VO₂ Kinetics in Supra-Aerobic Threshold Constant Tests Allow the
Visualisation and Quantification of the O₂ Saving after Cytochrome C Oxidase
Inhibition by Aerobic Training or Nitrate Administration

Domenico Maione,¹ Arrigo F.G. Cicero,¹ Stefano Bacchelli,¹ Eugenio Cosentino,¹
Daniela Degli Esposti,¹ Roberto Senaldi,² Enrico Strocchi,¹ Sergio D’Addato¹, Claudio
Borghi ¹

¹Medical and Surgical Sciences Dept., University of Bologna, Italy
²Sport Medicine Institute, University of Bologna, Italy

Running title: O₂ saving after COX inhibition

Corresponding author:

Prof. Claudio Borghi

Medical and surgical sciences Dept.
S.Orsola-Malpighi University Hospital
Via Albertoni 15 - Pad. 2
40138 Bologna, Italy
Tel. +39-0516363243 - FAX +39-051391320

e-mail: claudio.borghi@unibo.it
Summary
We tested whether the known cytochrome c oxidase (COX) inhibition by nitric oxide (NO) could be quantified by VO$_2$ kinetics during constant load supra-Aerobic Threshold (AT) exercises in healthy trained or untrained subjects following aerobic training or nitrate administration. In cycle ergometer constant load exercises supra-AT, identified in previous incremental tests, VO$_2$ kinetics describe a double exponential curve, one rapid and one appreciably slower, allowing the area between them to be calculate in O$_2$ L. After training, with increased NO availability, this area decreases in inverse ratio to treatment efficacy. In fact, in 11 healthy subjects after aerobic training for 6-7 weeks, area was decreased on average by 51%. In 11 untrained subjects, following the assumption of an NO donor, 20 mg isosorbide 5 mononitrate, area was decreased on average by 53%. In conclusion, supra-AT VO$_2$ kinetics in constant load exercises permit the quantification of the inhibitory effect NO-dependent on COX after either physical training or nitrate assumption.

Key-words
Oxygen consumption, anaerobic threshold, VO$_2$ kinetics, cytochrome c oxidase inhibition, nitrate, training
Introduction

Nitric oxide (NO), in appropriate concentrations, interacts with cytochrome c oxidase (COX), and causes an “early reduction” in the mitochondrial respiratory chain and a backlog of electrons in the upstream respiratory chain cytochromes because it partially and reversibly inhibits COX. Such an effect is modulated by both an increase in electrons turnover (ET) and by the normally high capacity of COX for processing electrons (Leterlier et al. 1994, Sharpe and Cooper 1998), allowing both cellular respiration and energy supply to be maintained. Experimentally, if NO production is strongly increased in cells, compensation will be gradually weakened, because the ability of COX to oxidize to water electrons, coming from cytochrome c, will approximate its saturation. This consequently decreases the O_2 consumption rate, i.e. mitochondrial respiration (Brown and Cooper, 1994, Brunori et al. 2005, Cleeter et al. 1994, Cooper and Giulivi, 2007, Erusalimsky and Moncada, 2007, Mason et al. 2006, Moncada and Ersalimsky, 2002, Palacios-Callender et al. 2007, Schweizer and Richter, 1994). This mechanism can predict that cells with low energy demand, with normal O_2 concentration [O_2], are respiring with a low ET rate. In this way, the COX catalytic sites, richer in oxidized species, will "consume" NO, oxidizing it to nitrate. In contrast, cells with high ATP demand, such as muscle fibers during intense physical effort, will respire with a high ET rate, and COX catalytic sites, richer in reduced species, will not consume NO, which, synthesized in quantity, will bind to and inhibit the COX catalytic sites with high affinity (Giuffré et al. 2000, Palacios-Callender et al.2007).

Such a mechanism has been also reported by other authors to explain the VO_2 cost reduction during high-intensity exercises after dietary nitrate (Bailey et al. 2009, Lansley et al. 2010; Larsen et al. 2010) or L-arginine supplementations (Bailey et al. 2010), and to explain the opposite effect after infusion by L-NAME (Jones et al. 2003). We also
previously described this effect (Maione et al. 1998; Maione et al. 2000; Maione et al. 2001a; Maione et al. 2001b).

