

PATHWAYS OF OXIDATIVE DAMAGE TO SKELETAL MUSCLE AFTER AN
ACUTE BOUT OF CONTRACTILE CLAUDICATION

By

ANDREW JUDGE

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Abstract of Dissertation Presented to the Graduate School
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Andrew Judge

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A limited number of studies have shown an increase in products of lipid peroxidation in the plasma of claudicants after exercise. Previously, we used an animal model to mimic the condition of exercise claudication to show oxidative damage and edema within the muscle. Using this same model, we investigated the sources of this oxidative damage in the gastrocnemius muscle, focusing on xanthine oxidase and neutrophils, in addition to determining the role iron plays in the exercising claudicant.

The increase in lipid hydroperoxides, seen in claudicant muscle, was attenuated with independent inhibition of xanthine oxidase activity, depletion of neutrophils, and chelation of iron. An additional marker of lipid peroxidation, 4 hydroxy-2-nonenal (HNE), was also attenuated by depletion of neutrophils. The HNE is formed from oxidant-induced decomposition of lipid hydroperoxides, but may bind to amino acid side chains of proteins, potentially affecting their function.

Protein oxidation, indicated by an increased level of protein carbonyls, was significantly increased in claudicant muscle, but attenuated by depletion of neutrophils. Since oxidants have the potential to modify membrane macromolecules, we also investigated LDH activity, as an indicator of muscle cell membrane permeability; and wet/dry ratio as a marker of edema – reflective of vascular membrane permeability. The LDH activity was decreased in claudicant muscles, reflecting loss of the cytosolic enzyme due to membrane alterations. This loss was attenuated with independent inhibition of xanthine oxidase activity, depletion of neutrophils, and chelation of iron. Edema, however, was only attenuated by neutrophil depletion.

Our findings suggest that neutrophils are the predominant source of oxidants after exercise claudication; and a major cause of edema. However, we also show that xanthine oxidase-derived oxidants contribute to lipid peroxidation and are chemotaxic to neutrophils. Finally, we show that chelation of iron also attenuates lipid peroxidation, despite an increase in xanthine oxidase and neutrophils, demonstrating iron's role in propagating oxidant reactions.

CHAPTER 1 INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Oxidative stress, due to ischemia followed by reperfusion (I-R) may cause significant muscle damage and dysfunction [1,2]. Most of the research regarding skeletal muscle I-R injury has used prolonged periods of ischemia followed by reperfusion. These studies found convincing evidence of oxidant generation and associated muscle damage. In addition, it has been shown that various countermeasures can attenuate this oxidative damage [2,3].

Recently, exercise claudication has been classified as a form of I-R; and accumulating evidence associates oxidative stress and muscle damage with this condition [4,5]. Using an *in vivo* rat model to mimic the condition of exercise claudication, we found evidence of protein oxidation, lipid peroxidation, and reduction of total glutathione in both the soleus and gastrocnemius muscles. This further substantiates the claim of oxidative damage after exercise claudication.

At present, only two studies have attempted to attenuate this oxidative stress in exercising claudicants, using antioxidant supplementation. Wijen et al. [5] administered Vitamins C and E together orally, over a 4 week period, and attenuated the oxidative stress in exercising claudicants. In a similar study, Silvestro et al. [6] infused Vitamin C intravenously, 5 minutes before the exercise bout; and were likewise successful in attenuating the oxidative stress.

While this therapy shows the potential for sequestering oxidants before they can elicit their damaging effects, the antioxidants used were not specific; and therefore may not be the most appropriate countermeasures. Recently, we found evidence of elevated xanthine oxidase activity and activated neutrophils after contractile claudication. Therefore, specifically blocking or inhibiting these pathways of oxidant generation should provide greater protection. In addition, iron mobilization occurs during prolonged I-R [7], and likely occurs after exercise claudication. Since iron is a powerful catalyzing agent in redox reactions, its chelation should provide protection from oxidative damage.

Our study attempted to inhibit xanthine oxidase and induce neutropenia, as well as chelate free iron, to establish the significance of each regarding oxidative damage.

Xanthine oxidase can be effectively inhibited with allopurinol — a purine analog whose metabolite, oxypurinol, forms a tight binding complex with the enzyme, rendering it inactive. Allopurinol-treated animals appear to have attenuation of I-R-induced muscle damage in a variety of tissues [8].

Neutropenia may be induced with cyclophosphamide – a compound activated enzymatically in the body to phosphoramidate mustard, a powerful and unstable DNA-alkylating agent. Cyclophosphamide has been shown to significantly reduce the number of circulating polymorphonuclear neutrophils [9], thereby diminishing the inflammatory response, and providing protection against I-R-induced injury [10]. This is presumed to occur through a reduction in polymorphonuclear neutrophil superoxide production.

Deferoxamine is a powerful iron chelator, and may be used to prevent iron from participating in redox reactions. Deferoxamine has effectively been used to prevent oxidative stress and muscle damage after prolonged I-R [3].

To date, no studies have used these countermeasures in the exercising claudicant. In fact, until recently no study had measured oxidative stress in muscle after exercise claudication, or considered the potential sources. We administered each of the countermeasures and measured oxidative stress after contractile claudication; and determined muscle edema, as evidenced by an increase in the wet/dry ratio.

It is imperative to gain a better understanding of the relative contributions of the various sources of oxidants produced during exercise claudication. At present, the lack of research in this area means that we are relying on somewhat speculative information, based on non specific research, and thus relying on assumptive preventative measures. It seems prudent to gain a specific understanding of the sources of oxidants so that the most appropriate countermeasures can be implemented to attenuate the stress and edema, and reduce further disability from this cardiovascular disease.

Review of Literature

Ischemia-Reperfusion

Ischemia may be defined as “a rate of blood flow to an organ that is inadequate to supply sufficient oxygen and maintain aerobic respiration in that organ” [11]. Therefore, skeletal muscle becomes ischemic when there is a mismatch between blood supply and blood demand resulting in insufficient oxygen delivery to the muscles. This may result from cardiovascular disease, severe trauma, vascular occlusions, or even strenuous or unaccustomed exercise. Although ischemia may cause muscle damage and, if prolonged enough, necrosis, it appears to be the re-oxygenation, or reperfusion of tissue that causes

the major damage. Reperfusion occurs through restoration of blood flow so that oxygen delivery can once again match the demand. Since this is essential to tissue survival, the potential damage appears to be the lesser of two evils.

Intermittent Claudication

Skeletal muscle ischemia due to disease is highly dependent on the degree of vessel blockage, and may manifest itself as pain at rest in the most severe cases (or more commonly, as claudication). The term claudication derives from the latin word *claudicatio*, meaning to limp; and describes a condition whereby pain is felt during exercise or activity (sufficient to require termination of the exercise), with relief upon rest. This exercise-induced pain is a result of the inability to deliver the required substrates to meet the demands of the working muscles – a similar condition to that which occurs during strenuous or unaccustomed exercise. Intermittent claudication refers to the continual process of pain upon exercise, and relief with rest.

The prevalence of intermittent claudication ranges from 2.9%, in the Speedwell Prospective Heart Disease Study [12] to 4.5% in the Edinburgh Artery Study [13]. The range probably reflects the differences in defining criterion. However, these numbers are likely an underestimate of the true incidence, since only 10 to 50% of people with claudication symptoms consult their general practitioner [14].

Peripheral Arterial Disease (PAD) is probably the most common cause of intermittent claudication. This disease is associated with atherosclerotic lesions, reducing blood flow to the area distal to the lesions. Therefore, the locality of pain can indicate which vessel might be restricted – calf pain is most frequently a consequence of femoral artery disease; whereas pain in the thigh/buttock area indicates proximal restriction, most likely due to aortoiliac disease [15].

Except in the most severe cases, the restricted blood flow does not cause ischemia of a resting skeletal muscle, due to its low metabolic demands. However, during physical activity, the metabolic demands of the exercising muscle increase greatly, and blood flow is required to increase many-fold to meet this demand. Although blood flow may be able to increase some, it is often not enough to match the increased demand, rendering the limb ischemic. This is illustrated in a study by Pernow et al. [16], who measured femoral artery blood flow in patients with claudication, both before and during a standard exercise test. During exercise, blood flow in claudicants increased almost threefold; however, this was still only 50% of the exercise blood flow in control subjects.

Recently, intermittent claudication has been referred to as a form of low-grade I-R [17]. This is not identical to the traditional I-R, whereby blood flow to a muscle is occluded for a given period (enforcing absolute ischemia) and then restored, allowing re-oxygenation of the tissue. During claudication, the ischemia is relative and the reperfusion does not occur due to restoration of blood flow, but rather due to a reduction in blood demand during the recovery period, allowing the oxygen demands of the muscle to once again be met. However, parallels between the two conditions certainly exist, which has led to the realization that intermittent claudication may generate the same damage that occurs after I-R (contractile dysfunction, oxidative injury [18], cell dysfunction and cell death [19]).

