Skeletal Muscle Mitochondrial DNA Injury in Patients With Unilateral Peripheral Arterial Disease

Hari K. Bhat, PhD; William R. Hiatt, MD; Charles L. Hoppel, MD; Eric P. Brass, MD, PhD

Background—Patients with peripheral arterial disease (PAD) have exercise limitation due to claudication-limited pain and metabolic alterations in skeletal muscle. PAD is also associated with oxidative stress, which is a known cause of mitochondrial DNA (mtDNA) injury. The present study was designed to test the hypothesis that PAD is associated with mtDNA injury, as reflected by an increased frequency of a specific 4977-base pair (bp) mtDNA deletion mutation.

Methods and Results—The deletion frequency was quantified in gastrocnemius muscle of 8 patients with unilateral PAD and 10 age-matched control subjects with the use of polymerase chain reaction methodologies. Muscle from the hemodynamically unaffected (less affected) PAD limb showed an 8-fold increased deletion frequency and the hemodynamically affected (worse affected) PAD limb had a 17-fold increased deletion frequency compared with muscle from control subjects. The frequency of the 4977-bp deletion in the worse-affected limb was positively correlated with the age of the patients but not the claudication-limited exercise performance of the patients. Total mtDNA content, citrate synthase activity, and cytochrome c oxidase activity were not different in the muscle from the 3 limb populations. However, the ratio of citrate synthase to cytochrome c oxidase was higher in the worse- versus less-affected limbs of PAD patients.

Conclusions—The present study demonstrates a large increase in the frequency of the mtDNA 4977-bp deletion in patients with PAD but in a distribution not limited to the hemodynamically affected limb. (Circulation. 1999;99:807-812.)

Key Words: peripheral vascular disease ■ aging ■ metabolism
Methods

Patients
Eight patients with unilateral PAD and 10 age-matched, sedentary control subjects were recruited. A history and physical examination were performed, and subjects underwent assessment of ankle/brachial index (ABI; ratio of systolic blood pressures in ankle to arm). Unilateral PAD was identified as previously described.28 The limb with hemodynamically determined PAD is referred to as the “worse-affected” limb, and the limb with ABI in the normal range is referred to as “less affected.” All subjects underwent graded treadmill testing and peak oxygen uptake (V̇O2) measurement.13,28,29 PAD subjects underwent needle biopsies of each gastrocnemius muscle at rest as described.6,13 Eight control subjects had a muscle sample taken from 1 leg, and 2 control subjects had a muscle sample taken from both legs. Samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. All protocols were approved by appropriate Institutional Review Boards, and subjects gave informed consent.

4977-bp Deletion Frequency

Total cellular DNA was extracted from 10 to 20 mg of frozen muscle by use of a QIAamp kit (Qiagen, Inc). Before polymerase chain reaction (PCR) amplification, DNA samples were digested with PstI (acting at nucleotide pairs 6910 and 9020) and HindIII (acting at nucleotide pairs 6203, 11680, and 12567) (Promega, Inc) for 16 hours at 37°C to linearize the mtDNA and cut the sequence within the 4977-bp deletion region.19 The digested DNA was extracted with phenol/chloroform, precipitated, and washed with ethanol, dissolved in Tris-HCl, 10 mmol/L, pH 9.0, and remeasured before dilution and use.

The proportion of total mtDNA containing a specific 4977-bp deletion (deletion of 8469 to 13447 nucleotide pairs16) was determined with a serial dilution PCR method modified from Corral-Debrinski et al.19 Two sets of PCR reactions were performed on each sample. For the first amplification, primers amplified a region not affected by the 4977-bp deletion or any other known mtDNA deletions (5′-TTC AAA TTC CTC CCT GTA CG-3′ [primer 1], complementary to bases 3108 to 3127, and 5′-TTG GCC TAC TGC TCG CAG TG-3′ [primer 2], complementary to bases 3701 to 3720). This amplification yields a 613-bp product from both wild-type and mtDNA containing the 4977-bp deletion and is termed the total mtDNA product. The second set of primers flanked the 4977-bp deletion and yielded a 593-bp product only if the 4977-bp deletion was present (5′-CTC TAG AGC CCA CTG TAA AGC TAA-3′ [primer 3], complementary to bases 8283 to 8305, and 5′-GGG GAC TGC TGC GTG TA-3′ [primer 4], complementary to bases 13832 to 13851). PCR amplifications (100 µL total volume) contained 200 µmol/L of each dNTP, 1.5 mmol/L MgCl2, 10 mmol/L Tris-HCl, and 50 mmol/L KCl, 0.3 µmol/L primers, and 5 U of Taq polymerase (Boehringer Mannheim). For PCR amplification of the 613-bp total mtDNA product, conditions were 2 minutes of denaturation at 94°C (reduced to 1 minute in the second cycle and 30 seconds in 33 subsequent cycles), 2 minutes of annealing at 51°C, and 2 minutes of polymerization at 72°C. After 35 cycles, a 7-minute extension at 72°C was performed. Amplification of the 593-bp deletion product was conducted under the same conditions except that annealing was performed at 56°C.19 Ethidium bromide-stained 2% agarose size-fractionation gels were photographed, and negative films were quantified with a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories). Each gel included a DNA mass ladder (Gibco BRL) in duplicate. The signal for the 800-bp fragment was used as a standard and to normalize the signal for each PCR product. For each sample, the PCR reactions were run at a minimum of 6 different DNA template concentrations (typically varying over the range of 2 to 200 ng per tube for the deletion amplification and 0.001 to 5 ng per tube for the total mtDNA amplification), which permitted generation of a product optical density (OD) versus template concentration curve. The ratio of the template DNAs required to yield the same amount of product was used as a measure of the relative amounts of deleted mtDNA versus total mtDNA in the sample (Figure 1).

