Decreased NADH dehydrogenase and ubiquinol-cytochrome c oxidoreductase in peripheral arterial disease

ERIC P. BRASS,1 WILLIAM R. HIATT,2 ANDREW W. GARDNER,3 AND CHARLES L. HOPPEL4
1Department of Medicine, Harbor-University of California Los Angeles Medical Center, Torrance, California 90509; 2Section of Vascular Medicine, Divisions of Geriatrics and Cardiology, University of Colorado Health Sciences Center, and The Colorado Prevention Center, Denver, Colorado 80203; 3Division of Gerontology, University of Maryland, and Geriatric Research and Education Clinical Center, Baltimore Veterans Affairs Medical Center, Baltimore, Maryland 21201; and 4Departments of Pharmacology and Medicine, Case Western Reserve University, and Geriatric Research and Education Clinical Center, Cleveland Veterans Affairs Medical Center, Cleveland, Ohio 44106

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Brass, Eric P., William R. Hiatt, Andrew W. Gardner, and Charles L. Hoppel. Decreased NADH dehydrogenase and ubiquinol-cytochrome c oxidoreductase in peripheral arterial disease. Am J Physiol Heart Circ Physiol 280: H603–H609, 2001.—Peripheral arterial disease (PAD) is associated with muscle metabolic changes that may contribute to the disability in these patients. However, the biochemical defects in PAD have not been identified. The present study was undertaken to test the hypothesis that PAD is associated with specific defects in skeletal muscle electron transport chain activity. Seventeen patients with PAD and nine age-matched controls underwent gastrocnemius muscle biopsies. There were no differences in the mitochondrial content per gram of skeletal muscle as assessed by citrate synthase activity between the PAD patients and the control subjects. Skeletal muscle NADH dehydrogenase activity was decreased by 27% compared with controls when expressed per unit of citrate synthase activity. Expression of enzyme activities normalized to cytochrome c-oxygen oxidoreductase activity confirmed a 26% decrease in NADH dehydrogenase activity and also demonstrated a 38% decrease in ubiquinol-cytochrome c oxidoreductase activity. Thus PAD is associated with specific changes in muscle mitochondrial electron transport chain activities characterized by relative decreases in NADH dehydrogenase and ubiquinol-cytochrome c oxidoreductase activities, which may contribute to the metabolic abnormalities and decreased exercise performance in these patients.

mitochondria; exercise

PERIPHERAL ARTERIAL DISEASE (PAD) is a common atherosclerotic disorder associated with substantial physical disabilities. Patients with PAD frequently suffer from muscle pain with exercise (claudication), which limits their ambulatory activity. While the initial process in PAD is clearly atherosclerotic, peripheral hemodynamics are poor predictors of claudication-limited treadmill exercise performance (11, 27). PAD-induced changes in skeletal muscle metabolism have been hypothesized to contribute to the pathophysiology of claudication (7).

Skeletal muscle from patients with PAD demonstrates a number of abnormalities consistent with metabolic dysfunction. Intermediates of oxidative metabolism accumulate, and the magnitude of accumulation correlates with functional capacity (16, 18). The kinetics of respiration as assessed by 31P magnetic resonance spectroscopy and O2 uptake (VO2) kinetics are perturbed in PAD (3, 22). Taken together, the observations in PAD are analogous to those seen in mitochondrial myopathies and suggest the presence of a functionally significant acquired metabolic myopathy (6, 7).

A specific biochemical defect in the muscle mitochondria of PAD has not been identified. Muscle content of mitochondria as assessed by mitochondrial DNA content, cytochrome c oxidase activity, and citrate synthase activity is normal or increased in PAD (5, 8, 37). In an analogous system, myocardial ischemia and ischemia-reperfusion have been associated with deficits in electron transport chain function thought to result from oxidative injury to critical polypeptides or membrane lipids (16, 29, 30, 35). PAD is also associated with increased oxidative stress (4, 19), and the repetitive ischemia with exercise in these patients parallels the ischemia in heart models.

The present study was undertaken to test the hypothesis that PAD is associated with specific defects in the mitochondrial electron transport chain. The results demonstrate relative decreases in NADH dehydrogenase and ubiquinol-cytochrome c oxidoreductase activities in skeletal muscle of patients with PAD.

