Apolipoprotein E−/− mice have delayed skeletal muscle healing after hind limb ischemia–reperfusion

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Introduction: Classic studies of limb ischemia–reperfusion injury have been performed using young healthy mice. However, patients with peripheral vascular disease are older and often exhibit metabolic derangements that may delay healing after revascularization. Mice with genetic deletion of apolipoprotein E (ApoE−/−) have been used as a model in various experimental scenarios of hypercholesterolemia. These experiments evaluated the inflammatory response and changes in skeletal muscle morphology during the acute and chronic phases of limb ischemia–reperfusion injury in aged ApoE−/− mice.

Methods: Age-matched ApoE−/− and wild-type (Wt) mice underwent 1.5 hours of unilateral hind limb ischemia, followed by 1, 7, or 14 days of reperfusion (DR). Histologic analysis of skeletal muscle fiber injury was assessed at 1DR. Morphologic evidence of muscular fiber maturation was assessed at 14DR. Levels of MyoD and myogenin, markers of skeletal muscle differentiation, were assessed at 7 and 14DR using Western blots. Markers of inflammation, including myeloperoxidase, macrophage inflammatory protein-2 (MIP-2), monocyte chemotactic protein-1 (MCP-1), and osteopontin, were assayed using enzyme-linked immunosorbent assay and chemokine (C-C motif) receptor 2 (CCR2) using Western blots at 1, 7, and 14DR. After 1DR, tissue adenosine 5′-triphosphate (ATP) levels were measured to assess metabolic activity. Unpaired t test and Mann-Whitney test were used for comparisons.

Results: Histologic evaluation of skeletal muscle after 1DR showed no difference in the degree of injury between Wt and ApoE−/− mice. However, at 14DR, ApoE−/− mice had higher percentage of immature muscle fibers than Wt mice. Myogenin level was lower in the ApoE−/− mice at 7DR. Injured skeletal muscle of ApoE−/− mice had lower levels of myeloperoxidase than Wt mice at 7 DR and higher levels of MCP-1 at 14DR. There was no difference in the levels of tissue ATP, MIP-2, osteopontin, or CCR2 at all experimental intervals.

Conclusion: Although there was no difference between the injured muscle of Wt and ApoE−/− mice during the acute phase of reperfusion, ApoE−/− mice showed delay in skeletal muscle healing during the chronic phase of reperfusion. This lag in muscle regeneration was associated with lower levels of myogenin at 7DR and an increased level of MCP-1 at 14DR in the ApoE−/− mice. The delay in skeletal muscle healing in the ApoE−/− mice may have broader implications for poor tissue healing and functional recovery in elderly patients who have vascular risk factors such as hypercholesterolemia. (J Vasc Surg 2008;48:701-8.)

Clinical Relevance: These studies demonstrate that hypercholesterolemia, a major risk factor for peripheral arterial disease, does not alter the acute consequences of skeletal muscle ischemia–reperfusion injury. Hypercholesterolemia did significantly compromise skeletal muscle healing during the chronic phase of reperfusion in these mice, which is temporally coincident with wound complications and poor functional recovery in humans.

Peripheral arterial disease (PAD) is estimated to affect anywhere from 3% to 30% of the adult population in the United States.1–5 Major risk factors for PAD include age, hypertension, hypercholesterolemia, diabetes mellitus, chronic renal insufficiency, and smoking. Often, an acute thrombotic or embolic event may prompt hypercholesterolemic patients to seek help in managing their lower extremity vascular disease.
terol levels can be >500 mg/dL. In contrast, wild-type (Wt) control strains, C57BL/6, have cholesterol levels of 60 to 90 mg/dL. As such, ApoE–/– mice have been used extensively to study the effects of hypercholesterolemia in various disease processes such as atherosclerosis and stroke.

The following studies were designed to study the effects of hypercholesterolemia after acute hind limb ischemia–reperfusion injury in aged ApoE–/– mice and age-matched C57BL/6 mice as controls. The local metabolic and inflammatory markers, indices of skeletal muscle differentiation, and muscle morphology were compared during the acute and chronic phases of reperfusion.

METHODS

Animal protocol and limb ischemia reperfusion. These experiments used 8- to 10-month-old, female ApoE–/– and their control strain, C57BL/6 mice (Jackson Laboratory, Bar Harbor, Me). Animal care and experimental procedures were performed 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) and approved by the Massachusetts General Hospital’s Subcommittee on Research Animal Care.

