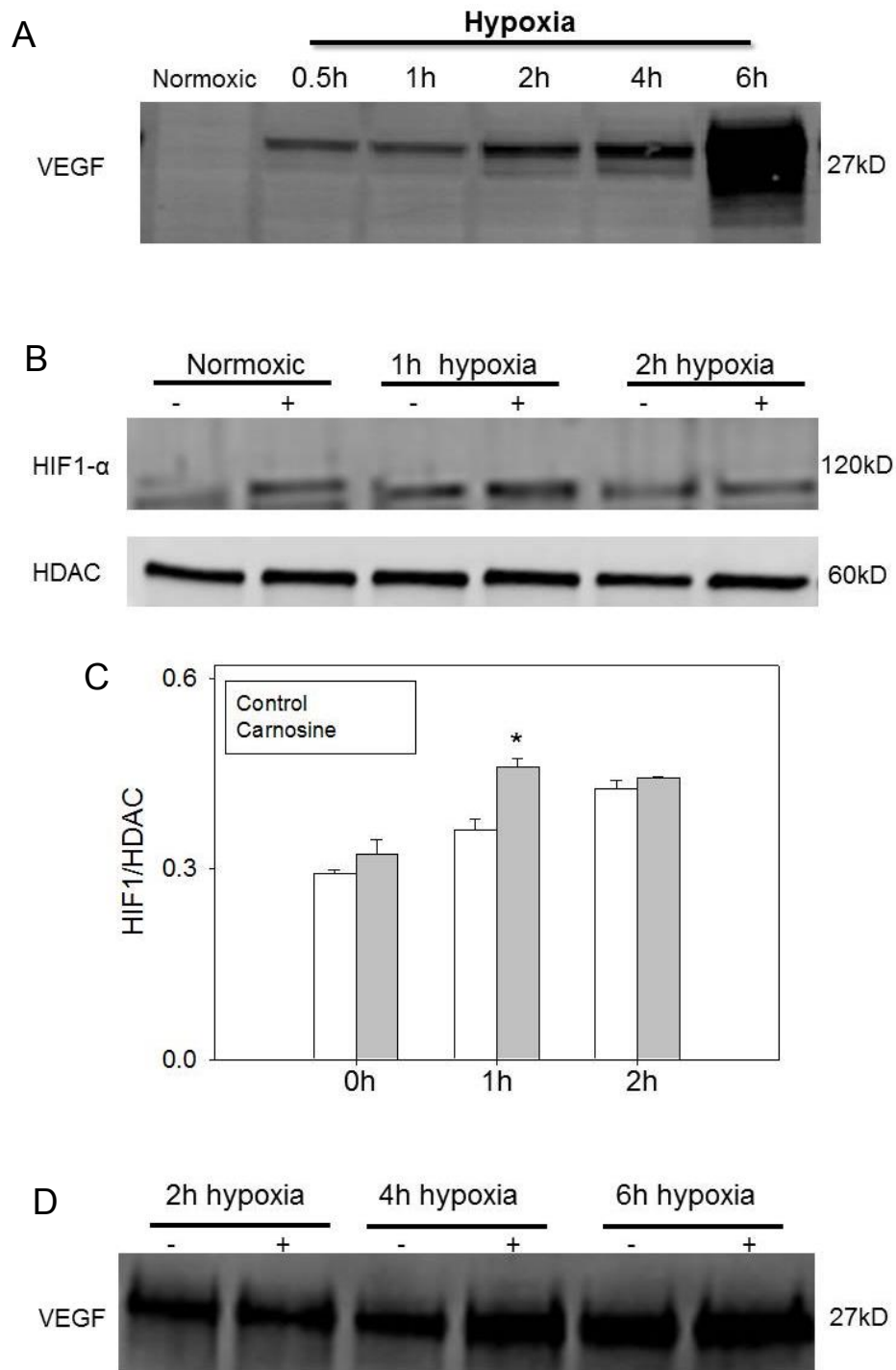
**Figure 19: Methyl carbinine (a synthetic analog of carnosine) has reduced Fe<sup>2+</sup> chelation. (A)** Structures of different histidine dipeptides: **(A)** Methyl carbinine, **(B)** anserine and **(C)** carnosine. **(D)** Fe<sup>2+</sup> chelation properties of the different dipeptides monitored by taking the absorbance at 560nm for different peptide concentrations.