METHODS

Patients. A total of 17 patients with PAD and 9 healthy controls were recruited and evaluated at the Maryland Veterans Affairs Health Care System or the University of Colo-

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rado Health Services Center. Patients with PAD were identified on the basis of an appropriate history of claudication and an abnormal ankle-brachial index (ABI), as previously described (15). All data refer to the limb with the lowest ABI in each patient. PAD patients had no exercise limitations other than claudication. Healthy controls were consecutive, unselected volunteers who provided informed consent, were >45 yr of age, were able to perform treadmill exercise, were healthy on the basis of history (including the absence of diabetes, claudication, or other cardiopulmonary disease), and had normal ABI measurements (>0.95).

All participants were characterized with respect to age, gender, race, height, weight, and smoking history. Patients were classified as smokers, as former smokers (history of smoking, but had not smoked during the preceding 12 mo), or as never having smoked. The average number of packs per day smoked and the total number of years of smoking were recorded to estimate the cumulative pack-year history of smoking. Body mass index was calculated as weight (kg)/[height (m)]².

Peak $V\dot{O}_2$ in control subjects ($V\dot{O}_2_{max}$) and PAD patients (claudication − limited peak $V\dot{O}_2$) were determined using progressive treadmill exercise, as previously described (10, 15). Gastrocnemius muscle biopsies were obtained using a Bergstrom needle and immediately frozen in liquid nitrogen, as previously described (17, 18).

The appropriate institutional review boards approved all procedures. Written informed consent was obtained from each participant.

**Enzyme assays.** Homogenates of frozen skeletal muscle (10 mg wet wt/ml) were prepared in 200 mM mannitol, 70 mM sucrose, 2 mM EDTA, 1% potassium cholate, and a protease inhibitor cocktail (pH 7.4) using a Polytron homogenizer. Homogenates were kept on ice, and assays were completed on the day of homogenation.

Electron transport chain activities were measured as specific donor-acceptor oxidoreductase activities. Donors and acceptors were chosen to span specific regions of the complete electron transport chain (Fig. 1). Thus NADH dehydrogenase reflects the proximal activity of complex I, NADH-cytochrome $c$ oxidoreductase measures activity of complexes I and III, succinate dehydrogenase is a measure of the proximal activity of complex II, succinate-cytochrome $c$ oxidoreductase measures activity of complexes II and III, ubiquinol-cytochrome $c$ oxidoreductase is a measure of complex III activity, and cytochrome $c$-oxygen oxidoreductase (or cytochrome oxidase) is a measure of complex IV activity. Citrate synthase (a non-electron transport chain mitochondrial enzyme) was measured as a marker of mitochondrial content.

Specific spectrophotometric enzyme assays were completed as previously described (21, 23, 24). NADH-cytochrome $c$ oxidoreductase activity was measured as the rotenone-sensitive reductase and succinate-cytochrome $c$ oxidoreductase as the antimycin-sensitive reductase. Ubiquinol-cytochrome $c$ oxidoreductase was measured as the antimycin-sensitive decyubiquinol-cytochrome $c$ oxidoreductase. Cytochrome $c$ oxidase activity was expressed as the first-order rate constant, as described by Wharton and Tzagoloff (38). Citrate synthase activity was determined using the method of Srere (33). All assays were completed for each biopsy, except in three biopsies from PAD patients. For these three patients, insufficient sample precluded completing all assays, and the cytochrome $c$ oxidase, NADH dehydrogenase, or succinate dehydrogenase assay was not performed on the biopsy (1 assay was omitted for each of the 3 biopsies).

**Statistical analyses.** Unpaired $t$-tests were used to compare variables between the PAD and control groups. Linear regression analysis was used to assess the relationship between biochemical and physiological parameters. Statistical significance was set at $P < 0.05$ (2-tailed). Values are means ± SE except where noted.