This method demonstrated similar PCR amplification efficiencies for the deletion-specific and wild-type reactions, and no 593-bp deletion product was detected in DNA from young healthy subjects (<0.01% frequency). The coefficient of variation was found to be 20% when a single sample was run multiple times on separate days. The validated lower limit of detection was 0.01%, and thus all estimates that yielded deletion frequencies <0.01% were conservatively reported as 0.01%.

Total mtDNA Content

A competitive PCR strategy was used for the measurement of total mtDNA.26 A 500-bp internal standard was generated with a PCR-MIMIC construction kit from Clontech with a nonhomologous DNA fragment of known sequence. The internal standard fragment was generated with 2 rounds of PCR amplification reactions, the first with composite downstream primers and the second with mtDNA-specific primers. Composite (40-mers) upstream and downstream primers were designed such that each composite primer consisted of a 20-nucleotide base mtDNA template-specific sequence, followed by a 20-nucleotide base sequence complementary to the MIMIC DNA fragment (5′-TTC AAA TTC CTC CCT GTA CGC AGT TTC CTC CGC TG-3′, composite upstream primer 5, and 5′-TTG GCC TAC TGC TCG CAG TGG GTT TAC ATA CTC TGG AGC A-3′, composite downstream primer 6). The PCR product generated was then used in a second round of PCR amplification with mtDNA-specific primers (primers 1 and 2 used for the amplification of the 613-bp total mtDNA product, discussed above) to provide the internal standard.

Various amounts of internal standard DNA fragment (typically 6.0×108 to 6.0×109 molecules, including at least 6 different concentrations) were added to a known amount of total muscle DNA (typically 0.01 to 0.1 ng). PCR amplification was performed for 30 cycles with primers 1 and 2 under conditions for the amplification of the 613-bp mtDNA product as described above, except that the primer concentration was 0.6 µmol/L. This reaction thus yielded the 613-bp product from mtDNA and a 500-bp fragment from the competitor template. The PCR reaction products were separated on 2% agarose gel, negative films of the ethidium bromide stained gels were measured by densitometry, and the logs of the ratio of the
mDNA-specific product and internal standard ODs were plotted against the log of molecules of internal standard added to the reaction. We calculated the mDNA content by determining the x intercept for the point at which the ratio of the 613- and 500-bp products was 1 (log 0).

Enzyme Assays
Muscle homogenates (10 mg/mL) of frozen tissue prepared in 220 mmol/L mannitol, 70 mmol/L sucrose, 10 mmol/L MOPS, and 1% cholate (pH 7.0) were used for enzyme and protein assays. Citrate synthase activity was determined by the method of Srere,31 cytochrome c oxidase by the method of Wharton and Tzagoloff,32 and noncollagenase protein by the method of Lilienthal et al.33

Statistical Analysis
Data are expressed as mean±SEM. Student’s t test (paired) was used to compare data for the worse- and less-affected limbs of PAD patients, and unpaired t tests were used for comparison between PAD and control subjects. Analyses of the 4977-bp deletion frequency data were prospectively based on log-transformed values to yield a normally distributed population. The values for both legs were averaged before population analyses were performed for control subjects biopsied in each leg.

Results
All PAD and control subjects were male and of similar age (Table 1). PAD patients had a peak oxygen consumption only 50% that of the control subjects. The ABI of the less-affected limb in the PAD subjects did not differ from controls, whereas the worse-affected limb had an ABI 50% of the control subjects. The ABI of the less-affected limb was 0.50±0.05 in PAD patients as compared with 0.90±0.06 in control subjects (P<0.05). All PAD and control subjects were male and of similar age.