Figure 20



**Figure 20: Time dependent increase in intracellular dipeptide levels:** (A) *LC/MS/MS* analysis of intracellular carnosine concentrations in C2C12 cells treated with carnosine at the indicated times. (B) *LC/MS/MS* analysis of intracellular carnosine and ODC levels after in cells treated with ODC at indicated times. NT indicates non treated cells

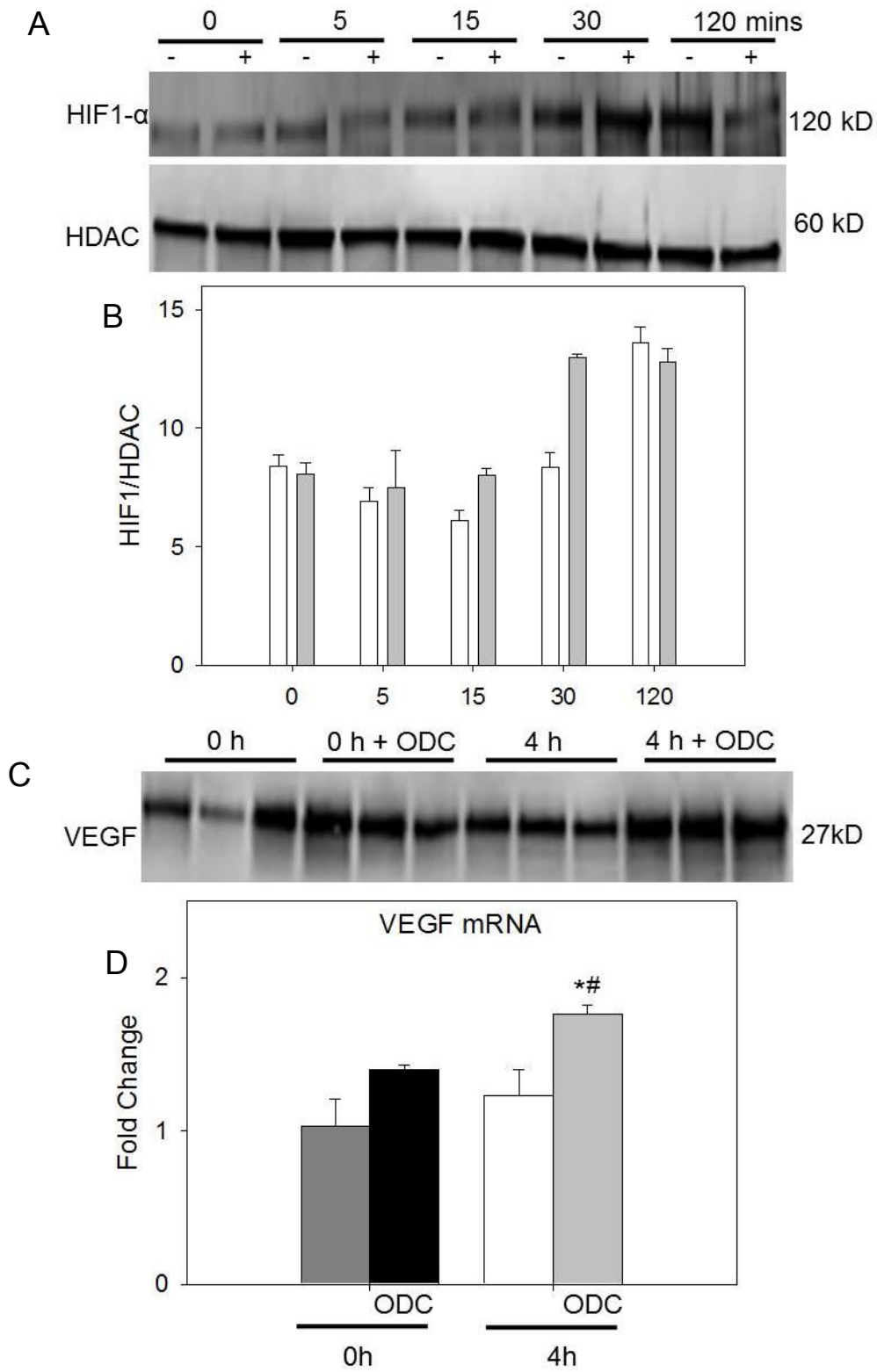
Figure 21





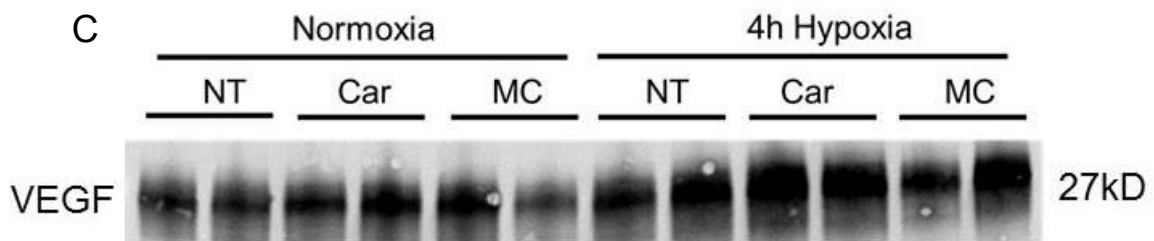
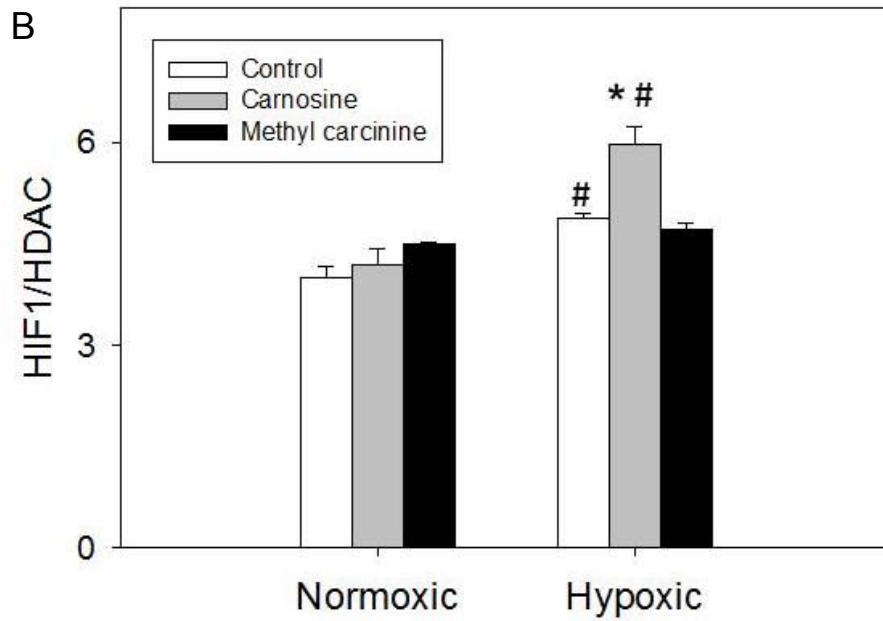
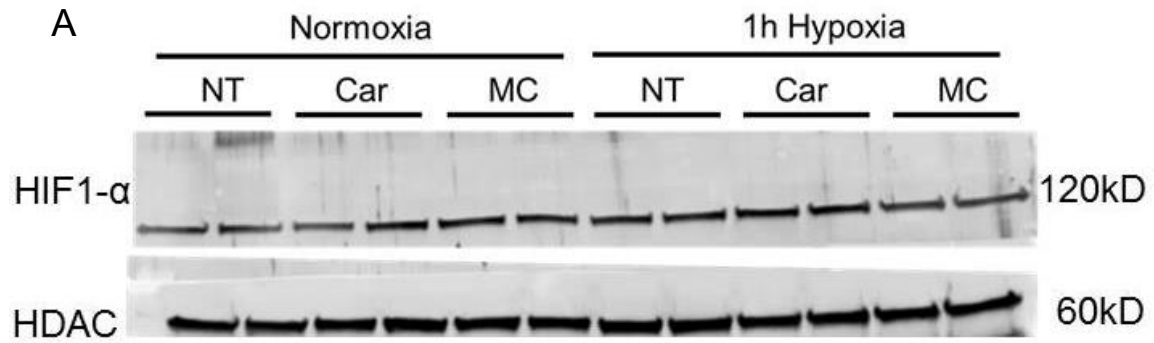
**Figure 21: Carnosine treatment results in increase HIF1- $\alpha$  nuclear translocation and VEGF secretion in hypoxic C2C12 cells.** (A) Time dependent changes in VEGF secretion into conditioned media following hypoxia in C2C12 cells. (B) Nuclear HIF1- $\alpha$  levels in hypoxic C2C12 cells with and without carnosine treatment. (C) Normalization of nuclear HIF1- $\alpha$  levels to HDAC. (D) VEGF levels in conditioned media of treated and non-treated hypoxic cells at indicated times. Experiments were repeated at least 3 times with n=4 for each experiment. \*p <0.05 vs non-treated hypoxic cells n=4