**RESULTS**

Nine control subjects and 17 patients with PAD underwent assessment of peripheral hemodynamics, maximal exercise testing, and needle biopsy of a gastrocnemius

![Fig. 1. Electron transport chain. Reducing equivalents from NADH are removed by the NADH dehydrogenase (NADH DH) in complex I and from succinate by the succinate dehydrogenase (SDH) of complex II. Electrons flow from complex I or II to convert ubiquinone to ubiquinol, which donates them to complex III. Cytochrome $c$ accepts electrons from complex III and transfers them to complex IV. Complex IV is responsible for the conversion of $O_2$ to water. Enzymatic activities in the electron transport chain can be measured as specific oxidoreductase activities on the basis of the specific donor and acceptor sites used. Thus NADH-cytochrome $c$ oxidoreductase is a measure of complex I and III function, succinate-cytochrome $c$ oxidoreductase is a measure of the function of complexes II and III, ubiquinol-cytochrome $c$ oxidoreductase is specific for complex III, and cytochrome $c$-oxygen oxidoreductase (or cytochrome $c$ oxidase) is a measure of complex IV function. NADH DH and SDH measure the initial portion of complexes I and II, respectively, by using NADH and succinate as donors but artificial acceptors.](http://ajpheart.physiology.org/)}
muscle from the most hemodynamically affected limb as determined by the ABI. The control and PAD groups were well matched for age, gender, race, and body mass index, except the PAD group included a higher proportion of African-Americans (Table 1). The PAD population included more smokers and, as expected, had a lower ABI in the biopsied limb. The maximal, claudication-limited \( V_\text{O}_2 \) in the PAD group was only 67% of the maximal \( V_\text{O}_2 \) in the control population.

Citrate synthase \([19.8 \pm 3.1 \text{ and } 21.4 \pm 2.0 \text{ mmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}\] in controls \((n = 9)\) and PAD patients \((n = 17)\), respectively\) and cytochrome \(c\) oxidase \([180 \pm 50 \text{ and } 160 \pm 10 \text{ min} \cdot \text{g}^{-1}\] in controls \((n = 9)\) and PAD patients \((n = 16)\), respectively\) activities did not differ between the control and PAD populations. Thus, consistent with standard practice, these activities were used as measures of mitochondrial content to normalize other electron transport chain activities.

The activity of NADH dehydrogenase per citrate synthase activity was reduced by 27% in PAD patients compared with controls (Table 2). Ubiquinol-cytochrome \(c\) oxidoreductase (Fig. 2) and succinate dehydrogenase (Table 2) activities tended to be lower in PAD patients (Fig. 2), while succinate-cytochrome \(c\) oxidoreductase activity was 38% higher \((P < 0.10)\) in PAD than in control muscle (Table 2).

### Table 1. Patient demographics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n)</td>
<td>9</td>
<td>17</td>
</tr>
<tr>
<td>Age, a, yr</td>
<td>68(46–77)</td>
<td>71(51–82)</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>8/1</td>
<td>15/2</td>
</tr>
<tr>
<td>Race, white/African-American</td>
<td>8/1</td>
<td>11/6</td>
</tr>
<tr>
<td>Body mass index, a, kg/m(^2)</td>
<td>29.4(20.8–31.0)</td>
<td>26.6(21.6–41.7)</td>
</tr>
<tr>
<td>Smoking, f</td>
<td>never/former/current</td>
<td>3/2/4</td>
</tr>
<tr>
<td>Smoking history, a, b pack-yr</td>
<td>21.6(12–64)</td>
<td>42.2(23–96)</td>
</tr>
<tr>
<td>ABI c</td>
<td>1.13</td>
<td>0.64 ± 0.44</td>
</tr>
<tr>
<td>Peak (V_\text{O}_2), a, d ml·min(^{-1})·kg(^{-1})</td>
<td>21.4(16.5–32.3)</td>
<td>14.4(9.3–24.3)</td>
</tr>
</tbody>
</table>

a Values are means, with range in parentheses. b Includes only patients with smoking history. c Resting ankle-brachial index (ABI, means ± SE) of biopsied limb. d Maximum \(O_2\) consumption (\(V_\text{O}_2\)) for control and PAD groups.