Oxidative Stress

There is considerable evidence of radical production induced by prolonged ischemia of skeletal muscle, with subsequent reperfusion [1,2,20]. This has applicability to surgical situations where it may be necessary to occlude blood flow for hours, before reperfusion is either allowed, or necessary. These studies have provided evidence of

elevated lipid hydroperoxides, decreased protein thiols, and a loss of glutathione. However, few studies have considered the idea that radicals may be generated after short-term I-R, as would be the case in the exercising claudicant. This form of I-R may be of a lower grade, and skeletal muscle has been shown to be fairly resistant to I-R injury. Indeed, Kadambi et al. [21] found no increase in lipid peroxidation after 30 min of ischemia followed by 1 h of reperfusion. However, when the metabolic demands of a muscle are increased while blood flow to the muscle is reduced, the mismatch between supply and demand becomes greater. This may cause a greater disruption to redox balance. Work from our lab shows convincing evidence of oxidative damage to both lipids and proteins, as well as a reduction in total glutathione; and an increase in wet/dry weight, which is indicative of edema, after a 30-minute bout of contractile claudication (Table 1). Other studies lend support to this, showing increased lipid peroxides [4] and increased *ortho*-hydroxyantipyrine — an indicator of oxidative stress [5].

Table 1. Markers of oxidative stress and edema after 30 min contractile claudication

	Soleus		Gastrocnemius	
	Sham-Stimulated	Ligated-Stimulated	Sham-Stimulated	Ligated-Stimulated
Lipid Hydroperoxides (mmol/g wet weight)	8.92 ± 1.43	13.76 ± 1.39 *	7.39 ± 0.97	10.88 ± 0.86 *
Protein Carbonyls (nmol/mg protein)	1.80 ± 0.05	2.83 ± 0.07 #	1.87 ± 0.13	2.89 ± 0.13 #
Total Glutathione (mM)	1.44 ± 0.03	1.29 ± 0.05 *	0.90 ± 0.01	0.76 ± 0.04 +
Wet/Dry Ratio	N/A	N/A	4.51 ± 0.08	4.72 ± 0.05 *

Values are mean ± SEM; N=8

*Significant at $p < 0.05$; +Significant at $p < 0.01$; #Significant at $p < 0.001$

Just as strenuous exercise in healthy individuals causes significant oxidative stress, while moderate intensity exercise does not [22], this too may be the case in claudicants. Silvestro investigated the effects of maximal and submaximal exercise on parameters of oxidative stress in claudicants; and discovered that only maximal exercise caused

significant lipid peroxidation and an increase in intracellular adhesion molecule-1 (ICAM-1). Expressed by endothelial cells, ICAM-1 is a receptor for the neutrophil adhesion molecules CD11/CD18, and is therefore important in determining neutrophil-endothelium cell adhesion. Adherence of these molecules primes the neutrophil for migration into the tissue.

These findings are interesting since claudicants are instructed, therapeutically, to exercise until near maximal pain. However, based on Silvestro's findings, it might seem logical for claudicants to exercise less intensely. This would presumably cause a milder ischemic insult, reducing oxidative stress and neutrophil infiltration, while potentially still providing the cardiovascular benefits of exercise that claudicants so desperately need.

Sources of Oxidants after Exercise Claudication

There are a variety of potential biological sources of oxidants. However, most research regarding skeletal muscle I-R has focused on the xanthine oxidase pathway and activated neutrophils. Work from our lab has shown xanthine oxidase activity and activated neutrophils to be elevated after a short bout of contractile-induced skeletal muscle ischemia (Table 2). Thus these potential sources are considered in greater detail.

Xanthine oxidase (XO) pathway. During ischemia, or very strenuous exercise, ATP catabolism occurs within the muscle cell, yielding hypoxanthine and xanthine. Normally these metabolites are oxidized via xanthine dehydrogenase (XDH) using NAD as the electron acceptor. However, XDH may be converted to XO by oxidation of thiol groups, or by calcium-dependent proteolytic attack. Since both ischemia and strenuous exercise can disrupt calcium homeostasis, the environment may favor this dehydrogenase to oxidase conversion. XO is still able to oxidize hypoxanthine and xanthine; however,

molecular oxygen acts as the electron acceptor instead of NAD, the consequence being the generation of large amounts of superoxide anion [23]. The necessary components for ROS production are, therefore, provided. The only missing component is molecular oxygen, provided on reperfusion.

Table 2. Potential sources of oxidants after 30 min contractile claudication.

	Soleus		Gastrocnemius	
	Sham-Stimulated	Ligated-Stimulated	Sham-Stimulated	Ligated-Stimulated
Xanthine Oxidase Activity (mU/g wet weight)	2.85 ± 0.09	3.39 ± 0.19 *	2.73 ± 0.08	3.29 ± 0.15 *
Myeloperoxidase Activity (U/g wet weight)	0.80 ± 0.02	1.15 ± 0.03 #	0.66 ± 0.08	1.03 ± 0.06 #
Mitochondrial Hydrogen Peroxide Release (nmol/min/mg protein)	N/A	N/A	0.48 ± 0.04	0.49 ± 0.06

Values are mean ± SEM; N=6

*Significant at $p < 0.05$; #Significant at $p < 0.001$

Neutrophils. It has been shown that neutrophil activation is significantly increased after intermittent ischemia of skeletal muscle [23-25]. Plasma neutrophil levels have been shown to peak within 5 min post-exercise in claudicants [25,26]; however infiltration into tissues takes longer. Smith et al. [27] showed what they referred to as a “dramatic” increase in tissue neutrophils after just 15 min of reperfusion after 4 h ischemia. However, Prem et al. [28] found that although tissue neutrophils increased after 30 min of reperfusion, after 2 h ischemia, the levels peaked after 4 h of reperfusion. This peak in neutrophil infiltration after a longer period of reperfusion is in agreement with others [29].

The mechanisms of neutrophil activation are unquestionably complex, and indeed remain largely unknown. However, it is interesting to note that oxidants themselves have been implicated as chemotactic to neutrophils [30]. This is supported by the observation

that treatment with radical inhibitors before reperfusion prevents tissue neutrophil infiltration [31]. In addition, the lipid peroxidation product 4-hydroxy-trans-2-nonenal has also been shown to be chemotactic to neutrophils [32], weaving a tighter web between oxidant production and propagation of the inflammatory process.

Role of Iron

Although the generation of superoxide and hydrogen peroxide are potentially damaging, they are poorly reactive and cannot alone explain the toxic effects of superoxide generating systems [33]. Instead, it seems that the highly damaging hydroxyl radical is largely responsible for oxidative injury. The hydroxyl radical may be generated by the reaction of superoxide with hydrogen peroxide (Haber-Weiss reaction), yet this proceeds too slowly to be of biological significance. However, in the presence of a suitable metal catalyst, for example iron or copper, the reaction proceeds much more rapidly. Iron appears to be the best candidate *in vivo*; and the iron-catalyzed Haber-Weiss reaction proceeds with a rate constant of $76 \text{ M}^{-1} \text{ s}^{-1}$, compared to almost zero without a catalyst. In addition to catalyzing hydroxyl radical formation, iron may also reinitiate lipid oxidation by converting lipid hydroperoxides to reactive alkoxyl or peroxy radicals [7].

Fortunately, under normal physiological conditions, virtually all iron is bound either to proteins, membranes, nucleic acids, or low-molecular-weight chelating agents [33]. Within the muscle cell, iron is stored mainly within ferritin molecules, a protein of 24 polypeptide chains capable of storing up to 4,500 iron atoms [34]. However, iron may be mobilized from ferritin and other storage forms, making the metal redox active. Release of iron from ferritin requires reduction of the ferric iron stored in the molecule, a feat the superoxide anion and other reducing agents are capable of. Biemond et al. [34]

stimulated polymorphonuclear leukocytes to produce superoxide; and observed significant iron mobilization from ferritin. Addition of SOD prevented this, providing convincing evidence that the iron mobilization occurred in a superoxide-dependent manner. Likewise, iron release from ferritin has been shown to be possible with xanthine and xanthine oxidase, a well known source of superoxide [35]. It has also been suggested that NADH and NADPH, which accumulate during ischemia, might interact with ferritin to release ferrous iron [36].

Furthermore, the rate of ferritin iron mobilization by reducing agents is accelerated by acidification. Brain homogenate incubated under aerobic and anaerobic conditions at three different pH's (7, 6 and 5) showed far greater iron delocalization under anaerobic conditions, which was exacerbated with a decrease in pH [36].

Iron has been shown to be an important pro-oxidant in prolonged I-R. Chiao et al. [7] provided evidence of iron delocalization after 2 h ischemia and 30 min reperfusion; and this was associated with membrane dysfunction and lipid peroxidation. Although no study has considered the role of iron in promoting oxidant reactions in the exercising claudicant, the conditions seem appropriate for iron delocalization. Exercising claudicants rely heavily on anaerobic respiration because of reduced oxygen delivery, causing an increase in reducing equivalents and increased lactic acid production. Acidosis, accumulation of reducing equivalents, and superoxide production could release iron from ferritin stores, thereby becoming available to catalyze free radical reactions [37].

