# Abnormal mitochondrial respiration in skeletal muscle in patients with peripheral arterial disease

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***Objective:*** **Discrete morphologic, enzymatic and functional changes in skeletal muscle mitochondria have been demonstrated in patients with peripheral arterial disease (PAD). We examined mitochondrial respiration in the gastrocnemius muscle of nine patients (10 legs) with advanced PAD and in nine control patients (nine legs) without evidence of PAD.**
***Methods:*** **Mitochondrial respiratory rates were determined with a Clark electrode in an oxygraph cell containing saponin-skinned muscle bundles. Muscle samples were obtained from the anteromedial aspect of the gastrocnemius muscle, at a level 10 cm distal to the tibial tuberosity. Mitochondria respiratory rate, calculated as nanoatoms of oxygen consumed per minute per milligram of noncollagen protein, were measured at baseline ($V_0$), after addition of substrates (malate and glutamate; ($V_{SUB}$), after addition of adenosine diphosphate (ADP) ($V_{ADP}$), and finally, after adenine nucleotide translocase inhibition with atractyloside ($V_{AT}$). The acceptor control ratio, a sensitive indicator of overall mitochondrial function, was calculated as the ratio of the respiratory rate after the addition of ADP to the respiratory rate after adenine nucleotide translocase inhibition with atractyloside ($V_{ADP}$/ $V_{AT}$).**
***Results:*** **Respiratory rate in muscle mitochondria from patients with PAD were not significantly different from control values at baseline (0.31 ± 0.06 vs 0.55 ± 0.12; $P = .09$), but $V_{sub}$ was significantly lower in patients with PAD compared with control subjects (0.43 ± 0.07 vs 0.89 ± 0.20; $P < .05$), as was $V_{ADP}$ (0.69 ± 0.13 vs 1.24 ± 0.20; $P < .05$). Respiratory rates after atractyloside inhibition in patients with PAD were no different from those in control patients (0.47 ± 0.07 vs 0.45 ± $P = .08$). Compared with control values, mitochondria from patients with PAD had a significantly lower acceptor control ratio (1.41 ± 0.10 vs 2.90 ± 0.20; $P < .001$).**
***Conclusion:*** **Mitochondrial respiratory activity is abnormal in lower extremity skeletal muscle in patients with PAD. When considered in concert with the ultrastructural and enzymatic abnormalities previously documented in mitochondria of chronically ischemic muscle, these data support the concept of defective mitochondrial function as a pathophysiologic component of PAD. (J Vasc Surg 2003;38:827-32.)**

Insufficient oxygen supply secondary to reduced blood flow is presumed to be the main physiologic cause for the manifestations of peripheral arterial disease (PAD). More recently, however, a number of investigators have proposed the presence of mitochondriopathy in chronically ischemic skeletal muscle.[1-10] Their findings suggest that in addition to reduced oxygen supply, suboptimal energy production from defective mitochondria may be a factor in PAD pathogenesis.

Over the last decade a number of studies have documented that mitochondria in chronically ischemic muscle have abnormal ultrastructure,[1] damaged DNA,[2] altered enzyme expression and activity, and abnormally high intermediates of oxidative metabolism.[3,4] In these studies mitochondria were evaluated in fixed or frozen muscle samples, which precluded simultaneous study of mitochondrial respiratory function. As a result, it is difficult to draw any definitive conclusions about the functional significance of these findings. Our laboratory and others have used phosphorus 31 magnetic resonance spectroscopy ($^{31}$P MRS)[6-10] to study the bioenergetics of chronically ischemic skeletal muscle during various types of exercise. These in vivo studies of mitochondrial function demonstrate an intrinsic defect in mitochondrial respiration, similar to that in the mitochondrial myopathies.[5,11] The main limitation of $^{31}$P MRS is that the extramitochondrial milieu cannot be measured or controlled. In addition, unless the exercise protocol is carefully designed and regulated, it can introduce the confounding influence of blood flow differences (at rest and with exercise) on bioenergetics, making data interpretation a challenge.