### Table 2. Mitochondrial enzyme activities normalized to citrate synthase activity

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH dehydrogenase</td>
<td>3.35 ± 0.34(9)</td>
<td>2.45 ± 0.15(16)</td>
</tr>
<tr>
<td>NADH-cytochrome (c) oxidoreductase</td>
<td>0.099 ± 0.035(9)</td>
<td>0.072 ± 0.013(17)</td>
</tr>
<tr>
<td>Succinate-cytochrome (c) oxidoreductase</td>
<td>0.108 ± 0.011(9)</td>
<td>0.151 ± 0.014†(17)</td>
</tr>
<tr>
<td>Ubiquinol-cytochrome (c) oxidoreductase</td>
<td>1.47 ± 0.33(9)</td>
<td>0.95 ± 0.18(17)</td>
</tr>
<tr>
<td>Cytochrome (c) oxidase</td>
<td>9.34 ± 1.70(9)</td>
<td>7.85 ± 0.62(16)</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>0.083 ± 0.010(8)</td>
<td>0.063 ± 0.005†(17)</td>
</tr>
</tbody>
</table>

Values are means ± SE of number of observations in parentheses. Units are dimensionless except for cytochrome \(c\) oxidase, which is in mmol·min\(^{-1}\)·g\(^{-1}\)·min\(^{-1}\)·g\(^{-1}\). *\(P < 0.05\) (2-tailed); †0.1 < \(P < 0.05\) (2-tailed).

When expressed per cytochrome \(c\) oxidase activity, the activity of NADH dehydrogenase and ubiquinol-cytochrome \(c\) oxidoreductase was 26 and 38% lower, respectively, in muscle from PAD patients than in muscle from healthy controls \((P < 0.05); \text{Figs.}\ 2B\ \text{and}\ 3\). When expressed per citrate synthase activity, succinate-cytochrome \(c\) oxidoreductase activity per cytochrome \(c\) oxidase activity was 50% higher in PAD patients than in healthy controls \((P < 0.05); \text{Fig.}\ 3\).

Smoking status (current, former, or never) did not have demonstrable effects on any of the electron transport chain activities in the control population, although the number of subjects in each group was small. Similarly, smoking status in PAD patients did not affect the mean NADH dehydrogenase activity normalized per citrate synthase activity \([2.39 ± 0.23 \text{ and } 2.53 ± 0.20\) in former \((n = 9)\) and current \((n = 7)\) smokers,
respectively, \( P = 0.667 \) or per cytochrome \( c \) oxidoreductase activity \([0.32 \pm 0.02 \text{ and } 0.30 \pm 0.04 \text{ mmol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}\cdot\text{min}^{-1}\cdot\text{g}^{-1}\) in former \((n = 9)\) and current \((n = 6)\) smokers, respectively, \( P = 0.726 \). Ubiquinol-cytochrome \( c \) oxidoreductase activity in PAD subjects was also not influenced by smoking status when normalized to citrate synthase activity \([0.78 \pm 0.25 \text{ and } 1.18 \pm 0.26 \text{ in former } (n = 10) \text{ and current } (n = 7) \text{ smokers, respectively}, \ P = 0.288 \) or to cytochrome \( c \) oxidoreductase activity \([0.092 \pm 0.017 \text{ and } 0.119 \pm 0.024 \text{ mmol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}\cdot\text{min}^{-1}\cdot\text{g}^{-1}\) in former \((n = 10)\) and current \((n = 6)\) smokers, respectively, \( P = 0.377 \). To evaluate the effects of smoking on enzyme activities, former smokers were compared with current smokers, regardless of PAD status. There was no effect of smoking on NADH dehydrogenase activity per citrate synthase activity \([2.58 \pm 0.23 \text{ and } 2.70 \pm 0.27 \text{ in former } (n = 11) \text{ and current } (n = 10) \text{ smokers, respectively}, \ P = 0.732 \]. Similar findings were observed for ubiquinol-cytochrome \( c \) oxidoreductase activity per citrate synthase activity \([0.90 \pm 0.23 \text{ and } 1.27 \pm 0.24 \text{ in former } (n = 12) \text{ and current } (n = 10) \text{ smokers, respectively}, \ P = 0.281 \).

Mitochondrial content per gram wet weight of muscle, as assessed by citrate synthase or cytochrome \( c \) oxidase activity, was strongly correlated with peak rates of \( \dot{V}O_2 \) in controls but not PAD patients (Table 3). Peak rate of \( \dot{V}O_2 \) in the control subjects was also correlated with age \((r = 0.777, P < 0.05)\), but no similar relationship was observed in the PAD population \((r = 0.055)\). Neither mitochondrial content as assessed by citrate synthase activity nor the NADH dehydrogenase or ubiquinol-cytochrome \( c \) content expressed per mitochondrial marker activity was correlated with claudication-limited peak rates of \( \dot{V}O_2 \) or ABI in the PAD patients (Table 3).