Figure 22



**Figure 22: Octyl-D-Carnosine (ODC) treatment increases HIF1- $\alpha$  nuclear levels with a concomitant increase in VEGF secretion and expression. (A, B)** Time course of nuclear levels of HIF1- $\alpha$  with and without ODC treatment, **(C)** VEGF secretion in conditioned media of hypoxic C2C12 cells **(D)** VEGF mRNA expression in hypoxic C2C12 cells. Experiments were repeated at least 3 times with n=4 for each experiment. \*p <0.05 vs non-treated hypoxic cells n=4

Figure 23



**Figure 23: Metal chelation by carnosine promotes HIF1- $\alpha$  stabilization:** (A, B) HIF1- $\alpha$  nuclear levels in C2C12 cells subjected to 1h of hypoxia with carnosine (car), methyl carbinine (MC) or no treatment (NT). **(C)** VEGF secretion in conditioned media of hypoxic C2C12. \*p <0.05 vs non-treated hypoxic cells n=4

## Discussion

The major goal of this study was to identify the mechanisms by which carnosine regulates HIF1- $\alpha$  and increases VEGF levels. Using an *in-vitro* model of hypoxia and nutrient deprivation we observed that HIF1- $\alpha$  nuclear levels and VEGF secretion was enhanced with increasing length of hypoxia and peaked at 1h and 6h respectively. This effect was significantly enhanced by carnosine treatment suggesting that carnosine stabilizes HIF1- $\alpha$ .

We investigated the contribution of metal chelation by carnosine to HIF1- $\alpha$  stabilization. Carnosine is a multifunctional compound with functions such as metal chelation, pH buffering and aldehyde quenching [202]. NMR studies have shown that carnosine interacts with divalent metals through 2 imidazole nitrogen, peptide bond, carboxylic acid and primary amino group [196]. However, one imidazole nitrogen and the amino group are critical for pH buffering and aldehyde quenching respectively [250, 251]. Therefore, to prevent metal chelation without influencing aldehyde quenching or pH buffering, we synthesized methyl carbinine an analogue of carnosine without a carboxylate group and with a methyl group on imidazole nitrogen. Analysis of pKa values shows that pH buffering was not altered significantly with these modifications since a pKa of 6.61 was observed for methyl carbinine compared to 6.75 for carnosine. Metal chelating capacity of this compound was significantly decreased compared to carnosine.

Using in vitro model of ischemia we found that, HIF1- $\alpha$  nuclear content and VEGF secretion was significantly increased with carnosine treatment, whereas for hypoxic cells treated with methyl carbinine, no change in HIF1- $\alpha$  levels was

observed compared to the non- treated cells. Our results are in line with previous studies showing that  $\text{Fe}^{2+}$  chelation promote recovery from HLI [198]. However, the use of carnosine has an added benefit because this dipeptide targets diverse pathways such as ROS-induced inflammation, lipid peroxidation and acidosis.

Carnosine can be hydrolyzed to  $\beta$ -alanine and histidine by carnosinase. Previous studies have shown that decarboxylation of histidine generates histamine [252] which is an important regulator of hematopoiesis, immune cells function and revascularization [253]. Hence, to determine the possible contribution of carnosine hydrolysis in hypoxic adaptation we used a non-hydrolysable octyl D-isoform of carnosine (ODC). We observed a similar effect of ODC on HIF-1 $\alpha$  levels and VEGF transcription as observed with the L-isoform suggesting that histidine-decarboxylation did not contribute to increase in HIF1- $\alpha$  activity. This is particularly important because the effect of this axis on carnosine's anti-ischemia effect is conflicting [252, 254].

In summary, our study identified that  $\text{Fe}^{2+}$  chelation by carnosine is important for regulating HIF1- $\alpha$  nuclear content. The increase in HIF1- $\alpha$  nuclear levels likely increases revascularization in carnosine treated HLI mice.

## CHAPTER V

### CONCLUSIONS AND FUTURE DIRECTIONS

#### **Concluding discussion**

The purpose of this study was to investigate the therapeutic potential of carnosine, a naturally occurring component of food, in critical limb ischemia. Recent data from developed and developing countries estimated that >200 million people worldwide and approximately 8 million people in United States have PAD [2, 6]. Both symptomatic and asymptomatic PAD patients have an increased risk of mortality, morbidity and lower quality of life [7]. With the increasing incidence of type 2 diabetes (T2D) and rising aging population the number of PAD patients is likely to increase [2]. Because PAD is an under-recognized disease, few medications are available to improve functional performance in these patients. Although surgical revascularization is an amenable treatment, low graft patency and restenosis limits its utility in these patients [175]. Therefore to adequately compensate these patients current emphasis is to increase therapeutic angiogenesis and arteriogenesis in the ischemic limb. This is expected to improve walking ability and quality of life in these patients. Hence, we tested the hypothesis that carnosine will promote post-ischemic angiogenesis and arteriogenesis through aldehyde quenching and metal chelation. To address this hypothesis, we determined whether carnosine supplementation would increase tissue reperfusion after hindlimb ischemia surgery.