To further define and characterize the functional defect in mitochondria in PAD, we performed an in vitro evaluation of mitochondrial respiration in freshly harvested and processed skeletal muscle samples. Specimens were treated with mechanical dissection and chemical permeabilization to obtain skinned muscle fibers. The mitochondrial respiratory function of these fibers was then evaluated in vitro with polarography. This processing removes the sarcolemmal membrane and the cytosol, but preserves the ultrastructure of mitochondria and other intracellular or-

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ganelles. The total mitochondrial population is accessible with this technique, thereby enabling direct investigation of mitochondrial respiratory function with complete control of the mitochondrial milieu.[12-15]

We studied patients with more advanced PAD than have been studied previously, focusing on patients with severe claudication and critical limb ischemia in need of either revascularization or amputation. Patients with advanced lower extremity PAD usually have very short distance claudication (muscle pain with minimal amount of exercise, which is relieved by rest) or critical limb ischemia (rest pain and tissue loss, usually involving the foot and toes). These patients have indications for either revascularization or, in the most severe cases, amputation to control complications of ischemia. Defective muscle cell bioenergetics may contribute to muscle dysfunction of claudication and the production of rest pain and tissue loss or gangrene in the severely ischemic limb.

## METHODS

The experimental protocol and consent form were approved by the institutional review board, and all subjects gave informed consent.

**Study group.** We recruited nine patients (10 legs) undergoing lower extremity operations because of advanced ischemia (life-style limiting claudication, rest pain, or tissue loss) (Table I). Patient mean age was 65 years. Each patient had, on average, 2.5 risk factors for atherosclerosis. Risk factors included diabetes, hypertension, smoking, dyslipidemia, coronary artery disease, obesity, and family history of complications of atherosclerotic disease. Six patients (six legs) underwent femoropopliteal or femorotibial bypass grafting, and in the other three patients (four legs) a major amputation procedure was performed. The diagnosis of PAD was objectively established on the basis of a combination of history, positive findings at physical examination, and significantly decreased ankle-brachial index (ABI; mean, 0.4) or toe pressure. In addition, angiographic documentation of PAD was available for seven of the nine patients. The diagnostic workup revealed evidence of femoropopliteal occlusive disease in five patients (five legs), and combined femoropopliteal and crural occlusive disease in the other four patients (five legs).

**Control group.** Nine patients (nine legs) undergoing lower extremity operations because of indications other than PAD were recruited. Patient mean age was 47 years. Each patient had, on average, one risk factor for atherosclerosis. Six patients had lower extremity venous disease, two patients had lower extremity orthopedic problems, and one patient had lower extremity trauma. These patients had no history of PAD symptoms, and all had normal lower extremity pulse at examination and noninvasive testing (ABI $\geq$1.0 at rest and after exercise). All control patients had a sedentary lifestyle.

**Biopsy technique.** Muscle biopsy samples were obtained from the gastrocnemius muscle, exposed through the incision made for the primary procedure. All muscle samples were obtained from the anteromedial aspect of the gastrocnemius muscle belly, at a level 10 cm distal to the tibial tuberosity. Identical operative technique was used for muscle harvest in all patients.

