Limb Blood Flow During Exercise Is Dependent on Nitric Oxide

Andrew J. Maxwell, MD; Eric Schauble, BS; Daniel Bernstein, MD; John P. Cooke, MD, PhD

Background—we have recently reported that hypercholesterolemia reduces aerobic exercise capacity in mice and that this is associated with a reduced endothelium-dependent vasodilator function, endothelium-derived nitric oxide (EDNO) production, and urinary nitrate excretion. These findings led us to test the hypothesis that EDNO production contributes significantly to limb blood flow during exercise and to determine whether loss of EDNO production is responsible for the decline in exercise capacity observed in hypercholesterolemia.

Methods and Results—Twelve-week-old wild-type (E\(^+\); n=9) and apoE-deficient (E\(^-\); n=9) C57BL/6J mice were treadmill-tested to measure indices defining exercise capacity on a metabolic chamber–enclosed treadmill capable of measuring oxygen uptake and carbon dioxide excretion. Urine was collected before and after treadmill exercise for determination of vascular NO production assessed by urinary nitrate excretion. The wild-type mice were then given nitro-\(\text{L}\)-arginine (E\(^-\)LNA) in the drinking water (6 mg/dL) for 4 days before undergoing a second treadmill testing and urinary nitrate measurement. An additional set of 12-week-old wild-type mice was divided into 2 groups: 1 receiving regular water (E\(^+\); n=8) and 1 administered LNA for 4 days (E\(^-\)LNA; n=8). These mice, along with an additional set of E\(^-\) mice (n=8), underwent treadmill testing to determine maximal oxygen uptake (\(V\dot{O}_2\max\)). The mice were then cannulated such that the tip of the tubing was positioned in the ascending aorta. Fluorescent microspheres (20 000) were infused into the carotid cannula while the mice were sedentary and again while approaching \(V\dot{O}_2\max\). When the mice were euthanized, the running muscles were collected and fluorescence intensity was measured to determine the peak-exercise redistribution of blood flow to the running muscles (expressed as percentage of total cardiac output, %COrm) during both states. Both E\(^-\)LNA and E\(^-\) mice demonstrated a markedly reduced postexercise urinary nitrate excretion, aerobic capacity, and %COrm at \(V\dot{O}_2\max\) compared with E\(^+\).

Conclusions—EDNO contributes significantly to limb blood flow during exercise. Conditions that reduce EDNO production disturb the hyperemic response to exercise, resulting in a reduced exercise capacity. (Circulation. 1998;98:369-374.)

Key Words: oxygen ■ vasculature ■ apolipoproteins ■ nitric oxide ■ microspheres

We recently reported that hypercholesterolemic mice manifest a reduction in indices of aerobic exercise capacity during treadmill testing, including a decline in \(V\dot{O}_2\max\), AT, distance run to exhaustion, aerobic work capacity, and RQ at exhaustion.\(^1\) This is true of both diet-induced and genetically prone (caused by an apoE deficiency) hypercholesterolemia. Furthermore, the reduction in exercise capacity is associated with an endothelium-dependent vasodilator dysfunction, attenuated elaboration of aortic EDNO, and a reduced exercise-induced NO production assessed by urinary nitrate excretion. These findings lead us to speculate that EDNO production contributes significantly to exercise-induced hyperemia and that a loss of EDNO-mediated hyperemia results in a decreased oxygen transport capacity of the vasculature and an attenuated aerobic capacity.

The present study was performed to determine whether inhibition of EDNO production, either pharmacologically or by hypercholesterolemia, reduces exercise-induced hyperemia and aerobic capacity.

Methods

Animals

Eight-week-old female E\(^+\) and E\(^-\) C57BL/6J mice (Jackson Laboratories, Bar Harbor, Me, and DCM) were entered into experimental protocols after a 1-week period of acclimation in the housing facilities of the Stanford DCM. All mice were inspected before the study by the DCM veterinarian and monitored daily by DCM technicians and investigators. All experimental protocols were approved by the Administrative Panel on Laboratory Animal Care of Stanford University and were performed in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care. All mice were housed 3 to 4 per cage. They were maintained on a 12-hour light/dark cycle and given unlimited access to food and water for the duration of the study. All mice were handled daily and taught to run on a treadmill with...
shock-plate incentive (Exer-4 Treadmill, Columbus Instruments) but were otherwise confined to cages for the duration of the study.

