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*Am J Physiol Regul Integr Comp Physiol* 289:1144-1154, 2005. First published Jul 14, 2005;  
doi:10.1152/ajpregu.00229.2005

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## Functional coupling of adenine nucleotide translocase and mitochondrial creatine kinase is enhanced after exercise training in lung transplant skeletal muscle

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Submitted 1 April 2005; accepted in final form 26 May 2005

**Guerrero, Karen, Bernard Wuyam, Paulette Mezin, Isabelle Vivodtzev, Marko Vendelin, Jean-Christian Borel, Rachid Hacini, Olivier Chavanon, Sandrine Imbeaud, Valdur Saks, Christophe Pison.** Functional coupling of adenine nucleotide translocase and mitochondrial creatine kinase is enhanced after exercise training in lung transplant skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 289: R1144–R1154, 2005; doi:10.1152/ajpregu.00229.2005.—Mechanisms responsible for limitation of exercise capacity in lung transplant recipients (LR) and benefits gained by exercise training were studied. Mitochondrial respiration parameters, energy transfer, and cell structure were assessed in vastus lateralis biopsies using the permeabilized fiber technique with histochemical and morphometric measurements. Twelve male controls (C) and 12 LR performed exercise training over 12 wk. Before exercise training, there were strong correlations between exercise capacity (maximal O<sub>2</sub> consumption and endurance time at 70% maximal power output) and cellular events, as assessed by percentage of type I fibers and apparent K<sub>m</sub> for exogenous ADP. Anticalcineurins were not involved in LR exercise limitation, since there were no differences in maximal mitochondrial rate of respiration before exercise training and no abnormalities in respiratory chain complexes compared with C. Training resulted in a significant increase in physiological parameters both at the cellular (apparent K<sub>m</sub> for exogenous ADP and stimulating effect of creatine) and integrated (maximal O<sub>2</sub> consumption, power output at ventilatory threshold, maximal power output, and endurance time at 70% maximal power output) levels in LR and C. After the training period, improvements in maximal O<sub>2</sub> consumption and in maximal mitochondrial rate of respiration were noted, as well as changes in endurance time and percentage of type I fibers. Because there were no changes in diameters and fiber types, baseline alteration of apparent K<sub>m</sub> for exogenous ADP and its improvement after training might be related to changes within the intracellular energetic units. After the training period, intracellular energetic units exhibited a higher control of mitochondrial respiration by creatine linked to a more efficient functional coupling adenine nucleotide translocase-mitochondrial creatine kinase, resulting in better exercise performances in C and LR.

mitochondria; oxidative phosphorylation; creatine/phosphocreatine shuttle; pulmonary transplantation; rehabilitation

LUNG TRANSPLANTATION (LT) has become an established option for end-stage respiratory diseases (35). Among such unre-

solved issues as organ shortage and development of progressive chronic dysfunction is limitation of maximal O<sub>2</sub> consumption ( $\dot{V}_{O_{2\max}}$ ) after LT, which was been studied since the early 1990s without definitive explanations (7, 9, 10, 16, 17, 20, 22, 34, 41, 42) (see Ref. 43 for review).  $\dot{V}_{O_{2\max}}$  is broadly determined by three major factors: ventilatory capacity, maximal cardiac output, and O<sub>2</sub> consumption by the working muscles. Lung transplant recipients (LR) exhibit normal cardiac and ventilatory responses to exercise; therefore, skeletal muscle dysfunction is thought to explain limitations (7, 9, 10, 16, 17, 20, 22, 34, 41, 42, 43). Systemic effects of chronic respiratory failure before LT (2, 26) and potential effects of immunosuppressants, especially the vehicle of cyclosporin A (15), have been proposed as explanations for limitations in  $\dot{V}_{O_{2\max}}$  and decreased exercise capacity after LT. However, the molecular mechanisms of the limitations in O<sub>2</sub> consumption in this acquired myopathy remain obscure for LR, even if reductions in skeletal muscle mass and type I fibers play an important role (2, 9, 26, 41, 43). Energy metabolism is thus involved in the genesis of the impairment of exercise capacity. At present, there exists a reliable technique to determine the mitochondrial oxidative capacity in human skeletal muscle: the permeabilized or skinned fiber technique (29, 44). This technique explores mitochondrial oxidative phosphorylation in situ without isolation of these organelles. This allows the measurement of mitochondrial respiration in situ in small biopsy samples. The content of mitochondria in the tissue and their functional capacities can easily be evaluated. In particular, this technique has already been used for studies of mitochondrial function in inspiratory muscles of patients with chronic obstructive pulmonary disease (25) and skeletal muscles of heart transplant recipients before and after rehabilitation (46).

The aims of the study were to look for alterations in the intracellular organization in energy metabolism, i.e., mitochondrial respiration and energy transfer via phosphocreatine shuttle and, if present, the relevance of mitochondrial bioenergetics in limitations of skeletal muscle performance in LR (3) and

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whether home exercise training can improve these abnormalities (4, 34).

## METHODS

### Subjects and Experimental Design

Twelve healthy males, recruited from acquaintances of investigators and free of any medications, were enrolled in the study as a control group (C). Twelve LR ( $35 \pm 28$  mo post-LT) were also included. Antirejection maintenance treatment consisted of prednisone, 0.1 mg/kg on average, tacrolimus, and mycophenolate mofetil (Table 1). Most LR received antihypertensive treatments such as calcium blockers or angiotensin-converting enzyme inhibitors. LR were free of acute and/or significant chronic rejection (except the 49-yr-old LR) or infection during the study. C and LR were examined before and after a 3-mo period of exercise training. Participants gave their voluntary consent. The protocol was approved by the ethics committee of our institution.

### Body Composition, Pulmonary Function, and Exercise Testing

Fat-free mass was measured with bioelectrical impedance analysis at 50 kHz, and the equation from Kyle et al. (15) was used. Pulmonary function was measured using standard techniques according to American Thoracic Society/European Respiratory Society recommendations (23). Lung function measurements consisted of 1) forced expiration from which 1-s forced expiratory volume, forced vital capacity, and expiratory flow-volume loops were derived and 2) thoracic gas volumes, which were determined in a plethysmograph (Medical Graphics, St. Paul, MN). Pulmonary function measurements are shown in Tables 1 and 2.

Maximal incremental and constant workload cycle exercise was performed as detailed below.

First, for maximal incremental cycling exercise, subjects were seated on an electrically braked ergocycle (Ergometer 900PC, Ergoline) driven by Vmax system 229 software (SensorMedics, Yorba Linda, CA). Gas exchanges were analyzed by the standard open-circuit method with an automated computer analysis system (Vmax 2130 automated spirometer, SensorMedics). Continuous monitoring of gas exchange provided measurements of  $\dot{V}O_2$ ,  $\dot{V}E$ ,  $\dot{V}E/\dot{V}O_2$ , and minute ventilation ( $\dot{V}E$ ). The expired  $O_2$  and  $CO_2$  fractions were analyzed breath by breath with zirconium electrochemical and infrared cell gas analyzers, respectively. Expiratory flow was measured with a hot wire pneumotachograph held with a mouthpiece. After 5 min of resting and 2 min of warm-up at 20 W, a progressive stepwise exercise was performed up to the individual's maximal capacity. Workload increments were 5 and 15 W/min for LR and C, respectively. Subjects were considered to have achieved maximal  $\dot{V}O_2$  if at least three of the four following criteria were met: 1) no further increase in  $\dot{V}O_2$  with increasing workload, 2) respiratory exchange ratio  $>1.1$ , 3) maximal heart rate  $\geq 90\%$  of the maximal predicted value, and 4) plasma lactate concentration  $>8$  mmol/l. In other cases, tests were considered as symptom-limited exercise testing. Power output at the ventilatory threshold ( $\dot{W}_{ventilatory\ threshold}$ ) was determined as the power output for which an abrupt and nonlinear increase in  $\dot{V}E$  (minimal value of  $\dot{V}E/\dot{V}O_2$ ) was observed over the incremental test.