**Tissue preparation for mitochondrial respiration measurement.[12]** After a brief rinse in ice-cold Ringer solution, the tissue specimen was transferred to ice-cold relaxing solution A, and minced with sharp forceps into bundles approximately 0.3 to 0.4 mm in diameter. Relaxing solution A consisted of 10 mmol/L of ethylene glycol tetraacetic acid (EGTA), 3 mmol/L of free $Mg^{++}$, 20 mmol/L of taurine, 0.5 mmol/L of dithiothreitol, 20 mmol/L of imidazole, 0.16 mol/L of potassium 4-morpholine ethanesulfonate (pH, 7.2), 5 mmol/L of adenosinotriphosphate (ATP) magnesium salt, and 15 mmol/L of phosphocreatine sodium salt. To remove intracellular calcium, the muscle bundles were softly agitated for 2 hours in relaxing solution A. Bundles were then skinned in relaxing solution A containing 100 $\mu$g/mL of saponin. Saponin is a glycoside of plant origin that in very low concentrations selectively removes the sarcolemma (myocyte membrace) without any visible effect on mitochondria or other intracellular structures. Saponin selectivity for sarcolemma lies in its hydrophobic core, which has high affinity for cholesterol. Sarcolemma is rich in cholesterol, whereas the membranes of mitochondria and endoplasmic reticulum contain little cholesterol.[13] The 20-minute skinning procedure was performed while the sample was being vigorously stirred at 4°C. After skinning, specimens were washed for 30 minutes in solution B to remove saponin and to reduce intracellular concentrations of high-energy phosphates and nicotine adenine nucleotide–dependent substrates. Solution B consisted of 10 mmol/L of EGTA, 3 mmol/L of free $Mg^{++}$, 20 mmol/L of taurine, 0.5 mmol/L of dithiothreitol, 20 mmol/L of imidazole, 0.16 mol/L of potassium 4-morpholine ethane sulfonate (pH, 7.2), 3 mmol/L of phosphate, and 10 mg/mL of fatty acid–free bovine serum albumin. Specimens were washed again for 30 minutes in fresh solution B before being placed in the oxygraph chamber for determination of mitochondrial respiration.

The mitochondrial yield and quality in the skinned fiber preparation has been extensively investigated by our team and others. Transmission and scanning electron microscopy of skinned muscle bundles demonstrate complete removal of the sarcolemma, with preservation of the total number of mitochondria.[12,14-19] In addition, embedment-free electron microscopy demonstrates that these mitochondria are morphologically intact and maintain undamaged contacts with the cytoskeleton and other subcellular organelles.[20] Furthermore, biochemical evaluation of skinned muscle bundles demonstrates no solute barriers between the intracellular and extracellular spaces, enabling complete control of mitochondrial physiology with the addition of external components.[12,16,18] Most important, the mitochondrial respiration in skinned muscle fibers is functionally comparable to that of high-quality isolated mitochondria preparations.[12,21,22]

**Measurement of mitochondrial respiratory rates.** Mitochondrial respiratory rates were determined with a Clark electrode (Yellow Springs Instruments, Yellow Springs, Ohio) in an oxygraph cell containing 15 to 20 muscle bundles in 3 mL of solution B at 34°C, with continuous stirring. The solubility of oxygen at 34°C was taken to be 386 natoms/mL. Respiratory parameters measured included basal respiratory rate ($V_0$), respiratory rate after addition of substrates (0.7 mmol/L of malate, 1.7 mmol/L of glutamate; ($V_{SUB}$), respiratory rate after further addition of 1 mmol/L of ADP ($V_{ADP}$), and respiratory rate after addition of 0.35 mmol/L of atractyloside ($V_{AT}$). Atractyloside is an inhibitor of the adenosine triphosphate (ATP)–ADP translocase, a mitochondrial membrane protein that exports ATP to the cytosol in exchange for ADP, which is used in the mitochondria to manufacture additional ATP. Inhibition of ATP-ADP translocase halts entry of ADP into the mitochondria, enabling testing of their respiratory function in the absence of ADP.

The most important components of this investigation are $V_{ADP}$, and $V_{AT}$. $V_{ADP}$ represents a state of maximal mitochondrial stimulation, or state 3 respiration, whereas $V_{AT}$ represents a state of maximal inhibition, or atractyloside-sensitive oxidative phosphorylation. Measurements obtained at baseline and after substrate addition alone have little or no significance to the overall evaluation. Specifically, at baseline the processed muscle fibers have no cytosol, and therefore minimal residual substrate and ADP. The similarity of respiratory rates at baseline reflects that specimen preparation in both groups removed the cytosol and most of the substrates and ADP within it. The acceptor control ratio, a sensitive indicator of mitochondrial function, was calculated as the ratio of state 3 respiration ($V_{ADP}$) to respiratory rate after ATP-ADP translocase inhibition ($V_{AT}$) with atractyloside ($V_{ADP}/V_{AT}$). This ratio is a sensitive indicator of overall mitochondrial function, and represents the relative difference between the two extremes of mitochondrial function that occur in the presence and absence of ADP.

