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A Functional Murine Model of Hind Limb Demand Ischemia

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Abstract

Introduction—To date murine models of treadmill exercise have been used to study general exercise physiology and angiogenesis in ischemic hind limbs. The purpose of these experiments was to develop a murine model of demand ischemia in an ischemic limb to mimic claudication in humans. The primary goal was to determine whether treadmill exercise reflected a hemodynamic picture which might be consistent with the hyperemic response observed in humans.

Methods—Aged hypercholesterolemic ApoE null mice (ApoE^{-/-}, n=13) were subjected to Femoral Artery Ligation (FAL), and allowed to recover from the acute ischemic response. Peripheral perfusion of the hind limbs at rest was determined by serial evaluation using laser Doppler imaging (LDI) on days 0, 7, and 14 following FAL. During the duration of the experiments, the mice were also assessed on an established 5 point clinical ischemic score which assessed the degree of digital amputation, necrosis, and cyanosis as compared to the non ischemic contralateral limb. After stabilization of the LDI ratio (ischemic limb flux/contralateral non ischemic limb flux) and clinical ischemic score, mice underwent two days of treadmill training (10 min @ 10 m/min, incline of 10°) followed by 60 minutes daily treadmill exercise (13 m/min, incline of 10°) through day 25. An evaluation of pre-exercise and post exercise perfusion using LDI was performed on two separate occasions following the onset of daily exercise. During the immediate 15 minute post exercise evaluation, LDI scanning was obtained in quadruplicate, to allow identification of peak flux ratios. Statistical analysis included unpaired t-tests and ANOVA.

Results—After FAL, the LDI Flux ratio reached a nadir between days one and two, then stabilized by day 14 and remained stable through day 25. The clinical ischemic score stabilized at day 7, and remained stable throughout the rest of the experiment. Based on stabilization of both the clinical ischemic score and LDI ratio, exercise training began on day 15. The peak 15 minute post exercise LDI ratio increased significantly as compared to pre-exercise ratio on day 17 (0.48 ± 0.04 vs. 0.34 ± 0.04 , $p < 0.05$) and day 25 (0.37 ± 0.03 vs. 0.27 ± 0.03 , $p < 0.01$). Within 2 hours of exercise, the LDI ratio returned to pre-exercise levels on both day 17 and 25.

Conclusions—Clinical and hemodynamic stabilization of limb perfusion is evident by 14 days after FAL. FAL followed by demand ischemia results in a reversible relative hyperemic response similar to those observed in exercising human claudicants. A murine model of FAL associated

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with demand ischemia may be a useful model to evaluate the metabolic, inflammatory and flow related changes associated with claudication in humans.

Introduction

Studies of lower extremity claudication in humans have revealed alterations in blood flow and energy metabolism associated with exercise. These alterations are believed to be related to fundamental changes in the mitochondrial activity, chronic inflammation and intermittent local ischemia reperfusion (IR) injury¹⁻⁴. Furthermore, there is evidence of significant coincident coronary artery disease⁵⁻⁷ and cerebrovascular morbidity^{8, 9} in patients with claudication. In recognition of this association between claudication and cardiovascular mortality the National Institute of Health has funded the CLEVER (Claudication: Exercise Vs. Endoluminal Revascularization) study, which is a prospective, multicenter, randomized, controlled clinical trial evaluating the relative efficacy, safety, and health economic impact of four treatment strategies for people with aortoiliac peripheral arterial disease and claudication¹⁰. While there are many laboratory models developed to evaluate the effect of ischemia on muscle reperfusion injury, and angiogenesis, there is little literature to evaluate the effect of hind limb demand ischemia, i.e. claudication on murine skeletal muscle. The purpose of these experiments was to develop a murine model of hind limb demand ischemia which mimics parameters of claudication in humans.

