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The VO_2-on Kinetics in Constant Load Exercise Sub-Anaerobic Threshold Reflects Endothelial Function and Dysfunction in Muscle Microcirculation

IN MEMORY OF GREAT PHYSIOLOGIST PROF. BRIAN JAMES WHIPP.

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Summary

To propose a test to evaluate endothelial function, based on VO_2 on-transition kinetics in sub-anaerobic threshold (AT) constant load exercise, we tested healthy subjects and patients with ischemic-hypertensive cardiopathy by two cardiopulmonary tests on a cycle ergometer endowed with an electric motor to overcome initial inertia: a pre-test and, after at least 24 h, one 6 min constant load exercise at 90 % AT. We measured net phase 3 VO_2-on kinetics and, by phase 2 time constant (τ), valued endothelial dysfunction. We found shorter τ in repeated tests, shorter time between first and second test, by persisting endothelium-dependent arteriolar vasodilatation and/or several other mechanisms. Reducing load to 80 % and 90 % AT did not produce significant changes in τ of healthy volunteers, while in heart patients an AT load of 70 %, compared to 80 % AT, shortened τ (Δ=4.38±1.65 s, p=0.013). In heart patients, no correlation was found between NYHA class, ejection fraction (EF), and the two variables derived from incremental cycle cardio-pulmonary exercise, as well as between EF and τ; while NYHA class groups were well correlated with τ duration (r=0.92, p=0.0001). Doxazosin and tadalafil also significantly reduced τ. In conclusion, the O_2 consumption kinetics during the on-transition of constant load exercise below the anaerobic threshold are highly sensitive to endothelial function in muscular microcirculation, and constitute a marker for the evaluation of endothelial dysfunction.

Key words

Anaerobic threshold • Constant load exercise • Endothelial function • O_2 consumption

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Introduction

At the onset of constant load, sub-anaerobic threshold (AT) cycle cardio-pulmonary exercise (CPX), a sudden and rapid increase in VO_2, usually lasting around 15-20 s, is observed; this is called the 1st phase (φI). The appearance of φI is so immediate, and its duration is so short, that VO_2 during this phase is believed to reflect, in large part, the early increase in pulmonary blood flow caused by an increase in heart rate and myocardial contractility, which are mediated both neurologically and by an increased venous return (Casaburi *et al.* 1989). This phase frequently shows artifacts that prevent it from being analyzed systematically. The 2nd phase (φII) follows the 1st one with a very variable delay, occasionally more than 30 s from the imposition of the load, and is characterized by

a slower increase in VO_2 according to an approximately exponential time course. This is followed, after around 3 min, by the 3rd phase (φIII), which coincides with the steady-state (Fig. 1) (Linnarsson *et al.* 1974, Whipp and Wasserman 1972, Whipp 1987).