After completion of all measurements, tissue was processed for determination of the amount of non-collagen protein, with the method of Lowry et al.[23] Respiratory rate was expressed as nanoatoms of oxygen consumed per minute per milligram of non-collagen protein. For quality control, mitochondrial respiratory rates were measured on each study day in myocardial specimens obtained from normal Swiss Webster mice. A 30% to 50% increase in mitochondrial state 3 respiration beyond that achieved after addition of substrates was considered a normal response and confirmation of proper assay and equipment performance.

**Statistical analysis.** Descriptive statistics were computed for the variables of interest in the two groups. To test for differences between control and PAD groups in respiratory rate at different stages of the polarography experiment and in the acceptor control ratios, a *t* statistic for two means was used. For this test, $P < .05$ was considered significant. Data are reported as mean ± SEM.

## RESULTS

Chronically ischemic mitochondria from patients with PAD demonstrated similar respiratory rate at baseline, compared with that in control patients. $V_{SUB}$ was significantly lower in patients with PAD compared with control patients. Similarly, $V_{ADP}$ remained significantly lower in patients with PAD than in control patients. $V_{AT}$ in patients with PAD was similar to that in control patients. Mitochondria from patients with PAD, compared with control patients, had a significantly lower acceptor control ratio (Table II; Figure).

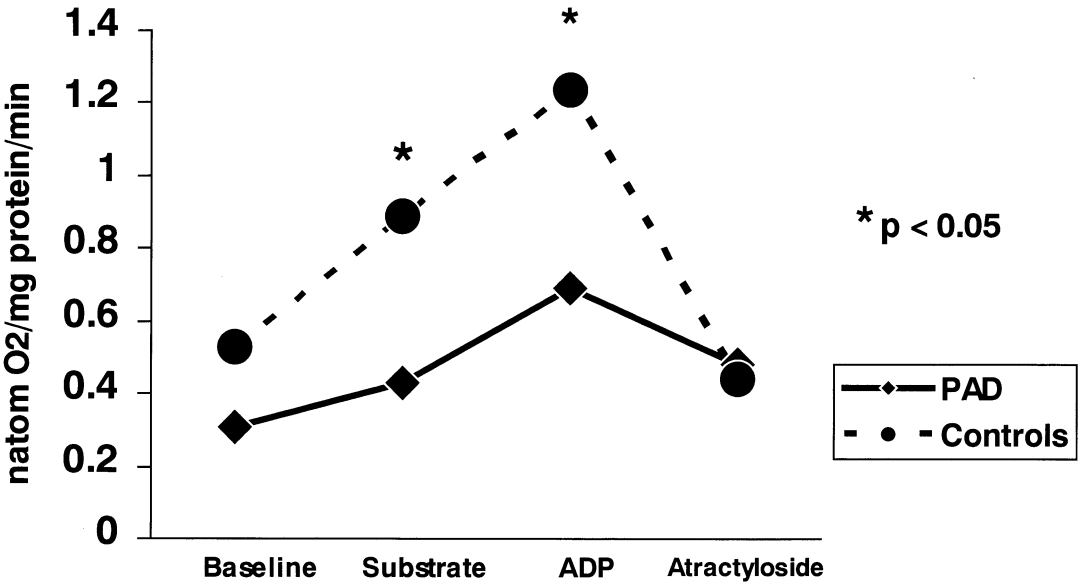
No attempt was made to correlate level of mitochondrial function with clinical manifestations of PAD in our study patients. It was thought that such a correlation would not be fruitful, because the group was small and homogeneous, containing only patients with very advanced disease (two patients with very short distance claudication and seven with rest pain or tissue loss). In such a group, differences in clinical manifestations are too small to be correlated, even with results obtained at conventional noninvasive testing and arteriography.

## DISCUSSION

Mitochondria in chronically ischemic muscle have abnormal ultrastructure,[1] extensively damaged DNA,[2] altered expression and activities of enzymes, and abnormally high intermediates of oxidative metabolism.[3,4] More remarkable, an intrinsic defect in mitochondrial function similar to that in mitochondrial myopathies[5] has been demonstrated by our team and others when the bioenergetics of chronically ischemic skeletal muscle was studied with $^{31}$P MRS.[6-10] When legs with different levels of ischemia were evaluated with $^{31}$P MRS, it was found that extremities with severe ischemia had the most defective oxidative function.[9] Analysis of $^{31}$P MRS findings[6] suggests that a combination of factors related to intensity and frequency of "ischemic insult," and possibly the ischemia-reperfusion associated with it, may be responsible for defective mitochondria.