Interaction

It is doubtful that ROS are derived from a single source in the exercising claudicant. Rather, there is likely some overlap and interaction among the different

sources. This is shown (after prolonged I-R) by Seekamp et al. [29], who measured tissue injury and vascular permeability. Neutrophil depletion, administration of catalase and superoxide dismutase, allopurinol, dimethylthiourea, dimethylsulfoxide, and complement depletion were all individually implemented as countermeasures; and each significantly attenuated muscle damage to some degree. Importantly, pretreatment with antioxidants also significantly reduced neutrophil infiltration, emphasizing the chemotactic potential of ROS.

Edema

The alteration of cell membranes due to post-ischemic oxidative stress has a strong correlation with the presence of intramuscular edema [38], characterized by fiber swelling, fiber destruction, and an increase in muscle wet weights. Neutrophil depletion or hydroxyl radical scavenging have been shown to significantly reduce both oxidative stress and edema [2], strongly implicating ROS in the muscle damage.

We have previously shown significantly increased muscle wet weights in the gastrocnemius and soleus muscles (and a significant increase in the wet/dry ratio in gastrocnemius muscles) of exercise claudication animals, possibly due to oxidative stress-induced edema.

Muscle Cell Membrane

Lactate Dehydrogenase resides in the cytoplasm of the muscle cell and has a molecular weight of 140,000 kDa, making it highly impermeable to the muscle cell membrane. Therefore, a decrease in LDH may indicate a change in membrane permeability, such that some of the enzyme has escaped into the extracellular compartment. This may be explained by an alteration in cellular ATP levels, which could

increase intracellular calcium levels, activating proteases and lipases, thereby altering normal membrane permeability. Given the degree of ATP catabolism that appears to occur during skeletal muscle ischemia [39] this is a possibility. Alternatively, it could be the result of oxidative modification of membrane lipids and proteins, as we previously showed to occur after contractile claudication.

This loss in LDH activity associated with oxidative stress has been reported previously. After downhill running Dawson [40] noted an increase in lipid peroxidation and myeloperoxidase activity; and a decrease in LDH activity in the gastrocnemius muscle, concluding that oxidative modification to the muscle cell membrane made it leaky. In a separate study, Jones [41] stimulated muscles *in vitro* for 30 min and quantified LDH release into the medium over a 3 h period. Enzyme release peaked 1 h post-stimulation in the soleus muscle under both normoxic and hypoxic conditions, but was significantly more elevated in the hypoxic conditions. In an attempt to distinguish whether ATP depletion or membrane disruption was responsible for this loss of LDH, Jones incubated with iodoacetate and cyanide, to reduce ATP; and then with deoxycholic acid, a detergent, to disrupt the membrane. Both treatments caused even greater release of LDH, confirming both as possibilities. The conclusion drawn was that both metabolic changes and physical damage could play a part.

Countermeasures

Allopurinol

Allopurinol is a purine analog whose metabolite, oxypurinol, forms a tight binding complex with xanthine oxidase, rendering the enzyme inactive. Since xanthine oxidase is a potential source of ROS, inactivating the enzyme could have protective effects against

ROS-induced damage. Animals treated with allopurinol appear to have attenuation of I-R-induced muscle damage in a variety of tissues [8].

Vina et al. [42] showed that blood XO levels were significantly elevated after exhaustive exercise; and that allopurinol administration prevented exercise-induced glutathione oxidation. Although exercising claudicants do not typically perform exercise that would be exhaustive to a healthy individual, even light exercise usually brings about shortness of breath, perspiration, and a general sense of fatigue [26]. Work from our lab, using an animal model of unilateral femoral artery blockage, showed that a mild stimulation protocol elicits much greater metabolic stress to the occluded limb compared to the control, as evidenced by a decline in force production over time.

Several studies have shown that oxidative stress is derived from xanthine oxidase during prolonged ischemia, followed by reperfusion, in skeletal muscle. Asami et al. [43] showed that muscular xanthine and malondialdehyde (MDA) levels were elevated during reperfusion after 5 h of ischemia. MDA is an indicator of lipid peroxidation, and its increase was attenuated by allopurinol administration, which strongly implicates XO as the source of the radical-induced lipid damage. Similarly, McCutchan et al. [44] showed that XO activity was significantly elevated with 3 h of ischemia followed by reperfusion; and that this was associated with hydrogen peroxide generation. Administration of either allopurinol or tungsten (which replaced molybdenum in xanthine oxidase's active site) reduced XO activity and hydrogen peroxide generation.

Cyclophosphamide

Cyclophosphamide is a derivative of the nitrogen mustard family, compounds originally developed as chemical weapons. Soldiers exposed to sulfur mustard suffered from low white-blood-cell counts (especially lymphocytes), apparently due to the

mustard's cytotoxic effects on dividing tissues. After that observation, nitrogen mustard, a similar but less toxic agent, was developed to treat cancer; and later used as an immunosuppressant. Cyclophosphamide is activated enzymatically in the body to phosphoramidate mustard, a powerful and unstable DNA-alkylating agent, interfering with DNA synthesis, therefore proving cytotoxic to dividing lymphocytes [45].

Several studies have used cyclophosphamide to induce neutropenia in rat models. Kuwabara et al. [9] noted the number of polymorphonuclear neutrophils was reduced to 20 per μL of blood 4 days after injection, compared to 1224 per μL of blood in control animals. Seekamp et al. [29] used cyclophosphamide to induce neutropenia 3 days before I-R exposure and found a protective effect against muscle permeability. This protection from cyclophosphamide-induced neutropenia has also been shown to be effective against an increase in microvascular permeability associated with short-term I-R [10].

Deferoxamine

Deferoxamine is a straight-chained molecule with three hydroxamic acid groups. When a ferric ion comes into contact with deferoxamine, the molecule twines itself around the ion, attaching it to its three hydroxamic acid groups. The molecule, therefore, provides a shell, surrounding the iron and becoming a very stable complex ([46].

The powerful iron-chelating properties of deferoxamine mean that it may inhibit lipid peroxidation and the generation of the hydroxyl radical from superoxide and hydrogen peroxide in biological systems where ferrous iron is free. Deferoxamine has also been shown, in high concentrations, to block the conversion of xanthine dehydrogenase to xanthine oxidase in cultured endothelial cells [47]. The mechanism for this is unknown, however the xanthine oxidase protein has four redox-active sites:

molybdenum, flavin adenine dinucleotide (FAD), and two iron sulfur centers of the ferredoxin type [48]. It was speculated that enzyme activity would be impaired by deferoxamine if it bound the iron cofactor.

Since myeloperoxidase contains a heme iron essential for its activity, the possibility exists that deferoxamine could inhibit MPO activity if it chelated the heme iron. However, since adding deferoxamine has no effect on the absorption spectrum of MPO, it doesn't appear to work in this manner [49]. Deferoxamine can, however, be oxidized by MPO and hydrogen peroxide, thereby competing with other electron donors; and deferoxamine has been shown to react with and degrade the highly oxidizing hypochlorous acid – a product of the MPO system [49].

Deferoxamine has a relatively low molecular weight (656.79 Da), which facilitates its entry into cells. In fact, deferoxamine has been shown to enter skeletal muscle cells in significant concentrations with a greater intracellular than extracellular distribution [50].

Administration of deferoxamine after prolonged I-R has been shown to prevent lipid peroxidation and to attenuate membrane dysfunction [3]. Additionally, Smith et al. [51] showed that either deferoxamine or apotransferrin (an iron-binding protein) administration prevented an increase in microvascular permeability associated with prolonged I-R.

Summary

In summary, it has been shown that an acute bout of contractile claudication causes an increase in protein oxidation, lipid peroxidation, and edema; and a loss of total glutathione. This is associated with an increase in xanthine oxidase activity and neutrophil infiltration. In addition, free iron is elevated after prolonged I-R; and is likely elevated after an acute bout of contractile claudication due to favorable conditions for

iron mobilization. Therefore, inhibition of xanthine oxidase activity, induction of neutropenia, and iron chelation have the potential to attenuate the oxidative stress and edema associated with an acute bout of contractile claudication. In addition, reactive oxygen species have themselves been implicated as chemotactic to neutrophils. Therefore, inhibition of xanthine oxidase and iron chelation, have the potential to attenuate neutrophil infiltration into tissue.

Purpose

Our purpose was to determine if inhibition of xanthine oxidase activity, induction of neutropenia, or iron chelation will protect against oxidative stress and muscle edema induced by an acute bout of contractile claudication. We also determined whether inhibition of xanthine oxidase activity and iron chelation, attenuate the increase in neutrophil infiltration associated with an acute bout of contractile claudication.

Rationale

We showed that oxidation of proteins and lipids occurs after an acute bout of contractile-induced skeletal muscle ischemia; and that this is associated with an increase in muscle wet weight, likely due to edema. We also showed an increase in xanthine oxidase activity and infiltration of neutrophils after the same conditions. It was shown by others that administering allopurinol inhibits xanthine oxidase activity; and therefore oxidant production [44]. It was also shown that induction of neutropenia by cyclophosphamide attenuates neutrophil infiltration and protects against oxidative stress-induced muscle damage [29]. Thus, it is speculated that allopurinol administration will inhibit xanthine oxidase activity, and that cyclophosphamide administration will attenuate neutrophil infiltration; and that both will independently attenuate protein and lipid oxidation and edema.