The E− mice were generated from targeted disruption of the apoE gene in the 129 embryonic stem cell line. Germ-line chimeras were mated and backcrossed for 10 generations with C57BL/6J wild-type mice.2

Experimental Protocol

To determine the effects of EDNO inhibition on aerobic capacity, a set of mice underwent the following treadmill studies. Eight-week-old E− (n = 9) and E+ (n = 9) mice were kept sedentary for 4 weeks. At 12 weeks of age, each mouse was treadmill-tested to measure indices defining exercise capacity. Urine was collected before and after treadmill exercise for determination of urinary nitrate excretion. The E− mice were then given LNA (Sigma Chemical Co) in the drinking water (6 mg/100 mL; E−LNA). This dose of LNA is similar to that shown to attenuate basal urinary nitrate excretion,4 and similar molar doses of LNA methyl ester have been shown to suppress the release of EDNO and the excretion of cGMP and nitrate in other animal models.5 After 4 days, the mice underwent a second treadmill testing and urinary nitrate measurement. Mice were euthanized in random order after treadmill testing by overdose of methoxyflurane (Pitman-Moore) inhalation anesthesia. Blood was collected from the right atrium for measurement of serum total cholesterol levels.

To determine blood flow distribution during exercise, an additional set of mice underwent a microsphere study. Eight-week-old wild-type mice and E− mice (n = 8) were kept sedentary for 4 weeks. At 12 weeks of age, the wild-type mice were divided into 2 groups, one receiving regular water (E+: n = 8) and one given LNA in the drinking water (6 mg/100 mL) for 4 days (E−LNA; n = 8). Each mouse underwent treadmill testing to determine V02max. The aorta was then surgically cannulated, and after overnight recovery, the mice underwent a microsphere delivery study (described below). Mice were euthanized after microsphere delivery by overdose of methoxyflurane inhalation anesthesia. The hindlimb muscles were collected and weighed for determination of microsphere density.

Indices of Exercise Capacity

V02max is defined as the plateau in V02 despite increasing work intensity. Commonly in exercise testing, the V02max is not measurable because the subject becomes exhausted or feels too much discomfort to continue before a plateau in V02 is reached. The treadmill protocol used in this study was designed so that the mice would quickly attain a plateau, reaching their V02max before exhaustion. In a few cases when a plateau was not reached, the V02max was approximated by the peak V02 attained by the animal before exhaustion.

The AT is an independent measure of aerobic capacity expressed in units of V02. For each mouse, the AT was determined from computer analysis (confirmed by blinded observer) of V02/V01 plots by the V-slope method of Beaver.1 In situations in which the slope of V02/V01 did not increase at higher work rates, the V02max was taken as the AT.

The distance run to exhaustion is taken as an approximate measure of overall work performance and is the total distance run. Aerobic work capacity was determined by the summation of minute oxygen uptake above basal rate over the course of treadmill running until exhaustion. This was multiplied by the constant 20 J/mL O2 to convert oxygen uptake to aerobic work.6,7

The RQ is the VCO2/V02 at any given time and at exercise intensities above AT is used as an indirect indicator of anaerobic work performance. At high work rates, anaerobic work supplements aerobic work. Lactic acid, produced from anaerobic metabolism, is buffered by serum bicarbonate, resulting in a stoichiometric increase in CO2 output over O2 uptake.8,9 Thus, RQ begins to rise after AT is attained and continues to rise with increasing anaerobic workload until exhaustion.10

Treadmill Testing

At the time of treadmill testing, each mouse was placed on a treadmill at a constant 8° angle enclosed by a metabolic chamber capable of measuring oxygen and carbon dioxide outflow once every minute (model CT-2, Columbus Instruments). After a 15-minute period of acclimation, basal measurements were obtained over a period of 7 minutes. The treadmill was then started at 10 m/min, and the speed was incrementally increased 1 m/min every minute until the mouse reached exhaustion. Exhaustion was defined as spending time on the shocker plate without attempting to reengage the treadmill. Data on oxygen uptake (V02), carbon dioxide output (VCO2), RQ, and distance run to exhaustion were collected and stored on hard disk (Oxymax Software, Columbus Instruments).