Revascularization of a chronically ischemic limb does not reverse mitochondrial oxidative dysfunction until several months after the intervention.[9] In addition, revascularization does not normalize exercise performance in the leg with claudication.[24] However, treatment with pentoxifylline (first drug approved by the US Food and Drug Administration for treatment of claudication) in a selected group of patients with moderate claudication improved leg exercise performance in a fashion directly proportional to improvement in muscle mitochondrial function.[7] These studies suggest that impaired arterial perfusion may not be the only factor that determines leg function in PAD. Treatment of mitochondriopathy in chronically ischemic skeletal muscle may be an independent therapeutic method capable of producing significant functional improvement with or without concomitant revascularization.[11,25]

We attempted to further evaluate the function of mitochondria in chronically ischemic muscle. Maximal mito-




Comparison of gastrocnemius muscle respiratory rate in patients with peripheral arterial disease *(PAD)* and control patients. *Baseline,* Respiratory rate at baseline; *Substrate,* respiratory rate after addition of malate and glutamate; *ADP,* respiratory rate after further addition of adenosine diphosphate; *Atractyloside,* respiratory rate after adenine nucleotide translocase inhibition with atractyloside. $P < .05$.

**Table I.** Demographic data for patients with PAD

| *Patient* | *Age (y)* | *Gender* | *Presentation* | *ABI* | *Extent of PAD* | *Operation* | *Risk factors* | $V_{ADP}$ |
|---|---|---|---|---|---|---|---|---|
| 1 | 51 | Female | Tissue loss | 0.3 | FP | Revascularization | 3 | 1.10 |
| 2 | 76 | Female | Calf claudication | 0.4 | FP | Revascularization | 2 | 1.17 |
| 3 | 65 | Male | Tissue loss | 0.5 | FP | Revascularization | 3 | 0.61 |
| 4 | 62 | Male | Tissue loss | 0.3 | FP + crural | Amputation | 3 | 1.33 |
| 5 Left leg | 75 | Female | Tissue loss | IC | FP + crural | Amputation | 3 | 0.57 |
| 5 Right leg | | Female | Tissue loss | IC | FP + crural | Amputation | 3 | 0.86 |
| 6 | 63 | Male | Tissue loss | 0.6 | FP | Revascularization | 1 | 0.40 |
| 7 | 67 | Female | Rest pain | 0.4 | FP + crural | Revascularization | 3 | 0.23 |
| 8 | 87 | Female | Tissue loss | 0.3 | FP + crural | Amputation | 2 | 0.40 |
| 9 | 42 | Female | Calf claudication | 0.5 | FP | Revascularization | 3 | 0.25 |

*PAD,* Peripheral arterial disease; *ABI,* ankle brachial index; *IC,* incompressible; *FP,* femoropopliteal segment; *VADP,* respiratory rate in presence of substrates (malate and glutamate) and adenosine diphosphate.

chondrial respiratory rate ($V_{ADP}$) after stimulation of the electron transport chain with the electron donor substrates malate and glutamate in the presence of ADP (final electron acceptor in oxidative phosphorylation) was significantly higher in control mitochondria than in PAD mitochondria. When respiration was inhibited by depriving the mitochondria of ADP, after atractyloside inhibition of ATP-ADP translocase, the rate returned to similar levels in both ischemic and control muscle. This difference between the two extremes in mitochondrial function was reflected in a significantly lower acceptor control ratio in mitochondria from patients with PAD compared with control patients. Both diminished response to stimulation with substrates and ADP, and lower acceptor control ratio, document significant mitochondrial dysfunction in chronically ischemic skeletal muscle.

