Mitochondrial complex I defect resulting from exercise-induced lower limb ischemia in patients with peripheral arterial disease

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Mitochondrial complex I defect resulting from exercise-induced lower limb ischemia in patients with peripheral arterial disease. J Appl Physiol 125: 938–946, 2018. First published May 24, 2018; doi:10.1152/japplphysiol.00059.2018.—This study aims to compare the structural and mitochondrial alterations between muscle segments affected by exercise-induced ischemia and segments of the same muscle without ischemia, in the same subject. In a prospective analysis, 34 patients presenting either peripheral arterial disease or chronic coronary syndrome without any evidence of peripheral arterial disease were eligible for inclusion based on findings indicating a need for either a femoro-popliteal bypass or a saphenous harvesting for coronary bypass. Before surgery, we assessed the level of exercise-induced ischemia in proximal and distal sections of the thigh by the measurement of transcutaneous oxygen pressure during an exercise treadmill test. Distal and proximal biopsies of the sartorius muscle were procured during vascular surgical procedures to assess mitochondrial function and morphometric parameters of the sartorius myofibers. Comparisons were made between the distal and proximal biopsies, with respect to these parameters. Thirteen of the study patients that initially presented with peripheral arterial disease had evidence of an isolated distal thigh exercise-induced ischemia, associated with a 35% decrease in the mitochondrial complex I enzymatic activity in the distal muscle biopsy. This defect was also associated with a decreased expression of the manganese superoxide dismutase enzyme and with alterations of the shapes of the myofibers. No functional or structural alterations were observed in the patients with coronary syndrome. We validated a specific model ischemia in peripheral arterial disease characterized by muscular alterations. This “Distal-Proximal-Sartorius Model” would be promising to explore the physiopathological consequences specific to chronic ischemia.

NEW & NOTEWORTHY We compared proximal versus distal biopsies of the sartorius muscle in patients with superficial femoral artery stenosis or occlusion and proof of, distal only, regional blood flow impairment with exercise oximetry. We identified a decrease in the mitochondrial complex I enzymatic activity and antioxidant system impairment at the distal level only. We validate a model to explore the physiopathological consequences of chronic muscle ischemia.

INTRODUCTION

Peripheral artery disease (PAD) is a major public health challenge. Most patients remain asymptomatic, partially because of their low levels of physical activity (4). When symptomatic, most patients experience claudication (Fontaine stage 2, Rutherford Grade 1). At equivalent levels of arterial impairment, the level of physical activity of patients is likely to be the major determinant of the biochemical modifications and adaptations of the muscle (27). Furthermore, when the consequences of an arterial occlusion are estimated, a precise quantification of any impairment of blood flow below the lesion, particularly during exercise, is of major importance because the collateral circulation can compensate for the occlusion of the arterial trunk. An ideal approach to studying the pathophysiology of exercise-induced ischemia in muscle, in patients with PAD and claudication, should address the following: 1) compare a muscle of patient with PAD to the same muscle not subjected to exercise-induced ischemia (but undergoing the same level of activity and physical strain), and 2) provide proof of and quantify the exercise-induced blood flow impairment at the level of muscle biopsies.

The sartorius muscle, the longest muscle in the human body, is innervated by a single nerve arising from a branch of the femoral nerve, with a heterogeneous vascularization. The proximal portion of the muscle is perfused by branches of the profunda femoris artery, while the distal portion is perfused by branches of the superficial femoral artery (7). The maximum length of each single sartorius muscle fiber does not exceed one-third of the muscle’s total length (14). Additionally, all of the muscular fibers support the same intensity and volume of contractions in the muscle but may differ in terms of their blood supply. The superficial femoral artery is one of the most commonly affected arterial trunks in patients with PAD. Its occlusion may induce ischemia in the distal segment of the sartorius muscle during exercise, while no changes in perfusion occur in the proximal part of this muscle. Animal models inducing acute or chronic ischemia in the sartorius are available (28), but, to the best of our knowledge, no such study is available to date in human patients with PAD. The proximal...
and distal portions of the sartorius muscle are readily accessible for biopsies during femoro-popliteal bypasses or saphenous vein harvesting.