Second, for endurance cycling exercise, subjects were seated on the ergocycle, and gas exchange was analyzed under the same conditions as previously described. After 5 min of resting and 2 min of warm-up at 20 W, a steady-state exercise was performed up to exhaustion. Exercise intensity was set at 70% of the maximal power output ( $\dot{W}$ ) reached by the subjects during their initial maximal incremental exercise. After exercise training, the maximal exercise test was repeated. The endurance test was also repeated on the same day, with the same load as the initial test, and the patient was allowed to

Table 1. Clinical characteristics of LR and C before exercise training

Age, yr	Preoperative Diagnosis	Type of Procedure	Months Post-LT	FEV <sub>1</sub> , liters	FEV <sub>1</sub> /FVC	$\dot{V}E$ /MVM	$\dot{V}E/\dot{V}O_{2\ max}$	$\dot{V}O_{2\ max}$ l/min	$\dot{W}_{max}$ , W	Max HR, % predicted
20	Cystic fibrosis	Double	22	4.20	0.84	0.55	36.6	2.42	165	90
37	Cystic fibrosis	Double	5	3.59	0.96	0.52	42.5	2.03	100	79
38	Emphysema	Double	60	3.72	0.87	0.54	48.2	2.20	120	77
40	Cystic fibrosis	Double	25	2.77	0.74	0.56	37.0	2.14	150	92
45	Emphysema	Double	59	2.16	0.79	0.63	43.2	1.49	55	69
47	Emphysema	Double	5	2.04	0.63	0.68	41.4	2.03	60	73
47	Histiocytosis	Double	10	3.53	0.88	0.47	47.0	2.30	105	95
49	Cystic fibrosis	Double	82	0.94	0.37	0.48	63.2	2.24	34	68
57	COPD	HL	76	2.27	0.77	0.57	40.7	1.97	80	66
58	PAH	Double	2	1.90	0.95	0.63	55.6	1.16	40	70
60	IPF	Single	51	1.63	0.70	0.46	46.8	2.16	70	75
70	Emphysema	Single	36	1.36	0.70	0.72	39.1	1.80	50	63
<b>47±13</b>			<b>35±28</b>	<b>2.51±1.04*</b>	<b>0.77±0.16</b>	<b>0.64±0.23</b>	<b>46.2±8.6*</b>	<b>1.25±0.56*</b>	<b>85±43*</b>	<b>76±11*</b>
22	C			4.87	0.86	0.58	29.2	2.89	285	94
22	C			4.67	0.85	0.44	31.1	3.11	225	85
26	C			4.53	0.90	0.62	36.2	2.46	210	99
30	C			5.45	0.81	0.68	28.8	3.16	315	99
30	C			4.71	0.82	0.45	27.2	3.22	315	99
32	C			3.79	0.83	0.53	36.6	2.90	180	91
35	C			5.00	0.84	0.56	31.4	3.43	165	85
44	C			4.55	0.89	1.29	35.0	2.74	180	101
44	C			3.14	0.79	0.59	32.1	2.51	165	105
50	C			4.83	0.78	0.62	37.2	2.76	253	88
54	C			3.91	0.76	0.79	29.5	2.08	180	96
55	C			4.07	0.75	0.53	40.9	2.84	210	81
<b>37±12</b>				<b>4.46±0.63</b>	<b>0.82±0.05</b>	<b>0.57±0.08</b>	<b>32.9±4.1</b>	<b>2.88±0.67</b>	<b>225±56</b>	<b>94±7</b>

Values in bold are means  $\pm$  SD. COPD, chronic obstructive pulmonary disease; PAH, pulmonary arterial hypertension; IPF, interstitial pulmonary fibrosis; HL, heart lung transplant; LT, lung transplant; FVC, forced vital capacity;  $\dot{V}E$ , external ventilation volume; MVM, maximal volume exchanged per minute [37  $\times$  1-s forced expiratory volume (FEV<sub>1</sub>)];  $\dot{V}O_{2\ max}$  = maximal  $O_2$  consumption;  $\dot{W}_{max}$  = maximal power output; Max HR, maximal heart rate. \* $P \leq 0.0002$  for comparisons between lung transplant recipients (LR) and controls (C) before exercise training.



Table 2. Effects of exercise training

	Exercise Training		Before vs. After training <i>P</i>	C vs. LR <i>P</i>
	Before	After		
BMI, kg/m <sup>2</sup>				
C	24 ± 3.4	24 ± 2.7	0.58	0.05
LR	22 ± 2.7	22 ± 2.0	0.25	
FEV <sub>1</sub> , %predicted				
C	108 ± 12	106 ± 9.7	0.20	0.0003
LR	74 ± 24	77 ± 26	0.44	
FVC, %predicted				
C	109 ± 13	109 ± 11	0.82	<0.0001
LR	78 ± 16	82 ± 18	0.19	
W <sub>max</sub> , W				
C	224 ± 56	255 ± 67	<0.001	<0.0001*
LR	85 ± 43	98 ± 42	0.04	
V <sub>ventilatory threshold</sub> , W				
C	112 ± 36	125 ± 42	0.09	<0.0001
LR	42 ± 21	52 ± 25	0.02	
V <sub>O<sub>2max</sub></sub> , l/min				
C	2.9 ± 0.67	3.1 ± 0.70	0.01	<0.0001
LR	1.2 ± 0.56	1.4 ± 0.51	0.06	
V <sub>O<sub>2max</sub></sub> , %predicted				
C	101 ± 16	113 ± 23	0.02	<0.0001
LR	63 ± 20	68 ± 19	0.07	
Endurance time at 70% W <sub>max</sub> , min				
C	36 ± 19	41 ± 13	0.08	0.04
LR	20 ± 10	31 ± 14	0.01	
Fiber type I, %				
C, <i>n</i> = 11	42 ± 21	36 ± 8.6	0.37	0.0008
LR, <i>n</i> = 11	16 ± 6.1	24 ± 17	0.14	
V <sub>0</sub> , nmol O <sub>2</sub> ·min <sup>-1</sup> · mg dry wt <sup>-1</sup>				
C, <i>n</i> = 11	0.51 ± 0.34	0.75 ± 0.52	0.10	0.51
LR	0.68 ± 0.36	0.79 ± 0.56	0.57	
V <sub>max</sub> , nmol O <sub>2</sub> ·min <sup>-1</sup> · mg dry wt <sup>-1</sup>				
C, <i>n</i> = 11	3.5 ± 1.5	4.3 ± 2.9	0.38	0.50
LR	3.2 ± 1.7	2.9 ± 1.3	0.56	
V <sub>atr</sub> , nmol O <sub>2</sub> ·min <sup>-1</sup> · mg dry wt <sup>-1</sup>				
C, <i>n</i> = 10	0.71 ± 0.38	1.5 ± 1.0	0.04	0.49
LR	0.79 ± 0.35	1.1 ± 0.68	0.12	

Values are means ± SD. BMI, body mass index; V<sub>0</sub>, basal mitochondrial respiration in the presence of glutamate (5 mM) and malate (2 mM); V<sub>max</sub>, maximal mitochondrial respiration rate; V<sub>atr</sub>, mitochondrial respiration rate in the presence of atractyloside, an inhibitor of adenine nucleotide translocase (ANT). ANOVA with 2 factors: 1) effects of exercise training and 2) group effect. \*Significant interaction between the effects of exercise training and the groups: LR vs. C.

continue to exercise to the limits of tolerance (to evaluate any potential improvement in exercise endurance).

#### Exercise Training Program

LR and C underwent exercise sessions, three times per week, for 12 wk at home on a cycle ergometer (Ergometer 900PC, Ergoline). They were directly supervised for the first sessions by a physiotherapist at home and via phone each subsequent week. For security reasons and control purposes, subjects had a cardiofrequency meter (S610i, Polar, Kempele, Finland) with memory. Every month, these monitors were downloaded to a personal computer to check that planned exercise sessions were achieved. Exercise consisted of cycling at 50% and progressively increasing to 80% of previously determined W<sub>max</sub> for 10 min followed by 5 min at 30% W<sub>max</sub>, three times per session in C and LR.

#### Skeletal Muscle Biopsy and Muscles Studies

Local anesthesia (1% xylocaine) was injected into the skin of the thigh without crossing the muscle aponeurosis to avoid mitochondrial uncoupling. One part of the biopsy sample was immediately immersed in ice-cold relaxing medium (*solution A* complemented with 0.2% BSA) and used for preparation of fibers (see *Preparation of permeabilized muscle fibers*). The second part was immediately frozen in isopentane, which was cooled by liquid nitrogen for enzymatic, morphological studies. The third part of the biopsy sample was fixed with 2.5% glutaraldehyde for electron microscopic study.