Mitochondrial dysfunction may have two major negative consequences. First, PAD mitochondria do not have the capacity to produce as much ATP as normal skeletal muscle mitochondria do. Patients with PAD are thus in double jeopardy; not only do they have a decreased supply of nutrients and oxygen, as a result of diseased arteries, but the concurrent mitochondrial respiratory defect leads to even less ATP production with the limited supplies of oxygen and nutrients present. Second, dysfunctional mitochondria are a source of abnormally high levels of reactive oxygen species, which may be independently injurious to the mechanisms that sustain normal cellular structure and function. The end result of this process is a significant decrease in energy production and a potentially increased level of destructive oxygen radicals in the skeletal myocytes of patients with PAD.[26]

**Table II.** Respiratory rate* in muscle mitochondria from patients with PAD compared with control patients

| | *PAD* | *Control subjects* | P |
|---|---|---|---|
| $V_0$ | 0.31 ± 0.06 | 0.55 ± 0.12 | .087 |
| $V_{SUB}$ | 0.43 ± 0.07 | 0.89 ± 0.20 | .038 |
| $V_{ADP}$ | 0.69 ± 0.13 | 1.24 ± 0.20 | .029 |
| $V_{AT}$ | 0.47 ± 0.07 | 0.45 ± 0.08 | .860 |
| Acceptor control ratio | 1.41 ± 0.10 | 2.90 ± 0.20 | .001 |

*PAD,* Peripheral arterial disease; $V_0$, rate at baseline; $V_{SUB}$, rate after initial stimulation with substrate (malate and glutamate) addition; $V_{ADP}$, rate after further stimulation with adenosine diphosphate; $V_{AT}$, rate after adenine nucleotide translocase inhibition with atractyloside; *acceptor control ratio,* $V_{ADP}/V_{AT}$.

*Nanoatoms of oxygen per milligram of non-collagen protein per minute; values represent mean ± SE.

It is important to point out that this study was designed to demonstrate the association between PAD and dysfunctional skeletal muscle mitochondria. A cause-and-effect relationship between the two cannot be established on the basis of these data. Additional work, with an animal model of hind limb ischemia, is currently under way in our laboratory, to clarify whether atherosclerosis specifically or ischemia alone from other disease is the cause of this dysfunction.

The skinned fiber technique is the ideal tool for assessment of mitochondrial respiratory efficiency when the sample is small (in our study, wet weight was 50-100 mg), as is usually the case in clinical studies in human subjects. A limitation of this technique is that the results are normalized to non-collagen protein and not to citrate synthase activity. The denominator of choice would have been citrate synthase activity, which is more specific than non-collagen protein for amount of mitochondria in a muscle sample. However, measurement of citrate synthase levels requires a substantially larger volume of muscle tissue than was available in this clinical study. More important, work from several laboratories has identified increased mitochondria content, reflected in increased citrate synthase levels per unit of non-collagen protein, in the muscle of patients with PAD compared with control subjects.[27-30] Therefore, if anything, use of citrate synthase for calculation of respiratory rate would have further increased the differences demonstrated between PAD muscle and control muscle, making them even more significant.

Another potential limitation of our study is that mean age of our patients with PAD was 65 years, compared with 47 years in the control group. This age difference could be important if mitochondrial function declines with age. However, the available data in human subjects show no age-related difference in malate-based $V_{ADP}$ in patients with age range 40 to 90 years.[31,32] Similarly, when mitochondrial function was extensively tested in an animal model of aging, no differences in malate-based $V_{ADP}$ rates could be demonstrated between adult and older animals.[33] Therefore it is unlikely that the mitochondrial dysfunction in our PAD muscle specimens is secondary to the age difference between the PAD and control groups.

In conclusion, our results demonstrate defective mitochondrial respiratory activity in chronically ischemic gastrocnemius muscle. Although the mechanisms responsible for this mitochondrial respiratory defect are not fully understood, our observations suggest that impaired efficiency of one or more components of the electron transport chain and oxidative phosphorylation may be the central underlying mechanism. A better understanding of these mechanisms may enable development of improved therapy for PAD through prevention or treatment of mitochondrial disease. Such therapy has the potential to produce significant improvement in PAD-related morbidity and mortality.

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