Measurement of Urinary Nitrogen Oxides

Mice were placed in metabolic chambers for basal and postexercise urinary nitrate collection.11 For the basal state, mice were confined to cages for >24 hours, and for the postexercise state, mice were treadmill-exercised for 22 minutes to a final treadmill speed of 32 m/min. Metabolic chambers were constructed as described previously.12 Urine was collected in test tubes containing 100 μL of isopropyl alcohol submerged in ice water for the duration of the 5-hour collection period. Urine was centrifuged at 4000 rpm for 5 minutes, and the supernatant was collected, diluted 1:9 in distilled water, and stored at −80°C for measurement of nitrogen oxides and creatinine.

Nitrogen oxides in the urine were measured with a commercially available chemiluminescence apparatus (model 2108, Dasibi Corp) as previously described.13 The samples (50 μL) were injected into boiling acidic vanadium (III) chloride. This technique uses acidic vanadium (III) chloride at 98°C to reduce both NO2− and NO−3 to NO, which is then detected by the chemiluminescence apparatus after reacting with ozone. Signals from the detector were analyzed by computerized integration of curve areas. Standard curves for NaN03/NaNO2 were linear over the range of 50 pmol/L to 10 nmol/L.

Urine creatinine was measured by the modified method of Slot developed by Sigma Diagnostics.14

Regional Blood Flow Determination

Regional blood flow to hindlimb muscles was determined as a percentage of cardiac output with a modification of previously described techniques.14,15

Surgical Preparation

Mice were anesthetized with isoflurane (Ohmeda Caribe) inhalation. An incision was made in the ventral midline of the neck. After the carotid sheath was exposed, the carotid artery was separated from the neurovascular bundle and secured by two 4-0 silk sutures. An incision was made in the carotid artery, and a 30-cm length of PE10 tubing (Becton Dickinson) tapered at one end by gentle stretch was filled with heparin (100 U/mL, Elkins-Sinn), introduced into the carotid artery, and advanced to the ascending aorta just distal to the aortic valve. The incision was oversewn, and the tubing was tunneled subcutaneously to a pouch under the skin on the back. The mice were then given a single dose of ampicillin (100 mg/kg diluted in saline 10 mg/mL IP). After overnight recovery, the mice were assessed for

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<td>AT = anaerobic threshold</td>
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<td>% COIrm = redistribution of blood flow to running muscles as percentage of total cardiac output</td>
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<td>DCM = Department of Comparative Medicine</td>
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<td>E− = apoE-deficient (apoE-knockout) mice</td>
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<td>E+ = wild-type mice</td>
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<td>E−LNA = wild-type mice receiving LNA</td>
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<td>EDNO = endothelium-derived nitric oxide</td>
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<tr>
<td>LNA = N^+nitro-L-arginine</td>
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<td>RQ = respiratory quotient</td>
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<td>V02max = maximal rate of oxygen uptake</td>
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running ability. Mice that could not attain 80% \( \dot{V}_O_2 \)max as determined previously were eliminated from the study.