The 2nd phase has been attributed to a combination of continuous increase of venous return from active muscles and a significant reduction of O_2 content in blood which has been subjected to gas exchange (Barstow and Molé 1987, Barstow *et al.* 1990, Hughson 1990, Sietsema *et al.* 1989). In this context, the φII time constant (τ) is the time taken during φII for VO_2 to reach 63 % of the rise to its steady state net value (φIII). It has been demonstrated that VO_2 measured in φII and φIII closely reflects the intramuscular O_2 consumption (Barstow and Molé 1987, Barstow *et al.* 1990, 1994, Berg *et al.* 1997, Grassi *et al.* 1996). The time constant τ can be reduced with training (Sietsema *et al.* 1989). ATP demand, net of rest quota, is initially met in part thanks to muscle endogenous energy sources, oxymyoglobin and oxyhaemoglobin, but chiefly by phosphocreatine splitting (PCr), whose fall mirrors inversely the rise of VO_2. This keeps the contribution of anaerobic glycolysis in ATP production at a negligible level (Mahler 1985, Marsh *et al.* 1993, Rostow *et al.* 1987). This phenomenon was described by Cerretelli *et al.* (1979) as "early lactate", it is always present and has been linked both to flow misdistribution of muscle microcirculation (Delp and Laughlin 1998, Delp 1999, Hughson 1990, Laughlin 1987, Schoemaker 1999, Remensnyder 1962, Tschakovsky and Hughson 1999), or to inertia in the mitochondrial oxidative metabolic machinery due to activation times for enzymes in the Krebs cycle and electron transport chain activity (Tschakovsky and Hughson 1999, Yoshida *et al.* 1995). In fact, during the first few seconds of a stress test two conditions that can cause flow misdistribution of muscle microcirculation coexist: on one hand, sympathetic hypertonia at rest limits the influx of blood to muscle microcirculation for the condition needs, and subsequently increases during stress (Whipp and Ward 1990); on the other hand, the initial hyperemia is misdistributed because it occurs without changes in vascular conductance so prefunding still resting as well as already activated fibers (Delp and Laughlin 1998, Delp 1999, Laughlin 1987, Rådegran and Saltin 1998, Remensnyder 1962, Tschakovsky and Hughson 1999). This misdistribution causes mitochondrial PO_2 to drop in active myocytes towards values that produce the “early lactate” phenomenon (Cerretelli *et al.* 1979, Tschakovsky and Hughson 1999) and moderates the rate of VO_2 increase during the very beginning of φII. Vasodilation is progressively reinforced both by local adenosine release and, more importantly, by endothelium-mediated substances, especially nitric oxide (NO) and prostacyclin, which diffuse to underlying vascular smooth muscle causing it to relax, and dilate nearby arterioles (Flammer and Lüscher 2010, Hughson 1990, Maiorana *et al.* 2003, Tschakovsky and Hughson 1999), thus creating rapid, widespread "functional sympatolysis" (Remensnyder *et al.* 1962). The resulting increase in conductance of vascular muscle microcirculation drives increased flow to dilated vessels, thus both improving the match between blood flow and the metabolic rate of active muscle cells and keeping the φII time constant short. The duration τ can therefore yield a purely indirect measure of endothelial function and, if lengthened, also can be considered as a marker of endothelial dysfunction. In cardiovascular disease, myocyte perfusion is often hindered due to a combination of factors: strong reinforcement of sympathetic tone, both centrally-mediated and secondary to renin-angiotensin system stimulation; endothelial dysfunction, resulting in reductions in both NO synthesis/activity and the signaling pathway from NO to cyclic guanosin monophosphate (cGMP) *via* soluble guanylyl-cyclase (sGC); increased release of powerful vasoconstrictors such as endothelium-derived contracting factors including several prostanoids, as prostaglandins and thromboxane A2 (arachidonic acid metabolism-derived by cyclooxygenase), endothelin-1 and reactive oxygen species generated by several sources, which, along with other substances, can produce oxidative stress, destroying NO and increasing vascular tone (Vanhoutte 2011, Virdis *et al.* 2010, 2013). For these reasons O_2 uptake is hampered in contracting fibers so that they are forced to increase the contribution of anaerobic lactacid metabolism to meet the increased energy requirements, and this prolongs τ duration (Barstow and Molé 1987, Hughson 1990, Maiorana *et al.* 2003). Therefore, τ lengthening can be considered a marker of endothelial dysfunction. In this paper, to evaluate endothelial function and dysfunction, we propose a test which uses VO_2 on-transition kinetics in sub-AT constant load exercise, always preceded by an incremental CPX, conducted on a different day to determine the load needed to reach AT.

Methods

Population

We screened more than 360 normal subjects and patients with heart disease (NYHA class II and III)

(overall called heart patients), in stabilized treatment according to ESH-ESC guidelines (McMurray *et al.* 2012), and 62 (M. 52, F. 10) agreed to participate in our researches. These represent examples of the method's applicability.

Exercise protocol

All tests were performed in the morning, after 2 h of resting in a room adjacent to our laboratory, for reasons described in discussion, using the Medifit1000 (Holland) cycloergometer, calibrated before each session; an ULTIMA-CPX (Medical Graphics Corporation, USA) ergospirometer, calibrated before each test, interfaced with a Case16 (Marquette, USA) electrocardiograph. The subjects first performed a maximal ramping CPX, preceded by a 3 min rest, starting from 10-15 W, as first load, following by load increments: 15-20 W/min, depending on the status of the patient: healthy and moderately active, sedentary, or heart failure (NYHA functional class II or III). Details of load increments are shown in Table 1. For the purposes of this study, sedentary subjects are those not participating in activities with a training effect. Then, in a second session, at least 24 h later, subjects performed a single 6 min cycle CPX at constant intensity, preceded by a 3 min rest. For each subject the load was calculated at 90 % of the AT (except in the second trial as noted in Table 1), identified in the incremental test by the V slope method (Beaver *et al.* 1986). No warm-up phase was included in any tests.

During both the incremental and the constant load test, we used a Rudolph mask (7930-7940 series, USA) and sealed the space between mask and face with a special gel modeled on the internal geometry of the mask (Ultimate Seal, Rudolph, USA). To further improve adhesion between face and mask, we replaced the cap and fastening straps of the Rudolph mask with PVC ones. To overcome the flywheel inertia at the onset of exercise, the cycle ergometer was equipped with an electric motor that accelerated the flywheel to its planned rotational velocity, namely 60 rpm for both incremental and constant load tests, while the pedals remained at rest, and this was turned off when pedaling began. Pedaling frequency during exercises was maintained constant with the help of a digital metronome.

All studies were conducted on volunteers in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from all study participants. Protocols were approved by the local ethical committee.