Our aim was to study the consequences of chronic ischemia on the human sartorius muscle by assessing the mitochondrial function and morphology of myofibers. We compared biopsy specimens from ischemic (exercise-induced blood flow impairment in the distal thigh) and nonischemic (absence of blood flow impairment in the proximal thigh) areas of the sartorius muscle in patients with PAD presenting with superficial femoral severe stenosis or occlusion and claudication.

MATERIAL AND METHODS

We performed a prospective, single-center study. From October 2013 to October 2016, selected patients scheduled for cardiovascular surgery at the University Hospital of Angers (France) were recruited. These patients were fully informed as to the study and its procedures, and all patients signed a written consent document. This research and all procedures were performed in compliance with the principles outlined in the Declaration of Helsinki. The study was approved by our institutional Ethic Committee and registered in ClinicalTrials.gov under Ref. No. NCT02834351.

Experimental Design

The studied population consisted of two groups:

1) The ISCH (ischemia) group comprising selected patients with Rutherford grade 1 (Fontaine stage 2) symptomatic peripheral arterial disease (PAD) and were scheduled for arterial femoro-popliteal bypass. Patients were included if the initial assessment showed a superficial femoral stenosis or occlusion with proof of exercise-induced ischemia at the distal but not at the proximal level of the thigh.

2) The CONT (control) group comprising selected patients suffering from chronic coronary artery disease (CAD) and scheduled for coronary artery surgery with saphenous vein harvesting for venous bypass. These patients served as controls, so they were included only if there was no evidence of PAD, exertional limb pain, or exercise-induced ischemia at the distal and proximal levels of the thigh.

All patients were over 18 yr of age, and there was no maximum age limit. Exclusion criteria were pregnancy, any legal constraint, or current participation in another clinical trial.

Initial Assessment

At the time of the initial assessment, we recorded the patients’ body weight, height, sex, comorbidities, previous vascular interventions for lower limb PAD, smoking habits, and treatment history from the patients’ medical records. For all patients, noninvasive vascular examinations were performed including 1) ankle to brachial systolic pressure index (ABI) calculated from the ankle and brachial systolic blood pressure measurements using Doppler methods, and 2) an analysis of any radiological images confirming the presence of a stenosis or occlusion of the superficial femoral arteries (in patients with PAD) or any recordings of Doppler-sonography of the superficial femoral artery (in patients with chronic CAD). PAD was defined by the presence of a severe stenosis or occlusion at the superficial femoral level, and an ABI <0.90. The absence of PAD was defined by an absence of stenosis or occlusion at the superficial femoral level and an ABI >0.90 on both legs. The evaluations of exercise-induced ischemia were estimated using transcutaneous oximetry (Ex-TcPO2) obtained during treadmill exercise, as previously described (1, 2). TcPO2 is a useful technique that measures the local skin oxygen partial pressure under probes heated to 44.5°C to improve local perfusion and oxygen transcutaneous diffusion. Ex-TcPO2 provides evidence of exercise-induced regional blood flow impairment (RBFI) at various levels (1, 2, 5). We positioned one probe on the proximal thigh (facing the proximal portion of the sartorius muscle), another probe 5–10 cm above the knee joint at the anterior and internal distal part of the thigh (facing the distal portion of the sartorius muscle), and a probe on the chest for reference. Other probes were eventually used on the buttock and the calf. The treadmill tests were conducted until maximum pain was recorded in patients with PAD and for a maximum of 15 min in patients suffering from chronic CAD. From the TcPo2 recorded values, we calculated the decrease from rest of oxygen pressure (DROP) at each limb site as previously reported (1, 2). The minimum value of the DROP is proportional to the regional severity of RBFI (1). The presence or absence of ischemia was defined as a DROP lower or higher than −15 mmHg, respectively.