In this context, the present study aims to test whether it is possible visualize and quantify the interaction between NO and COX by recording VO$_2$ kinetics during cycle ergometer constant-load exercises (or square wave) supra-Aerobic Threshold (AT) in a population of 11 healthy subjects after aerobic training and in 11 healthy subjects after administration of an organic nitrate acting as a NO donor.

**Methods**

Two groups of healthy volunteers were enrolled for the study. The first group included 11 healthy subjects: 9 young students and 2 amateur cyclists 68 and 72 old years (8 males, 3 females; mean age = 30.2 ± 20.1 years; BMI = 24.9 kg/m$^2$); the second group included 11 healthy untrained subjects (6 males, 5 females; mean age = 36.5 ± 8.9 years; BMI = 25.6 kg/m$^2$) from the Dept of Medical and Surgery Sciences, University of Bologna.

All subjects underwent a maximal cardiopulmonary test with a ramp protocol, preceded by a 3-minute rest period, and a 3-minute warm-up at the load of the first step ramp, which therefore began at the second step. Load progression during the ramp was adapted to the expected functional capacity of each subject, and loads of square wave tests were selected on the basis of previous incremental tests. Nevertheless, ramp test durations were significantly different for subjects in the second group, who were dissimilar in physical characteristics and functional capabilities. However, the objective of this study was only to demonstrate the ability of our method to illustrate and quantify the consequences of the interaction between NO and COX.

Subjects in the first group carried out two similar tests at constant load before and after a 7-weeks aerobic training program, performed on cycle ergometer. Four subjects also underwent a subsequent detraining of equal duration. Therefore, each subject in this group
performed a cardiopulmonary test before and after training, at constant intensity, and the four detraining subjects performed a third square wave test, with the same procedure and the same load. Each test was executed with the load at the point of "respiratory compensation to metabolic acidosis" (RC) (Wasserman et al. 1967; Wasserman et al. 1999), which was identified during the previous incremental tests.

The subjects of the second group carried out two similar square wave tests: before and after 1 dose of 20 mg of isosorbide-5-mononitrate, at the RC load, which was reduced by an average of 10.6% (range 4-40%). This large range is the result of attempts to identify the most effective load intensity in highlighting the interaction NO-COX. In any case, the first test was performed as a control, and the second, one day later, after nitrate at timing of its maximum effect: 1 to 1.5 hour after its assumption.

For all tests, a Medifit 1000 cycle ergometer (Holland), calibrated before each session and an ULTIMA-CPX (Medical Graphics Corporation - MGC) ergospirometer, calibrated before each test, were used and interfaced with a CASE 16 (Marquette, USA) electrocardiograph.

We used a Rudolph mask (7930-7940 series, USA) and, only in the second group, we sealed the space between the mask and the face with a special gel, modelled on the internal geometry of the mask (Ultimate Seal, Rudolph, USA). To further improve adhesion between face and mask, again only in the second group, we replaced the cap and fastening straps of the Rudolph mask with PVC ones. A constant pedalling frequency during each test was set at 60 rpm and maintained with the help of a digital metronome.

The raw breath-by-breath VO₂ data were averaged over 5 on 7 breaths. In addition, VO₂ data derived from the constant load tests were treated with a moving average filter in order to reduce the sampling noise, temporally aligned to the exercise beginning, and interpolated to 0.01 s.

A biexponential mathematical model was fitted to the data, using custom-built software, Cardio Pulmonary Exercise Parameters Estimator (CPEPE). This iteratively optimizes the
model parameters to fit to the VO₂ experimental data. The mathematical model was biexponential because in this kind of tests the VO₂ time-course presents two exponential kinetic components: a virtual one, extrapolated via software, which is earlier and rapid, and one corresponding to the actual VO₂ during the test, which is appreciably slower [the so-called “slow component” (SLC)] and overlaps to the previous one (Fig. 1, above to left) (Barstow and Molé 1991; Casaburi et al. 1987; Pool et al. 1994; Whipp and Ward 1990, Whipp 1994). The integral of the area between two exponentials was calculated, expressed in litres (l) of O₂. All square wave tests were run to enable the computing of the area during 6 minutes from the point of separation of two exponentials, except for a shorter duration in a single subject of the second group.