Table 1. Load protocol for different groups of subjects referred to in text.

Group	Incremental test load (W)			Constant test load (% AT)	
	Subgroup	Step 1	Later steps	Trial 2	Trials 1,3-5
Without heart failure	Active	15	20	Day 1: 90 %	90 %
	Sedentary	15	15	Day 2: 80 %	
NYHA class II	Active	15	15	Day 1: 90 %	
	Sedentary	10	15	Day 2: 70 %	
NYHA class III	-	10	10		

Data analysis

A monoexponential model was fitted to the raw unaveraged breath-by-breath VO_2 data of the constant load tests, interpolated every 0.01 s, by our software package: Cardio Pulmonary Exercise Parameters Estimator (C.P.E.P.E.), written in the Matlab language. This iteratively optimizes the model parameters to fit to the VO_2 experimental data (Fig. 1), but φI is excluded from the fitting process to reduce artifacts. To further improve the fit and make the test suitable for clinical use, our software started curve fitting from a baseline point, hidden by φI, that was identified as the start of the 2nd phase, using trends in O_2 end-expiration partial pressure ($PETO_2$), the corresponding CO_2 value ($PETCO_2$), and respiratory exchange ratio to mouth (RER). In fact, according to Whipp *et al.* (1982) these three variables throughout φI typically were maintained reasonably stable at their prior control values, on occasion hyperventilation, a very frequent condition, was evidenced in φI, causing $PETO_2$ and RER to increase and $PETCO_2$ to decrease. Thereafter, $PETCO_2$ rose to a new steady level during φII, which was maintained in φIII. $PETO_2$ and R decreased in φII, exhibiting transient undershoots.

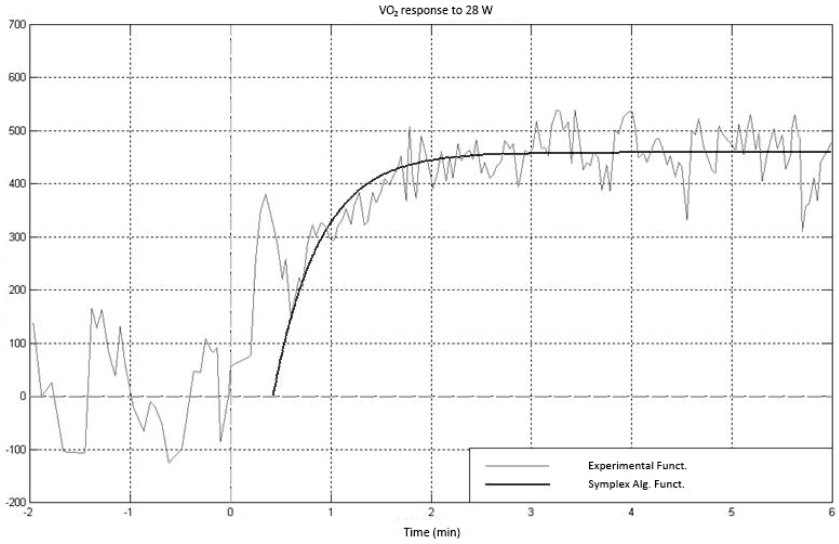

Fig. 1. In grey the on-transition VO_2 kinetics in ml.min^{-1} of rectangular exercise sub-AT; in black the fitting curve superimposed on 2nd and 3rd phase. Raw breath-by breath data.