Anesthesia was induced by intraperitoneal injection of sodium pentobarbital (50 mg/kg), and maintained by administering a subsequent dose of 10 mg/kg as needed. After induction of anesthesia, unilateral hind limb ischemia was achieved by applying 4.5-oz orthodontic rubber bands (American Orthodontics, Sheboygan, Wis) on the hind limbs using the McGivney Hemorrhoidal Ligator Applicator (George Percy McGown, Brooklyn, NY) as previously described. Orthodontic rubber bands were then removed after 90 minutes of ischemia. Mice were placed on a heating pad during the ischemic and immediate recovery period to maintain normal body temperature of 37°C.

Once the mice were awakened from anesthesia, they were returned to their cages and allowed free access to water and food. After reperfusion periods of 1, 7, or 14 days, mice were euthanized with intraperitoneal injection of sodium pentobarbital (200 mg/kg). Blood and skeletal muscle tissue were harvested from each mouse. For histologic studies, posterior compartments of calf skeletal muscles from each mouse were dissected out, snap frozen in liquid nitrogen, and stored at −80°C until extraction.

Serum cholesterol levels. Serum cholesterol levels of Wt and ApoE–/– sham mice were measured to verify their cholesterol profile. Whole blood was collected from Wt and ApoE–/– sham mice. Blood samples were allowed to clot at room temperature for approximately 60 minutes and then centrifuged at 3000g for 20 minutes. The resulting supernatant was stored at −80°C until extraction. Serum cholesterol levels for Wt and ApoE–/– mice were measured using Infinity Cholesterol Kit (Thermo Electron Corporation, Louisville, Colo) according to the manufacturer’s protocol.

Histology. Limbs from mice that underwent hind limb ischemia and reperfusion were fixed in 4% paraformaldehyde for 8 hours. The gastrocnemius muscle from each limb was dissected out, rinsed in Dulbecco Phosphate Buffered Saline (PBS) for 1 hour, and serially dehydrated in graded acetone. Each sample was embedded using JB-4 Embedding Kit (Polysciences Inc, Warrington, Pa) under vacuum conditions, cut in cross-section at 2-μm thickness using a motorized microtome (Leica Microsystems Inc, Bannockburn, Ill), and stained with Masson trichrome for 1 day reperfusion samples and hematoxylin and cosin for 14 day reperfusion samples.

Stained slides were examined under light microscopy at ×200 magnification (Nikon E600 Upright Microscope, Tokyo, Japan). Images were acquired from the entire muscle section, and each image was assigned a serial number using SPOT Insight Digital Camera (Diagnostic Instruments, Sterling Heights, MI). The blinded observer then examined the images of each muscle in random order using a random number generator (www.randomizer.org) until a minimum of 1200 muscle fibers per section had been scored.

For limbs that underwent 1 day of reperfusion (DR), muscle fibers were scored as uninjured or injured based on the morphology of the individual fibers. Uninjured fibers were characterized as having well-defined borders, uniform texture and colors, and easily identifiable satellite cells and pericellular nuclei. Injured fibers, on the other hand, had interrupted or ragged borders, inconsistent texture and color, breaks in the cytoplasm, and nuclei detachment. Histologic assessment of muscle fibers at 14DR was based on the presence of mature skeletal muscle fibers (small peripheral nuclei) vs immature proliferative skeletal muscle myoblasts (large lobulated central nuclei). For 1DR samples, scores were summarized as percentage of injured fibers, whereas for 14DR samples, scores were summarized as percentage of immature fibers.

Tissue MyoD, myogenin, and chemokine (C-C motif) receptor 2 (CCR2) levels. Total protein (40 μg) was solubilized with an equal volume of Laemmli sample buffer (0.25M Tris-HCl, pH 6.8; 8% sodium dodecyl sulfate, 40% glycerol, 0.4M dithiothreitol, and 0.04% Bromophenol Blue; BioRad, Hercules, Calif), boiled for 5 minutes, and loaded onto a 12% density Tris-HCl polyacrylamide-sodium dodecyl sulfate gel. Samples underwent electrophoresis, followed by electroblotting transfer to a nitrocellulose membrane (BioRad). The membranes were blocked in PBS containing 0.05% Tween-20 (PBS-T) supplemented with 5% nonfat dried milk for 1 hour at room temperature. The blots were then incubated with rabbit polyclonal anti-MyoD antibody, rabbit polyclonal antimyogenin antibody, or goat polyclonal anti-CCR2 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) in blocking buffer at optimum dilution of 1:1000 for 1 hour.