Mobilization of iron from its stored sources can catalyze the Haber-Weiss reaction, generating the highly reactive hydroxyl radical; and reinitiating lipid peroxidation, creating the alkoxyl and peroxy radicals. Delocalization of iron was shown to occur after prolonged I-R; and is associated with lipid peroxidation [7]. In addition, chelation of iron by deferoxamine was shown to prevent lipid peroxidation [3]. Thus, it is expected that deferoxamine administration will attenuate protein and lipid oxidation and edema.

Reactive oxygen species have been shown to be chemotactic to neutrophils [30]; and inhibiting their generation has attenuated neutrophil infiltration [31]. Thus, it is anticipated that iron chelation and xanthine oxidase inhibition will reduce the amount of ROS produced; and therefore attenuate neutrophil infiltration.

Questions and Hypotheses

Question 1. Does an increase in xanthine oxidase activity cause oxidative stress and muscle edema, generated by an acute bout of contractile claudication?

Hypothesis 1. Reduction of xanthine oxidase activity will attenuate lipid peroxidation and protein oxidation, as well as muscle edema, induced by an acute bout of contractile claudication.

Question 2. Does an increase in xanthine oxidase activity cause neutrophil infiltration into muscle after an acute bout of contractile claudication?

Hypothesis 2. Reduction of xanthine oxidase activity will attenuate neutrophil infiltration into muscle after an acute bout of contractile claudication.

Question 3. Does neutrophil infiltration cause the oxidative stress and muscle edema induced by an acute bout of contractile claudication?

Hypothesis 3. Reducing neutrophil infiltration into tissue will attenuate lipid peroxidation and protein oxidation, as well as muscle edema, induced by an acute bout of contractile claudication.

Question 4. Does reduction of free iron attenuate the oxidative stress and muscle edema induced by an acute bout of contractile claudication?

Hypothesis 4. Reduction of free iron will attenuate lipid peroxidation and protein oxidation, as well as muscle edema, induced by an acute bout of contractile claudication.

Question 5. Does reduction of free iron attenuate neutrophil infiltration induced by an acute bout of contractile claudication?

Hypothesis 5. Reduction of free iron will attenuate neutrophil infiltration into tissue, induced by an acute bout of contractile claudication.

CHAPTER 2 METHODS

Animals

All experiments were performed on male Sprague Dawley rats (120 d old) to avoid any antioxidant protection from estrogen. They were fed rat chow, given water ad libitum, and maintained on a 12-h light/dark photoperiod for 7 days before the beginning of these experiments. During this 7 day period, animals were handled daily to prevent a stress hormone-induced reduction in body weight at the beginning of the experiments. Animals were then randomly assigned to one of four experimental groups: Control (CON); Allopurinol supplemented (ALLO); Cyclophosphamide supplemented (CYCLO) and; Deferoxamine supplemented (DFO). The limbs of each rat were then randomly assigned to a ligated/stimulated (LS) or a sham ligated /stimulated (SS) group for the study.

Supplementation Protocol

Control

Animals were injected intraperitoneally with 0.5 mL saline twice daily, beginning 2 days before ischemia. On the day of the experiment, the second injection was given 30 min before the contractile-induced ischemia.

Allopurinol

Animals were given an intraperitoneal injection of 50 mg of allopurinol per kg body weight twice daily, beginning 2 days before the ischemia. On the day of the experiment, the second injection was given 30 min before the contractile-induced

ischemia. This quantity of allopurinol has been shown to yield extracellular fluid concentrations of 10 μ M, a concentration sufficient to cause an >80% inhibition of xanthine oxidase activity without a scavenging effect [52]. The allopurinol was dissolved in normal saline by adding 1 N sodium hydroxide; and administered slowly.

Cyclophosphamide

Animals were given intraperitoneal injections of 20 mg of cyclophosphamide per 100 g of body weight, 4 days before contractile-induced ischemia. This dose has been shown to reduce the circulating leukocyte count by 85 to 90%, inhibiting microvascular damage [10]; and, more specifically, to reduce neutrophils to 1.4% of normal levels [53].

Deferoxamine

Animals received an IP injection of 100 mg deferoxamine per kg body weight twice a day, beginning 2 days before the ischemia. On the day of the experiment, the second injection was given 30 min before the contractile-induced ischemia. Deferoxamine was dissolved in normal saline and administered slowly. This quantity of deferoxamine has been shown to reduce neutrophil infiltration and muscle damage [54].

Ligation Procedure

After isofluorane anesthesia (5% for induction, 1.5 to 2.5% for maintenance), a small incision was made directly above the inguinal fold; and the femoral artery was exposed and isolated by blunt dissection. Two ligatures were placed tightly around the vessel and the vessel was cut between the ties. This procedure produces a 60% to 70% reduction in blood flow during muscle contraction [55,56]. Topical antibiotic powder was placed on the wound before closure with sutures. The sham surgery limbs underwent the identical procedure except the femoral artery was left intact.

In Vivo Stimulation

Twenty-four hours post-ligation both hindlimbs were stimulated in vivo for 30 min, and force production was measured. Animals were placed in a prone position in a specially fabricated Plexiglas apparatus that allows the animal to be secured in a reproducible position with limited mobility of the lower leg except at the tibiotarsal joint. Animals were kept warm by an incandescent light, and core temperature maintained from 35 to 38° C, measured with a rectal thermistor probe. A calibrated force/displacement ergometer was secured to the forefoot between the first and second footpads by a lightweight chain such that the tibiotarsal angle is 90°. The voltage signal from the force transducer was processed via a computerized data acquisition system (LabView, National Instruments, Austin).

A stainless steel stimulating electrode was placed transcutaneously near the sciatic nerve midway between the posterior ischeal spine and the greater femoral trochanter. Another stainless steel stimulating electrode (anode) was inserted 3 mm subdermally in the midline of the lower back. The sciatic nerve was then stimulated proximally with 100V, 1.0 pulses per second, and a stimulus time of 0.05 ms (Grass Instruments).

Tissue Removal and Storage

One hour post-stimulation the gastrocnemius muscle was removed. Each muscle was dissected free, immediately placed in cold antioxidant buffer (100 μ m EDTA, 50 mM Na₂HPO₄, 1 mM BHT), blotted dry, weighed, and rapidly frozen in liquid nitrogen and stored at -80°C until assayed.

Biochemical Assays

Protein Concentrations

Protein content of muscle homogenates was determined using the biuret technique [57].

Muscle Water Content

Total water content of the gastrocnemius muscles was determined by using a freeze drying technique incorporating a vacuum pump with a negative pressure of ~1 mm Hg. A precise frozen wet weight was measured, and then tissues placed in a freeze-dry unit (Virtis Sentry Benchtop 3 L). The dry weight was terminated when the same weight was recorded three times in succession during a six-hour interval.

Protein Oxidation

Protein carbonyls was measured spectrophotometrically as described by Reznick and Packer [58], with modifications reported by Yan et al. [59]. Briefly, samples were incubated in dinitrophenylhydrazine (DNPH) dissolved in HCl, with blanks incubated in HCl only. Following reaction with DNPH, proteins was precipitated in 20% TCA, washed in ethyl acetate-ethanol (1:1 vol/vol) and dissolved in 6 M guanidine hydrochloride, pH 2.3. Tissue protein carbonyl content was quantified by determining the absorbance at 370 nm and using an extinction coefficient of 22,000 M⁻¹. Protein concentrations was determined using a BSA standard curve in guanidine HCl with absorbance read at 280 nm.

Lipid Peroxidation

Lipid hydroperoxides were measured using the ferrous oxidation/xylene orange technique reported by Hermes-Lima et al. [60]. Briefly, samples were homogenized in 100% methanol, centrifuged, and the resulting supernatant mixed in solution with an iron

source (FeSO_4), an acid (H_2SO_4) and a reactive dye (xylenol orange). In this mixture, the membrane peroxides oxidize Fe^{2+} to Fe^{3+} and the peroxides are reduced. The Fe^{3+} reacts with xylenol orange to form a Fe^{3+} -Xylenol orange complex, yielding a colored product that is accompanied by an absorbance change at 580 nm.

4-Hydroxy-2-Nonenal (HNE)

Proteins were separated on a 4-20% precast polyacrylamide gel (BMA, Rockland), using 30 μg of protein per well, and then transferred onto a nitrocellulose membrane. The membrane was then blocked overnight using a blocking solution containing 0.05% Tween and 5.0% milk. Membranes were incubated for 1 hour with the primary antibody (Alpha Diagnostics, San Antonio) using a 1:500 dilution, thoroughly washed and then incubated for 1 hour in anti-rabbit IgG horseradish peroxidase (Amersham Life Science, United Kingdom) using a 1:1000 dilution. Blots were developed using ECL (Amersham Pharmacia Biotech, United Kingdom), and imaged using an Image Station (Eastman Kodak Company, model 440cF). Arbitrary OD units were calculated by multiplying the area of each band by its optical density and then normalized to the control group (CON SS), which we made to 100%.