**Electron microscopy.** Semifine cross sections (1–2 μm) were prepared with a Leica Microsystems microtome and stained with toluidine blue. Fine sections (0.07 μm) were prepared with a Reichert-Jung microtome, stained with uranyl acetate and lead citrate, mounted on 300-mesh grids, and examined under a CM10 Philips electron microscope.

**Muscle fiber types.** The muscle cross sections (10 μm) were preincubated at pH 4.35. After they were stained for myosin ATPase, fibers were visually counted to determine the percentage and the mean diameter of each fiber type (8). Morphometric measurements were performed with Histo Biocom software (Les Ullis, France).

**Preparation of permeabilized muscle fibers.** The nonfrozen parts of the biopsy samples that had been immediately immersed in ice-cold relaxing medium (*solution A* complemented with 0.2% BSA) were used in these experiments. Thin bundles of fibers were then separated by sharpened needles in ice-cold *solution A* with 0.2% BSA. After dissection, fibers were incubated in *solution A* with 50 μg/ml saponin, 30 min, at 4°C and mild stirring. They were then washed three times in *solution B* with 0.2% BSA, 10 min, at 4°C and mild stirring to eliminate the detergent and the endogenous metabolites (29).

**Determination of the rate of mitochondrial respiration in skinned fibers.** The main mitochondrial reactions studied to characterize the muscle energy metabolism are schematically shown in Fig. 1. The

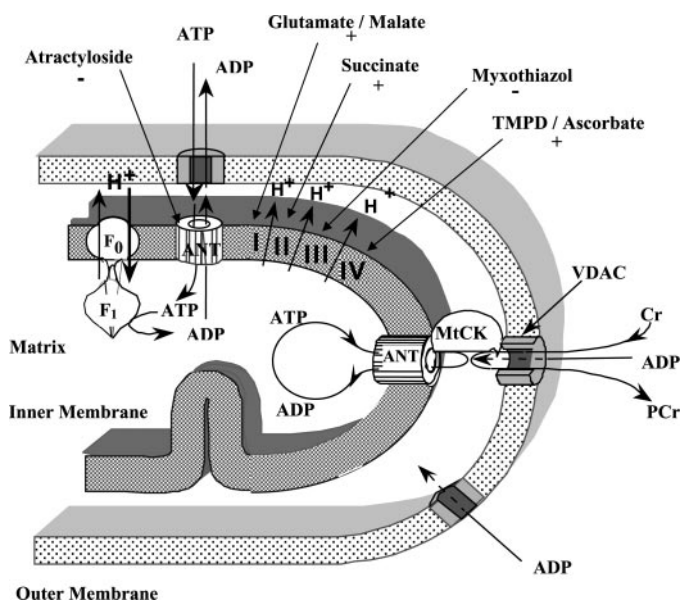


Fig. 1. Representation of the different mitochondrial compartments (matrix and intermembrane space) delimited by the outer and the inner membranes. Mitochondrial respiratory chain (localized in the inner membrane) as well as its substrates (+) and inhibitors (–) consume O<sub>2</sub> and create the proton gradient necessary for the synthesis of ATP. The functional coupling of adenine nucleotide translocase (ANT)-mitochondrial creatine kinase (MiCK) is also represented to show the control of respiration by creatine (Cr) in the case of oxidative muscle by the regeneration of local ADP in the intermembrane space. On the outer mitochondrial membrane, porin allows ADP and Cr to go inside mitochondria. PCr, phosphocreatine; TMPD, tetramethylpentadecane; VDAC, voltage-dependant anion channel; F<sub>0</sub>F<sub>1</sub>, mitochondrial ATPase synthase.

rates of  $\dot{V}_{O_2}$  were recorded by using a two-channel, high-resolution respirometer (Oroboros Oxygraph, Paar, Graz, Austria) in solution B (measurement of ADP kinetics of respiration) or using KCl solution for cytochrome *c* test (for composition, see below). Activity of respiratory chain complexes was determined in KCl solution with 2 mg/ml BSA plus 5 mM glutamate, 2 mM malate, 2.5  $\mu$ M rotenone, 5 mM succinate, 32  $\mu$ M myxothiazol, 5 mM ascorbate, 1 mM tetramethylpentadecane, and 5 mM KCN. Determinations were carried out at 25°C; solubility of  $O_2$  was taken as 215 nmol/ml (14).

**Solution A** contained (in mM) 1.9 CaK<sub>2</sub>EGTA, 8.1 K<sub>2</sub>EGTA (free calcium concentration of 0.1  $\mu$ M), 9.5 MgCl<sub>2</sub>, 0.5 DTT, 50 potassium-MES, 20 imidazole, 20 taurine, 2.5 Na<sub>2</sub>ATP, and 15 phosphocreatine, pH 7.1, adjusted at 25°C. **Solution B** contained (in mM) 1.9 CaK<sub>2</sub>EGTA, 8.1 K<sub>2</sub>EGTA, 4.0 MgCl<sub>2</sub>, 0.5 DTT, 100 potassium-MES, 20 imidazole, 20 taurine, and 3 K<sub>2</sub>HPO<sub>4</sub>, pH 7.1, adjusted at 25°C.

KCl solution for cytochrome *c* test contained (in mM) 125 KCl, 20 HEPES, 3 magnesium acetate, 5 KH<sub>2</sub>PO<sub>4</sub>, 0.4 EGTA, and 0.3 DTT, pH 7.1, adjusted at 25°C.

For respiration rate determinations, 2 mg/ml BSA was added; 5 mM glutamate and 2 mM malate were added as substrates.

**Reagents.** All reagents were purchased from Sigma except ADP, which was obtained from Boehringer-Mannheim.

#### Statistical Analysis

Values are expressed as means  $\pm$  SD. Comparisons were made using two-way ANOVA, with one factor corresponding to comparisons between C and LR and the second comparing before and after exercise training. Significant interactions within these two factors are reported.  $P < 0.05$  was taken as the level of significance.

## RESULTS

### Comparisons Between C and LR Before Exercise Training

Table 1 reports clinical characteristics in LR and C. The male-to-female ratio was 12:0 for C and 10:2 for LR. Body-mass index was lower, as well as 1-s forced expiratory volume and forced vital capacity in LR (Table 2). No subject exhibited ventilatory limits

during maximal cycling exercise test. As expected,  $\dot{W}_{max}$ ,  $\dot{W}_{ventilatory}$  threshold,  $\dot{V}_{O_2 max}$  (in l/min or %predicted), and endurance times were much lower in LR compared with C (Tables 1 and 2).

Histochemical determination of fiber types is reported in Fig. 2A for C and Fig. 2C for LR. LR had a significantly lower percentage of type I fibers compared with C (Table 2). Figure 3, A and C for C and LR, respectively, shows examples of mitochondrial arrangement in the fibers along the direction of sarcomere orientation analyzed by electron microscopy. Most mitochondria were localized close to the *z*-line, as characteristic for the glycolytic and oxidative-glycolytic fibers. In some places, a small amount of intermyofibrillar mitochondria was seen.

### Exercise Training Effects in C and LR

Exercise training had physiological effects in both groups with regard to  $\dot{W}_{max}$ ,  $\dot{W}_{ventilatory}$  threshold, and  $\dot{V}_{O_2 max}$  (Table 2), with a trend toward more pronounced effects in LR. Endurance time increased by 55% in LR, whereas it did not reach significance in C. External ventilation significantly decreased after exercise training for comparable workloads in C and LR (data not shown). Interestingly, the fiber-type distribution (Fig. 3B for C and Fig. 3D for LR) and diameters of both fiber types were not significantly changed by exercise training. Analysis of multiple electron micrographs did not reveal any visible changes in the arrangement of mitochondria in the cells after exercise training (Fig. 3B in C and Fig. 3D in LR).

