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Analysis of Ischemic Muscle in Patients with Peripheral Artery Disease using X-Ray Spectroscopy

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Abstract

Background—Peripheral artery disease (PAD) is a vascular disease caused by atherosclerosis resulting in decreased blood flow to the lower extremities. The ankle brachial index (ABI) is a standard PAD diagnostic test, but only identifies reduced blood flow based on blood pressure differences. The early signs of PAD manifest themselves not only clinically, but also at an elemental and biochemical level. However, the biochemical and elemental alterations to PAD muscle are not well understood. The objective of this study was to compare fundamental changes in intracellular elemental compositions between control, claudicating, and critical limb ischemia muscle tissue.

Materials and methods—Gastrocnemius biopsies from three subjects including one control (ABI 0.9), one claudicating (0.4 ABI<0.9) and one critical limb ischemia patient (ABI<0.4) were evaluated using a scanning electron microscope and energy dispersive X-ray spectroscopy to quantify differences in elemental compositions. Spectra were collected for 5 myofibers per specimen. An analysis of variance was performed to identify significant differences in muscle elemental compositions.

Results—This study revealed that intracellular magnesium and calcium were lower in PAD compared to control myofibers while sulfur was higher. Magnesium and calcium are antagonistic,

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AUTHOR CONTRIBUTIONS

K.C, G.C. and I.P. conception and design of research; I.P., R.B., N.D. and K.C. performed experiments; R.B., N.D. and K.C. analyzed data; R.B. and K.C. interpreted results of experiments; R.B. and K.C. prepared figures; R.B. and K.C. drafted manuscript; R.B., G.C., I.P. and K.C. edited and revised manuscript; R.B., G.C., I.P. and K.C. approved final version of manuscript.

DISCLOSURES

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meaning, if magnesium concentrations go down calcium concentrations should go up. However, our findings do not support this antagonism in PAD. Our analysis found decreases in sodium and potassium, in PAD myofibers.

Conclusions—These findings may provide insight into the pathologic mechanisms that may operate in ischemic muscle and aid in the development of specialized preventive and rehabilitative treatment plans for PAD patients.

Keywords

Atherosclerosis; X-ray microanalysis; Scanning electron microscopy; muscle damage; vascular disease

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INTRODUCTION

Peripheral artery disease (PAD) is a chronic disease caused by atherosclerosis, affecting more than 8 million lives in United States (1). It can result in the narrowing or blockage of major arteries that supply the lower extremities, and if left untreated, the severity of the disease can lead to limb amputation (2). The most common symptom of PAD is intermittent claudication (IC), which is characterized by lower extremity pain that is exhibited during mild exercise such as walking, but resolves after rest (3). However, some patients with PAD fail to mention these symptoms to a physician which contributes to the underdiagnoses of PAD (4). If the disease continues to progress, tissue damage becomes more severe, and the patient experiences critical limb ischemia (CLI), a condition characterized by chronic pain at rest and tissue loss (2). Once the disease has reached this end stage, hypoxic tissue damage is significant, and the viability of the limb is poor, frequently requiring amputation in the absence of revascularization (5, 6).

The pathophysiology of PAD is complex and involves changes occurring on both physical and biochemical levels such as reduced blood flow, altered metabolic processes, skeletal muscle degeneration, and oxidative damage (7–11). The Ankle brachial index (ABI) is a simple and noninvasive test physicians use to diagnose PAD, and is performed by determining the ratio of systolic blood pressure in the ankle to that in the arm. However, this method has limitations in monitoring progression or regression of PAD; both abnormal blood flow and its effects on skeletal muscle need to be measured in order to determine the degree of muscle damage or repair. There is a fundamental lack of detail concerning muscle degeneration as well as recuperation in response to vascular reperfusion surgeries. Hence, there is a need to investigate the effects of PAD on skeletal muscle at a cellular and molecular level.

Previous work from our group studied morphological parameters of myofibers including area, roundness, perimeter, diameter, major and minor axes, solidity and fiber density of muscles classified as control, claudicating, and critical limb ischemia samples (8). We have also performed spectroscopic studies, aimed at developing techniques for identifying new spectral biomarkers and elucidating biochemical mechanisms critical to hypoxic cell damage (7).