Xanthine Oxidase Activity

Xanthine oxidase was measured using a modified version of Amplex Red Xanthine/Xanthine Oxidase Assay Kit from Molecular Probes. In this assay xanthine oxidase catalyzes the oxidation of purine bases, hypoxanthine, or xanthine to uric acid and superoxide. Superoxide spontaneously degrades to hydrogen peroxide, which in the presence of horseradish peroxidase, reacts stoichiometrically with Amplex Red reagent to generate the red-fluorescent oxidation product resorufin. The emission wavelength was set at 590 nm and excitation fluorescence measured at 560 nm.

Myeloperoxidase Activity

MPO activity was assayed according to methods by Belcastro [61]. Briefly, tissue was homogenized in 0.5% hexadecyltrimethyl ammonium bromide in 50 mM potassium phosphate buffer, pH 6.0. Homogenate was then be sonicated, freeze-thawed three times, sonicated once more, and subsequently centrifuged at 1,300 g for 15 mins. Ten μ l of supernatant was removed and incubated with 290 μ l of 50 mM potassium phosphate buffer with 0.6mM hydrogen peroxide and 167 μ g *o*-dianisidine dihydrochloride per ml. One unit of MPO was defined as a change in absorbance of 1.0 at an optical density of 480 nm at a temperature of 25°C.

Lactate Dehydrogenase Activity

LDH activity was determined according to methods by Bergmeyer et al [62]. This assay uses pyruvate and muscle homogenate (containing LDH), to oxidize NADH to NAD, which is accompanied by a change in absorbance at 340 nm. Since the NADH being oxidized is equimolar to the pyruvate being reduced, the change in absorbance is directly proportional to the LDH activity in the sample.

Statistical Analysis

All data was analyzed using a two-way analysis of variance, and the contractile data was analyzed using a two way ANOVA with repeated measures. Significance was established at the $P < 0.05$ level, and a Bonferroni post-hoc test was used where necessary.

CHAPTER 3 RESULTS

Overview of Experimental Findings

This study examined the pathways of oxidative damage after an acute bout of contractile claudication. By inhibiting each of the pathways individually, a cause and effect relationship could be established. The major findings of the study are that neutrophil depletion and iron chelation attenuated the oxidative damage associated with contractile claudication, while edema was only attenuated with neutrophil depletion. Inhibition of xanthine oxidase activity significantly attenuated lipid peroxidation, but not protein oxidation or edema.

Morphological Measurements

Each of the countermeasures was well tolerated by the animals, with no mortalities or visual side effects noted. The body weights of ALLO and CYCLO animals significantly decreased, by 7% and 13% respectively, from the time of group assignment (pre-injection) to the time of sacrifice, and the muscle weights of CYCLO animals were significantly lower than the muscle weights of CON LS, DFO SS and DFO LS. However, neither the muscle weight/body weight ratio nor total protein concentration was different across any of the groups (Table 1).

Contractile Function

Force generation from the triceps surae muscle group significantly decreased in each of the ligated-stimulated (LS), or claudicant, limbs during the thirty-minute stimulation period (Figure 1). Compared to their sham-stimulated limbs, both the LS

CON and DFO groups were significantly decreased after 3 minutes of stimulation, and the ALLO and CYCLO group after just 2 minutes. However, neither the LS nor SS limbs were different across treatments, providing evidence that each group was made ischemic to the same degree and that administration of the various countermeasures had no effect on force generation during the contractile claudication period itself.

Lipid Hydroperoxides (LOOH)

LOOH's were significantly elevated ($p < 0.001$) in the LS limbs of control and ALLO supplemented animals, compared to sham. However, the LS limb of ALLO animals was significantly ($p < 0.05$) less than the LS limb of the control group. There were no differences in LOOH levels between the LS and SS limbs of the CYCLO and DFO groups (Figure 2).

4-Hydroxy-2-Nonenal Levels

CON SS limbs were used as a control for comparisons of CON LS, ALLO LS, CYCLO LS, and DFO LS. Total HNE binding was significantly attenuated in the CYCLO LS only (Figure 3).

Protein Carbonyls

Both the control group and ALLO supplemented animals showed a significantly increased ($p < 0.05$) protein carbonyl content in the LS limb compared to SS. There were no differences between the LS limbs of ALLO animals and control. DFO and CYCLO supplementation both attenuated this increase in protein carbonyls in the LS limb (Figure 4).

Xanthine Oxidase Activity

In control, DFO supplemented and CYCLO supplemented animals, xanthine oxidase activity significantly increased ($p < 0.001$) in the LS limb, compared to sham. This increase was attenuated in the ALLO supplemented group (Figure 5).

Myeloperoxidase (MPO) Activity

Control ($p < 0.001$), ALLO supplemented ($p < 0.01$), and DFO supplemented ($p < 0.05$) animals had significantly elevated MPO activity in the LS limb compared to SS. However, MPO activity in LS limbs of both the ALLO group ($p < 0.01$) and the DFO group ($p < 0.05$) was significantly lower than the LS limb of control animals. There was no increase in MPO activity in CYCLO supplemented animals (Figure 6).

Wet/Dry Ratio

The control ($p < 0.01$), ALLO ($p < 0.05$), and DFO ($p < 0.05$) groups showed a significant increase in muscle wet/dry ratio following contractile claudication, in the LS limb compared to SS (Figure 7). This increase was attenuated in the LS limbs of CYCLO animals.

Lactate Dehydrogenase (LDH) Activity

The LS limbs of control animals showed a significant decrease ($p < 0.05$) in LDH activity. This reflects “damage” to the muscle cell membrane since the enzyme may “leak” from its cytosolic residence into the vasculature, thereby decreasing enzyme activity within the muscle. The decrease in LDH activity was attenuated in the ALLO, CYCLO, and DFO groups (Figure 8).

Table 3. Body weight, wet muscle weight, and protein concentration from all groups. Values are expressed as mean \pm SEM. **a** indicates significantly different ($p < 0.05$) from the treatment pre-injection body weight. **b** indicates significantly different ($p < 0.05$) from CYCLO SS, and **c** indicates significantly different ($p < 0.05$) from CYCLO LS. (SS = sham-stimulated; LS = ligated-stimulated).

Treatment	Pre-injection body weight (grams)	Body weight at time of sacrifice (grams)	Group	Muscle weight (grams)	Muscle weight/body weight ratio	Total protein concentration (mg/gram wet weight)
CON	356.3 \pm 6.46	350.5 \pm 6.840	SS	2.05 \pm 0.03	5.84 \pm 0.12	139.6 \pm 3.26
			LS	2.11 \pm 0.04 b c	6.04 \pm 0.15	140.1 \pm 4.98
ALLO	349.2 \pm 5.15	324.2 \pm 8.432 a	SS	1.97 \pm 0.04	6.07 \pm 0.09	136.5 \pm 6.04
			LS	2.04 \pm 0.07	6.28 \pm 0.15	142.0 \pm 5.95
CYCLO	365.0 \pm 2.88	319.2 \pm 6.395 a	SS	1.84 \pm 0.05	5.75 \pm 0.16	128.1 \pm 1.96
			LS	1.82 \pm 0.06	5.71 \pm 0.21	132.4 \pm 4.05
DFO	370.3 \pm 5.91	357.3 \pm 7.126	SS	2.12 \pm 0.06 b c	6.09 \pm 0.11	131.2 \pm 3.93
			LS	2.26 \pm 0.05 b c	6.31 \pm 0.14	130.0 \pm 2.99

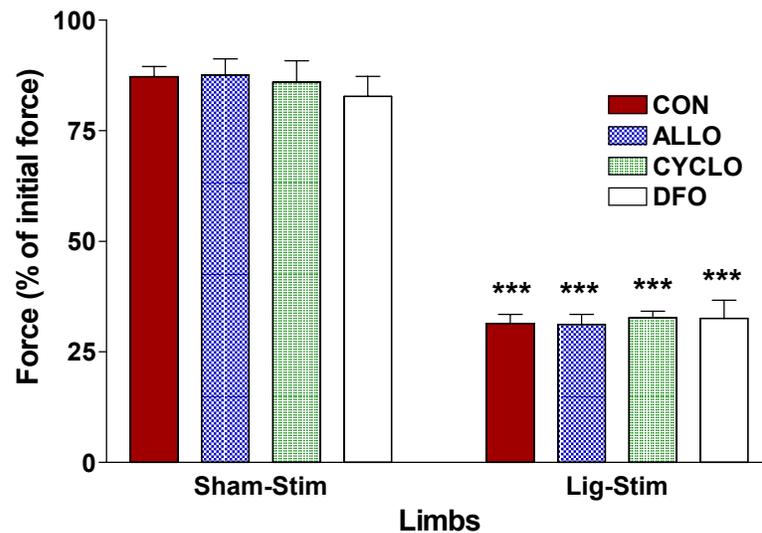


Figure 1. Force production from the triceps surae muscle group during the last minute of the 30-minute stimulation period. Values are means \pm SEM. * indicates significantly different ($p < 0.001$) from sham-stim limbs undergoing the same treatment.