Muscle biopsies. During surgery, distal and proximal biopsies of the sartorius muscle were obtained by vascular surgeons before arterial clamping (patients with PAD) or saphenous vein harvesting (patients with CAD). Distal and proximal sartorius samples were procured from above the popliteal space and at the femoral triangle, respectively. The muscle biopsies were performed using a scalpel blade (electrocautery for excision was avoided) during the leg surgery under general anesthesia. The volume of each sample was ~1 cm3. Biopsies were immediately frozen in liquid nitrogen and kept at −80°C before analysis.

Ex vivo analyses. Ex vivo investigations of muscle biopsies were performed in the department of Biochemistry and Genetics at University Hospital in Angers in order to characterize the mitochondrial function and the morphological structure of the muscular fibers. Biologists performing ex vivo analyses were blinded to the results of clinical investigations. Any differences observed between the distal and proximal biopsies taken from the same sartorius muscle were analyzed.

Citrate synthase and complexes I, II, III, and IV enzymatic activities. Each muscle fragment was weighed and then homogenized in 10 times its weight in mannitol buffer with a glass–glass Potter in a cold room. Centrifugation was performed at 650 g and 4°C for 20 min. The supernatant was decanted and retained; the pellet was suspended in 10 vol of mannitol buffer and subjected to the same procedure. Both supernatants were pooled and used for the assays. The protein concentration was measured using the BCA protein assay kit (Thermo Scientific). The activities were measured according to standard methods at 37°C with a UV mc2 spectrophotometer (SAFAS), as validated in our laboratory (22).

Western blot analysis. The tissue preparation was performed as explained above for the analysis of the mitochondrial activity. The muscle homogenate proteins were denatured at 50°C using mercaptoethanol, after the addition of a 1% protease inhibitor cocktail (Sigma-Aldrich). For the detection of nicotinamide adenine dinucleotide ubiquinone oxidoreductase subunit B6 (NDUFB6), a subunit of complex I (CI), cytoplasmic copper- and zinc-containing superoxide dismutase (Cu-ZnSOD) and mitochondrial manganese-dependent superoxide dismutase (MnSOD), as well as α-tubulin and voltage-dependent anion channel (VDAC) proteins (loading controls) were separated by SDS-PAGE using a 12.5% SDS-Tris gel and 20 μg of the proteins per well. The separated proteins were transferred onto an immunoblot nitrocellulose membrane (Trans Blot Turbo; Bio-Rad). The membrane was blocked for 2 h at room temperature (Odyssey blocking buffer; Li-Cor) and then incubated overnight in a cold room with a cocktail of monoclonal antibodies at a 1:1000 dilution. Rabbit anti-superoxide dismutase 1 (SOD1; ab13533; Abcam, Paris, France) and rabbit anti-superoxide dismutase 1 (SOD1; ab13499; Abcam) were used for antioxidant enzyme detection. Mouse anti-NDUF6 (ab68331; Abcam) was used for CI detection, and anti-rabbit α-tubulin (ab59680; Abcam) and anti-mouse VDAC (ab61273; Abcam) were used as loading controls. Fluorescent secondary antibodies at a 1:10,000 dilution (rabbit anti-mouse 680 nm and goat anti-rabbit 790 nm; Abcam) were incubated for 2 h at room temperature, and the fluorescence detection was performed using a Li-Cor Odyssey appa-
results (Li-Cor Biotechnology, Bad Homburg, Germany) with a dual wavelength fluorescence detection (700 and 800 nm). The means of each spot were normalized against the loading controls.

**Histological analysis.** The frozen biopsy specimens were sectioned into 4-μm units using a cryostat. We performed several different types of analysis including 1) morphological analysis by hematoxylin-eosin-stained sections and 2) inflammatory cell infiltration assessment by immunohistochemistry (19, 20). The morphometric parameters were measured with ImageJ software. As described in detail by Cluff et al. (8), the morphometric parameters include the following 1) the myofiber cross-sectional area, 2) the myofiber perimeter, 3) myofiber roundness, and 3) myofiber solidity. With regard to the myofiber diameter, an equivalent diameter was defined as the diameter of a circle with the same diameter as the segmented myofiber region, calculated as described by Cluff et al. (8). Inflammatory cell infiltration was assessed by immunohistochemistry and staining for human leukocyte antigen (HLA)-A/B/C (myosin heavy chain class I) expression, following a standard immunohistochemistry protocol. Quantification was performed relative to a standard muscle preparation of the laboratory used for all HLA quantification analyses of the laboratory of histology.