Scanning electron microscopy and elemental microanalysis techniques have been extensively applied in the field of engineering and biological sciences for analyzing and quantifying all the elements, with the exception of H, He and Li (12). Energy dispersive X-ray spectroscopy (EDS) is a method used in analytical chemistry to detect and characterize the elemental composition of a given sample. By exciting a material using a high energy beam, characteristic X-rays are emitted from the probed atom, and is measured as a spectral profile. Scanning electron microscope (SEM) EDS uses the electron beam formed from the instrument to excite the sample and produce X-rays. This form of X-ray microanalysis has been used in past research as a sensitive method to determine elemental concentrations in skeletal muscle (13, 14). Studies have also investigated the role of ionic concentration changes during cell injury, tenotomy in rat muscles, and human skeletal muscle after knee surgeries, and have provided strong evidence that the elemental fluctuations are due to disturbed homeostasis of intracellular concentrations in cells (15–17). Thus, SEM-EDS was selected as a candidate to ascertain the effects of PAD on muscle function, by determining the change of elemental composition in myofibers.

The central hypothesis for this study was that key changes in the elemental composition can be correlated with the progression of PAD in affected patients. The objective of this study was to compare changes in intracellular elemental compositions such as calcium (Ca), sodium (Na), potassium (K), magnesium (Mg), and sulfur (S) in control, claudicating, and critical ischemic muscle. Ca, Na, K, Mg, and S were selected as the elements of interest in this study due to their biological importance in muscle function. Further, these electrolytes were shown to undergo significant changes in diseased cardiac muscle (18), driving the motivation to determine if similar changes occurred in PAD muscle.

MATERIAL AND METHODS

TISSUE COLLECTION

The tissue sample collection protocol was approved by the Institutional Review Board of the VA Nebraska-Western Iowa and the University of Nebraska Medical Center and all subjects gave informed consent. Muscle tissue specimens were collected from one patient with clinically diagnosed critical limb ischemia (CLI) ($ABI < 0.4$), one patient with clinically diagnosed claudication ($0.4 \leq ABI < 0.9$), and one control patient (no lower limb impairment or symptoms of PAD and a resting $ABI \geq 0.9$) (19). Demographic details are listed in Table 1. The control patient had 25 pack-years smoking, and quit 2 years prior to the study. The claudicating patient was at 40 pack-years smoking, and was down to 1 pack a month for the last 5 years. The CLI patient was at 34 pack-years smoking, and is currently down to half a pack per day. Muscle biopsies were collected with a 6 mm Bergstrom needle from the anteromedial aspect of the gastrocnemius, approximately 10 cm distal to the tibial tuberosity. Tissue samples were sectioned at 4 μm , fixed in methacarn, and embedded in paraffin in accordance with traditional tissue processing methods (20, 21). Prior to collecting scanning electron microscopy images, the specimens were deparaffinized with xylene and a series of ethanol washes and allowed to air dry. The muscle samples were myosin-labeled for morphometric analysis, the methods of which are outlined in a previous study by our group (8).

DATA COLLECTION – SEM EDS MICRO-ANALYSIS

Using a scanning electron microscope, equipped with an energy dispersive X-ray spectrometer, the existence and atomic percentage of ions in the muscle tissue samples were examined. The SEM system (Carl Zeiss Sigma VP Field Emission Scanning Electron Microscope), contained a scientific grade high-resolution in-lens detector, equipped with variable pressure and an energy dispersive x-ray spectrometer to characterize the elemental composition of the specimen. The number of X-ray counts produced from the sample were compared to the number of known X-ray counts for specific elements of interest, and from this, the atomic percent mass fraction of the element in the sample was derived. X-rays were generated from the electron beam focused on the desired area. Imaging and elemental analysis were carried out in a specimen chamber filled with nitrogen gas, at a variable pressure of 60 Pascal with an accelerating voltage of 17.5 kV, using INCA (Oxford Instruments) software for the EDS analysis (peak identification, elemental composition assessment and processing of the measured signal). Elemental maps were collected at the muscle fascicle level to visualize the elemental distributions. For intracellular ion assessment, *Point & ID* (a feature of the INCA software) was used to specify a rectangular region of interest covering the interior of the myofiber, and was used for identifying spectral peaks and determining atomic percent of the identified elements. Using this software X-ray micro-analysis was performed, followed by subsequent elemental atomic percent assessments.