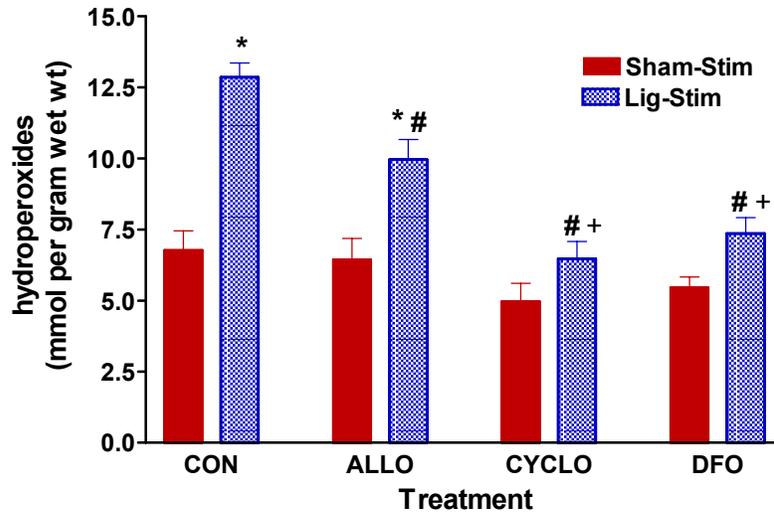


Figure 2. Lipid Hydroperoxide levels; values are expressed as mean \pm SEM. * indicates significantly different ($p < 0.001$) from the sham-stim group undergoing the same treatment. # indicates significantly different ($p < 0.05$) from CON Lig-Stim. + indicates significantly different ($p < 0.05$) from ALLO Lig-Stim.

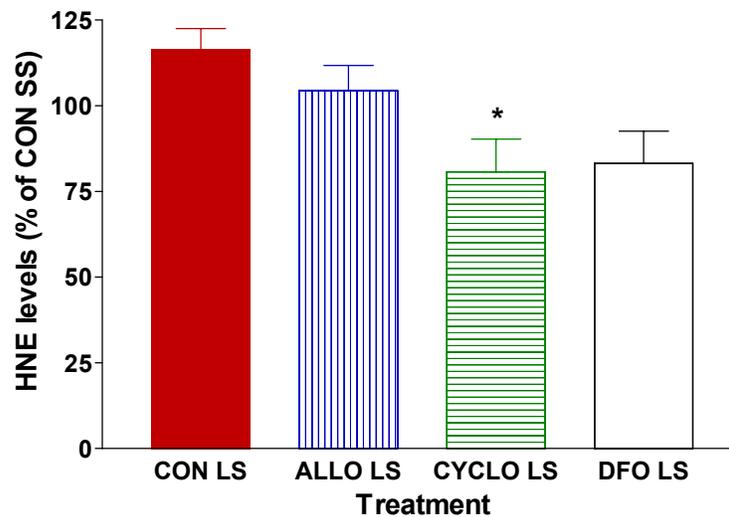


Figure 3. Total HNE levels in CON LS, ALLO LS, CYCLO LS, and DFO LS limbs. Values are expressed as a percentage of CON SS levels. * indicates significantly different from CON LS.

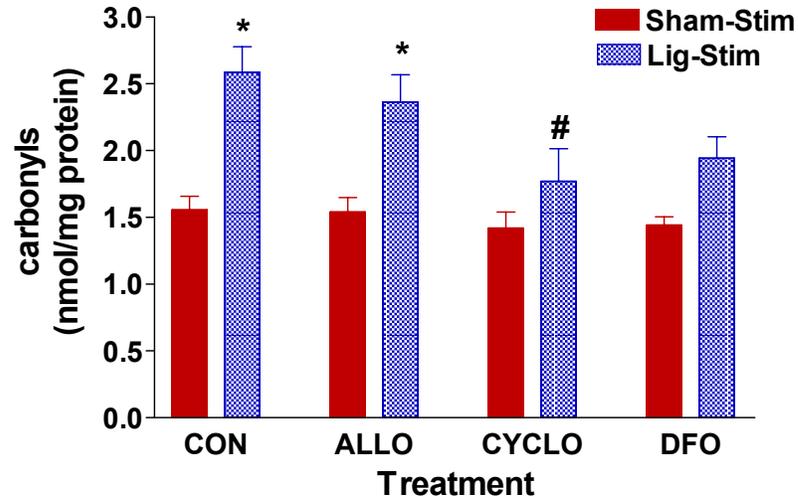


Figure 4. Protein carbonyls; values are expressed as mean \pm SEM. * indicates significantly different ($p < 0.01$) from the sham-stim group undergoing the same treatment. # indicates significantly different ($p < 0.05$) from CON Lig-Stim.

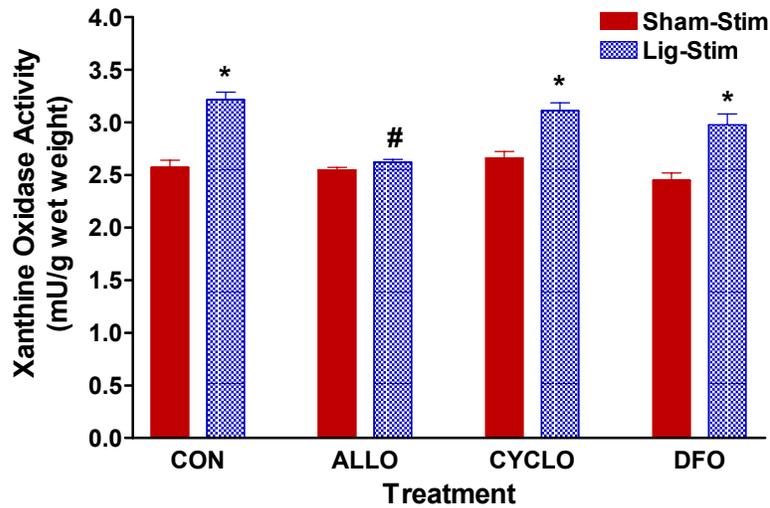


Figure 5. Xanthine oxidase activity; values are expressed as mean \pm SEM. * indicates significantly different ($p < 0.001$) from the sham-stim group undergoing the same treatment. # indicates significantly different ($p < 0.001$) from CON Lig-Stim.

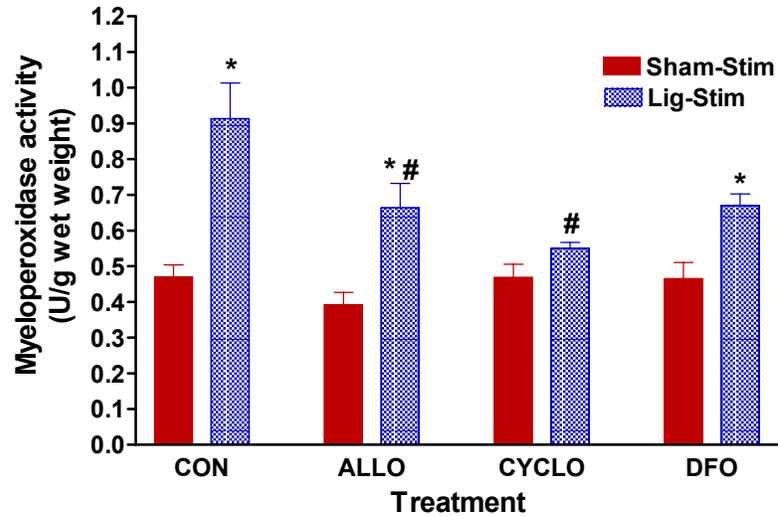


Figure 6. Myeloperoxidase activity; values are expressed as mean \pm SEM. * indicates significantly different ($p < 0.05$) from the sham-stim group undergoing the same treatment. # indicates significantly different ($p < 0.05$) from CON Lig-Stim.

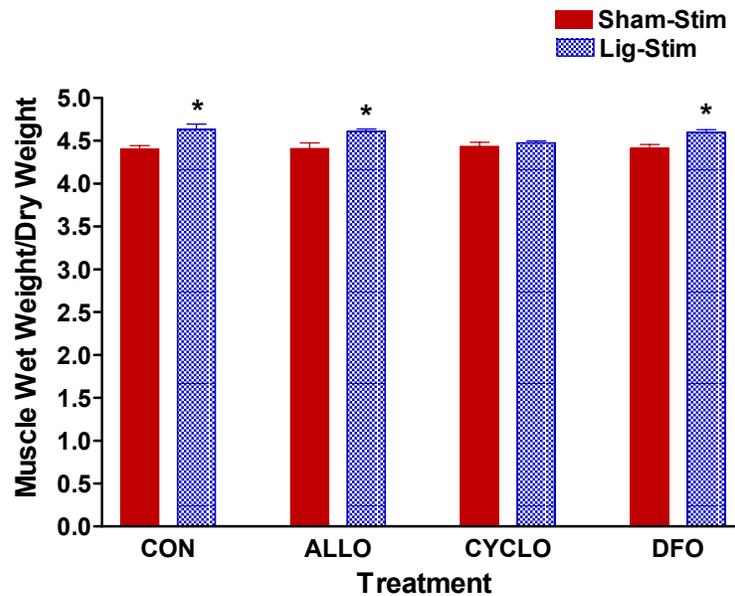


Figure 7. Muscle Wet/dry ratio; values are expressed as mean \pm SEM. * indicates significantly different ($p < 0.05$) from the sham-stim group undergoing the same treatment.