As expected, the gastrocnemius muscle from the control subjects showed a low (0.05%) 4977-bp deletion frequency (Table 2). In contrast, the less-affected PAD limbs showed an 8-fold higher deletion frequency than controls (P<0.05), and the worse-affected PAD limbs demonstrated a 17-fold higher deletion frequency than control subjects (P<0.01; Table 2).

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The frequency of the 4977-bp deletion in the worse-affected limbs from the PAD population was positively correlated with age (Figure 3). A similar correlation of lesser slope was seen in the less-affected limb (log deletion frequency=0.0468×age−5.78; r=0.735). The low deletion frequency in the control muscles precluded a meaningful correlation analysis in this population. There were no significant relationships between the 4977-bp deletion frequency in the worse-affected limbs and either the patients’ ABI or claudication-limited exercise performance.

Muscle mitochondrial expression is closely linked to total mDNA content.34,35 No significant differences in the total mtDNA content were found between control subjects and the less- or worse-affected limbs of PAD patients (Table 2). No differences in the activity of citrate synthase or cytochrome c

### TABLE 1. Characteristics of Control Subjects and PAD Patients

<table>
<thead>
<tr>
<th></th>
<th>Control (n=10)</th>
<th>PAD (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>61±3</td>
<td>63±4</td>
</tr>
<tr>
<td>Smoking status, n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smokers</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Former smokers</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>ABI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>1.10±0.02</td>
<td>1.09±0.03</td>
</tr>
<tr>
<td>After exercise, ND</td>
<td>Less: 1.00±0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Worse: 0.50±0.05†</td>
<td></td>
</tr>
<tr>
<td>Peak V̇O₂, mL·min⁻¹·kg⁻¹</td>
<td>28.4±2.0</td>
<td>14.3±0.8†</td>
</tr>
</tbody>
</table>

Less indicates less-affected limb; Worse, worse-affected limb; and ND, not determined.

Values are mean±SEM.

*P<0.05 less vs worse; †P<0.05 vs control.

### TABLE 2. Frequency of the 4977-bp mDNA Deletion

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>Less-Affected Limb</th>
<th>Worse-Affected Limb</th>
</tr>
</thead>
<tbody>
<tr>
<td>% mDNA with 4977-bp deletion</td>
<td>0.05±0.01</td>
<td>0.43±0.28†</td>
<td>0.88±0.33*</td>
</tr>
<tr>
<td>mDNA, molecules×10⁶/μg DNA</td>
<td>1.15±0.14</td>
<td>1.01±0.22</td>
<td>0.89±0.12</td>
</tr>
</tbody>
</table>

*P<0.05 compared with control values. Values are mean±SEM.
oxidase were detected in control subjects versus PAD-affected limbs or between the less- or worse-affected limbs of PAD patients (Table 3). However, the ratio of citrate synthase to cytochrome c oxidase was increased in muscle from the worse-affected PAD limbs compared with the least-affected limbs ($P<0.05$).

**Discussion**

Although PAD is a consequence of atherosclerosis-induced blood flow limitations, lower extremity hemodynamics do not predict peak exercise performance.$^{3,4,12}$ Metabolic changes have been defined in skeletal muscle of PAD patients with many features similar to those in mitochondrial myopathies.$^{5–8,10–13}$ The molecular basis for altered muscle metabolism in PAD is unknown. The current study demonstrates that the frequency of the 4977-bp mtDNA deletion is increased in PAD but is not specific for the ischemic limb.

The 17-fold increase in frequency of the 4977-bp deletion in the worse-affected limb of the PAD subjects compared with controls is consistent with the increased deletion frequency seen in ischemic myocardium$^{23,24}$ and the preliminary observations of Egawhary et al.$^{16}$ in nonmuscle cells in PAD. The most severely affected individual demonstrated a deletion frequency 88-fold higher than the control group mean (Figure 2) and 440-fold higher than the individuals at the low end of the control group range (data not shown). The 4977-bp deletion arises from alternate pairing during mitochondrial genome replication.$^{17}$ and the frequency of this deletion is increased in a variety of settings, including enhanced free radical generation.$^{24,37}$ Ischemia and ischemia/reperfusion are associated with increased free radical generation from the mitochondrial electron transport chain,$^{38,39}$ and PAD is associated with a state of increased oxidative stress.$^{26,27}$

There was a strong trend for a higher deletion frequency in the worse- versus less-affected limb in individual patients (2-fold higher absolute mean deletion frequency, $P=0.119$, with 6 of 8 paired limbs demonstrating the relationship; Figure 2). The current study was not powered to detect differences in deletion frequency of this small magnitude (a total of $\sim$20 patients would be required), and thus the lack of statistical significance may be misleading. Nonetheless, the major increase in mtDNA injury appears to be independent of local ischemia in the PAD population. Smoking is prevalent in the PAD population and has been hypothesized to accelerate mtDNA injury.$^{40}$ However, the control group contained smokers, and no difference in 4977-bp deletion frequency within the control group as a function of smoking was identified (data not shown). Alternatively, mtDNA injury in the less-affected limb may reflect subclinical arterial disease that is sufficient to induce injury mechanisms despite the lack of symptoms and ABIs in the normal range. This is unlikely given the strong relationship between the degree of mtDNA injury in the less-affected limb and the injury in the worse-affected muscle (Figure 2). Thus, the oxidative stress associated with PAD$^{26,27}$ appears to induce mtDNA damage in a distribution beyond the ischemic vascular bed, which may be further aggravated by ischemia in the affected limb.