**Statistical Analysis**

The baseline characteristics of both groups were compared using an unpaired t-test. All data are presented as a means ± SD. Each participant acted as his or her own control, so in each group the parameters measured from the proximal and distal muscular portions were compared using a paired t-test. Mitochondrial complex activities are expressed as a ratio of the citrate synthase (CS) activity. Protein expression obtained by Western blot is also expressed as a ratio in relation to the expression of either the anti-rabbit α-tubulin or anti-mouse VDAC loading controls. Therefore, the t-test was performed with log-converted values for these results.

All analyses were performed using the statistical software Prism 7 (Graph Pad). P < 0.05 was considered to be statistically significant.

**RESULTS**

**Peripheral Arterial Assessment of the Studied Population**

Among the 38 eligible patients, a total of 34 patients were included in the study, as described in the flowchart (Fig. 1). Twenty-two patients presented with Fontaine stage II, which was established on the basis of the medical history and physical examination. ABIs in these 22 patients with PAD were <0.90, and the arterial lesions were confirmed by the presence of either a stenosis or an occlusion of the superficial femoral artery. The other twelve patients presented with chronic CAD. The CAD patients had no history of claudication or lower limb arterial surgery and had a normal ABI and no superficial femoral artery stenosis or occlusion in the leg where the saphenous vein harvesting was performed.

After obtaining the Ex-TcP02 results were obtained, 13 patients were excluded. Seven of the 22 patients with PAD presented no significant evidence of distal thigh ischemia (a DROP index greater than −15 mmHg despite a severe stenosis or occlusion of the superficial femoral artery (imaging and DROP results of 1 of these patients are illustrated in Fig. 2A). Two of the 22 patients showed evidence of a significant proximal ischemia (DROP index less than −15 mmHg) as a result of significant iliac lesions. In the group of patients suffering from chronic CAD, four patients with no previous history of PAD or of exercise-related lower limb symptom were excluded because they exhibited exercise-induced ischemia at the proximal or distal thigh level during our testing.

Finally, 13 patients with PAD and 8 patients with chronic CAD fulfilled our inclusion criteria and were analyzed as the ISCH and CONT groups, respectively. A typical example of a patient of the ISCH group is presented in Fig. 2B. The mean ABI in the ISCH group was 0.60 ± 0.1 (vs 1.22 ± 0.3 in

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**Fig. 1. Study flowchart.** DROP, decrease from rest of oxygen pressure; ISCH, ischemia; CONT, control; TcP02, transcutaneous oximetry; ABI, ankle brachial indexes.
CONT group, \( P < 0.001 \), Fig. 3A), and all patients had stenosis or occlusion of the superficial femoral artery. Moreover, in the distal portion of the thigh (facing the distal portion of the sartorius muscle) in the ISCH group, we obtained a mean DROP index of \(-24 \pm 10 \) mmHg, which was lower than the cutoff value of \(-15 \) mmHg that indicates the presence of exercise-induced ischemia. The DROP at the distal portion of the thigh was significantly different from the DROP obtained in the proximal portion (\(-10 \pm 4 \) mmHg, \( P < 0.001 \), Fig. 3B). In the CONT group of patients with no history of PAD, the average DROP index at the proximal and distal thigh levels were higher than the cutoff value of \(-15 \) mmHg (\(-6 \pm 2 \) and \(-7 \pm 4 \) mmHg, respectively, Fig. 3B). The other baseline characteristics of the analyzed patients are presented in Table 1. Few differences were observed between the ISCH and CONT groups with respect to the baseline characteristics. The ISCH patients were more often active smokers (\( P < 0.01 \)) but were prescribed \( \beta \)-blockers less frequently (\( P < 0.01 \)) than CONT patients.