Methods

Animal Protocol

Experiments with ApoE null (ApoE^{-/-}) mice were approved by the Massachusetts General Hospital's subcommittee on research animal care in accordance with, the "Principles of Laboratory Animal Care" (Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication No. 86-23, Revised 1996). The ApoE^{-/-} mice were subjected to femoral artery ligation on the left and sham ischemia (femoral cut down but no arterial ligation) on the right. The mice were fed normal chow during the course of these experiments.

Hind limb Femoral Artery Ligation

8–10 month-old female ApoE^{-/-} mice (n=13, Jackson Laboratory, Bar Harbor, Maine) were fed a normal diet. Mice were anesthetized by IP administration of 40–50mg/kg Pentobarbital, and placed on a heating pad to maintain their core temperature at 37±0.5°C by monitoring rectal temperature. A 0.5–1 cm skin incision was made longitudinally on the anterior thigh of the left hind limb. The femoral artery was exposed under a surgical microscope and dissected from the femoral vein and nerve. The femoral artery and visible branches were ligated proximal to the superficial epigastric artery and above the bifurcation of the saphena and popliteal arteries with 8.0 silk ligatures (FAL). The arterial segment between the ligatures was excised¹¹. Incision was closed with a 5.0 polypropylene suture. In the right leg, there was a surgical incision and dissection but no ligation. The incision was closed with 5.0 polypropylene sutures. Mice received 0.05–0.1mg/kg Buprenorphine HCL sc for pain relief. Hind limb blood flow was assessed immediately before and after ligation using Laser Doppler Imaging.

Laser Doppler Imaging

A laser Doppler imager (LDI, Moor Instruments, Wilmington, DE) was used to assess limb perfusion^{12, 13} by acquiring flow images of the ischemic and the contralateral foot at 37–38.0°C core temperature under Isoflurane anesthesia. Baseline LDI images were obtained before and immediately after the femoral cut downs were completed in both limbs as

described above. The LDI source was mounted on a movable track that was fixed exactly 10 cm above the mice limbs when the animals were restrained on a warming table. The laser beam (780 nm), reflected from moving red blood cells in nutritional arterioles, capillaries and venules, was detected and processed to provide a computerized, color-coded image. Image analysis software (Laser Doppler Perfusion Measure, V3.08, Moor Instruments) was used to calculate the limb mean flux units, which represents a quantitative analysis of tissue perfusion on a scale of 0 to 1000. Limb perfusion was expressed as the ratio of the Flux value of the ischemic limb relative to the value of the contralateral limb (Flux Ratio).

Exercise Protocol

Aged hypercholesterolemic ApoE null mice (n=10) were subjected to Femoral Artery Ligation (FAL), and allowed to recover for 15 days. Peripheral perfusion of the hind limbs at rest was determined by serial evaluation using laser Doppler imaging (LDI) on days 0, 7, and 14 following FAL. After stabilization of the LDI ratio (ischemic limb flux/contralateral non ischemic limb flux) at day 14, mice underwent two days of treadmill training (10 min at 10 m/min, incline of 10°) followed by 60 minutes daily treadmill exercise (13 m/min, incline of 10°) through day 26. On days 17 and 25 after FAL mice underwent pre-exercise, 15 minute post exercise and 2 hours post exercise evaluation of the ischemic and contralateral limb perfusion using LDI. During the immediate 15 minute post exercise evaluation, LDI scanning was obtained 4 times during this interval allowing measurement of the initial and peak flux ratios.

Clinical Ischemic Score

A five point scoring system described by Stabile et al.¹⁴ were utilized to characterize ischemic tissue changes in the limb after FAL. Any evidence of post FAL digital amputation was rated as 5. Frank tissue necrosis was scored as a 4. Mice with a clinical ischemic score of 4 or 5 within the course of the experiment were eliminated from subsequent Doppler Imaging and exercise analysis. Severe cyanotic discoloration was scored as a 3 and mild discoloration (pale appearance) was scored as 2. A completely normal appearance of the limb as compared to the non FAL limb was rated 1. Mice were examined at day 1 immediately prior to ligation, 6 hours after ligation, then 2, 7, 14, 21 and 25 days after ligation.