DATA ANALYSIS

Elemental compositions were collected from five myofibers based on visual inspection of each tissue specimen from the control, claudicating, and critical limb ischemia muscle samples. This was performed by the operator, who divided the images into five sectors and selected one myofiber with moderately irregular shape and size from each region in the PAD muscle. In the case of the control sample, one myofiber with normal appearing shape and size was selected from each region. An assumption was made that each myofiber was independent of each other. Candidate myofibers were identified by morphological characteristics that are present in PAD myopathy (abnormal myofiber shapes, irregular clustering, and group atrophy). These characteristics were quantified in our previous studies, and correlated with the progression of the disease (8, 22). Figure 1 displays the morphological changes skeletal muscle undergoes with increasing PAD severity. Means and standard deviations of the atomic percent of the elemental components were calculated. A statistical analysis of variance (ANOVA) was performed using Design-Expert (Stat-Ease, Inc.), followed by a Bonferroni adjusted multiple comparison analysis to identify significant differences in atomic percent of the elemental compositions between myofibers analyzed from the control patient, the claudicating patient, and the critical limb ischemia patient. The significance level was set at $\alpha=0.05$. Interpretations of the differences in elemental composition was guided by a literature review of the role and function of each elemental component.

RESULTS

Figure 2 (A–D) exhibits high resolution scanning electron microscope images of the PAD muscle tissue at the fascicle level, myofiber level, and myofibril level. Figure 3A displays elemental maps of Carbon (C), Oxygen (O), Calcium (Ca), Potassium (K), Sodium (Na), Magnesium (Mg), and Sulfur (S) of PAD muscle at the fascicle level. Using the energy dispersive X-ray spectrometer, the atomic percentages for Ca, Na, K, Mg, and S were compared among the control, claudicating, and CLI muscle specimens. Figure 3B presents a spectral profile of the elemental distribution from a region of interest inside the myofiber. The statistical ANOVA revealed significant differences in elemental compositions for calcium ($p=0.003$), magnesium ($p=0.0001$), potassium ($p=0.0094$), sodium ($p=0.0001$) and sulfur ($p=0.004$), among control, claudicating and critical limb ischemic muscle samples (Figure 4). Scanning electron microscopy and EDS were able to characterize changes in the elemental composition of PAD muscle, which may correlate with a clinical diagnosis of PAD.

DISCUSSION

The objective of this study was to compare fundamental changes in intracellular elemental compositions in myofibers from control, claudicating, and critical limb ischemia patients. When compared to the control sample, EDS X-ray microanalysis results from both the claudicating and CLI muscle tissue indicated significant changes in the measured elements. Specifically, we observed a decrease in potassium, sodium, magnesium, and calcium, and an increase in sulfur. In ischemic muscles such as those found in PAD patients, cell injury and necrosis are indicated by significant chemical fluctuations in the cells as the disease progresses (13, 23–26). In ischemic muscle cells there is a significant decrease in oxidative phosphorylation, which leads to a decline in available adenosine triphosphate (ATP) and altered diffusion of ions across the membrane (16). The physical changes in normal, healthy muscle tissues and subsequent repair or damage is in large controlled by key elements such as potassium, sodium, magnesium, sulfur, and calcium, and significant changes in the intracellular concentrations of any of these elements could have profound and lasting effects on the cell in PAD muscle tissue (26–30). The presence and distribution of these elements also influence the electrical properties of the cell by creating electrochemical concentration gradients (31), and control its osmolarity.