Mitochondrial Function Assessment

Mitochondrial biogenesis: CS activity. Mitochondrial content assessed by CS activity was comparable between the proximal and distal segments (278 \( \pm \) 52 and 312 \( \pm \) 170 nmol-min\(^{-1}\)-mg protein\(^{-1}\), respectively; \( P > 0.05 \)) in ISCH patients and in CONT patients (273 \( \pm \) 103 and 234 \( \pm \) 74 nmol-min\(^{-1}\)-mg protein\(^{-1}\), respectively; \( P > 0.05 \)).

Mitochondrial respiratory chain complex activities. CI enzymatic activity normalized to CS significantly decreased in the distal portion (0.093 \( \pm \) 0.03) of the sartorius muscle compared with the proximal portion (0.121 \( \pm \) 0.04) in the ISCH group (\( P = 0.004 \)), which was not observed in the CONT group (0.081 \( \pm \) 0.03 vs 0.085 \( \pm \) 0.04, respectively; \( P = 0.81 \), Fig. 4A). Activities of the three other complexes (complexes II, III, and IV) were not affected by chronic exercise-induced ischemia. Indeed, no significant difference was observed between proximal and distal portions of the sartorius muscle in ISCH patients (Fig. 4, B–D).

Western Blot Results

Expression of CI. The decrease of CI enzyme activity was not associated with a modification of the CI content. The Western Blot analysis showed a comparable protein expression of a subunit of CI, NDUFB6 (NADH:ubiquinone oxidoreductase subunit B6), in both muscular portions in the ISCH and the CONT groups (Table 2 and Fig. 5). Antioxidant defense. In the ISCH group, the antioxidant system was affected by a significant decrease in the expression of the mitochondrial MnSOD in the distal portion compared with the proximal portion of the muscle (\( P = 0.03 \)). In contrast, the cytoplasmic Cu-ZnSOD expression was comparable between both of the sartorius muscle portions in ISCH patients (Table 2 and Fig. 5). In CONT patients, MnSOD and Cu-
ZnSOD expressions were comparable between both muscular portions (Table 2 and Fig. 5).

**Histological Analysis**

**Myofiber morphology.** In the distal portion of the sartorius in the ISCH patients, the myofiber cross-sectional area, perimeter, and equivalent diameter, calculated as described by Cluff et al. (8), tended to decrease compared with the same measurements of the proximal portion, but the difference was not significant (Table 3 and Fig. 6A). However, exercise-induced ischemia induced an alteration of the myofiber shapes. Indeed, in the ISCH group, myofibers of the distal portion showed a significant decreased in roundness ($P = 0.03$) (Fig. 6, A and B) with no alteration of the myofiber solidity (Table 3). In CONT patients, the myofiber morphology was similar between the distal and proximal sartorius portions.

**Inflammatory cell infiltration.** The HLA-A/B/C expression was comparable in the proximal and distal portions of the sartorius biopsies in both groups, which revealed the absence of an inflammatory process in exercise-induced muscular ischemia.

**DISCUSSION**

Several clinical studies have highlighted both skeletal muscle dysfunction and increased oxidative stress as major pathways involved in muscle ischemia (8, 15, 16, 18, 25, 29). A usual approach to identifying these pathways involves comparisons between patients with claudication and healthy control subjects. Unfortunately, variability in muscle type within individuals has been well established, and this variability results from both genetic and environmental factors (3, 11) and physical activity levels (27). Indeed, the majority of patients with PAD are older. Age is a factor that induces modulations of mitochondrial homeostasis in skeletal fibers (9) and depresses the capacity for angiogenesis (21). Mitochondrial dysfunction and alterations of skeletal myofibers have been identified in patients with PAD (8, 15, 16, 29). Overall, the cardiovascular risk factors (e.g., age, smoking, diabetes, or hypercholesterolemia) clearly differ between patients with PAD and healthy control patients. However, in the latter noted studies, patients with PAD were compared with control patients who were not precisely matched based on comorbidities, smoking habits, and medication and activity levels, even though such factors have been reported to impair mitochondrial biogenesis (24) or skeletal muscle dysfunction (13).