**Treadmill Exercise Protocol**

The instrumented mice were placed on a treadmill in a metabolic chamber in random order. The tubing was fed through a hole in the chamber, and the carotid artery tubing was connected to a pressure transducer for continuous heart rate and blood pressure measurement. A 100-\( \mu \)L sample of blood was withdrawn for basal lactate measurement. After 20 minutes of acclimation and oxygen uptake analysis, blue-green microspheres were injected into the carotid tubing and infused with normal saline for regional blood flow determination at rest. An equal volume of blue-green microspheres was injected into a reference vial for a “100% of flow” control. The treadmill was then started at 10 m/min and increased 1 m · min⁻¹ while the oxygen uptake curve was followed. As the oxygen uptake curve began to plateau, yellow-green microspheres were injected into the carotid cannula and infused with normal saline. An equal volume of yellow-green microspheres was injected into a reference vial. A second 100-\( \mu \)L sample of blood was withdrawn for peak-exercise lactate measurement. After the completion of the yellow-green microsphere infusion, mice were euthanized by methoxyflurane overdose. The gastrocnemius, quadriceps, and both kidneys were removed, dissected free of fat and connective tissue, blotted dry, weighed, and placed in sample vials for fluorimetric determination.

**Microsphere Protocol**

Blue-green and yellow-green fluorescently labeled microspheres (15-\( \mu \)m diameter, Molecular Probes) were diluted to 20,000 microspheres/20 \( \mu \)L saline. At the time of injection, the microsphere solution was vortexed for 5 minutes. Microspheres (20 \( \mu \)L) were drawn into a microinjector syringe and transferred to the carotid artery tubing. The cannula was connected to a glass syringe mounted in an injector pump and filled with saline, which was used to flush the carotid cannula (100 \( \mu \)L over a period of 30 seconds).

**Fluorimetric Determination of Microsphere Number**

Tissues and reference samples were digested in 2N KOH in methanol overnight in a shaker bath at 40°C. The samples were centrifuged at 3000g, and the supernatant was removed to the level of the tissue plug. The tissue plug was resuspended twice in distilled water with 0.5% Tween80 (Fisher Scientific) and centrifuged, and the supernatant was decanted. The plug was then resuspended in methanol, centrifuged, and decanted. The remaining methanol was removed by evaporative drying. The remaining microsphere residue was dissolved in 2 mL of 2-ethoxyethylacetate (Arcon Organics) and measured by fluorimetry (model LS50B; Perkin-Elmer) using the recommended extinction and emission frequencies for microsphere fluorescence (\( \lambda_{ex} \), 425 nm and \( \lambda_{em} \), 468 nm for blue-green and \( \lambda_{ex} \), 490 nm and \( \lambda_{em} \), 505 nm for yellow-green).

Regional blood flow for resting and exercise states was calculated from fluorescent intensity as the percent of cardiac output to the tissue (%COt): \( %CO_{ts,i} = \frac{I_{ts,i}(WT_{tissue}/WT_t)}{I_{rs}(WT_{tissue})} \), where \( I_{ts,i} \) is the fluorescent intensity of the tissue sample residue from mouse i during state s (resting or exercise) and \( I_{rs} \) is the fluorescent intensity of the reference sample residue. Variability in fluorescence intensity due to variation in tissue blood collected is removed by normalizing the tissue weight, WTt, to the average tissue weight of all animals, WTt_tissue. Regional blood flow to the left and right kidneys was determined to assess adequacy of microsphere mixing. A >10% difference in regional blood flow between kidneys was a criterion for removing an animal from analysis. Of the 24 mice cannulated, 12 mice met the inclusion criteria for analysis (4 died, 3 did not achieve \( \dot{V}_O_2 \) goal, 1 catheter loss, and 4 with poor microsphere mixing).

**Hematology and Biochemistry**

Blood samples (100 \( \mu \)L) collected at the time of regional blood flow determination were placed in serum separator tubes containing fluoride/oxalate anticoagulant. The samples were immediately centrifuged at 400g for 10 minutes. The serum was kept on ice until analysis. Serum lactate was analyzed within the hour of collection by an enzymatic method developed by Sigma Diagnostics (procedure 739).

Blood samples were collected at the time the animals were killed. These were immediately centrifuged at 3000 rpm for 15 minutes. The serum was separated and stored at ~80°C until analysis. Total serum cholesterol was analyzed by the enzymatic method of Allain et al as developed by Sigma Diagnostics.

**Data Analysis**

Data are expressed as mean±SEM. Comparisons of single means from multiple populations were made by one- and two-factor univariate one-way ANOVA followed by Fisher’s protected least significant difference. A value of \( P<0.05 \) was accepted as statistically significant.