### Mitochondrial Respiration Before and After the Exercise Training Period

Before exercise training, LR mitochondrial respiratory chain capacity was similar to C. The basal rate of respiration was obtained in the presence of 5 mM glutamate and 2 mM malate, substrates of the complex I of the respiratory chain (Figs. 1 and 4). The usually low basal rate of respiration

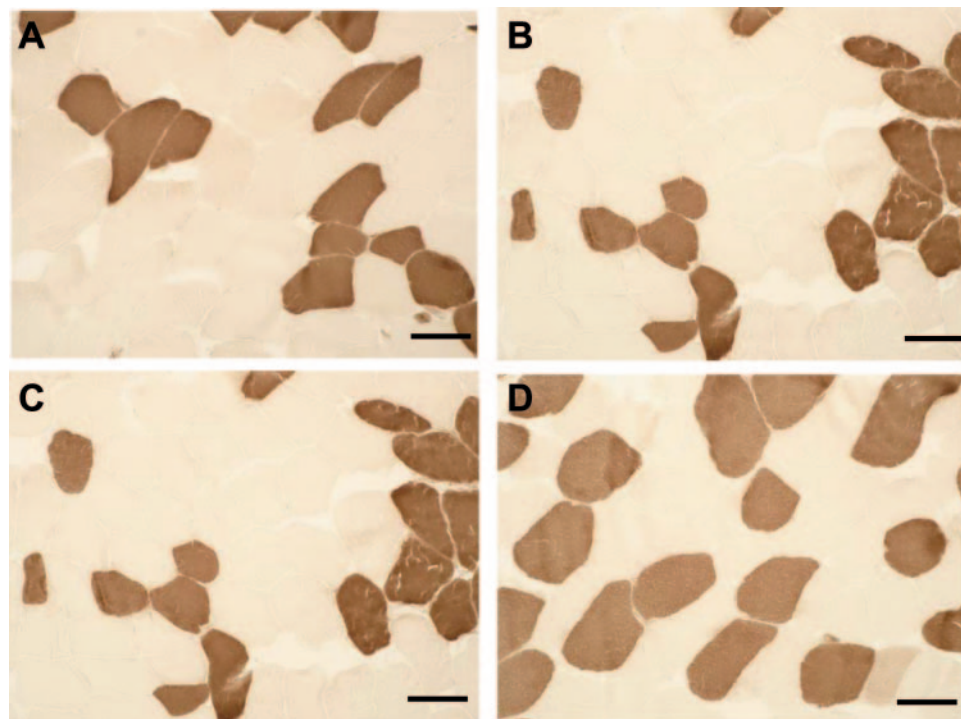


Fig. 2. Identification of fiber type according to ATPase reactivity. Type I fibers appear darker, whereas type II fibers appear brighter. A and B: fibers from controls (C) before and after rehabilitation, respectively. C and D: fibers from lung transplant recipients (LR) before and after exercise training, respectively. Note that LR have a higher proportion of type II fibers compared with C and that the proportion of each type of fiber does not change with training in both groups. Bars = 60  $\mu$ m.



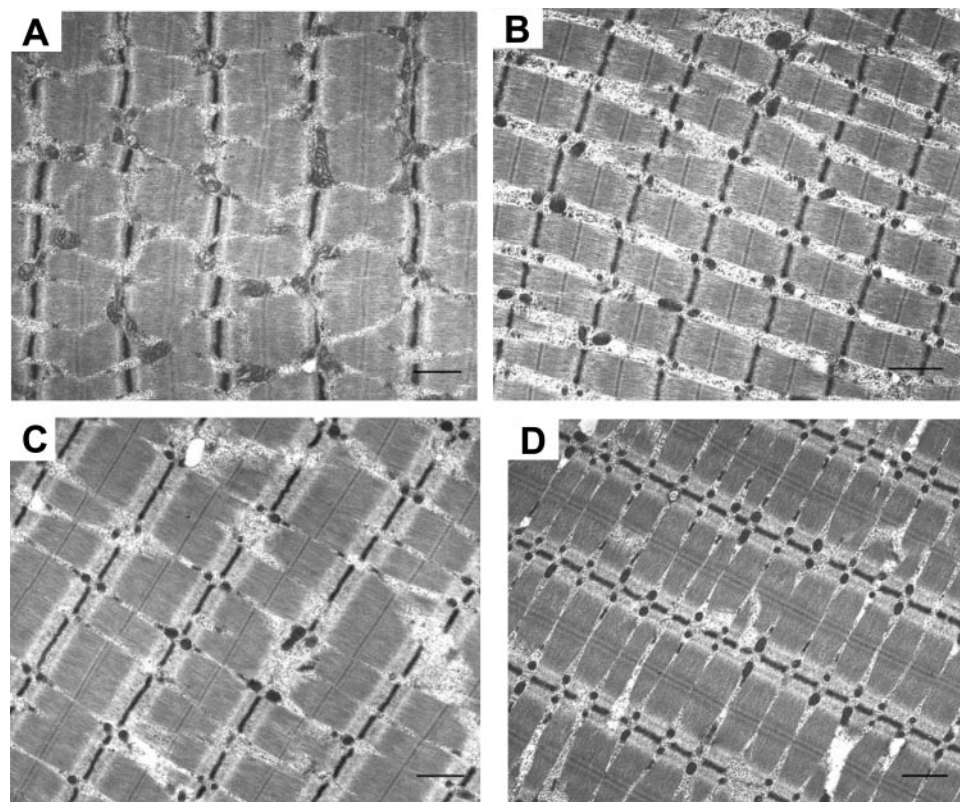


Fig. 3. Electron microscope images. *A* and *B*: regular organization of pairs of mitochondria at the z-line level in a control quadriceps muscle before and after training, respectively. *C* and *D*: samples were taken from LR quadriceps muscle before and after training. In this case, mitochondrial regular arrangement is conserved on both sides of the sarcomeres. Note that mitochondrial morphology is normal in all images. Bars = 1  $\mu\text{m}$ .

corresponds to the state 2 of the respiratory chain where the membrane potential (gradient of electrochemical potential of protons) is generated. After addition of ADP, transported into mitochondrial matrix in exchange for ATP by adenine nucleotide translocase (ANT) (Fig. 1), the membrane potential is used for the synthesis of ATP (state 3 of respiratory chain). Due to a decrease of the membrane potential, the respiration rate in state 3 is increased manyfold in the presence of ADP at a high concentration (2 mM) to achieve the maximal rate of respiration ( $V_{\max}$ ; Fig. 4D). This phenomenon is called acceptor control, and the ratio of the respiration rate in state 3 to that in state 2 is called the acceptor control ratio (ACR). Figure 4D shows that basal respiratory rate was very low and ACR exceeded 8, giving evidence for an intact inner membrane and effective coupling between oxidation and phosphorylation in the skeletal muscle mitochondria in LR and C. The integrity of the outer mitochondrial membrane was assessed by the cytochrome *c* test (14). The absence of the effect of exogenous cytochrome *c* (8  $\mu\text{M}$ ) on the respiration rate in the KCl medium showed integrity of this membrane (Fig. 4D). Addition of atractyloside resulted in a decrease of respiration to state 2 (Table 2, Fig. 4, *A* and *B*). In C, basal respiratory rate and  $V_{\max}$  values were close to those found in the literature (25, 46), and the ACR values indicated a high degree of coupling between oxidation and phosphorylation (data not shown). With glutamate and malate as substrates, the basal respiratory rate and  $V_{\max}$  values were not significantly different in C and LR (Table 2). Activities of respiratory chain complexes (by activating and inhibiting fragments of the respiratory chain) did not reveal any alteration of the oxidative phosphorylation in the mitochondrial inner membrane of LR before and after exercise training compared with C (data not shown).