A second approach would be to compare a symptomatic leg with an asymptomatic leg in the same patients with PAD. Nevertheless, asymptomatic ischemia in an asymptomatic leg can interfere with the results. Furthermore, bilateral muscle biopsies in patients with PAD can be associated with risk and also raise ethical concerns. A third, and slightly more satisfying, approach would be to study different muscles in the same leg (e.g., sartorius vs. gastrocnemius) above and below the level of an arterial occlusion, but this approach faces the problem of muscle heterogeneity (3). Furthermore, the presence of a severe stenosis or occlusion of the superficial femoral artery is not necessarily proof of exercise-induced ischemia.

**Table 1. Baseline characteristics of the ISCH and CONT groups**

| Characteristics | ISCH $(n = 13)$ | CONT $(n = 8)$ | $P$  |
|-----------------|----------------|---------------|-----|
| Sex: men/women | 13/0           | 7/1           |     |
| Age, yr         | $60 \pm 10$    | $68 \pm 7$    | NS  |
| BMI, kg/m$^2$   | $27 \pm 6$     | $30 \pm 1$    | NS  |
| History         |                |               |     |
| Peripheral arterial disease | 13 | 0 | $<0.01$ |
| Hypertension    | 7              | 4             | NS  |
| Dyslipidemia    | 7              | 6             | NS  |
| Diabetes        | 6              | 2             | NS  |
| CAD             | 4              | 8             | $<0.01$ |
| Stroke          | 1              | 0             | NS  |
| Vascular surgery| 8              | 0             | $<0.01$ |
| Obstructive sleep apnea | 2 | 1 | NS   |
| Chronic bronchitis| 4          | 0             | NS  |
| Active smoker   | 9              | 1             | $=0.01$ |
| Ongoing medications |      |               |     |
| Antiplatelet or anticoagulation medications | 13 | 7 | NS |
| β-Blockers      | 4              | 8             | $<0.01$ |
| Antidiabetic drugs | 5          | 2             | NS  |
| Lipid-lowering drugs | 11        | 8             | NS  |
| ACE inhibitors or ARBs | 11 | 6 | NS |
| Treatments for pulmonary diseases | 1       | 1           | NS  |

Results are expressed as means ± SD or number of observations. The values of ischemic (ISCH) and control (CONT) groups were compared using the unpaired Student’s $t$-test. BML, body mass index; NS, nonsignificant differences; CAD, coronary artery disease; ARBs, angiotensin II receptor blockers; ACE inhibitors, angiotensin-converting enzyme inhibitors.
below the lesion, due to the possibility of collateral vessels normalizing the overall flow, specifically in chronic diseases.

Last, regardless of the potential confounding factors, and the use of the symptomatic vs. the asymptomatic leg, or the sartorius vs. the gastrocnemius muscles for comparison, most of the previous studies evaluated patients with PAD on the basis of their medical history, the ABI value, or arteriography (15, 16, 29). None of these three clinical approaches provide evidence for the presence of exercise-induced ischemia.

In our study, we decided to assess the mitochondrial and structural muscle changes in the human sartorius muscle after confirmation of isolated distal (without proximal) thigh ischemia in patients with PAD with claudication. We compared these findings to the absence of such differences in control patients with comparable cardiovascular risk factors. The discrimination of proximal and distal RBFI at the thigh level allowed us to use patients as their own control by comparing two portions of the same muscle subjected to identical strains and levels of activity. This study has allowed us to associate a structural and functional muscle analysis with the objective estimation of regional impairment of oxygen supply during exercise in patients with PAD. ABI at rest or postexercise

Table 2. Results of the Western blot analysis for ISCH and CONT group patients

|                      | ISCH Group (n = 13) | CONT Group (n = 8) |
|----------------------|---------------------|--------------------|
|                      | Proximal portion    | Distal portion     | P       | Proximal portion | Distal portion | P       |
| NDUFB6               | 2.17 ± 1.67         | 2.66 ± 2.20        | NS      | 1.82 ± 2.03      | 1.63 ± 2.21   | NS      |
| MnSOD                | 0.60 ± 0.54         | 0.54 ± 0.66        | 0.03    | 0.81 ± 0.87      | 0.88 ± 1.36   | NS      |
| Cu-ZnSOD             | 4.13 ± 2.88         | 4.02 ± 2.72        | NS      | 6.13 ± 2.98      | 5.02 ± 2.97   | NS      |