In Chapter II, we demonstrated by Laser Doppler perfusion imaging and microfil perfusion-microCT analysis that carnosine treatment increases tissue perfusion and vascular volume. We also showed that tissue injury was reduced, and limb function significantly improved by carnosine feeding. Based on these observations we concluded that supplementation of carnosine improves revascularization after limb ischemia.

Having established that carnosine improves blood flow in the ischemic limb we next examined the effect of ischemia on endogenous carnosine synthesis, hydrolysis and transport. LC/MS/MS analysis of the ischemic limb showed that there was no change in carnosine levels by HLI surgery however, levels of carnosine were increased in the ischemic limb of mice supplemented with carnosine. qRT-PCR analysis demonstrated that levels of carnosine transporter, PHT1, was increased in the ischemic limb of carnosine treated mice. These results established that oral carnosine supplementation increases carnosine levels in the ischemic limb by increasing the expression of transporters.

Next we evaluated the effect of this increase in muscle carnosine on the adaptive response to ischemia. We observed an increase in VEGF levels which was associated with increased levels of circulating EPCs and decreased bone marrow EPCs. This suggests a possible VEGF dependent mechanism regulating EPC exodus from the bone marrow niche.

Urinary analysis of the mice subjected to HLI showed that extrusion of carnosine-acrolein conjugates was increased by carnosine supplementation. Similarly, levels of carnosinylated proteins adduct were increased in the ischemic limb of carnosine

treated mice. These results suggests that increased extrusion of aldehyde conjugates and carnosinylation of proteins decreases carbonyl stress in carnosine treated mice. These reports demonstrate a direct involvement of carnosine in the adaptive response to ischemia and suggest a direct potential benefit of increasing muscle carnosine levels.

As mentioned, ROS generation is increased in reperfusion [33, 49]. It has been suggested that some of the secondary and metastable products derived from ROS amplify their effects. In this process several highly reactive lipid peroxidation products are generated that can be removed by enzymes aldose reductase, aldehyde dehydrogenase, and glutathione S transferases. However, under diseased conditions these pathways are overwhelmed and thus unsaturated aldehydes increasingly react with amino acid residues of proteins. Recently, it has been shown that carnosine administration has a sparing effect on antioxidant systems such as serum superoxide and glutathione peroxidase since the levels of these enzymes are increased with carnosine treatment [245]. We also show that protein-HNE adducts that were increased with ischemia were reduced when carnosine was administered to the ischemic mice. Although carnosine is an effective quencher of HNE, glutathione S-transferaseA4 also affects this highly reactive metabolite through the process of glutathionylation. However, recent studies have shown that glutathione derivative of HNE can induce excessive macrophage inflammation through TNF- $\alpha$  and NF- $\kappa$ B pathways. Furthermore, use of these enzymes by viral transduction as therapies are still in infancy. Therefore, our results showing that supplementation of carnosine improves revascularization

has a selective advantage as a therapeutic option for ischemia [255]. Carnosine neither exacerbate nor attenuates the inflammatory response but still prevents tissue injury induced by ischemia and increases neovascularization response.

In Chapter III we demonstrate that VEGF levels are increased in the ischemic limb of the carnosine treated mice. Therefore, we investigated the role of metal chelation by carnosine on this process. We hypothesized that metal chelation by carnosine will stabilize HIF1- $\alpha$  through prolyl hydroxylase inhibition and increase VEGF expression. To determine the contribution of metal chelation we synthesized methyl carbinine, an analogue of carnosine which has diminished metal chelation ability. Using in vitro model of oxygen and nutrient deprivation our results show that there was no change in HIF1- $\alpha$  nuclear levels by methyl carbinine treatment compared to non-treated cells. We further determined whether hydrolysis of carnosine by carnosinase and subsequent decarboxylation of histidine to histamine is involved in regulating nuclear levels of HIF1- $\alpha$ . We used octyl-D-carnosine a non-hydrolysable isoform of carnosine. Our results show that octyl-D-carnosine was equally effective as carnosine in increasing nuclear HIF1- $\alpha$  levels.

Collectively our results demonstrate that carnosine increases revascularization in murine hindlimb ischemia. We show that HIF1- $\alpha$  stabilization and VEGF secretion may be critical for these responses. Further we established that metal chelation and aldehyde quenching by carnosine are critical for promoting revascularization.