**Results**

**Effect of LNA or Hypercholesterolemia on NO Production**

The body weights of the E⁻ and E₂ mice were the same, and the weight of the E⁻ mice did not change after 4 days of LNA administration (Table). The cholesterol levels of the E⁻ mice were significantly elevated compared with E₁ mice (94±114 versus 57±4 mg/dL, serum). Basal urinary nitrate values were similar in the E₁, E₁LNA, and E₂ mice (350±100, 250±50, and 200±10 pmol/mg creatinine, respectively, Figure 1). Postexercise urinary nitrate levels were increased in the E⁻ mice (550±40 pmol/mg creatinine); by contrast, exercise did not increase urinary nitrate levels in the E₁LNA or E⁻ mice (90±25 and 85±30 pmol/mg creatinine, respectively).

**Effect of LNA or Hypercholesterolemia on Exercise Capacity**

Suppression of NO by LNA resulted in a decrease in aerobic capacity compared with E⁻ mice as measured by \( \dot{V}_O_2 \)max (E⁻, 120±2; E₁LNA, 107±4 ml/min/kg; \( P<0.05 \) and aerobic work capacity (E⁻, 11.2±0.6; E₁LNA, 7±1 J/g; \( P<0.05 \)). There was also a trend toward a decline in running distance (E⁻, 475±29; E₁LNA, 397±30 m; \( P=NS \)) and anaerobic threshold (E⁻, 84±4; E₁LNA, 76±3 mlO₂/min/kg; \( P=NS \)). The magnitudes of reductions in the indices of aerobic capacity observed in E⁻ mice were similar to those seen in the E₁LNA mice (Table, Figure 2).

**Effect of LNA or Hypercholesterolemia on Exercise Hyperemia**

The basal heart rates and both basal and peak-exercise mean blood pressures of the three groups were not different from each other, although the mean blood pressure of the E₁LNA group tended to be higher than the other two, reflecting an effect of NO suppression (Table). Accurate peak-exercise heart rates were not obtained. Basal serum lactate did not differ between the three groups (13±3, 11±2, and 15±2 mg/dL for E⁻, E₁LNA, and E₂, respectively, \( P=NS \)). Peak-exercise lactate was significantly higher than basal in all three groups but, again, did not differ between groups (62±12, 68±21, and 77±24 mg/dL for E⁻, E₁LNA, and E₂, respectively, \( P=NS \)). This rise in serum lactate confirmed that the mice were exercising at an intensity above their anaerobic threshold at the time of the second microsphere injection.
Nitric Oxide and Exercise Hyperemia

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<td>Peak-exercise serum lactate, mg/dL</td>
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<td>(V_\text{O}_2\max), mL \cdot min(^{-1}) \cdot kg(^{-1})</td>
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<td>AT, mL \cdot min(^{-1}) \cdot kg(^{-1})</td>
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<td>Peak-exercise % cardiac output to running muscles</td>
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*\(P<0.05\), †\(P<0.01\), ‡\(P<0.005\) vs \(E^+\); §\(P<0.05\) vs basal.

Basal %\(CO_m\) did not differ between the three groups (6±2%, 5±2%, and 5±2% of total cardiac output for \(E^+\), \(E^\text{LNA}\), and \(E^-\), respectively, \(P=\)NS) (Figure 3). Peak-exercise %\(CO_m\) nearly doubled in the \(E^+\) mice (from 6±2% to 11±3%, \(P<0.05\)). In striking contrast, the %\(CO_m\) remained essentially the same as basal in the \(E^\text{LNA}\) and \(E^-\) mice (7±1% and 6±3% of total cardiac output for \(E^\text{LNA}\) and \(E^-\), respectively).

Discussion

The salient findings of this study are that (1) inhibition of NO synthase activity reduces exercise capacity; (2) inhibition of NO synthase activity reduces exercise-induced redistribution of blood flow to skeletal muscles; and (3) similar alterations in exercise capacity and exercise-induced limb blood flow are observed in hypercholesterolemia (which is known to inhibit NO activity).