The trial was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) on volunteers, after informed consent had been confirmed in writing. The protocol was approved by the local ethical committee. The normality of the distribution of the tested parameters was evaluated by the Kolmogorov-Smirnov test. Differences in area within every group, respectively before and after training, and before and after nitrate, were computed by the t-test for paired data, while in the 4 detrained subjects of the first group, differences were tested by the ANOVA test for repeated measures with Bonferroni correction. In all statistical analyses the significance level was set at p<0.05. All summary statistics are presented as means ± standard deviation (SD).

Results

After training, in the first group of subjects the area between the two exponential was reduced by the treatment, while it increased after detraining (Fig. 1). In these subjects the mean amplitude of the area before the training was 2.92 ± 0.96 l, while post-training it was 1.44 ± 0.90 l, with a difference of 1.47 ± 0.41 l (−51%; p<0.001); in the four detrained
subjects the mean amplitude of the area before and after training, and after the detraining was, respectively, 3.69 ± 0.57, 2.11 ± 0.65, and 3.04 ± 0.34 l. The mean difference between first and second tests was 1.59 l (-42.8%; p=0.006), between the first and third tests 0.65 l (-17.6%; p=0.020), between second and third tests 0.93 l (+44.1%; p=0.014). In subjects of the second group (Fig. 2), and the mean area between the two exponentials before nitrate assumption was 0.76 ± 0.42 l, and after nitrate 0.36 ± 0.28 l, with a difference of 0.40 ± 0.25 l (-53%; p<0.001).

Discussion

The exercise at a constant load supra-AT, executed in subjects of the first group after aerobic training effectively illustrates the consequences of NO-COX interaction (Fig. 1). The training increased vascular capacity to synthesize NO, which the square wave exercise produced, decreasing VO\textsubscript{2} thus sparing O\textsubscript{2}, and inhibiting the COX. Oxygen sparing also reduces both metabolic and respiratory stress, and hence the fatigue, induced by exercise, promoting O\textsubscript{2} diffusion out of muscle fibres mitochondria towards other fibres far away from vessels (Thomas DD et al. 2001, Victor MV et al. 2009). So, it is not surprising if in one individual subjects of both group, independently of age, sex and the stimulus used (training/nitrate), the inhibitory action on COX was so effective as to completely null the area between the two exponentials in the square wave test, flattening the VO\textsubscript{2} curve to assume a mono-exponential course, typical of square wave exercises sub-AT (Fig. 3). Due to this mechanism, and the evidence that O\textsubscript{2} sparing is not compensated by an equal increase in anaerobic metabolism, O\textsubscript{2} sparing retards the critical moment when short term effort is no longer bearable by the cardiovascular and pulmonary systems. Such an effect is very probably due to the activation of AMP-activated protein kinase (AMPK) (Dzeja and Terzic 2009; Steinberg and Kemp 2009), which occurs when the AMP/ATP ratio increases beyond a certain threshold, mainly as a consequence of
accelerated ATP lysis, as during exercise, making this ratio a sensitive indicator of reduced cellular energy state (Hardie and Sakamoto 2006; Jorgensen et al. 2006). Such muscle metabolic changes produced by AMPK activation are smaller after a single effort than after endurance training, and lie in increased insulin sensitivity (Etgen et al. 1997; Goodyear and Kahn 1998). In fact, translocation of the glucose carrier GLUT4 from intracellular stores into myocyte plasma membrane is increased (Kurt-Kraczek et al. 1999), causing both greater glucose uptake, and hexokinase activation (Holmes et al. 1999), which promotes glycolysis, supplying more substrates to the Krebs cycle. At the same time, there is an inhibition of glycogen synthesis (Carling and Hardie, 1989), without affecting glycogenolysis, during muscular effort (Mu et al. 2003). Furthermore, plasma fatty acids uptake is increased, largely through increased AMPK-dependent expression of carrier proteins on the plasma membranes (Bonen et al. 2007). Plasma fatty acids and the fatty acids stored in muscle fibers are then oxidized (Kiens 2006). All these mechanisms, together with increased concentration of the Krebs cycle enzymes in the muscle fibers mitochondria (Holloszy et al. 1970), lead to both increased aerobic metabolism and reduced recourse to anaerobic metabolism. Consequently, both the peak of the respiratory exchange ratio at the mouth (RER or R) and the area under the RER curve decrease after training and increase after detraining (Fig. 1). In fact, the effect of AMPK in increasing GLUT4 and plasma fatty acids carriers is relatively short-lasting, and regular muscle recruitment is essential to maintain their activity (Host et al. 1998; Koonen et al. 2004).