After washes in PBS-T, the membranes were probed with horseradish peroxide-conjugated goat antirabbit immunoglobulin G (IgG) at 1:4000 dilutions in blocking buffer for 1 hour at room temperature. Membranes were
RESULTS

Serum cholesterol Levels. Serum cholesterol levels were measured in Wt and ApoE−/− mice. ApoE−/− mice had significantly higher levels of serum cholesterol compared with Wt mice (104 ± 3 vs 491 ± 12 mg/dL; \( P < .0001 \)).

Histology. Histologic assessment of Wt and ApoE−/− gastrocnemius muscles was done at 1DR and 14DR to study the effects of transient ischemia during the acute and chronic phases of reperfusion. At 1DR, both Wt and ApoE−/− mice demonstrated a significant amount of muscle fiber injury, interstitial edema, and infiltrating leukocytes in the skeletal muscle. Representative images from both Wt and ApoE−/− gastrocnemius muscles are shown in Fig 1, a and b, respectively. Quantitative assessment of skeletal muscle revealed no difference in the level of injury between Wt and ApoE−/− mice at 1 day after reperfusion (Wt 48% ± 6% injured fibers vs ApoE−/− 42% ± 5% injured fibers; \( P = .444; \) Fig 2, a).

After 2 weeks of reperfusion, both Wt and ApoE−/− mice had evidence of skeletal muscle regeneration as indicated by the presence of immature fibers with centrally located nuclei. Representative images from Wt and ApoE−/− gastrocnemius muscles are shown in Fig 1, c and d, respectively. Unlike the acute phase of reperfusion, at 14DR, there was a significant difference in skeletal muscle morphology between Wt and ApoE−/− mice (Fig 2, b). Wt mice had 47% ± 5% immature fibers, whereas ApoE−/− mice had 68% ± 5% immature fibers (\( P = .007 \)).

Tissue MyoD and myogenin levels. Quantitative values of band densities for MyoD and myogenin are shown in Fig 3, a and b, respectively. Although no difference was noted in the level of MyoD between Wt and ApoE−/− at either time points, the level of myogenin in ApoE−/− mice was significantly lower at 7DR (Wt 13.9 ± 1.7 AU vs ApoE−/− 7.7 ± 1.2 AU; \( P = .014 \)). By 14DR, the difference in myogenin levels between Wt and ApoE−/− mice was not significant (Wt 6.8 ± 1.3 AU vs ApoE−/− 4.9 ± 0.9 AU; \( P = .299 \)).

Tissue CCR2 levels. Quantitative values of band densities for CCR2 were evaluated at 1DR, 7DR, and 14DR in Wt vs ApoE−/− mice. There was a significant increase in the level of CCR2 at 1DR compared with sham mice in both groups. However, there was no difference in the levels between the Wt and APO 2−/− groups, respectively, at any time points: 1DR, 450.4 ± 30.2 vs 436.5 ± 25.9 AU (\( P = .733 \)); 7DR, 74.6 ± 16.7 vs 79.7 ± 11.4 AU (\( P = .811 \)); and 14DR, 79.1 ± 52.68 vs 46.6 ± 20.5 AU (\( P < .0001 \)).

Inflamatory protein-2 (MIP-2),, monocyte chemotactic protein-1 (MCP-1),, osteopontin levels. Frozen muscle (200 mg) samples were homogenized with a polyclon homogenizer in a test tube containing 1 mL of Radioimmunoassay Precipitation Assay (RIPA) Buffer and 10 μL of protease inhibitor cocktail (Sigma P-8340, St Louis, Mo). The homogenized samples were then centrifuged at 16,000 \( \times \) g for 10 minutes, and the supernatants were frozen in aliquots at −80°C until analysis.

The hind limb skeletal muscle levels of MPO, MIP-2, MCP-1, and osteopontin were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s protocols (MPO, Cell Sciences, Canton, MA; MIP-2, MCP-1, osteopontin: R&D Systems, Minneapolis, Minn). The ELISA plates were read with a Spectraxmax-250 plate reader (Molecular Devices, Sunnyvale, Calif). Total protein concentration in each skeletal muscle extract was determined using the BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, Ill) with bovine serum albumin as the standard. MPO, MIP-2, MCP-1, and osteopontin concentrations were extrapolated off the standard curve and normalized to the total protein concentration.