EDS X-ray microanalysis of the PAD muscle samples revealed there was a significant decrease in potassium in the patients with claudication and critical limb ischemia compared to the control. Potassium plays a key role in cell growth, muscle contraction, electrolyte homeostasis, and muscle anabolism (32). Its importance is highlighted in the sodium potassium pump, or Na^+/K^+ -ATPase, which functions to maintain the cells resting membrane potential, thus affecting the membrane permeability to various ions. The potassium potential across the cell membrane causes faster depolarization, which speeds up the rate that the muscle fiber contracts (31). It also plays a significant role in regulating cell signaling pathways, as well as control of calcium concentrations inside the cell. Disruption of major homeostatic elements such as potassium can have generalized adverse effects, such

as muscle weakness due to the increased stimulus required to trigger action potentials in cells, a mechanism that is completely dependent on cell membrane potential (32).

In addition to the potassium deficiency, it was also observed that magnesium, calcium and sodium were decreased as well in both claudicating and CLI patients compared to the control. Magnesium deficiency can cause oxygen levels to decline in skeletal muscles, and has been shown to increase oxygen uptake and utilization to meet these demands in the body (33, 34). A decrease in magnesium impairs the Na^+/K^+ -ATPase as well as calcium ion channels and $\text{Na}^+/\text{Ca}^{2+}$ pumps in the cell membrane (35). Na^+/K^+ -ATPase is driven by ATP in the form of a magnesium-ATP complex; in ischemic conditions, ATP is quickly used up in the cell in an attempt to establish chemical equilibrium and reverse the cellular damage. Because of the low concentrations of ATP, magnesium can no longer bind to its complex and leaves the cell, causing further disruption of the membrane potential and impairment of the sodium potassium pump. The result is a marked decrease in potassium in the cell. Magnesium is also commonly known as a biological antagonist to calcium, exhibiting an inversely proportional relationship to the latter (36). This intracellular-extracellular activity is one of the key factors for the generation of nerve and muscle action potentials (31). Interestingly, while one would expect the Mg-Ca antagonistic relationship to cause an increase in Ca, the collected SEM-EDS data showed a decrease in both calcium and magnesium in the muscle samples from the claudicating and CLI patients. This is in contrast with published reports of a steady intracellular increase in Ca concentration for ischemic muscle cells (26, 37).

It is possible that this calcium reduction, or handling, could be caused by the actions of hydrogen sulfide (H_2S). Recently discovered and coined as the third known gasotransmitter, hydrogen sulfide has been the topic of many areas of research, including ischemic injury (38). Such research implicates that hydrogen sulfide can have strong myoprotective effects when administered during reperfusion preconditioning (39, 40). H_2S works both endogenously and exogenously; as an exogenous neuromodulator the gasotransmitter relaxes the smooth muscle cells of the arterial wall, increasing the lumen diameter, which increases blood flow to ischemic tissue. Endogenously, hydrogen sulfide exhibits anti-apoptotic and anti-inflammatory properties, as well as the ability to normalize high calcium concentrations the ischemic cell. It does this by inducing the expression of protein kinase C (PKC) in the cell. The protein accelerates reuptake of calcium into the sarcoplasmic reticulum of the cell as well as its extrusion out of the cell through sodium calcium channels (41). This transmitter is produced by 3 enzymes: cystathionine β -synthase (CBS), cystathionine γ -lyase (CGL), and 3-mercaptopyruvate sulfur transferase (3MST). These control how much and when hydrogen sulfide is expressed in cells (40). However little is understood about the mechanisms through which calcium is handled through hydrogen sulfides bio signaling pathway, such as how the expression of the key enzymes generating this gasotransmitter is affected by ischemic conditions.

The cause of the decrease in sodium is unclear, however these changes may implicate damage to key pathways governing sodium homeostasis. Aldosterone is a key regulating hormone for sodium concentrations in the cell, but little research has been done on how its regulation is affected by ischemic conditions (42). Many studies observed that during

simulated ischemia, ATP declines and anaerobic glycolysis increases the intracellular and extracellular pH to a state of acidosis, causing an increase in mitochondrial calcium. Sequentially, an increase in calcium will cause an increase in intracellular Na^+ through NHE exchanger (37). Likewise, the observed decrease in calcium could cause lowered sodium content in the cell.