Taken together, these considerations show that both the O₂ sparing and the RER decrease depend not only on the NO-COX interaction, but also on the consequential, complex and effective, metabolic changes, reported above.

Yet, other mechanisms link NO-COX interaction to AMPK activation. As a result of such interaction the electrons leak from complexes I and III increases, and one electron is transferred from NO to molecular O₂ to produce superoxide anion (O₂⁻) and then hydrogen
peroxide (H₂O₂), causing rapid depletion of ATP and an accumulation of AMP (Almeida et al. 2004). This activates AMPK, indicating H₂O₂ to be a major signal for AMPK regulation under oxidative stress (Choi et al. 2001). Again, during both NO-COX interaction (Woods et al. 2005), and muscular exercise (Rose and Hargreaves 2003) Ca²⁺ is released and, linked to calmodulin kinase kinase, activates AMPK. Besides, endothelial NO-synthase is also a downstream target of AMPK that is therefore able to influence the availability of NO. So, the pathway NO-cytochrome c oxidase-AMPK is a real signalling cascade and could represent a survival pathway during various pathophysiological conditions (Fig. 4).

In the second group, after administration of nitrate, we confirmed that NO-dependent COX inhibition is unambiguously direct, and that it can be quantified through this clinical test using VO₂ kinetics during constant intensity exercises supra-AT (Fig. 2). Before and after treatment, the remarkable differences in area between the two groups are due in part to lower loads of the square wave tests in second group. In fact, the workload selection used in the first group is preferable, because the greater area developed allows for easier inter-subject and inter-group comparison. A reduction in the contribution of anaerobic metabolism after nitrate assumption was also observed in square wave tests of the second group, albeit less pronounced compared to those of the first group, due to the loads, all selected below the RC point of the incremental test. However, the results of the square wave tests in the two groups were qualitatively quite similar, despite the different load intensities: both higher NO concentrations and metabolic changes improved tolerance to stress, reduced VO₂ and decreased RER during muscular effort, after physical training and nitrate administration.

The quantification, by our test, of the variable area, could be used to monitor the benefits of physical aerobic training, because we believe that more effective training should be followed by a greater decrease in area. In clinical and experimental pharmacology and in cases of pathology, drugs either directly activating AMP-kinase or acting in general on
mitochondria can also affect the main, respiratory, mitochondrial function, and the method, that we described, can provide information about this. This study is a starting point to implement this technique in clinical and experimental use, as well as in sports medicine.

In conclusion, on the basis of our preliminary data, the VO$_2$ kinetics supra-AT during constant load exercise allow the visualization and quantification of the NO inhibitory effect on COX after both physical training and nitrate assumption.

**Acknowledgements**

This trial was carried out with the supports of institutional funding from the University of Bologna.

**References**

ALMEIDA A, MONCADA S, BOLAÑOS JP: Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway. *Nat Cell Biol* 6: 45-52, 2004.

BAILEY SJ, WINYARD P, VANHATALO A, BLACKWELL JR, DIMENNA FJ, WILKERSON DP, TARR J, BENJAMIN N, JONES AM: Dietary nitrate supplementation reduce the O$_2$ cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J Appl Physiol* 107: 1144-1155, 2009.

BAILEY SJ, WINYARD PG, VANHATALO A, BLACKWELL JR, DIMENNA FJ, WILKERSON DP, JONES AM: Acute L-arginine supplementation reduces the O$_2$ cost of moderate intensity exercise and enhances high-intensity exercise tolerance. *J Appl Physiol* 109: 1394-1403, 2010.