Tissue adenosine 5′-triphosphate (ATP) levels. Samples of frozen muscle (200 mg) were homogenized with a polytron homogenizer in a test tube containing 10% trichloroacetic acid. Samples were centrifuged for 10 minutes, and the supernatants were frozen at –80°C until analysis.

The ATP levels were measured using ATPlite Luminescence Assay according to the manufacturer’s protocol (PerkinElmer Life, Boston, Mass). Top counts were read using a 1450 MicroBeta plate reader (PerkinElmer Life) Concentrations of the unknowns were extrapolated off the standard curve and expressed as nanomole per mg tissue weight. The ATP levels of the reperfused limbs were then divided by ATP levels of the respective contralateral limbs and expressed as percentage of contralateral limb ATP level.

Statistical analysis. All data were expressed as mean ± standard error of the mean and all analyses were performed using InStat 3 (GraphPad Software, San Diego, Calif). Comparisons between the ApoE−/− and Wt groups were made using the unpaired \( t \) test or Mann-Whitney test.
8.1 ± 0.7 ng/mg vs ApoE−/−, 5.3 ± 0.9 ng/mg; \( P = 0.043 \), but by 14DR, there was no difference in MPO levels between the two groups (Wt, 6.0 ± 1.8 ng/mg vs ApoE−/−, 8.3 ± 3.2 ng/mg; \( P = 0.879 \)).

Tissue MIP-2 levels were assessed as a measure of neutrophil chemotactic activity in the hind limbs of Wt and ApoE−/− mice after ischemia reperfusion injury. MIP-2 levels in both Wt and ApoE−/− mice were elevated at 1 day...
after reperfusion (Fig 4, b). Similar to MPO, there was no difference in MIP-2 levels between Wt and ApoE –/– mice at 1DR (5.8 ± 1.0 vs 5.8 ± 1.0 pg/mg; P = .982). By day 7 and 14 of reperfusion, MIP-2 levels were significantly decreased in both groups, and there was no difference between the Wt and ApoE–/– mice at either time points, respectively: 7DR, 1.5 ± 0.5 vs 1.2 ± 0.5 pg/mg (P = .397); and 14DR, 0.6 ± 0.3 vs 1.0 ± 0.1 pg/mg (P = .127).

Tissue MCP-1 levels were measured as an index of monocyte/macrophage chemotactic activity in the injured skeletal muscles. There was no difference in MCP-1 levels between Wt and ApoE–/– mice at 1 and 7DR (Fig 4, c). However, at 14DR, MCP-1 was significantly higher in the ApoE–/– mice (15.7 ± 1.7 vs 38.5 ± 3.9 pg/mg; P < .0001).

Because osteopontin is an important glycoprophospho-protein known to modulate immune response and muscle remodeling, tissue osteopontin levels were assessed at 1DR, 7DR, and 14DR. A transient increase in osteopontin levels occurred during early reperfusion, but there was no overall difference between the two strains at any time point during reperfusion (Fig 4, d).

**Tissue ATP levels.** ATP levels were measured at 1DR to assess the level of metabolic activity in the skeletal muscles of Wt and ApoE–/– mice after hind limb ischemia–reperfusion injury. There was no difference in skeletal muscle ATP levels between Wt and ApoE–/– mice (34% ± 8% vs 17% ± 5%; P = .099).

**DISCUSSION**

Our study shows that although there was no difference in the level of injury between Wt and ApoE–/– mice during the acute phase of reperfusion (Fig 2, a), there was delayed healing in the injured limbs of ApoE–/– mice during the chronic phase of reperfusion (Fig 2, b). This lag in skeletal muscle regeneration was reflected in the lower levels of myogenin in ApoE–/– mice at 7DR (Fig 3, b). Myogenin is a marker of terminal differentiation in skeletal muscle.19 The relative decrease in myogenin expression at 7DR preceded the histologic appearance of immature skeletal muscle fibers in ApoE–/– mice at 14DR (Fig 1, d).