*Effect of ADP and creatine on mitochondrial respiration before exercise training.* Successive additions of exogenous ADP in increasing concentrations up to 2 mM resulted in an increase of the respiration rate. For high concentrations of ADP (2 mM),  $V_{\max}$  was reached (Fig. 4, *A* and *B*). Apparent  $K_m$  for exogenous ADP was calculated from the double-reciprocal plot of these kinetic data (Fig. 4C). Apparent  $K_m$  for exogenous ADP represents the sensitivity of mitochondria for ADP, including on the one hand the permeability of the outer mitochondrial membrane (voltage-dependent anion channel) and on the other hand cytoplasmic diffusion restrictions for exogenous ADP induced by cellular structures (31, 36). When these experiments were repeated in the presence of 20 mM creatine, the kinetics of respiration regulation by exogenous ADP was changed, i.e., addition of small amount of ADP rapidly increased the respiration rate (Fig. 4, *B* and *C*) by decreasing the apparent  $K_m$  for exogenous ADP (increasing affinity for ADP). This is explained by the functional coupling of the mitochondrial creatine kinase (MtCK) with ANT (Fig. 1). The  $V_{\max}$  of respiration in the presence of creatine was the same or higher than in the absence of creatine (Fig. 4B). The functional coupling between MtCK and ANT is the natural mechanism of regulation of respiration by ADP locally produced from mitochondrial ATP by the MtCK localized at the outer surface of the inner mitochondrial membrane (Figs. 1 and 7). Dramatic differences were found in the apparent  $K_m$  values for exogenous ADP in regulation of respiration between C and LR before exercise training (Fig. 5). In C, apparent  $K_m$  for exogenous ADP ( $131 \pm 26 \mu\text{M}$ ) was characteristic of skeletal muscle having mixed fiber composition, with intermediate values of apparent  $K_m$  for ADP oxidative (250–300  $\mu\text{M}$ ) and glycolytic (10–20  $\mu\text{M}$ ) fibers. This average value of apparent

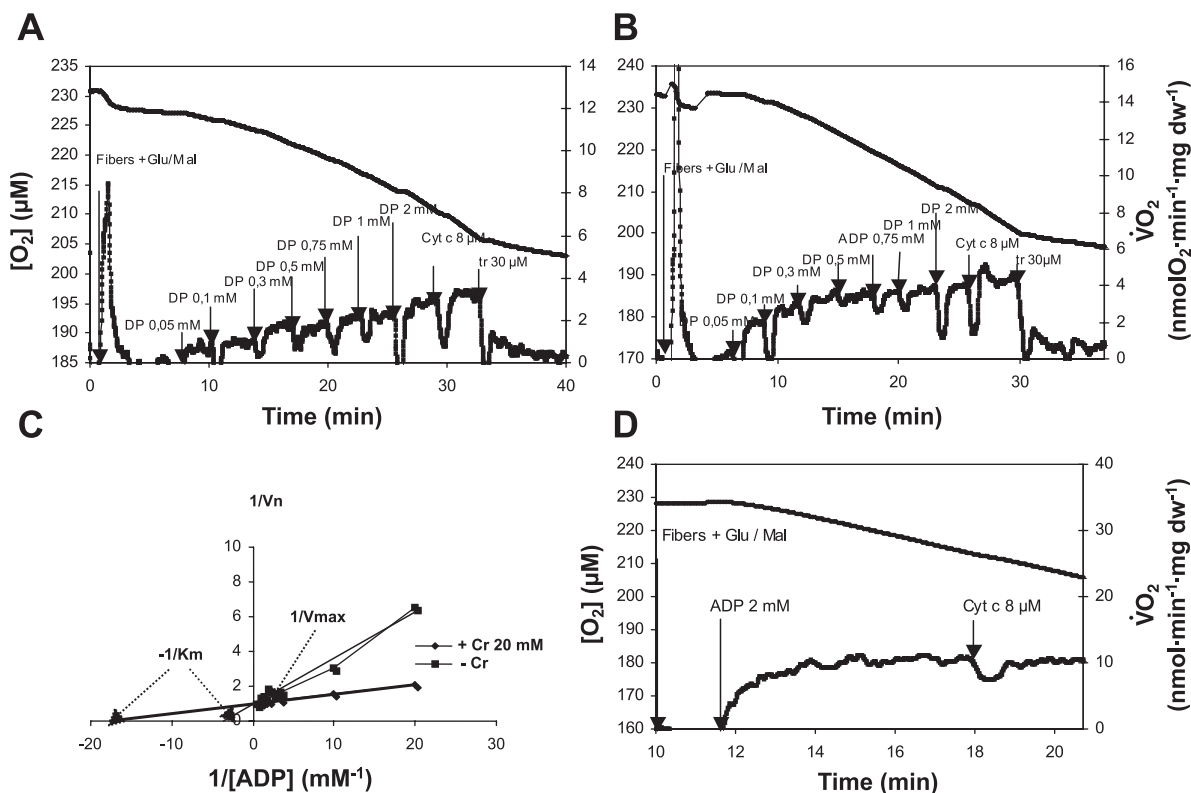


Fig. 4. Examples of  $\text{O}_2$  consumption ( $\dot{V}\text{O}_2$ ) by permeabilized quadriceps fibers. *Top traces* (A, B, D): changes in  $\text{O}_2$  concentration ( $[\text{O}_2]$ ) vs. time. *Bottom traces* (A, B, D): rate of respiration (derivative function of  $\dot{V}\text{O}_2$ ). Decrease in respiration after substrate addition is artifactual. A: kinetics of regulation of respiration by exogenous ADP in solution B. This experimental trace shows the dependence of respiration on concentration of ADP. Mitochondrial respiration increases with increasing concentrations of ADP in a range from 0.05 to 2 mM to reach the maximal rate of respiration ( $V_{\max}$ ). From this ADP-stimulated respiration, a Michaelis-Menten representation can be obtained as mitochondrial respiration rate is a function of ADP concentration (see Fig. 2C). At the end of the measure, cytochrome c (Cyt c) addition does not change the respiration, indicating that the outer membrane is intact. Moreover, atracyloside (Atr) addition results in a decrease of respiration back to the basal rate of respiration ( $V_0$ ), showing that we observe mitochondrial respiration. B: kinetics of the regulation of respiration by exogenous ADP in the presence of Cr in solution B. In the presence of Cr,  $V_{\max}$  is reached at lower concentrations of ADP. This is explained by the control of Cr on oxidative phosphorylation. At the end of the measure, Cyt c addition results in no damage of the outer membrane. Moreover, Atr addition results in a decrease in respiration back to  $V_0$ . C: double reciprocal plot of Michaelis-Menten graph: Lineweaver-Burk representation. This graph allows determination of the apparent affinity of mitochondria (apparent  $K_m$ ) for ADP. In the absence of Cr, apparent  $K_m$  value is rather high (more than 100  $\mu\text{M}$ ), meaning a low affinity of mitochondria for ADP. However, in the presence of Cr, apparent  $K_m$  is low, indicating a higher affinity of mitochondria for ADP.  $V_n$ , normalized rate of respiration. D: test of integrity of the outer mitochondrial membrane in KCl medium.  $V_0$  of fibers is obtained in the presence of glutamate (Glu; 5 mM) and malate (Mal; 2 mM).  $V_{\max}$  is reached by the addition of 2 mM ADP. Cyt c was then added (8  $\mu\text{M}$ ). There is no change in  $\dot{V}\text{O}_2$ , thus demonstrating that the outer mitochondrial membrane is not altered; otherwise, there would have been an increase in the rate of respiration. dw, Dry weight.

$K_m$  corresponded to the muscle fiber-type distribution in control fibers (Table 2). In the presence of creatine (20 mM), the apparent  $K_m$  for ADP decreased significantly to  $57 \pm 31 \mu\text{M}$  because of the local regeneration of ADP by MtCK in the intermembrane space. This ADP was more efficient in stimulation of respiration than the exogenous ADP. This explains the drop in  $K_m$  and shows interaction between MtCK and ANT in skeletal muscle mitochondria. The creatine effect on the apparent  $K_m$  for exogenous ADP was calculated as the difference between  $K_m$  in the absence of creatine and in the presence of creatine.

In LR before exercise training, although there was no statistically significant difference with C regarding the respiration rates (basal respiration rate and  $V_{\max}$ ), the apparent  $K_m$  was significantly lower ( $94 \pm 34 \mu\text{M}$ ) compared with C. This low value of apparent  $K_m$  was accompanied by a decay of type I oxidative fibers in LR fiber (Table 2). Moreover, the stimulating effect of creatine was less apparent in LR (Fig. 5). The partial loss of the creatine effect resulted from the glycolytic metabolism, which does not rely on coupled energy transfer systems (25).

*Effects of ADP and creatine on mitochondrial respiration after exercise training.* Figure 5 shows the dramatic effects of physical training on apparent  $K_m$  for exogenous ADP in both C and LR. In C, apparent  $K_m$  for exogenous ADP increased from  $131 \pm 26$  to  $260 \pm 67 \mu\text{M}$ , whereas in the presence of creatine this parameter remained very low (Fig. 5). These data underline the increased efficiency of the respiration regulation by creatine in C. The same pattern is seen in LR: the apparent  $K_m$  increased from  $94 \pm 34$  to  $203 \pm 62 \mu\text{M}$  and the creatine effect became highly significant after exercise training (Fig. 5).  $V_{\max}$  values were unchanged after exercise training in all conditions, but  $V_{\max}$  values with creatine increased by 58% in LR ( $P = 0.05$ ) (data not shown).