Statistical Analysis

All data was expressed as mean $\pm$ SEM and all analyses were performed using InStat 3.0 software (GraphPad Software, San Diego, CA). Comparisons along the time course of the LDI monitoring, the Clinical Score, and exercises was done with an ANOVA, and Tukey-Kramer post tests.

Results

Temporal Profile of Limb Perfusion after FAL

There was a significant difference over time in the LDI ratio after FAL ($p < 0.0001$, ANOVA). There was a sharp immediate decrease in LDI ratio after FAL between days 1 and 2 (1.0087 ± 0.039 vs. 0.1862 ± 0.0128 , $p < 0.001$), days 1 and 7 (1.0087 ± 0.0128 vs. 0.1651 ± 0.0103 , $p < 0.001$) and 1 and 14 days (1.0087 ± 0.0128 vs. 0.3429 ± 0.0381 , $p < 0.001$). There was a stabilization of the LDI ratio between days 14 and 21 (0.3248 ± 0.0381 vs. 0.3248 ± 0.0262 , $p > 0.05$) and days 21 and 25 (0.3248 ± 0.0262 vs. 0.2646 ± 0.0328 , $p > 0.05$).

Ischemic Score following FAL

There was a significant change in the clinical ischemic score over the 25 days after FAL ($p<0.0001$, ANOVA). By 6 (2.2 ± 0.133 , $p<0.001$) hours and 2 days (2.0 ± 0 , $p<0.001$) after FAL, there was a significant increase in the clinical ischemia scores as compared to day one prior to FAL (Figure 2). By days 7 through 25 following FAL, there was no difference in clinical ischemic scores as compared to day one prior to FAL. Three mice developed frank necrosis by day 3, and these mice were excluded from data analysis. No mice developed digital amputation. In the non-ischemic contralateral limb, there was no clinical evidence of ischemia throughout the entire experiment. On days 7 through 25, there was no difference in the clinical ischemic score between the ischemic limb and the contralateral limb.

Reactive Hyperemia Following Treadmill Exercise

The LDI flux ratio changed significantly over time when comparing baseline, peak exercise on day 17 ($p=0.008$, ANOVA) and day 25 ($p=0.002$, ANOVA). The peak 15 minute post exercise LDI flux ratio increased significantly as compared to pre-exercise ratio on day 17 (0.48 ± 0.04 vs. 0.34 ± 0.04 , $*p<0.05$) and day 25 (0.37 ± 0.03 vs. 0.27 ± 0.03 , $+p<0.01$), indicative of reactive hyperemia. Within 2 hours of exercise, the LDI ratio returned to pre-exercise levels on both day 17 (0.48 ± 0.04 vs. 0.33 ± 0.03 , $*p<0.05$) and day 25 (0.37 ± 0.03 vs. 0.21 ± 0.02 , $+p<0.01$).

Discussion

The experiments described in this manuscript indicate that murine femoral artery ligation associated with repeated treadmill exercise provides a clinical scenario and hemodynamic profile consistent with human claudication. In human claudicants, there is rarely evidence of significant limb threatening ischemia at rest, but after exercise the clinical symptom of pain is usually noted, and there is hemodynamic evidence of demand ischemia. There is no clinical evidence of limb threatening ischemia in these mice despite femoral artery ligation by day 7 (figure 2), which is consistent with claudication in humans^{1, 15}. There are numerous laboratory studies that have documented extensive defects in skeletal muscle metabolism and morphology in animal models, but these studies have employed chronic ischemia in sedentary mice, or electrical stimulation of isolated muscle until tetanic fatigue is reached^{16–19}. The physiological studies of human tissue are of course limited, but are consistent with an ischemic skeletal muscle myopathy. There are many publications using acute femoral artery ligation and treadmill exercise to study angiogenesis in rodent models of hind limb ischemia, however most of these experiments started treadmill exercise shortly after femoral artery ligation^{20–23}. Laboratory studies indicate that there is a robust inflammatory response associated with acute FAL^{24, 25} thus these experiments were designed to start exercise after stabilization of both the clinical and objective measures of limb ischemia. These experiments are necessary since there is increasing amounts of data to suggest that skeletal muscle is in fact a large endocrine organ^{26, 27}. If muscle is in fact a major endocrine model, it could be an important source of systemic inflammatory mediators during demand ischemia. Thus experiments were undertaken to study the clinical and hemodynamic profile of demand ischemia after femoral artery ligation in a hypercholesterolemic murine model. The hypercholesterolemic ApoE $-/-$ mouse was used because hypercholesterolemia is a risk factor for atherosclerosis in humans^{28, 29}.