Accumulation of mtDNA mutations occurs at a slow rate in normal aging.$^{25}$ PAD may be viewed as accelerating this process. In the PAD limb, the frequency of the 4977-bp deletion was correlated with the patient’s age (Figure 3). The 4977-bp deletion is only one of many deletion and point somatic mutations that have been identified in human mtDNA. Thus, the quantitative assessment of mtDNA injury demonstrated here does not reflect the full extent of mtDNA damage but rather the lower limit of injury and the relative degree of injury between samples.

Somatic mtDNA mutations have been associated with a number of degenerative diseases.$^{14,15}$ However, the causative role of the mtDNA injury in disease pathophysiology has not been well established in any clinical model. The patient with unilateral PAD illustrates the difficulty in interpreting mtDNA injury as a mechanism in disease pathophysiology. Patients with unilateral PAD clearly lateralize symptoms.

**Table 3. Enzyme Activities for Citrate Synthase and Cytochrome Oxidase**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>Less-Affected Limb</th>
<th>Worse-Affected Limb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate synthase, $\mu$mol $\cdot$ min$^{-1}$ $\cdot$ g protein$^{-1}$</td>
<td>133±13</td>
<td>108±10</td>
<td>145±22</td>
</tr>
<tr>
<td>Cytochrome c oxidase, $\min^{-1}$ $\cdot$ mg$^{-1}$</td>
<td>2.12±0.29</td>
<td>1.86±0.31</td>
<td>1.96±0.29</td>
</tr>
<tr>
<td>Citrate synthase/cytochrome c oxidase, $\mu$mol citrate synthase $\cdot$ min$^{-1}$</td>
<td>71±10</td>
<td>66±9</td>
<td>78±7*</td>
</tr>
</tbody>
</table>

* $P<0.05$ compared with less-affected limb. Values are mean±SEM.
hemodynamics, and biochemical abnormalities between the 2 limbs. Assessment of mtDNA injury in only the diseased limb might have suggested that mtDNA injury was relevant to the pathogenesis of the myopathy of PAD. However, examination of the less-affected limb makes this hypothesis less tenable. The high degree of mtDNA injury in the less-affected limb of some subjects and the overlap in deletion frequency in the less- versus worse-affected limb population (Figure 2) make a primary role for mtDNA injury less tenable. The high degree of mtDNA injury in the worse-affected limb might have suggested that mtDNA injury was relevant to the disease in PAD patients with unilateral disease. The trend is also seen in Table 3. In mitochondrial myopathies, expression of nuclear-encoded mitochondrial enzymes is often increased compared with mtDNA-encoded enzymes. Consistent with this concept in PAD patients, the ratio of citrate synthase (nuclear encoded) to cytochrome c oxidase (dependent on mtDNA-encoded subunits) activities was increased in the worse- versus less-affected limb. Again, the present study was not powered to detect differences in this ratio between the control and PAD limbs due to the greater intersubject versus intrasubject variability.

Although mitochondrial content varies in muscle with training and detraining, the relative amounts of each of the mitochondrial constituents remains constant. Citrate synthase activity is increased in PAD-affected muscle and this trend is also seen in Table 3. In mitochondrial myopathies, expression of nuclear-encoded mitochondrial enzymes is often increased compared with mtDNA-encoded enzymes. Consistent with this concept in PAD patients, the ratio of citrate synthase (nuclear encoded) to cytochrome c oxidase (dependent on mtDNA-encoded subunits) activities was increased in the worse- versus less-affected limb. Again, the present study was not powered to detect differences in this ratio between the control and PAD limbs due to the greater intersubject versus intrasubject variability.

In summary, PAD is associated with accumulation of mtDNA injury, as evidenced by the high 4977-bp mtDNA deletion frequency, but this injury is not limited to the worse-affected limb in patients with unilateral disease. mtDNA injury in the less-affected limb and the relative preservation of mtDNA content and cytochrome c oxidase activity make it unlikely that a simple model of mtDNA injury causing decreased expression is responsible for the metabolic derangements seen in PAD.

Acknowledgment

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References


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