BARSTOW TJ, MOLÉ PA: Linear and non linear characteristics of oxygen uptake kinetics during heavy exercise. *J Appl Physiol* 71: 2099-2106, 1991.
BONEN A, CHABOWSKI A, LUIKEN JJFP, GLATZ JFC: Mechanisms and regulation of protein-mediated cellular fatty acid uptake: molecular, biochemical, and physiological evidence. Physiology 22: 15-29, 2007.

BROWN GC, COOPER CE: Nanomolar concentrations of nitric oxide reversibly synaptosomal respiration by competing with oxygen at cytochrome oxidase. FEBS Lett 345: 295-298, 1994.

BRUNORI M, GIUFFRÉ A, SARTI P: Cytochrome c oxidase, ligands and electrons. J Inorg Biochem 99: 324-336, 2005.

BRUNORI M, FORTE E, ARESE M, MASTRONICOLA D, GIUFFRÉ A, SARTI P: Nitric oxide and the respiratory enzyme. Biochim Biophys Acta. 1557: 1144-1154, 2006.

CARLING D, HARDIE DG: The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. Biochim Biophys Acta 1012: 81-86, 1989.

CASABURI R, STORER TW, BEN-DOV I, WASSERMAN K: Effect of endurance training on possible determinants of VO₂ during heavy exercise. J Appl Physiol. 62: 199-207, 1987.

CHOI SL, KIM SJ, LEE KT, KIM J, MU J, BIRNBAUM MJ, KIM SS, HA J: The regulation of AMP-activated protein kinase by H₂O₂. Biochem Biophys Res Commun 287: 92-97, 2001.

CLEETER MW, COOPER JM, MONCADA S, SCHAPIRA AH: Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. FEBS Lett 345: 50-54, 1994.

COOPER CE, GIULIVI C: Nitric oxide regulation of mitochondrial oxygen consumption II: molecular mechanism and tissue physiology. Am J Physiol Cell Physiol 292: C1993-C2003, 2007.
DZEJA P, TERZIC A: Adenylate kinase and AMP signalling networks: metabolic monitoring, signal communication and body energy sensing. *Int J Mol Sci* **10**: 1729-1772, 2009.

Erusalimsky JD, Moncada S. Nitric oxide and mitochondrial signalling. From physiology to pathophysiology. *Arterioscler Thromb Vasc Biol* **27**: 2524-2531, 2007.

ETGEN GJ, JENSEN J, WILSON CM, HUNT DG, CUSHMAN SW, IVY JL: Exercise training reverses insulin resistance in muscle by enhanced recruitment of GLUT4 to the cell surface. *Am J Physiol* **272** (*Endocrinol. Metab. 35*): E864-E869, 1997.

GIUFFRÉ A, BARONE MC, MASTRONICOLA D, D’ITRI E, SARTI P, BRUNORI M: Reaction of nitric oxide with the turnover intermediates of cytochrome c oxidase: reaction pathway and functional effects. *Biochemistry (Mosc)* **39**: 15446-15453, 2000.

GOODYEAR LJ, KAHN BB: Exercise, glucose transport, and insulin sensitivity. *Annu Rev Med* **49**: 235-261, 1998.

HARDIE DG, SAKAMOTO K: AMPK: a key sensor of fuel and energy status in skeletal muscle. *Physiology* **21**: 48-60, 2006.

HOLLOSZY JO: Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J Biol Chem* **242**: 2278-2282, 1967.

HOLLOSZY JO, OSCAI LB, DON IJ, MOLÉ PA: Mitochondrial citric acid cycle and related enzymes; adaptive response to exercise. *Bioch Biophys Res Comm* **40**: 368-1373, 1970.

HOLMES BF, KURTH-KRACZEK EJ, WINDER WW. Chronic activation of 5’-AMP-activated protein kinase increases GLUT4, hexokinase, and glycogen in muscle. *J Appl Physiol* **87**: 1990-1995, 1999.
HOST HH, HANSEN PA, NOLTE LA, CHEN MM, HOLLOSZY JO: Rapid reversal of adaptative increases in muscle GLUT4 and glucose transport capacity after training cessation. *J Appl Physiol* **84**: 798-802, 1998.

JONES AM, WILKERSON DP, WILMSHURST S, CAMPBELL IT: Influence of L-NAME on pulmonary O₂ kinetics during heavy-intensity cycle exercise. *J Appl Physiol* **96**: 1033-1038, 2003.