**DISCUSSION**

The exercise limitation in PAD is not well predicted by hemodynamics, indicating a complex pathophysiology of claudication \((11, 27)\). For example, patients with

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### Table 3. Relationship between mitochondrial enzyme activities and physiological parameters

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variable</th>
<th>Univariate Correlation Coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Peak ( \dot{V}O_2 )</td>
<td>Cytochrome ( c )-oxygen oxidoreductase</td>
<td>0.791*</td>
</tr>
<tr>
<td>Peak ( \dot{V}O_2 )</td>
<td>Citrate synthase</td>
<td>0.733*</td>
</tr>
<tr>
<td>Peak ( \dot{V}O_2 )</td>
<td>NADH-dehydrogenase normalized to citrate synthase</td>
<td>0.299</td>
</tr>
<tr>
<td>Peak ( \dot{V}O_2 )</td>
<td>Ubiquinol-cytochrome ( c ) oxidoreductase to cytochrome oxidase</td>
<td>0.364</td>
</tr>
<tr>
<td>ABI</td>
<td>Citrate synthase</td>
<td>0.364</td>
</tr>
<tr>
<td>ABI</td>
<td>NADH-dehydrogenase normalized to citrate synthase</td>
<td>0.134</td>
</tr>
<tr>
<td>ABI</td>
<td>Ubiquinol-cytochrome ( c ) oxidoreductase to cytochrome oxidase</td>
<td>0.159</td>
</tr>
</tbody>
</table>

\*\( P < 0.05 \).
PAD have many features consistent with an acquired metabolic myopathy, including accumulation of metabolic intermediates and perturbed regulation of mitochondrial respiration in the affected muscle of PAD patients (3, 6, 7, 16, 18, 22). The present studies confirm no difference between PAD patients and healthy subjects in muscle mitochondrial content as assessed by marker enzymes. However, the activities of key components of the electron transport chain are decreased per mitochondrial content in the muscle of patients with PAD.

The activity of NADH dehydrogenase (the initial enzyme activity in the NADH-ubiquinone oxidoreductase or complex I of the electron transport chain, Fig. 1) expressed per either of the mitochondrial markers, citrate synthase or cytochrome c oxidase, was decreased in PAD (Table 2, Fig. 3). Ubiquinol-cytochrome c oxidoreductase (complex III of the electron transport chain) was statistically decreased in PAD per cytochrome c oxidoreductase activity, with a similar trend when expressed per citrate synthase activity. Review of the ubiquinol-cytochrome c oxidoreductase activity for individual subjects expressed per either marker (Fig. 2) emphasizes the frequency of very low complex III activities in the PAD group. For example, 11 of 17 PAD subjects had a value <1.0 vs. 3 of 9 controls expressed per citrate synthase, and 9 of 16 PAD patients had a value <0.1 vs. 1 of 9 controls expressed per cytochrome c oxidase activity. These changes appear specific, inasmuch as succinate-cytochrome c oxidoreductase activity was increased per mitochondrial marker activity in PAD patients vs. controls. Importantly, changes in the relative activities of electron transport chain components may lead to increased electrochemical gradients and free radical leakage.

Patients with claudication have normal skeletal muscle blood flow at rest but develop muscle ischemia with exercise. Normal blood flow is restored when the patient discontinues exercise secondary to claudication. This creates an environment for ischemia-reperfusion injury analogous to that seen in the heart, and PAD is well documented to be associated with increased oxidative stress (4, 19). Interestingly, complexes I and III have been suggested to be vulnerable to free radical injury in several models, including ischemia-reperfusion (29, 30, 35, 36). NADH dehydrogenase is vulnerable to oxidative inactivation in free radical-generating systems (40). Structural studies have suggested that the NADH dehydrogenase component of complex I may be exposed to the matrix environment, while the remainder of the complex is embedded in the membrane (12). This may contribute to a unique vulnerability to oxidative injury. Complex III is a major source of mitochondrial free radical leakage and has been shown to be damaged in cardiac ischemia-reperfusion (37, 39). Thus the observed decreased electron transport chain activities in PAD may result from oxidative damage and contribute to a positive-feedback injury, inasmuch as impaired electron transport function results in increased free radical leakage (1, 31).