#### *Correlations of $\dot{V}\text{O}_{2\max}$ and Endurance Time at 70% $\dot{W}_{\max}$ Before and After Exercise Training*

Before exercise training in C and LR ( $n = 24$ ),  $\dot{V}\text{O}_{2\max}$  ( $\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) was correlated with percentage of type I



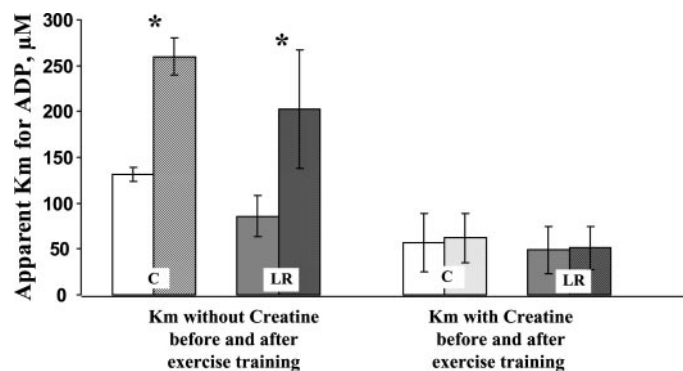


Fig. 5. Comparison of ADP sensitivity of mitochondrial respiration of C and LR skeletal muscle before and after exercise training with and without Cr. In the absence of Cr, apparent  $K_m$  for ADP in C is characteristic for mixed skeletal muscle (open bars). That of the LR group, however, is significantly lower (gray bars), closer to that of glycolytic fibers. In the presence of Cr, apparent  $K_m$  for ADP decreases in C as well as in LR. After rehabilitation (hatched bars), in the absence of Cr, apparent  $K_m$  for ADP increases 2-fold in C and LR. In the presence of Cr, apparent  $K_m$  for ADP decreases in C and LR. Values are means  $\pm$  SD. \* $P < 0.001$  before vs. after exercise training for  $K_m$  for ADP without Cr. Right:  $K_m$  for ADP with and without Cr are highly significant ( $P = 0.0005$  in C,  $P = 0.0009$  in LR), whereas these difference are more pronounced in C vs. LR ( $P = 0.02$ ).

fibers ( $r = 0.69$ ,  $P < 0.001$ ), apparent  $K_m$  for exogenous ADP ( $r = 0.64$ ,  $P = 0.001$ ), and  $V_{max}$  ( $r = 0.44$ ,  $P = 0.03$ ) (Fig. 6).  $\dot{W}_{max}$  was correlated with percentage of type I fibers ( $r = 0.77$ ,  $P < 0.0001$ ) and the apparent  $K_m$  for exogenous ADP ( $r = 0.58$ ,  $P = 0.004$ ). Endurance time at 70%  $\dot{W}_{max}$  was related to percentage of type I fibers ( $r = 0.72$ ,  $P = 0.0001$ ), fat-free mass ( $r = 0.53$ ,  $P = 0.007$ ), and the apparent  $K_m$  for exogenous ADP ( $r = 0.48$ ,  $P = 0.02$ ).

After exercise training,  $\dot{V}O_{2max}$  (%predicted) changes were related to  $V_{max}$  changes in C and LR ( $r = 0.55$ ,  $P = 0.007$ ).

**DISCUSSION**

Using the saponin-skinned fiber technique, we provided new insights in the mechanisms of limitation of exercise capacity and the benefits of exercise training in LR in relation to C. Before exercise training, LR mitochondrial respiratory rates did not reveal any alterations compared with C rates, excluding deleterious effects of anticalcineurins. Before exercise training, there were strong correlations between exercise capacity ( $\dot{V}O_{2max}$  and endurance time) and cellular events, as assessed by the percentage of type I fibers (apparent  $K_m$  for exogenous ADP). Exercise training resulted in significant improvements in bioenergetics at both the cellular (apparent  $K_m$  for exogenous ADP and stimulating effect of creatine) and the integrated level ( $\dot{V}O_{2max}$ ,  $\dot{W}_{max}$ , endurance time), which were initially lower in LR compared with C. Changes in  $\dot{V}O_{2max}$  and  $V_{max}$  were related. Changes in endurance time and percentage of type I fibers were also correlated. Because there were no changes in diameters and fiber types, baseline alteration of apparent  $K_m$  for exogenous ADP and its improvement after training might be related to changes within the intracellular energetic units (ICEUs) (Fig. 7). After training, as shown by ICEUs, there was a higher control of mitochondrial respiration by creatine linked to a more efficient functional coupling of ANT-MtCK, resulting in better exercise performances in C and LR.

*Comparisons Before Exercise Training Between LR and C*

Before exercise training, LR achieved lower  $\dot{V}O_{2max}$ ,  $\dot{W}_{max}$ , and endurance time compared with C (Table 2) as previously reported by Williams et al. (42, 43) and others (7, 9, 10, 16, 17, 20, 22, 34, 41). Pre- and posttransplant factors may explain impairment in exercise capacity in LR. Pretransplant factors include long-standing deconditioning, poor nutritional status, direct and indirect effects of chronic hypoxia, and corticosteroid exposure (see Refs. 2 and 3 for reviews) and mainly result in a reduction of type I fibers in skeletal muscles (11, 26). End-stage respiratory diseases via a systemic inflammation and hypoxia (2, 3) lead to an inhibition of anabolic transcription

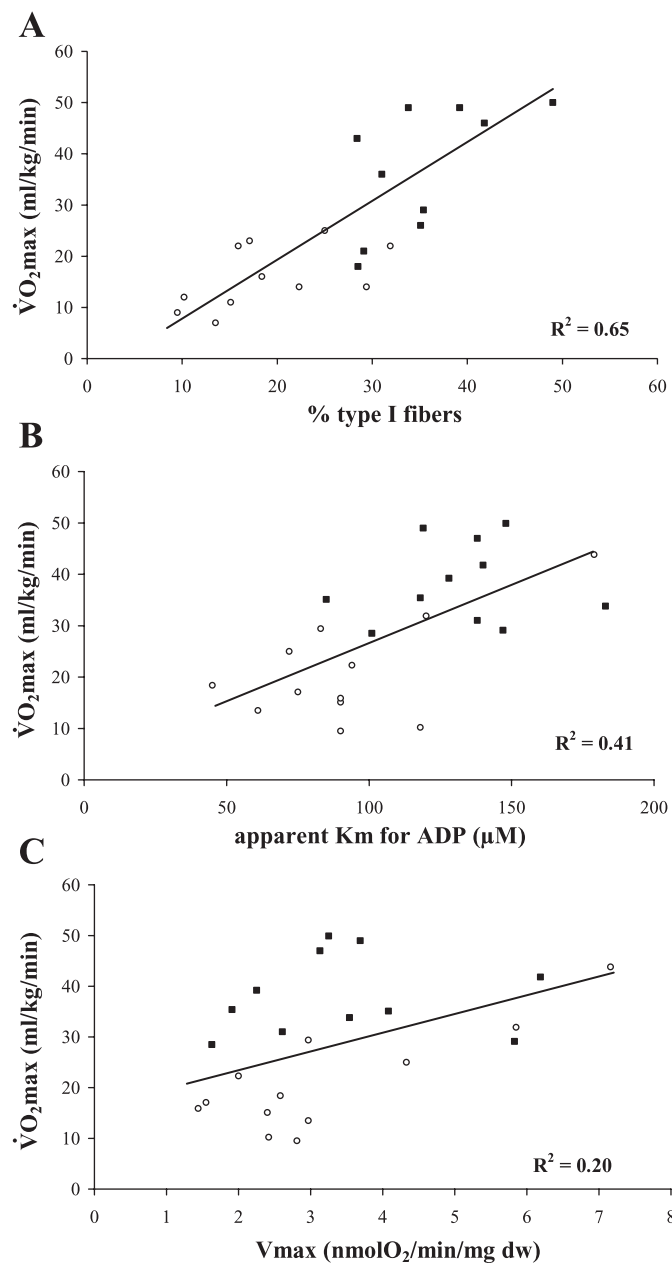


Fig. 6. Correlations for C (■) and LR (○) before exercise training ( $n = 24$ ) between maximal  $O_2$  consumption ( $\dot{V}O_{2max}$ ) and bioenergetics parameters. A: percentage of type I fibers. B: apparent  $K_m$  for exogenous ADP. C: mitochondrial  $V_{max}$ .