The main limitation of this study was the small sample size that was analyzed. However, this pilot study was able to demonstrate the utility of SEM-EDS as a feasible means of observing changes in key elements in muscle fibers present at varying degrees of myopathy, and warrants a larger scale study to confirm these findings. Further investigation is needed to determine if these findings are decisive or conclusive. Furthermore, the control methodology utilized in this study could be further strengthened by the use of a sample from a non-ischemic muscle from each patient as an additional control. Including non-ischemic muscles from the same PAD patients can help to determine whether the biochemical changes we are describing are secondary to the effects of PAD on the affected leg or due to some other patient co-morbidity such as global atherosclerotic burden, aging, or active smoking. The muscle biochemical mechanisms that are altered under ischemic conditions are significant, and many are still unknown. Investigation of the mechanisms explored in this analysis may also provide a better understanding of the effects of disrupted elemental composition in ischemic cells.

CONCLUSIONS

In this study, we demonstrated that SEM-EDS may be a useful analytical tool for evaluating the changes of elemental compositions in PAD muscle. These changes may correlate with the degree of myopathy present in patients with the disease. To our knowledge, this is the first study of its kind, and may provide a broader picture of the pathologic mechanisms operating in the leg muscles of PAD patients which are chronically exposed to repeated episodes of ischemia and ischemia/reperfusion. If successful, further investigation of these mechanisms could lead to the discovery of biomarkers that may improve staging and possibly prognosis for a large spectrum of patients with PAD. This research also expands on the rapidly growing library of work characterizing the effects of ischemia on muscle cells through spectral imaging, increasing our knowledge and providing a more comprehensive picture of the pathophysiology of the disease.

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Figure 1.

Morphological changes that occur in PAD muscle as the disease progresses. Normal muscle morphometry consists of uniform myofiber size and shape. **A)** Representative myosin labeled images of control, claudicating, and CLI muscle with myofiber outlines are overlaid in green. **B)** Segmented binary image of the myofibers. In claudicating and CLI muscle, myofibers become more irregular, the endomysium and perimysium thicken, and atrophy is observed. In CLI muscle, there is an increase in muscle features that have been associated with stage of PAD. Specifically, key changes such as increased variation in size and shape and clustering of irregular smaller myofibers can be observed. **C)** Overlay image of the sarcolemma combined with the outlined myofibers.

Figure 2. Scanning electron microscope (SEM) images of CLI (A–B) and claudicating (C–D) PAD myofiber cross-sections. **A)** Presents a section at the fascicle level, **B–C)** presents muscle tissue at the myofiber level, and **D)** an image at the myofibril level.

Figure 3.

A) Elemental maps of Carbon (C), Oxygen (O), Calcium (Ca), Potassium (K), Sodium (Na), Magnesium (Mg), and Sulfur (S) of PAD muscle at the fascicle level. **B)** Energy dispersive X-ray spectroscopy (EDS) micro-analysis spectral profile from the intracellular elemental contents from a region of interest inside the myofiber. The region of interest that was measured is shown within the magenta box displayed in the scanning electron micrograph.

Figure 4.

An analysis of variance (ANOVA) identified significant differences in elemental composition between the muscle fibers analyzed from the critical limb ischemia patient, the claudicating patient, and the control patient. Decreases in **A)** Calcium ($p=0.003$), **B)** Magnesium ($p=0.0001$), **C)** Potassium ($p=0.0094$), and **D)** Sodium ($p=0.0001$), and an increase in **E)** Sulfur ($p=0.004$) were observed. (** represents a $P < 0.01$, *** represent a $P < 0.001$)

Table 1

Demographics of the control patient, the patient with claudication, and the patient with critical limb ischemia.

	Control	Claudication	CLI*
Age (years)	61	63	70
Ankle brachial index (ABI)	1.1	0.4	0.1
Sex	Male	Male	Male
Obesity	Yes	No	Yes
Hypertension	Yes	Yes	Yes
Diabetes mellitus	No	No	No
Smoking	Former	Current	Current

* Critical Limb Ischemia