Expression of one subunit of complex I [NADH:ubiquinone oxidoreductase subunit B6 (NDUFB6)] and two superoxide dismutase isoenzymes (mitochondrial: MnSOD; and cytoplasmic: Cu-Zn SOD). Results are presented after normalization against the loading control. The value obtained in the distal thigh segment was compared with that of the proximal thigh segment using a paired Student’s t-test. ISCH, ischemia; CONT, control; NS, nonsignificant.

Fig. 4. Mitochondrial respiratory chain complex activities related to citrate synthase (CS) activity in the ischemic (ISCH, n = 13) and control (CONT, n = 8) groups, comparing the activity in the distal (Dist) sartorius portion with that in the proximal (Prox) portion, in each group. A–D: complex I (CI) related to CS (A); complex II (CII) related to CS (B); complex III (CIII) related to CS (C); and complex IV (CIV) related to CS (D). §§P < 0.01, by paired Student’s t-test; NS, nonsignificant.

Fig. 5. Representative fluorescent Western blot showing decreased MnSOD content in the distal (Dist) portion compared with the proximal (Prox) portion of Sartorius tissue from an ischemic (ISCH) subject, which was not observed in a control (CONT) subject. VDAC, voltage-dependent anion channel.
cannot account for exercise-induced ischemia in the proximal thigh vs. the distal thigh. Multilevel plethysmography or pressure measurements could provide some information about the hemodynamic consequences of arterial lesions at rest but cannot be performed during and after exercise. TcPO2 is a surface technique and not a direct quantification of muscle ischemia. With the use of multiprobe devices, Ex-TcPO2 reflects RBFI simultaneously at different levels during exercise (1, 2, 5). Furthermore, the use of these devices helps to exclude systemic hypoxemia as a potential factor that could interfere with walking ability (6). Once again, the technique does not quantify muscle ischemia itself, but it can quantify regional impairments of oxygen supply during exercise at the surface above the areas of the proximal and distal thigh muscle biopsies. Near-infrared spectroscopy could have been proposed as an alternative tool. Nevertheless, the technique is highly sensitive to skin blood flow (12) and fails to detect proximal ischemia, compared with Ex-TcPO2 (5), which is able to quantify proximal ischemia. In the absence of other available noninvasive techniques, the Ex-TcPO2 approach provides a unique surrogate marker of segmental ischemia that we used to optimally select a group of patients yet fulfilling all classical clinical criteria (history, ABI, and imaging). The last interest is to also exclude any significant exercise-induced ischemia resulting from moderate “considered nonsignificant” aorto-iliac lesions in the studied patients that may coexist with the superficial femoral artery lesions.

In our “Distal-Proximal-Sartorius Model”, the muscular portion exposed to exercise-induced ischemia in patients with PAD showed a significant functional alteration characterized by an impairment of mitochondrial complex I activity without a decrease in protein expression, and a significant decrease in mitochondrial anti-oxidant MnSOD expression. In our model, muscular alterations are more moderate than those shown in previous clinical studies. Various hypotheses can be made to explain this difference. First, our patients had moderate PAD (ABI at 0.60) and the respiratory mitochondrial chain alteration could depend on the severity of the PAD. Indeed, in the study of Koutakis et al. (15), patients with an ABI of ~0.55 had a decrease in the activity of complexes I and IV, whereas decreases in the activity of complexes I, III, and IV have been reported in a study with patient presenting more advanced PAD (ABI at 0.34) (25). Second, smoking have been reported to impair the skeletal muscle function, as Complex IV activity