## Limitations and Future Directions

### *Mechanistic Consideration*

The results from this study has revealed insights into the mechanisms by which carnosine can increase revascularization responses, however, other mechanistic questions remain to be answered. We showed that carnosine treatment increases nuclear levels of HIF1- $\alpha$  and observed an increase in VEGF levels. We therefore concluded that the increase in HIF1- $\alpha$  could account for the increase in VEGF since VEGF is a transcriptional target. Although VEGF transcription is highly documented to be regulated by HIF1- $\alpha$  we did not test this directly in our experiments. Pharmacological inhibition HIF1- $\alpha$  or used of HIF1- $\alpha$  knockdown cells will be essential to identify if changes in VEGF obtained with carnosine treatment is attributable to HIF1- $\alpha$  only.

Also, we identified that Fe<sup>2+</sup> chelation by carnosine is essential for regulating nuclear HIF1- $\alpha$  content however, it will be important to establish which upstream targets are being regulated by this process. Prolyl hydroxylases involved in targeting HIF1- $\alpha$  for proteasomal degradation require Fe<sup>2+</sup> of activity. Measurement of prolyl hydroxylase activity with and without carnosine treatment will strengthen our understanding and establish whether the increase in nuclear content was as a result direct inhibition of prolyl hydroxylase. Likewise using tagged HIF1- $\alpha$  we can directly measure its translocation in our model. Current dogma in the field states that following prolyl hydroxylase inactivation, HIF1- $\alpha$  is nuclearly translocated and binds to hypoxia response elements to induce

transcription of target genes [113]. Using labelled or tagged HIF1- $\alpha$  we can directly measure its stabilization and subsequent nuclear translocation in future studies.

Recent evidence has documented a role for NO and ROS in HIF1- $\alpha$  stabilization in an oxygen independent manner [256]. Divalent metal chelation by carnosine can limit reactive oxygen and nitrogen species generation [257] thereby increasing HIF1- $\alpha$  stability. It will be important to identify which of these processes regulates HIF-1 $\alpha$  stability.

Secondly, during ischemia the increased reliance on glycolysis leads to acidosis due to proton accumulation from lactic acid decomposition. Since carnosine is a pH buffering agent, supplementation may increase glycolysis in the ischemic tissues. Hence evaluating the role of pH buffering of carnosine in ischemic adaptations will provide insight into the mechanisms by which carnosine attenuates wound healing responses.

Our immunohistochemistry data shows that carnosine increases muscle regeneration after ischemia. Mammals have a highly orchestrated system of skeletal muscle regeneration. This process involves activation of muscle resident satellite cells through transcription factors MyoD and Myf5. Other factors such as Pax3 and Pax7 are also involved in regeneration [258]. Recently, signal transducer and activator of transcription 3 (STAT3) signaling pathway was shown to be involved in muscle regeneration. Studies have shown that this pathway is important to ensure survival, activation and proliferation of satellite cells into fully differentiated myotubules [259, 260]. Interestingly, carnosine has been shown to activate this pathway in neuronal cell after cerebral ischemia [261]. Thus,

understanding the regulation of this pathway by carnosine in the context of muscle ischemia may be important in understanding how it regulates muscle regeneration.

Lastly, as outlined in chapter I several cell types such as endothelial, smooth muscle, muscle and immune cells are involved in the ischemic response. In this study we determined the responses in ischemic muscle which promotes recovery by carnosine supplementation. Future studies are needed to establish how endothelial cells respond to carnosine treatment e.g. assays to measure tube formation and scratch assay. Similarly, paracrine signaling between muscle and endothelial cells can be determined by treating the myoblasts with carnosine and treating human umbilical vascular endothelial cells (HUVECs) with conditioned media from carnosine treated cell to determine changes in tube formation.