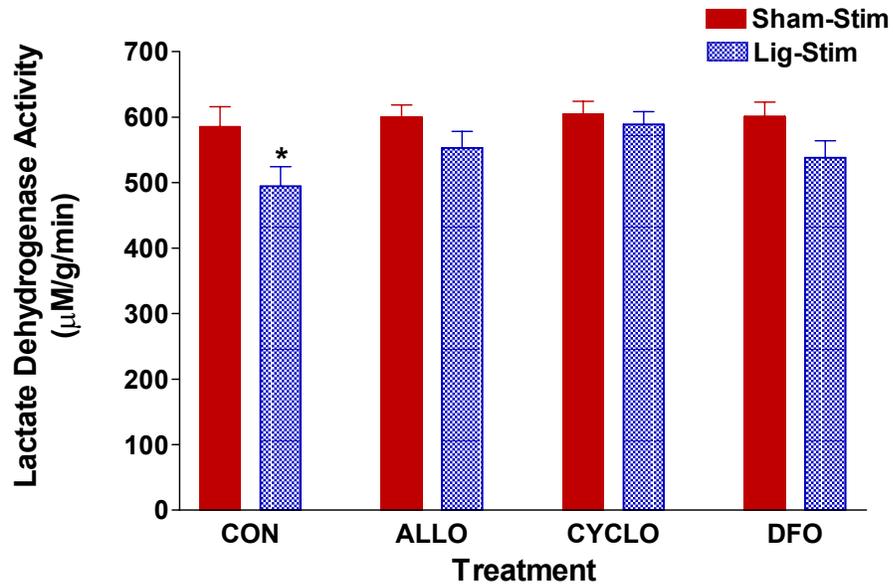


Figure 8. Lactate dehydrogenase activity; values are expressed as mean \pm SEM. * indicates significantly different ($p < 0.05$) from the sham-stim group undergoing the same treatment.

CHAPTER 4 DISCUSSION

Overview of Experimental Findings

This is the first study to examine the pathways of oxidative damage and edema after an acute bout of contractile claudication. The major findings of this study are twofold: inhibiting the increase in xanthine oxidase activity and chelation of iron significantly attenuates lipid peroxidation, but not protein oxidation or edema, after contractile claudication. Secondly, induction of neutropenia attenuated lipid peroxidation, protein oxidation, and edema after contractile claudication. Together, these findings indicate that activated neutrophils are the major source of oxidative damage after contractile claudication, and that this damage contributes to edema. In addition, the data suggest that iron plays a pivotal role in contributing to oxidative damage, presumably by being mobilized during contractile claudication from bound sources, thereby becoming available to partake in redox reactions.

Contractile Claudication

Consistent with our previous findings, an acute bout of contractile claudication causes a significant increase in lipid peroxidation, protein oxidation and edema. This is associated with an increase in xanthine oxidase activity, increased neutrophil infiltration, and a loss of lactate dehydrogenase activity. Since LDH is ordinarily confined to the muscle cell, the loss of LDH activity suggests the muscle cell membrane may be modified by oxidants, making it more permeable and contributing to loss of LDH.

Xanthine Oxidase Inhibition

Allopurinol is known to be an inhibitor of xanthine oxidase activity. However, we quantified the activity of the enzyme to ensure the dose administered was sufficient to inhibit its activity in this study. As expected, allopurinol supplemented animals showed no increase in xanthine oxidase activity after contractile claudication, whereas the control, cyclophosphamide and deferoxamine groups each showed significant increases. This confirms that the allopurinol served its purpose in inhibiting xanthine oxidase activity. In addition, DFO has been shown to attenuate the increase in xanthine oxidase activity in cultured endothelial cells exposed to radicals. Although the mechanism of this is unknown, speculation suggests that xanthine oxidase activity would be impaired by deferoxamine if it bound the iron cofactor at the enzyme's active site. Our findings, however, suggest that DFO has no effect on attenuating xanthine oxidase activity after contractile claudication. Therefore, this potential mechanism of protection may be excluded from the protective effects DFO had in this study.

Despite the lack of increase in xanthine oxidase activity in ALLO animals there was still significant lipid peroxidation, protein oxidation, and edema in these animals. However, the lipid peroxidation was significantly less than control animals, showing inhibition of xanthine oxidase activity provided some protection, and suggesting xanthine oxidase as a source of radicals. In addition, inhibition of xanthine oxidase activity attenuated the loss of LDH activity seen in control animals. With leakage of LDH reflective of a disruption to muscle membrane permeability, these data suggest xanthine oxidase-derived oxidants may target primarily membrane lipids. Since oxidants are non-discriminate in their attack, a logical explanation is that oxidants derived from xanthine oxidase are produced within the lipid membrane, where close proximity dictates their

modification. This is an attractive hypothesis since immunolocalization techniques demonstrate xanthine oxidase is concentrated in capillary endothelial cells [63], and histochemical localization studies indicate the enzyme is also localized in the sarcolemma [63]. Both locations are abundant in lipids and could help explain the loss of LDH and the increase in lipid hydroperoxides.

Since no other studies have measured xanthine oxidase activity, or the effects of the enzyme's inhibition after claudication, parallels can only be drawn from prolonged I-R studies. In one such study [44], the results were very similar to ours in that I-R caused an increase in lipid peroxidation and an associated increase in xanthine oxidase activity. With administration of allopurinol lipid peroxidation was significantly attenuated, confirming that xanthine oxidase-derived radicals can cause peroxidation of cell membranes.

If xanthine oxidase-derived radicals cause lipid peroxidation to endothelial cell membranes, membrane permeability might be altered, causing edema. Since CON animals exhibit increased xanthine oxidase, oxidative stress, and edema, this seems to be an attractive possibility. Indeed, this association has been found by others following prolonged I-R [51]. However, there was no attenuation of the increase in wet/dry ratio in the ALLO group, suggesting other factors contribute to the edema seen after an acute bout of contractile claudication.

In addition, it was hypothesized that inhibiting xanthine oxidase activity would attenuate neutrophil infiltration, and indeed although MPO activity was significantly elevated in the ALLO group, it was 33% lower compared to control animals. Therefore, it can be concluded that xanthine oxidase-derived oxidants are important in the

accumulation of neutrophils after contractile claudication. This chemotaxic potential of oxidants from xanthine oxidase is in agreement with Seekamp [29], who used allopurinol to inhibit xanthine oxidase activity and observed a significant reduction in MPO content following prolonged I-R. The chemotactic potential of xanthine oxidase-derived radicals has several possible explanations. When neutrophils infiltrate tissue from the vasculature, they must first be attracted to, and bind to, the endothelium. Since xanthine oxidase is localized to the endothelium, this places the enzyme at the scene of neutrophil adhesion. Indeed, isolated endothelial cells or isolated vessels exposed to hydrogen peroxide show increased sensitivity for neutrophils [64,65]. There are several potential mechanisms to explain this. One is that oxidants stimulate endothelial cells to synthesize and/or release chemoattractants, such as platelet activating factor and leukotriene B₄ [66]. Another potential mechanism is that oxidants may directly induce the expression of endothelial cell adhesion molecules. Indeed endothelial cells exposed to hydrogen peroxide have been shown to induce P-selectin expression [67], and neutrophils incubated in hydrogen peroxide increase their expression of CD11 and CD18 [68].

Neutropenia

The use of cyclophosphamide to deplete neutrophils was based on several studies using this agent to cause neutropenia both at baseline and during ischemia-reperfusion injury. Mackie [69] showed the neutrophil count of cyclophosphamide-injected animals was <10% of control animals after 4 days; Lee [70] measured circulating neutrophils at <1% of control, 5 days post-cyclophosphamide injection and; Bertuglia et al [10] showed that cyclophosphamide injected animals had leukocyte counts that were 7% of control animal levels, after 30 minutes of ischemia and 30 minutes reperfusion. This clearly shows the capacity for cyclophosphamide to induce neutropenia.

The CYCLO group in this study showed no significant increase in MPO activity after contractile claudication, confirming attenuation of neutrophil infiltration into the tissue. This lack of neutrophil infiltration attenuated lipid peroxidation, protein oxidation, edema, and muscle membrane damage, clearly demonstrating the ability of neutrophils to cause oxidative damage and edema. Although no previous studies have measured tissue neutrophil levels after claudication, or depleted neutrophils prior to claudication, our findings are in agreement with others showing attenuation of lipid peroxidation and edema [2]; and reduction of muscle permeability [29] with neutrophil depletion prior to prolonged I-R.

The attenuation of edema with neutropenia, observed here, has previously been shown after prolonged I-R [10]. It appears that as neutrophils migrate through the vascular endothelium into the muscle they may release lysosomal enzymes and/or oxidizing species. These molecules can damage the endothelium and alter membrane permeability, thereby resulting in edema. A second possibility is that the diapedesis process itself may widen endothelial gap junctions, thereby contributing to edema. Although neither of these potential mechanisms were addressed in this study, it is clear that neutrophils cause significant edema after contractile claudication.