To accomplish this goal, we first subjected the mice to femoral artery ligation and allowed the surgical stress associated with the ligation to stabilize. The nadir of hind limb hypoperfusion (Figure 1) occurred during days 2 through 7 following FAL, then stabilized at day 15 when the treadmill exercise experiments were started. The changes in the clinical scoring of tissue ischemia stabilized by day 7 following FAL (Figure 2), and remained stable

during the entire 26 days of the experiments. The stabilization of the flow to a mouse hind-limbs subjected to FAL has been described by other investigators^{11, 12, 25}. The gradual post ischemic stabilization of blood flow is thought to be related to increased collateral and compensatory flow due to angiogenesis and arteriogenesis, which are known to be abnormal in the setting of hypercholesterolemia^{12, 30}. Because of these factors, the treadmill experiments began during an interval where objective and subjective measurement of the FAL limb function and perfusion was stable.

In the clinical scenario of patient care, documentation of significant resting ischemia can be made by a combination of physical exam, imaging studies and non invasive testing. In claudicants, there is usually no evidence of significant resting ischemia thus the clinical exam may be unreliable in documenting the presence or absence of ischemia which could be contributing to pain with ambulation. Most non invasive tests document a decreased perfusion associated with treadmill exercise³¹. Laser Doppler imaging in humans^{32, 33} and animals^{30, 34} subjected to demand ischemia demonstrate a hyperemic response associated with exercise. In humans with critical limb ischemia, the hyperemic response to exercise is absent³⁵. Thus experiments were performed to determine whether the FAL mice developed a hyperemic response to demand ischemia caused by treadmill exercise. As Figure 3 clearly shows, on both day 17 and 25, the mice exhibited a vigorous hyperemic response to treadmill exercise. This is entirely consistent with demand ischemia. This response is equivalent to a decreased ankle brachial index or decreased PVR and is caused by extensive vasodilation. Based on this observation, ongoing biochemical and molecular analysis of moderately ischemic murine hind limb muscle subjected to sedentary and demand ischemia is needed to understand to what extent claudication might contribute to a systemic inflammatory response in humans.

Conclusion

In the setting of the known cardiovascular morbidity associated with claudication³⁶ and limb ischemia^{37, 38}, a hemodynamically relevant model is needed to test pathologic muscle dysfunction, regeneration, cytokine production, experimental therapeutics and the capacity for muscle subjected to demand ischemia to affect other organ systems. This model is a step in the direction needed to perform laboratory studies to address some of these issues.

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Figure 1. LDI Flux Ratio following Femoral Artery Ligation

On day one, the flux ratios were identical. By day 2, the LDI flux ratio fell significantly between days 2 through day 7. By day seven, the LDI increased significantly and remained stable throughout the experiment.

Figure 2. Clinical Ischemia Score

There was evidence of significant ischemia in the FAL limb at 6 hours and day2 after ligation. By day 7, the clinical scores stabilized in the ischemic limb throughout the rest of the experimental intervals. In contrast, the clinical ischemic score in the non ischemic contralateral limb was stable throughout the entire experiment.

Figure 3. Hyperemia Induced by Demand Ischemia

On days 17 and 25 the mice subjected to LDI prior to and after exercise showed evidence of a significant hyperemic response which is characteristic of demand ischemia.