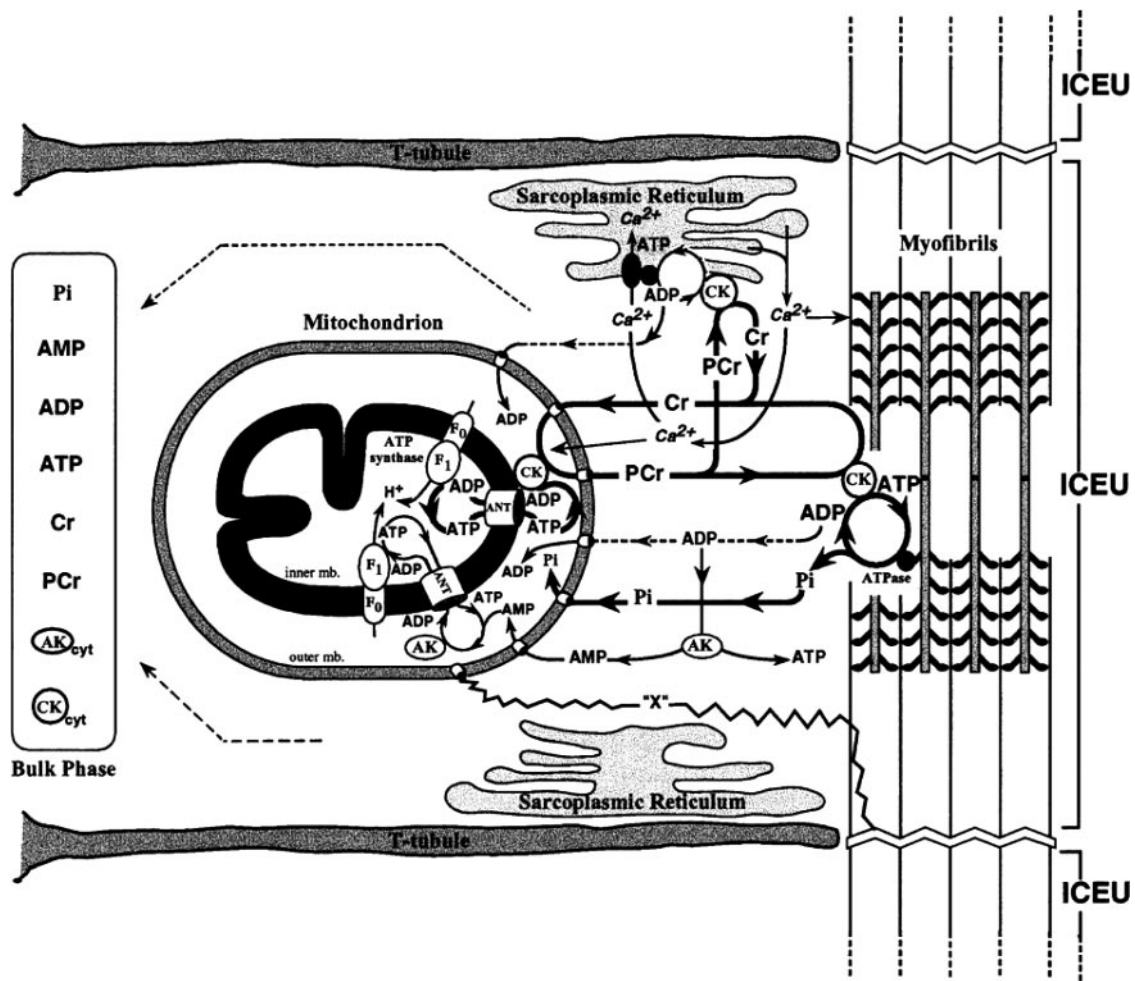


Fig. 7. Schematic representation of the intracellular energetic unit (ICEU). The mitochondria, the sarcoplasmic reticulum, and Mg-ATPase of myofibrils are interconnected by metabolic channeling of reaction intermediates and energy transfer within the ICEU by the creatine kinase (CK)-PCr and myokinase systems. The protein factors (still unknown and marked as "X"), most probably connected to cytoskeleton, fix the position of mitochondria and probably also control the permeability of the VDAC channels for ADP and ATP. This increases the microcompartmentation of adenine nucleotides within the ICEU. By interaction with cytoskeletal elements, the mitochondria and sarcoplasmic reticulum are precisely fixed with respect to the structure of the sarcomere of myofibrils between 2 z-lines and correspondingly between 2 T tubules. Adenine nucleotides within the ICEU do not equilibrate rapidly with adenine nucleotides in the bulk-water phase. ATP in the bulk-water phase may constitute a cellular metabolic reserve or serve some regulatory purposes. Adenine nucleotides within the ICEU and bulk-water phase may be connected by some more rapidly diffusing metabolites such as Cr and PCr. AK, adenylate kinase; AK<sub>cyt</sub>, cytosolic adenylate kinase; CK<sub>cyt</sub>, cytosolic creatine kinase.

factor, such as myoD, with a resulting preferential ubiquitination of the myosin heavy chain, as in cancer cachexia (1). Among posttransplant factors, cardiac and ventilatory limitations do not play a significant role because, in LT, the heart is not denervated and ventilatory limits were not reached in our patients, despite lower pulmonary function tests (Tables 1 and 2). In rats, it has been shown that anticalcineurins via the vehicle of cyclosporin A are responsible for reduced exercise endurance time by a decay in mitochondrial oxidative phosphorylation via reduced complex I and IV activities (28). In a clinical setting such as LT, after cardiac transplantation, immunosuppressive therapy effects are negligible on mitochondrial respiration (46) since  $V_{max}$  in LR was not different from C (Table 2). Moreover, LR patients received tacrolimus as anticalcineurin therapy and were not exposed to cyclosporin A (15). It appears that skeletal muscle dysfunction acquired before LT plays a central role in exercise limitation after LT, as shown by Wang et al. (41) in LR and Richardson et al. (26) in

chronic obstructive pulmonary disease. Our results confirmed that percentage of type I fibers plays a major role in  $\dot{V}O_{2max}$ ,  $\dot{W}_{max}$ , and endurance time in LR and C (26, 41) (Table 2 and Figs. 2 and 6).

Before exercise training, besides the important role of type I fibers, analyses of bioenergetic parameters such as apparent  $K_m$  for exogenous ADP,  $V_{max}$ , and stimulating effect of creatine on respiration give new insights into cell organization related to ATP production and channeling of energy transfer after the training period (Figs. 1, 6, and 7). Before exercise training in LR, our data showed that mitochondrial rates of respiration ( $V_{max}$ , basal rate, and mitochondrial respiration rate in the presence of atractyloside) were similar to control values (Table 2). Moreover, the activities of each respiratory chain complex gave normal responses to the different stimuli. This suggests that the intrinsic muscle oxidative capacities are normal in LR excluding complex I and IV inhibitions by anticalcineurins, as already reported in heart transplant recipients (46). On the

other hand, apparent  $K_m$  for exogenous ADP was significantly lower in LR compared with C (Fig. 5), mainly explained by the decreased percentage of type I fibers in LR skeletal muscle (Fig. 2C) (14).

#### Exercise Training Effects in LR and C

**Improvement of apparent  $K_m$  for exogenous ADP.** A sensitive parameter resulting from exercise training was the dramatic improvement in endurance time in LR. To our knowledge, it is the second report on benefits of exercise training in LR (34) and the first to use a home-based program for LR. We have found significant modifications of the cell energy metabolism parameters in permeabilized cells. After exercise training, a twofold increase in the apparent  $K_m$  for exogenous ADP without any significant fiber-type transition from glycolytic to oxidative muscles and no alterations in fiber diameter were observed in both groups (Figs. 2B and 5 and Table 2). This increase in apparent  $K_m$  for exogenous ADP resulted in more significant effects of creatine on mitochondrial respiratory parameters. Electron microscopic observations of biopsy materials showed in all cases similar cell structures and intracellular arrangement of mitochondria, mostly close to the  $z$ -line (Fig. 3). Therefore, the observed changes in biochemical parameters most probably indicated alterations in protein complexes in energy transfer pathways as discussed below.