Since LDH is confined to the muscle cell and its loss reflects an alteration in membrane permeability, the attenuated loss of this enzyme's activity with neutropenia implicates neutrophils. This is the first evidence to show the muscle cell membrane is, at least in part, being oxidized by neutrophil-derived oxidants after contractile claudication, thereby making the membrane more permeable.

Iron Chelation

Deferoxamine is a powerful iron chelator that is used broadly in preventing iron-dependent pro-oxidant reactions. It does this by preferentially removing iron from low molecular weight components, including amino acids, organic acids or carbohydrates [71]. Since deferoxamine has been shown to slowly penetrate the plasma membrane [46], thereby entering cells, its protection may be displayed at both the intracellular and extracellular level.

In this study, DFO animals showed significant attenuation of lipid peroxidation, neutrophil infiltration, and an attenuation of the loss in LDH activity. However, there was still significant edema in these animals. These findings show iron's function in redox reactions, after contractile claudication, but suggest iron has no role in causing edema.

Since superoxide and hydrogen peroxide are poorly reactive in an aqueous environment, it is generally thought the more potent, more damaging, hydroxyl radical is responsible for the majority of oxidant-induced cellular damage after skeletal muscle ischemia [72]. It is even suggested that hydroxyl radical formation is critical to cellular injury [73]. With the presence of a suitable transition metal catalyst, such as iron, necessary for the Fenton reaction to proceed at a significant rate, iron is clearly very important to the redox balance.

Our findings suggest iron is paramount in causing oxidative damage after contractile claudication. This conclusion is based on the fact that DFO animals have a significant increase in xanthine oxidase activity, and significant neutrophil infiltration, therefore large amounts of oxidants are still being produced. However, despite this oxidant production, significant protection is afforded with chelation of iron. Therefore, the findings of this study are in agreement with others [7], showing that when iron is

chelated, and disruption to the Fenton reaction is presumed, significant attenuation of oxidative damage occurs.

Since deferoxamine is thought to enter cells, its protective effects could conceivably occur on either side of the plasma membrane. This issue has been addressed by several investigators with conflicting results. However, those studies conducted in skeletal muscle tissue after I-R appear to lean on the side of extracellular protection. Smith et al [51] administered deferoxamine and apotransferrin independently to address this issue in ischemia-reperfused skeletal muscle. Since apotransferrin cannot cross the cell membrane its protective effects can only be exhibited in the extracellular space. With both compounds exhibiting the same degree of protection, a suggested conclusion was that the iron-catalyzed Haber-Weiss reaction occurs in the extracellular space. In another study of ischemia-reperfused skeletal muscle, Fantini et al [3] administered DFO and DFO conjugated to pentastarch independently to animals. This conjugation alters the physical properties of DFO, so that it cannot cross the cell membrane, while retaining its capacity to chelate. Since the two compounds exerted similar protective effects in inhibiting lipid peroxidation, an extracellular site of action was strongly suggested.

Although we are unable to determine from our study which side of the cell membrane DFO is exerting its protective effects, it is certainly a strong possibility that, similar to those studies just discussed, the protection is in the extracellular compartment. This is based on our findings of neutrophils being the predominant source of oxidants after contractile claudication. This would lead to extracellular oxidant production, and potentially extracellular hydroxyl radical formation. Therefore chelation of extracellular

iron would be beneficial in this situation. However, this speculation warrants further investigation.

Since we were unable to measure free iron in tissue, the protective effects of DFO could conceivably be explained by alternatives to iron chelation. DFO has been reported to react with $O_2^{\bullet -}$, however the rate constant for this reaction at physiological pH is about $10^2 M^{-1} s^{-1}$ [33], which is approximately eight orders of magnitude less than the overall rate of non-enzymatic dismutation of $O_2^{\bullet -}$ [74], and therefore this protective possibility is unlikely. A more likely artifact is DFO's scavenging of $\bullet OH$, which proceeds with a rate constant of approximately $10^{10} M^{-1} s^{-1}$ [33]. However, using a dose of DFO identical to ours, plasma concentrations appear to stabilize at less than 20 μM . This concentration is presumably the same in other extracellular fluids, and is suggested to be too low for significant scavenging of $\bullet OH$ or $O_2^{\bullet -}$ [75]. In other studies using a similar dose of DFO, and controls with ferrioxamine (which reacts with $\bullet OH$ with the same rate constant as DFO), no protection was afforded, reinforcing the conclusion that the protective effects cannot be due to radical scavenging [76].

In any case, as mentioned before, a suitable metal catalyst is necessary for formation of $\bullet OH$, and iron is the best candidate for this role *in vivo*. Since DFO is accepted to be a very powerful iron chelator, it is likely $\bullet OH$ formation is greatly reduced, thereby limiting the very compound for which DFO has the potential to scavenge.

4-Hydroxy-2-Nonenal

4-hydroxy-2-nonenal (HNE) levels were measured as a further marker of oxidative stress, in the LS limbs of all treatment groups, and compared to CON SS levels. Although this aldehyde is formed from the decomposition of lipid hydroperoxides, it may actually

binds to, and modifies, proteins by interacting preferentially with lysine, histidine, serine, and cysteine residues.

Since HNE is relatively stable, and can easily diffuse within the cell or escape the cell, it has the potential to interact with many different cellular proteins [77]. This was reflected in our western blot by the appearance of several bands at varying molecular weights. In this study, we summed the net intensity of each band in each lane, in an attempt to quantify total protein-bound HNE levels. Only the CYCLO group showed significant attenuation of total protein-bound HNE levels after contractile claudication.

Several studies have demonstrated that increased levels of HNE are potentially very cytotoxic, inhibiting enzymes, protein synthesis, protein degradation, calcium sequestration, and exhibiting chemotaxic potential to neutrophils [78-80]. In addition, HNE can significantly alter the cellular redox balance by rapidly conjugating with the reactive thiol groups of glutathione (GSH). Indeed, GSH is part of the endogenous cellular pathway of HNE metabolism [81]. However, since glutathione is believed to be the primary buffer against reactive oxygen species in skeletal muscle [82], its loss due to HNE conjugation may make the cell more susceptible to oxidative damage. This provides an interesting concept since we have previously shown total GSH levels to be decreased after contractile claudication.

CHAPTER 5 CONCLUSIONS

This is the first study to investigate the pathways of oxidant production after contractile claudication. To establish a cause and effect relationship, we inhibited the major pathways individually. This also provided information on the relative contributions of each pathway. We predicted that both the xanthine oxidase pathway and activated neutrophils are responsible for the oxidative damage and edema seen after contractile claudication. The data supported these expectations, and provided insight into the predominant pathway.

Inhibition of xanthine oxidase activity attenuated lipid peroxidation, the loss of LDH activity seen in control animals, and reduced neutrophil infiltration. The conclusion can therefore be made that xanthine oxidase-derived oxidants cause oxidative damage after an acute bout of contractile claudication. In addition, oxidants from this source are chemotactic to neutrophils.

Neutropenia reduced neutrophil infiltration by ~82% compared to the ligated-stimulated limb of control animals and had a protective effect on all parameters - attenuating lipid peroxidation, protein oxidation, edema and the loss in LDH activity. This clearly shows neutrophils are the predominant source of oxidants, and therefore oxidative damage, after contractile claudication. Although increased neutrophil levels have been documented in the vasculature following exercise claudication in humans, this is the first study to show neutrophil recruitment into skeletal muscle after this condition. This is in agreement with prolonged I-R studies in skeletal muscle, which have firmly

established the recruitment of leukocytes into tissue. This causes oxidative damage as the membrane bound NADPH oxidase oxidizes NADPH to NADP^+ while reducing molecular oxygen to superoxide.

Finally, data from the DFO group suggest iron is heavily involved in the oxidative damage seen after contractile claudication. This implies the Fenton reaction causes a large part of the oxidative damage. Indeed, lipid hydroperoxide levels were reduced by ~43% and protein carbonyl content by ~25% in the LS limb of the DFO group compared to the LS limb of the CON group.

Since we were unable to actually measure iron levels in this study we cannot definitively conclude that the protection afforded by deferoxamine is solely due to its chelating characteristics. Indeed, deferoxamine has radical scavenging capabilities, which could interfere with the results of this study. However, it is only a weak scavenger of superoxide, and at the dose used in this study the concentration is likely too low for significant scavenging of the hydroxyl radical. Therefore, the probable protective role DFO plays in this study is as an iron chelator.

Future research should focus on the infiltration of neutrophils into tissue after exercise claudication, and the “signals” leading to this infiltration. In addition, it should be determined whether oxidative damage to skeletal muscle after exercise claudication is necessary for, or a hindrance to, the muscular adaptations that occur with this condition.

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BIOGRAPHICAL SKETCH

Andrew R. Judge was born in Northampton, England and raised in the village of Harpole, just outside Northampton. He graduated from Loughborough University, Leicestershire, England in 1996 with a bachelor's degree. In 1997 he moved to Lake Charles, Louisiana where he attended McNeese State University. Here he received a master's degree with a specialization in Exercise physiology. Andrew began his Doctor of Philosophy degree in exercise physiology in 1999, at the University of Florida.