Smoking is associated with increased oxidative stress (27a), and this may contribute to cellular injury. Inasmuch as the PAD group in the present study was enriched with current and former smokers compared with the control group, smoking may have contributed to the changes in enzyme activities. Analysis of subgroups in the control (smokers vs. nonsmokers) and PAD (current smokers vs. former smokers) groups did not identify any effects of smokers on the activities of interest. Nonetheless, because of the small number of subjects and the imbalance between the control and PAD groups in smoking history, a contribution from smoking to the changes observed cannot be excluded.

The clinical and pathophysiological significance of the changes in enzyme activities observed here is unknown. Nonetheless, the magnitude of decreases in relative NADH dehydrogenase (26–27%) and ubiquinol-cytochrome c oxidoreductase (38%) activities in PAD are similar to those observed in other degenerative disorders, such as Parkinson’s disease. In these diseases, oxidative stress and impaired mitochondrial function are hypothesized to be important components of the pathophysiology (13, 32). Our understanding of the integrated regulation of oxidative metabolism in conditions such as exercise is inadequate to predict how changes of this magnitude would affect muscle function or oxidative metabolism. Nonetheless, Taylor and colleagues (34) reported that as little as 5% inhibition of complex III resulted in inhibition of succinate oxidation by intact rat muscle mitochondria, despite no effect on succinate-cytochrome c oxidoreductase activity. Similarly, Rossignol and colleagues (28) reported that a 15% inhibition of complex III activity in muscle mitochondria was associated with impaired respiration with pyruvate as substrate. Thus decreases in enzyme activities of the magnitude identified in PAD may impair mitochondrial respiration.

In contrast to NADH dehydrogenase and ubiquinol-cytochrome c oxidoreductase activities, the relative succinate-cytochrome c oxidoreductase activity is increased in PAD (Table 2, Fig. 3). This appears discrepant, inasmuch as succinate-cytochrome c oxidoreductase activity is dependent on ubiquinol-cytochrome c oxidoreductase activity (Fig. 1), which is decreased in PAD when normalized to cytochrome c oxidase activity. However, succinate-ubiquinone oxidoreductase activity may be rate limiting under the conditions of the succinate-cytochrome c oxidoreductase assay, masking the 38% decrease in the specific complex III activity. Consistent with this postulate, Taylor and colleagues (34) reported that 30–50% inhibition of complex III was required to inhibit succinate-cytochrome c oxidoreductase activity. Also, the absolute succinate-cytochrome c oxidoreductase activity was only 10% of the ubiquinol-cytochrome c oxidoreductase activity in control.
trol muscle (Fig. 3, note the 10-fold correction in succinate-cytochrome c oxidoreductase activity). Inasmuch as succinate dehydrogenase activity was not increased with PAD, the findings suggest a specific increase in the succinate-ubiquinone oxidoreductase complex distal to the dehydrogenase. Increased succinate-cytochrome c oxidoreductase activity has also been observed in congenital defects of the electron transport chain (2).

Mitochondrial content per gram of skeletal muscle as assessed by mitochondrial DNA content (37), citrate synthase activity (5, 37), or cytochrome c oxidase activity (5) does not differ between PAD patients and controls. Additionally, citrate synthase activity per mitochondrial content in PAD is observed, despite a 30–50% decrease of mitochondrial content. This normal mitochondrial content validates the use of this enzyme activity as a marker of mitochondrial content. Additionally, citrate synthase activity per mitochondrial DNA content is unaffected by PAD (37), validating the use of this enzyme activity as a marker of mitochondrial content. This normal mitochondrial content in PAD is observed, despite a 30–50% decrease in claudication-limited peak V\textsubscript{O2} compared with maximal rates of V\textsubscript{O2} in healthy controls (37). Maximal aerobic exercise capacity is a major determinant of mitochondrial content in healthy individuals (38) (Table 3), but regulation in PAD must include other factors.

In summary, PAD is associated with specific, relative defects in NADH dehydrogenase and ubiquinol-cytochrome c oxidoreductase activity in skeletal muscle. These enzyme changes are consistent with the hypothesis that impaired electron transport chain function contributes to the metabolic dysfunction and oxidative stress in PAD.

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