It was found by Ventura-Clapier's and Bigard's (18, 44, 45) groups that voluntary activity induced specific alterations in the fast-twitch skeletal muscle, among which was the very significant (several times) increase in the apparent  $K_m$  for exogenous ADP in the regulation of respiration of the skinned cardiac fibers. Our present results give new important information to understanding this phenomenon. Our data excluded some plausible explanations such as influence of muscle hypertrophy and increase of the diffusion distances for exogenous ADP (33) and allowed the assumption of exercise-induced alterations in cell structure and protein complexes.

**Creatine/phosphocreatine shuttle and metabolic channeling.** In the slow-twitch skeletal muscles, energy is transferred between mitochondria and myofibrils by the creatine/phosphocreatine shuttle, whereas in the fast-twitch muscles the energy for contraction is supplied mostly by the glycolytic system via equilibrium cytoplasmic creatine kinase and coupled myofibrillar MM-creatine kinase systems (12, 32, 37, 40). In the preparations of permeabilized fibers, a stimulating effect of creatine on ADP-stimulated respiration indicated a functional coupling between the MtCK and ANT by regeneration of local ADP (Fig. 1) (32). This local ADP, produced by MtCK in the vicinity of ANT, enters the mitochondrial matrix for rephosphorylation and activates respiration. These cycles are repeated many times, explaining the increase in the rate of respiration at much lower exogenous ADP concentrations in the medium in the presence of creatine, compared with those in its absence. The result is a decrease in apparent  $K_m$  for exogenous ADP in the presence of creatine, directly showing the functional coupling between MtCK and ANT.

#### ICEU and Mitochondrial Organization and Interactions

In the fast-twitch skeletal muscle, mitochondria are localized at the level of I band and close to  $z$ -lines and the T-tubular system (21, 38). This was mostly the case in the biopsy

samples that we studied (Fig. 3). Oxidative skeletal muscles occupied intermediate positions between cardiac and glycolytic muscles with respect to quantity of mitochondria, which were localized both at I- and A-band levels (21). The value of the apparent  $K_m$  for exogenous ADP is very different in oxidative and glycolytic muscles (14). In the muscle samples with mixed fiber types, the value of the apparent  $K_m$  for exogenous ADP is between two extreme values (300–400  $\mu$ M and 10–20  $\mu$ M), depending on the fiber composition (14). These differences that exist between different types of muscles may be explained by the different structural and functional organizations of energy metabolism of the cells that exist due to distinct interactions between mitochondria, sarcoplasmic reticulum, and sarcomeres with the cytoskeleton (30). Furthermore, in oxidative cells, the formation of functional complexes between these structures, which are called ICEUs (30), gives rise to the unitary nature of energy metabolism in these cells. The structure of these energetic units is described in detail in Fig. 7.

In skeletal muscles with different mitochondrial positions, the structures may be different (30). These complexes play an important role in metabolic channeling of endogenous ADP by an effective functional coupling between local ADP production by enzymatic systems that consume ATP and its utilization for ATP synthesis in the mitochondrial matrix (Fig. 7) (5). These channeling mechanisms were related to the increased heterogeneity of the intracellular diffusion of ADP and ATP and local restrictions of their intracellular diffusion, due to the specific structural organization of the ICEUs. Thus, according to this limitation in adenine nucleotide diffusion, the increased value of the apparent  $K_m$  for exogenous ADP in permeabilized fibers showed a higher degree of structural organization of energy metabolism (30).

The role of the structural organization of the cells was clearly seen in other experiments after a nonselective proteolytic treatment of permeabilized fibers from oxidative muscles with trypsin (1  $\mu$ M) (13). After this treatment, intracellular arrangement was lost and apparent  $K_m$  for exogenous ADP was decreased close to the value of isolated mitochondria (13). These data most probably show that some or many cytoskeletal proteins could be responsible for this specific cytoarchitecture and also for regulation of mitochondrial function within the muscle cells (6, 19).

Additionally, transgenic animals depleted of cytoskeletal proteins such as desmin, the predominant protein in muscle cells, display significant alterations of mitochondrial position in the cells and regulation of their respiration *in situ* (13). The proteins that may control the permeability of the mitochondrial outer membrane within the structure of ICEUs are shown in Fig. 7. The nature of the proteins that control mitochondrial position and function in the cells, however, is still not completely clear.

A complete picture will probably require the integration of data obtained from functional genomics approaches and modeling efforts. Progress toward this goal will come in the form of high-throughput methods to identifying the connections within the protein-protein and protein-DNA interaction networks. Thus the recent advances in these areas may provide us with a first glimpse of the overall structure of molecular interaction networks in this complex biological system. Their identification would be an interesting further task of muscle



cell proteomic analysis to find an explanation for the interesting phenomenon observed in this work.

Exercise activities may induce changes in the expression of these proteins and thus increase the restrictions of the exogenous ADP diffusion into the cells. The increase in restriction barriers may contribute to a better metabolic channeling within ICEUs, making the role of the creatine kinase-phosphocreatine pathway more important in the regulation of respiration. The observed statistically significant increase in the  $V_{\max}$  of mitochondrial respiration measured in the presence of creatine is consistent with this conclusion and may partially explain the increase in  $\dot{V}O_{2\max}$  in LR after exercise training (Table 2). These sensitive events could occur at an early stage and do not allow us to make any clear correlation with the integrated physiological parameters as they concern the intracellular level and do not involve muscle fiber changes (see *Limitations of the Study*). These processes could be compared with the early stage of cardiac ischemia, in which the functional coupling between MtCK and ANT is lost while the heart still has functional properties (27).

#### Limitations of the Study

Although two women were included in LR and none in C, this fact did not reach clinical and statistical significance and may not explain differences between LR and C before and after exercise training. More importantly, the 10-yr difference in favor of C compared with LR may explain the higher exercise capacity in C. Actually, as previously reported (24), the mitochondrial theory of aging does not explain the age-related decline of muscle performance in young and elderly healthy adults since mitochondrial oxidative capacity and ATP formation are unchanged.

Before exercise training, highly significant correlations between integrated physiological data and bioenergetic assessment at the cellular level were obtained, suggesting a role of the ICEUs in physical performance. This kind of correlation between improvement in effort tolerance and changes in intracellular metabolic channeling (apparent  $K_m$  for exogenous ADP and creatine effect) was not achieved after exercise training. This could be related to a type II error. Our hypothesis is that these changes, picked up at the cellular level, occur at the very early stages. This lack of correlation might be due to the methods that we used to assess muscular performance. In this regard, a segmental endurance muscular assessment would have resulted in better correlations between muscle performance and bioenergetic studies (26).

#### Conclusion

Muscle dysfunction in LR is mostly related to pretransplant factors because anticalcineurins did not result in mitochondrial dysfunction. Before exercise training, in LR and C, exercise capacity is related to skeletal muscle structure, i.e., fiber type and ICEU organization. Home exercise training is feasible and efficient in both LR and C. After exercise training, ICEUs exhibited a higher control of mitochondrial respiration by creatine linked to a more efficient functional coupling of ANT-MtCK, resulting in better exercise performances in C and LR.

#### ACKNOWLEDGMENTS

The Centre d'Investigation Clinique, Inserm of the Centre Hospitalier Universitaire de Grenoble, and Béatrice Rival and Cécile Chérion (research

assistants), as well as technicians from the pathology laboratory and the pulmonary function test laboratory, are acknowledged for logistic support. We are indebted to Elisabeth Maclat and technicians of AGIR à Dom, a home care association, for the excellent care they provided to the subjects for home exercise training. We are deeply indebted to our volunteers for enthusiastic participation. The contribution of Laurence Kay to some parts of the discussion is also gratefully acknowledged. We thank Dr. Dan Veale (Fellow of the Royal College of Physicians) for reviewing the English text.

#### GRANTS

This work was supported by grants from Conseil Scientifique of Association Nationale de Traitement à Domicile Innovation et Recherche, Conseil Scientifique of AGIRàDom, Comité départemental de Lutte contre les Maladies Respiratoires de l'Isère, Délégation à la Recherche Clinique du Centre Hospitalier Universitaire de Grenoble, Centre d'Investigation Clinique, Inserm, CHU Grenoble, and Programme Interdisciplinaire Complexité du Vivant et Action STIC-Inserm. This work was also supported in part by Marie Curie Fellowship of the European Community program "Improving Human Research Potential and the Socio-economic Knowledge Base" (M. Vendelin, HPMF-CT-2002-01914) and Estonian Science Foundation Grants 5515 and 6142.

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