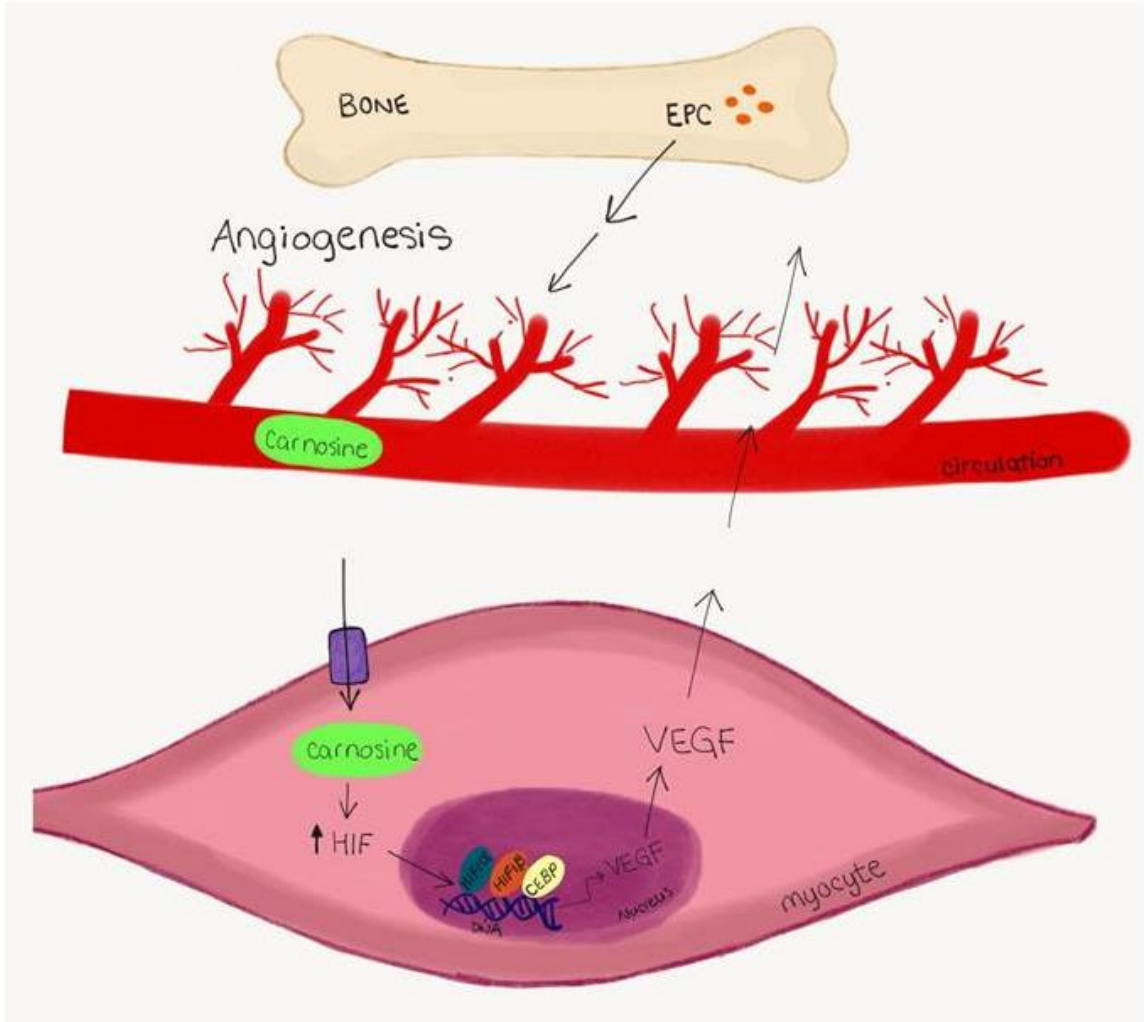
#### *Clinical Application*

In this study we laid the foundation for carnosine to be used as a viable therapy for critical limb ischemia however, several questions need to be answered before it can be fully translational. Further studies are needed to determine the optimal dose, mode and frequency of administration. One of the limitations of this hindlimb ischemia model is that the studies were conducted in healthy mice with no comorbid conditions. This clearly is not the case in the human patient populations. PAD patients have other diseases such as cardiovascular disease, diabetes which can inversely affect the reparative response. Further studies evaluating the effect of carnosine on revascularization in the presence of one or more of these risk factors will be important in elucidating the true therapeutic potential of carnosine.

Secondly,  $\beta$ -alanine is the rate limiting substrate for carnosine synthesis. This amino acid is predominantly obtained from pyrimidine degradative pathway. Diverse polymorphisms exist in human populations for the rate-limiting enzyme dihydropyrimidine dehydrogenase that is involved in the synthesis of  $\beta$ -alanine. These polymorphisms have been studied in the context of chemotherapy administration where deficiencies lead to severe toxicity and death [262]. Recent epidemiological work suggests that genetics could be playing an important role in the development of PAD however most of the candidate genes have not been identified [7]. Therefore, based on these established polymorphisms and the known benefits of carnosine and studies showing diminished carnosine levels in patients with cardiovascular risk factors like diabetes, it may be important to evaluate the possible contributions of these polymorphisms to carnosine levels. Also, associations between these polymorphisms and cardiovascular disease development may be important in predicting patients who may develop PAD and selecting patients which may benefit from carnosine therapy.

Lastly, most of the key pathways involved in muscle ischemia are similar in cardiac or cerebrovascular ischemia. Therefore, although this work studied carnosine in the context of critical limb ischemia it can also be utilized in other forms of tissue ischemia.