JØRGENSEN SB, RICHTER EA, JØRGEN F, WOJTASZEWSKI P: Role of AMPK in skeletal muscle metabolic regulation and adaptation in relation to exercise. *J Physiol* **574**: 17-31, 2006.

KIENS B: Skeletal muscle lipid metabolism in exercise and insulin resistance. *Physiol Rev* **86**: 206-243, 2006.

KOONEN DPY, BENTON CR, ARUMUGAM Y, TANDON NN, CALLES-ESCANDON J, GLATZ JFC, LUIKEN JJFP, BONEN A: Different mechanism can alter fatty acid transport when muscle contractile activity is chronically altered. *Am J Physiol Endocrinol Metab* **286**: E1042-E1049, 2004.

KURTH-KRACZEK EJ, HIRSHMAN MF, GOODYEAR LJ, WINDER WW: 5'-AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* **48**: 1667-1671, 1999.

LANSLEY KE, WINYARD PG, FULFORD J, VANHATALO A, BALLEY SJ, BLACKWELL JR, DIMENNA FJ, GILCHRIST M, BENJAMIN N, JONES AM. Dietary nitrate supplementation reduces the O₂ cost of walking and running: a placebo-controlled study. *J Appl Physiol* **110**: 591-600, 2010.

LARSEN FJ, WEITZBERG E, LUNDBERG JO, EKBLOM B. Effect of dietary nitrate on oxygen cost during exercise. *Acta Physiol* **191**: 59-66, 2007.
LARSEN FJ, WEITZBERG E, LUNDBERG JO, EKBLOM B: Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise. *Free Rad Biol Med* **48**: 342-347, 2010.

LETERRILLIER T, HEINRICH R, MALGAT M, MAZAT J-P: The kinetic basis of threshold effects observed in mitochondrial diseases: a systemic approach. *Biochem J* **302**: 171-174, 1994.

MAIONE D, SENALDI R, AZZOLINI PL, MONDARDINI P, TENTONI C, DRAGO E: Supra-threshold VO₂ kinetics of prepuberal children. Physical training effects. *Med Sport* **51**: 307-312, 1998.

MAIONE D, SENALDI R, GNUDI G, CUNA PR, MAIETTA P, MAIONE A: Training effects on VO₂, VCO₂, VE, Hr supra-threshold kinetics and on incremental test parameters. *Med Sport* **53**: 145-164, 2000.

MAIONE D, SENALDI R, GNUDI G, CUNA PR, AZZOLINI PL, MAIETTA P, DRAGO E, TENTONI C: VO₂ slow component adaptations by physical training induced on elder men. *Med Sport* **54**: 17-27, 2001a.

MAIONE D, SENALDI R, GNUDI G, MAIETTA P, MAIONE A, TENTONI C, DRAGO E: Our experience in VO₂ kinetics use for physical training monitoring. *Med Sport* **54**: 51-62, 2001b.

MASON MG, NICHOLLS P, WILSON MT, COOPER CE: Nitric oxide inhibition of respiration involves both competitive heme, and noncompetitive copper, binding to cytochrome c oxidase. *Proc Natl Acad Sci USA* **103**: 708-713, 2006.

MONCADA S, ERUSALIMSKY JD: Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nat Rev Mol Cell Biol* **3**: 214-220, 2002.

MU J, BARTON ER, BIRNBAUM MJ: Selective suppression of AMP-activated protein kinase in skeletal muscle: update on “lazy mice”. *Biochem Soc Trans* **31**: 236.241, 2003.
PALACIOS-CALLENDER M, HOLLIS V, FRAKICH N, MATEO J, MONCADA S.
Cytochrome c oxidase maintains mitochondrial respiration during partial inhibition by nitric oxide. *J Cell Sci* **120**: 160-165, 2007.

Poole DG, Barstow TJ, Gaesser GA, Willis WT, Whipp BJ. VO₂ slow component; physiological and functional significance. *Med Sci Sports Exerc.* **26**: 1354-1358, 1994.

ROSE AJ and HARGREAVES M. Exercise increases Ca²⁺/calmodulin-dependent protein kinase II activity in human skeletal muscle. *J Physiol.* **553**: 303-309.