### Table 3. Morphometric parameters of the distal and proximal portions of the sartorius myofibers, in the ISCH and CONT group patients

|                        | ISCH Group (n = 13)       | CONT Group (n = 8)       | P  |
|------------------------|--------------------------|--------------------------|----|
|                        | Proximal portion         | Distal portion           |    |
| Cross-sectional area   | 5,126 ± 1,454            | 4,451 ± 1,750            | NS |
| Perimeter              | 280 ± 41                 | 264 ± 55                 | NS |
| Equivalent diameter    | 79 ± 12                  | 73 ± 16                  | NS |
| Solidity               | 0.97 ± 0.01              | 0.96 ± 0.02              | NS |
|                        | 3,891 ± 600              | 4,377 ± 1,015            | NS |
| Perimeter              | 248 ± 23                 | 263 ± 31                 | NS |
| Equivalent diameter    | 69 ± 6                   | 73 ± 9                   | NS |
| Solidity               | 0.97 ± 0.01              | 0.97 ± 0.01              | NS |

The value obtained in the distal thigh segment was compared with that of the proximal thigh segment using a paired Student’s t-test. ISCH, ischemia; CONT, control; NS, nonsignificant.

![Fig. 6. A: representative images (×20) of hematoxylin and eosin-stained skeletal myofibers sections of distal (Dist) portion and proximal (Pros) portions in ischemic (ISCH: top) and in control group (CONT: bottom). B: roundness of the Sartorius’s myofibers, comparing the value obtained in the distal and proximal portions of the sartorius in each group (n = 13 in ISCH group and n = 8 in CONT group). §P < 0.05, by paired Student’s t-test.](https://www.jappl.org)
Concerning the morphological alterations, previous studies have highlighted myofiber degeneration, characterized by a decrease of the myofiber sizes and alterations in their shape (8, 15, 29). Our results are consistent with a model of ischemia that specifically affected complex I activity, leading to an energy production defect and an increase in the production of ROS at the portion of the muscle exposed to exercise-induced ischemia. Finally, our human model of chronic ischemia did not result in inflammation, which is consistent with preclinical models of chronic ischemia (18), contrary to models based on acute ischemia (10, 26). This result confirms the importance of a suitable model to explore tissue injuries associated with chronic exercise-induced ischemia.

There are several limitations to the present study. First, our small sample size, which is based on a stringent patient selection process enlisted to reduce the variability of the results. It could explain the observed absence of significant decreases in myofiber sizes in our exercise-induced ischemia model. Second, the stringent patient selection process resulted in a selection of patients with a less advanced pathological PAD stage, which could explain less severe tissue injuries than those observed in previous studies (8, 25, 29). Another limitation of this study is the assessment of muscular ischemia by a transcutaneous pressure measurement and the gradient of skin to tissue makes the estimation of absolute TcPO2 lower than at a proximal level. We can support this hypothesis by data not described in our results: in the ISCH group, at the calf level the DROP index was significantly lower than at the distal thigh portion (−46 ± 26 vs. −23 ± 9 mmHg, respectively, \( P = 0.008 \)).

Conclusion

Despite the final small number of patients studied, we have been able to specifically assess the consequences of a local muscular exercise-induced ischemia in patients with PAD and stage 2 claudication, using the sartorius muscle as its own control. These consequences are characterized by a limited mitochondrial respiratory dysfunction, with a decrease in complex I activity and a slight alteration of the muscular myofibers. This study confirms the great potential for studying the mitochondrial function in the same human muscle. This type of model would avoid most of the issues of variability in extrapolating animal study results to human populations and with respect to noncomparable control groups in human clinical studies. This model would help us to explore metabolomic changes and vascular remodeling consequent to a chronic ischemia in PAD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

I.S. and P.A. conceived and designed research; I.S., P.A., S.C., M.A., N.G., J.P., C.B., and M.D. performed experiments; I.S., P.A., S.C., N.G., F.L., P.R., and S.H. analyzed data; I.S., P.A., F.L., V.P., P.R., and S.H. interpreted results of experiments; I.S. and P.A. prepared figures; I.S., P.A., and P.R. drafted manuscript; I.S., P.A., S.C., M.A., N.G., F.L., J.P., C.B., M.D., V.P., P.R., and S.H. edited and revised manuscript; I.S., P.A., S.C., M.A., N.G., F.L., J.P., C.B., M.D., V.P., P.R., and S.H. approved final version of manuscript.

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