Figure 24





**Figure 24: Schematic overview of mechanism by which carnosine improves revascularization:** Carnosine supplementation increases muscle carnosine content. This leads to HIF-1 $\alpha$  stabilization presumably through PHD inhibition and VEGF expression. VEGF acting through its receptor increases EPC mobilization and homing to the ischemic tissue leading to neovascularization.

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## CURRICULUM VITAE

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### **Past Professional Experience**

09/2009- 07/2011      Research Assistant, Department of Microbiology, School of Medical  
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10/2008-08/2009      Teaching/Research Assistant, Department of Medical Biochemistry,  
University of Ghana Medical School, Accra.

### **Awards and Distinctions**

07/ 2014	2 <sup>nd</sup> place winner Research Louisville
2008	Dr. and Mrs. Adunyah Award for Best Graduating Biochemistry Student, KNUST
2008	Best Graduating Student College of Science, KNUST
2007	Provost Award for Best Student in Biochemistry
2007/2008	Dean Honor List, Faculty of Biosciences, KNUST

### **Society Membership**

2015-present	Member American Heart Association
2015-present	Member Peripheral Vascular Disease Council of the American Heart Association
2009- present	Member American Society of Microbiology
2012-present	Member, International Society for Infectious Disease

### **Publications**

#### **Peer-Reviewed Manuscripts**

Blancquaert, L., Baba, S. P., Kwiatkowski, S., Stautemas, J., Stegen, S., Barbaresi, S., Chung, W., **Boakye, A. A.**, Hoetker, J.D., Bhatnagar, A., Delanghe, J., Vanheel, B., Veiga-da-Cunha, M., Derave, W., Everaert, I. Carnosine and anserine homeostasis in skeletal muscle and heart is controlled by beta alanine transamination

Brainard RE, Watson LJ, DeMartino AM, Brittian KR, Readnower RD, **Boakye AA**, Zhang D, Hoetker JD, Bhatnagar A, Baba SP, Jones SP. (2013) High Fat Feeding in Mice Is Insufficient to Induce Cardiac Dysfunction and Does Not Exacerbate Heart Failure. PLoS ONE 8(12): e83174. doi:10.1371/journal.pone.0083174

## **Abstracts/Posters**

Hayley Scholl, Jaron Thomas, Deqing Zhang, **Adjoa A. Boakye**, Shahid P. Baba, Role of carnosine in obesity, Research Louisville, Louisville, KY

**Boakye AA**, Conklin D, Guo L, Zhang D, McCracken J, Bhatnagar A, Baba SP. Carnosine Supplementation Attenuates Ischemic Injury in Mouse Model of Hindlimb Ischemia. Research Louisville, 5<sup>th</sup> biannual symposium (2015), Department of Biochemistry and Molecular Biology, University of Louisville, Louisville, KY (**oral presentation**)

Blancquaert L, Stautemas J, Stegen, S., Barbaresi S, Chung W, **Boakye, A. A**, Baba SP, Bhatnagar A, Derave W, Everaert I. Inhibiting beta alanine transamination: a way to promote histidyl-dipeptide loading in mice. 2015) Symposium on carnosine and histidine dipeptides, Gabicce Mare, Italy

**Adjoa Agyemang Boakye**, Deqing Zhang, David Hoetker, Luping Guo, Yiru Guo, Daniel Conklin, Aruni Bhatnagar, Shahid Pervez Baba. Dipeptide Pill for ischemic injury. (2015) Symposium on carnosine and histidine dipeptides, Gabicce Mare, Italy

**Boakye AA**, Conklin D, Guo L, Zhang D, McCracken J, Bhatnagar A, Baba SP. Carnosine Supplementation Promotes Revascularization in Mouse Model of Hindlimb Ischemia. Research Louisville, Atherosclerosis Thrombosis and Vascular Biology/ Peripheral Vascular Disease Scientific Sessions (2015), San Francisco California, USA

**Boakye AA**, Conklin D, Guo L, Zhang D, McCracken J, Bhatnagar A, Baba SP. Carnosine Supplementation Promotes Revascularization in Mouse Model of Hindlimb Ischemia. Research Louisville, Sept 2014

Deqing Zhang, **Adjoa Agyemang Boakye**, David Hoetker, Daniel Conklin, Aruni Bhatnagar, Shahid Pervez Baba, Carnosine Supplementation Attenuates and Reverses Diet-Induced Obesity and Insulin Resistance. International Conference on Carnosine and Anserine, Toyko, Japan, 2014