SARTI P, GIUFFRÉ A, FORTE E, MASTRONICOLA D, BARONE MC, BRUNORI M: Nitric oxide and cytochrome c oxidase: mechanisms of inhibition and NO degradation. *Biochem Biophys Res Commun* **274**: 183-187, 2000.

SCHWEIZER M, RICHTER C: Nitric oxide potently and reversibly deenergizes mitochondria at low oxygen tension. *Biochem Biophys Res Commun.* **204**: 169-175, 1994.

SHARPE MA, COOPER CE: Interaction of peroxynitrite with mitochondrial cytochrome oxidase. Catalytic production of nitric oxide and irreversible inhibition of enzyme activity. *J Biol Chem* **273**: 30961-30972, 1998.

STEINBERG GR and KEMP BE: AMPK in health and disease. *Physiol Rev* **89**:1025-1078, 2009.

THOMAS DD, LIU X, KANTROW SP, LANCASTER JR: The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and O₂. *Proc Natl Acad Sci USA* **98**: 355-360, 2001.

VICTOR MV, NUÑEZ C, D'OÇÓN P, TAYLOR CT, ESPLUGUES JV, MONCADA S: Regulation of oxygen distribution in tissues by endothelial nitric oxide. *Circ Res* **104**: 1178-1183, 2009.

WASSERMAN K, VAN KESSEL A, BURTON CD: Interaction of physiological mechanisms during exercise. *J Appl Physiol* **22**: 71-85, 1967.
WASSERMAN K, HANSEN JE, SUE DY, CASABURI R, WHIPP BJ: Principles of exercise testing and interpretation, Baltimore, USA: Lippincott Williams & Wilkins (eds.), third edition, 1999, p. 76.

WHIPP BJ and WASSERMAN K: Oxygen uptake kinetics for various intensities of constant load work. J Appl Physiol 33: 351-356, 1970.

WHIPP BJ and WARD SA: Physiological determinants of pulmonary gas exchange kinetics during exercise. Med Sci Sports Exerc 22: 62-71, 1990.

WHIPP BJ: The slow component of O_2 uptake kinetics during heavy exercise. Med Sci Sports Exerc 26: 1319-1326, 1994.

WOODS A, DICKERSON K, HEATH R, HONG SP, MOMCILOVIC M, JOHNSTONE SR, CARLSON M, CARLING D: Ca^{2+}/calmodulin-dependent protein kinase kinase-β acts upstream of AMP-activated protein kinase in mammalian cells. Cell Metab 2: 21-33, 2005.
**Fig. 1.** In a young student of the first group, VO$_2$ kinetics during square wave test supra-AT with the area between the two exponentials: training and detraining effects. Light gray line: VO$_2$, continuous black line: fitting curve, dashed black line: first exponential extrapolated curve. Bottom unaveraged data: VO$_2$ in light grey line, R in black line.

**Fig. 2.** Supra-AT VO$_2$ kinetics in a 49-years old subject of the second group. Before (left) and 1 hour after (right) 20 mg of isosorbide-5-mononitrate: during 6 min area is decreased by 50%; bottom VO$_2$ in light gray, R in black.

**Fig. 3.** A 72-years old cyclo-amateur of the first group: pre- training (left), and after training (right). The area between the two exponential disappeared after training; bottom unaveraged data; VO$_2$ in light grey, R in black.

**Fig. 4.** The activating pathways AMPK and its effects on glucose and fatty acids metabolisms and on RER. Explanation in the test.
**Fig. 1**

O₂ area in l for 6 min: 3.6

1.5

2.8

Rmax 1.25
Pretraining

1.14
Posttraining

1.28
Detraining
Fig. 2
Fig. 3
Fig. 4

- $\uparrow H_2O_2$
- $\uparrow NO \rightarrow \downarrow COX \rightarrow \downarrow O_2$
- $\uparrow Ca^{2+} +$ calmodulin k
- muscular work $\rightarrow \uparrow (AMP/ATP)$
- AMPK
- Glut4 $\rightarrow \uparrow$ glucose uptake
- more substrate to Krebs cycle
- carrier proteins $\rightarrow \uparrow$ fatty acids uptake
- IRES