In addition to the differences observed in myogenin expression and skeletal muscle morphology, reperfused limbs of ApoE–/– mice showed significantly higher level of MCP-1 compared with Wt at 14DR (Fig 4, c), but showed no difference in CCR2 levels at any time points. MCP-1, a member of the CC chemokine family, is a potent recruiter of circulating monocytes and certain subsets of T cells. Its activity is mediated by its receptor, CCR2, a seven-transmembrane-spanning G protein-coupled receptor.20 MCP-1 is thought to play a key role in recruiting monocytes into early atherosclerotic lesions, in development of intimal hyperplasia after angioplasty, and in arteriogenesis.21 In addition, MCP-1 has been shown to be important in skeletal muscle regeneration after injury.22-25

The higher levels of MCP-1 at 14DR after ischemic injury may indicate a prolonged local inflammatory process is associated with the delay in skeletal muscle regeneration in the ApoE–/– mice. Because this higher level of MCP-1 is not associated with difference in the level of CCR2 expression, it is also possible that the relatively higher levels of MCP-1 observed in the ApoE–/– mice may be related to decreased function of CCR2 in the ApoE–/– mice.

Evidence to support this possibility comes from the work of Warren et al.23 who showed that mice treated with MCP-1 antibodies, as well as mice deficient in CCR2 (CCR2–/–), had delayed skeletal muscle recovery after freeze-induced injury. Furthermore, in the Warren study, the delay in skeletal muscle recovery was accompanied by a
prolonged inflammatory response in the injured skeletal muscle of CCR2−/− mice at 14 days after injury, as evidenced by extensive immunostaining with Mac-1 and Mac-3, markers of monocytes/macrophages.24 Interestingly, they did not observe any difference in MyoD or myogenin expression between CCR2−/− and Wt mice. Their failure to observe differences in MyoD or myogenin expression may be related to the nature of freeze-induced injury, rather than warm ischemia reperfusion.

Because recent evidence from both clinical and experimental studies demonstrates that inflammation plays a critical role in a wide variety of cardiovascular and vascular diseases,21,26-35 we performed an intensive evaluation of markers of inflammation and acute tissue injury after skeletal muscle reperfusion in Wt and ApoE−/− mice. Several studies of skeletal muscle ischemia–reperfusion injury indicate a temporal and quantitative relationship between ischemia, reperfusion, tissue injury, and levels of proinflammatory markers and cytokines.36-38 For these reasons, an analysis of tissue levels of MPO, MIP-2, MCP-1, and osteopontin were undertaken.

Under baseline, preischemic conditions, ApoE−/− mice exhibited greater MIP-2 levels than the Wt controls. However, these levels were of questionable biologic significance because they were measured at the lower end of the standard curve. MPO, MCP-1, and osteopontin levels were not different between these strains at baseline conditions. This observation is relevant primarily because these were aged mice (8 to 10 months old), where the chronic exposure to hypercholesterolemia in ApoE−/− mice could have influn-
ence their baseline inflammatory state and cytokine expression.

MPO levels were significantly greater than sham at 1DR for both Wt and ApoE−/− mice. By 7DR, there was slightly lower level of MPO in the ApoE−/− vs Wt mice, but this difference did not persist at 14DR. The biologic significance of the differences in MPO at 7DR is not certain because the levels were similar to baseline levels in the nonischemic, non-reperfused muscle. Throughout the reperfusion intervals that were analyzed, there were no significant differences in either MIP-2 or osteopontin levels.

Ischemia–reperfusion is a complex process that involves inflammatory and metabolic derangements as components of tissue injury. To evaluate the metabolic consequences of ischemia–reperfusion injury in these experiments, level of ATP at 1DR was compared between Wt and ApoE−/− mice. These levels were equally decreased at 1DR. Thus, it is unlikely that metabolic differences between Wt and ApoE−/− mice contributed to the process of delayed muscle regeneration in these experiments.

CONCLUSIONS

These experiments reveal evidence of delayed skeletal muscle regeneration in ApoE−/− mice after hind limb ischemia–reperfusion. Age-matched controls did not exhibit a similar pattern of delayed muscle healing. Delayed skeletal muscle regeneration may contribute in part to delayed functional recovery after lower extremity revascularization. Although our analysis of tissue inflammation and metabolism was not all-inclusive, it is unlikely that the delay in skeletal muscle healing is caused by differences in the degree of acute injury or inflammation. Rather, our data show that persistent inflammation is present during the chronic phase of reperfusion in the ApoE−/− mice. Further studies evaluating the relationship between this delay in skeletal muscle healing and the role of hypercholesterolemia phenotype and how hypercholesterolemia may modulate inflammatory response is warranted.

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AUTHOR CONTRIBUTIONS

Conception and design: JK, HA, VP, TA, WA, MW
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Final approval of the article: JK, HA, VP, TA, JY, WA, MW
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707