In this study, we set out to determine whether EDNO contributes significantly to exercise-induced hyperemia. We find that inhibition of EDNO activity markedly inhibits exercise-induced redistribution of blood flow to skeletal muscle. The data reveal the importance of EDNO in determining physiological regional shifts in blood flow. These data confirm our previous observation that hypercholesterolemia perturbs EDNO activity and reduces exercise capacity. The present study suggests a mechanism for this dysfunction.

Critique of Methods

To determine whether EDNO plays a significant role in exercise-induced hyperemia, we conducted microsphere experiments in our mouse model. Ideally, use of a double-cannulation method would have provided the most information about cardiac function and regional blood flow determination. However, there is no precedent for this technique in conscious mice, and studies using double cannulation in our laboratory proved to be impractical. In addition, we chose to position the tip of the catheter distal to the aortic valve rather than the traditional position within the left ventricle. This position can be expected to result in poor microsphere mixing at the level of the proximal branches of the aorta but more even mixing at the more distant branches, which was confirmed by the traditional method of comparing microsphere numbers between the left and right kidneys. This sacrifice in microsphere uniformity was made to preserve the competency of the aortic valve, which is almost
certainly important to the physiology of the exercising animal. Despite these limitations of this microsphere technique, information about the distribution of microspheres to the running muscles relative to the entire cardiac output was obtainable. Whereas the portion of the cardiac output to the running muscles during exercise nearly doubled in E\textsuperscript{-1}mice (6±2% to 11±3% of total cardiac output; \( P < 0.05 \)), there was no increase in the portion of cardiac output to the running muscles in hypercholesterolemic mice or mice given LNA. This doubling of the portion of cardiac output to the exercising muscles underestimates the absolute increase in blood flow to these muscles by not taking into account the increase in cardiac output, which, in a normal human subject, can increase 4-fold.\textsuperscript{18}

Relationship of EDNO, Exercise Hyperemia, and Exercise Capacity

Previous work has demonstrated the importance of endothelium-derived mediators for flow-dependent vasodilation in the microcirculation of skeletal muscle. Although earlier studies demonstrated that prostaglandin release mediated flow-dependent vasodilation in isolated skeletal muscle preparations,\textsuperscript{19,20} more recent studies have demonstrated at least an equal and possibly an even greater role for EDNO.\textsuperscript{21,22} These findings have been confirmed in vivo by Doppler flow studies of human forearm blood flow in response to hypoxia-induced hyperemia.\textsuperscript{23,24} However, there are conflicting reports on the role of EDNO in exercise-induced hyperemia after repetitive handgrip and wrist-flexion exercises.\textsuperscript{25–28} This study not only supports the importance of EDNO in exercise-induced hyperemia, but it is also the first to suggest that a loss of EDNO can be rate-limiting to oxygen delivery and exercise performance. This finding fits with previous work that supports the concept that vascular transport of oxygen can be rate-limiting to metabolic capacity.\textsuperscript{29–33} It is quite possible, then, that any disturbance in the rate-limiting mechanism of oxygen delivery, such as a defect in flow-mediated vasodilation, would affect exercise capacity.

It is possible that other mechanisms for decreased exercise capacity exist in the presence of systemic administration of LNA. NO plays a role in cardiac and skeletal myocyte function,\textsuperscript{34} and the role that neuronal NO plays in metabolic activity is yet to be investigated. Inhibition of NO within these systems may cause adverse consequences for exercise capacity. In myocytes, however, a reduction in NO stimulates mitochondrial respiration.\textsuperscript{34} The effects of hypercholesterolemia on exercise-induced hyperemia and aerobic capacity were remarkably similar to that of treatment with the NO synthase antagonist. We suspect that this is because hypercholesterolemia shares a common mechanism: perturbation of the endothelial NO synthase pathway.

To conclude, EDNO appears to play a significant role in limb blood flow during exercise. A pathological disturbance in EDNO activity like that observed in hypercholesterolemia results in a loss of blood flow redistribution and a reduction in exercise capacity.

Acknowledgments

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