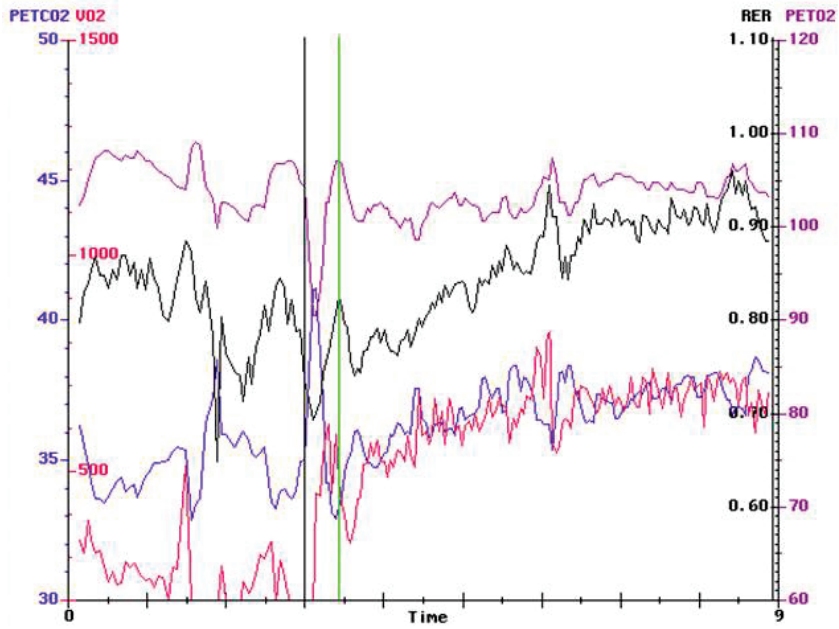

Fig. 2. Method of highlighting point from which fitting curve leaves basic line. In blue $PETCO_2$ – end-tidal PCO_2 mm Hg; in red VO_2-on kinetics sub-SA ml.min^{-1}; in black RER – respiratory exchange ratio; in violet $PETO_2$ – end tidal PO_2 mm Hg. Raw breath-by breath data.

A vertical line passing through the point where these latest changes occur, and that therefore corresponds to the start of φII, reaches the baseline in a point from which fitting curve starts. The fitted curve must pass through the point where φI meets φII, which then becomes visible (Figs 1 and 2). Net VO_2 at steady-state (φIII) was measured and the φII time constant was calculated on the fitting curve. If curve fitting was forced to detach from the baseline at the point where the load is applied, as has always been done in past, φI would be incorporated into the calculation of τ, whose duration would then be lengthened (Whipp *et al.* 1982).

Statistical analysis

In all cases the parameter of interest was τ, the exponential rise time in φII. The normality of the distribution of the tested parameters was evaluated by the Kolmogorov-Smirnov test. Differences in τ between first and repeated runs in the first trial were tested by the ANOVA test for repeated measures with Bonferroni correction; while in the other trials differences of τ before and after treatment were computed by the t-test for paired data. In the third trial the relationships between τ and the selected variables were obtained by linear correlation. In all statistical analyses the significance level was set at $p<0.05$. Summary difference statistics are presented as mean ± standard deviation (SD), and percentage differences and effect sizes, expressed as Cohen's d, are also given.

Table 2. Characteristics of study populations.

	n	Gender		Age (years)	Height (cm)	Body weight (kg)
Trial 1						
Normal subjects	3	M. 3	–	66.41±9.19	168.78±8.07	82.04±14.64
Patients	25	M. 22	F. 3			
Trial 2						
Normal subjects	5	M. 3	F. 2	56.80±3.42		
Patients	4	M. 4	–	72.50±1.73		
Trial 3						
Normal subjects	–					
Patients	17	M. 12	F. 5	69.4, range 50-80	160.73±5.10	74.80±7.98
Trial 4						
Normal subjects	–					
Patients	4	M. 4	–	73.8±5.12	164.8	75.5
Trial 5						
Normal subjects	–					
Patients	4	M. 4	–	65±4.40	170±3.74	84±5.56

Trial 1 – the effect of retest on τ

The effect of repeat testing on τ variation was examined in a set of 28 subjects: 3 healthy subjects and 25 treated heart patients NYHA class II (Table 2), separated in 3 equal number groups, based on the time separating two successive tests, approximately 30, 60 and 120 min. The tests, rigorously with a load corresponding to 90 % of the AT, were performed in different days with same constant load. 30 tests were performed because one of the patients was inserted in two groups, and another one was in all three groups; the normal subjects were distributed between all 3 groups.

Trial 2 – the dependence of τ on the load intensity

The study of the dependence of τ on the load intensity was conducted on five healthy subjects and four male treated NYHA class II heart patients (Table 2). Two exercises at constant intensity were performed on different days, with a load corresponding to 90 % and 80 % of the AT in healthy subjects and 90 % and 70 % in heart patients.

Trial 3 – the relationship between τ and some heart efficiency ratios

In 17 treated heart patients (Table 2) 12 in NYHA class II and 5 in NYHA III linear correlations were calculated between τ duration, and variables obtained from cycle CPX: peak VO_2 (pVO_2), measured as the % of its theoretical maximal value; VO_2 at AT, measured as the % on the theoretical minimum (40 % of theoretical pVO_2); echocardiographic ejection fraction (EF); and NYHA classes II and III, assigning a score to these of 2 or 4, respectively.

Trial 4 – the effect of α_1-blocker administration

2 mg/day doxazosin was administered to four NYHA class II heart patients, in standard stabilized treatment (Table 2). The constant τ was assessed immediately before treatment, and following one week of administration.

Trial 5 – the effect of tadalafil administration

20 mg tadalafil was administered to four NYHA class II heart patients (Table 2), in standard stabilized treatment. The constant τ was assessed immediately before treatment, and 28 h following administration.

Results

We found that τ values of healthy individuals do not exceed 13 s, regardless of age, as long as physically active, while values above 16 s are clearly pathologic; values within this range could be considered in a grey zone which may occur both in healthy people with a sedentary lifestyle and in patients with mild pathology.

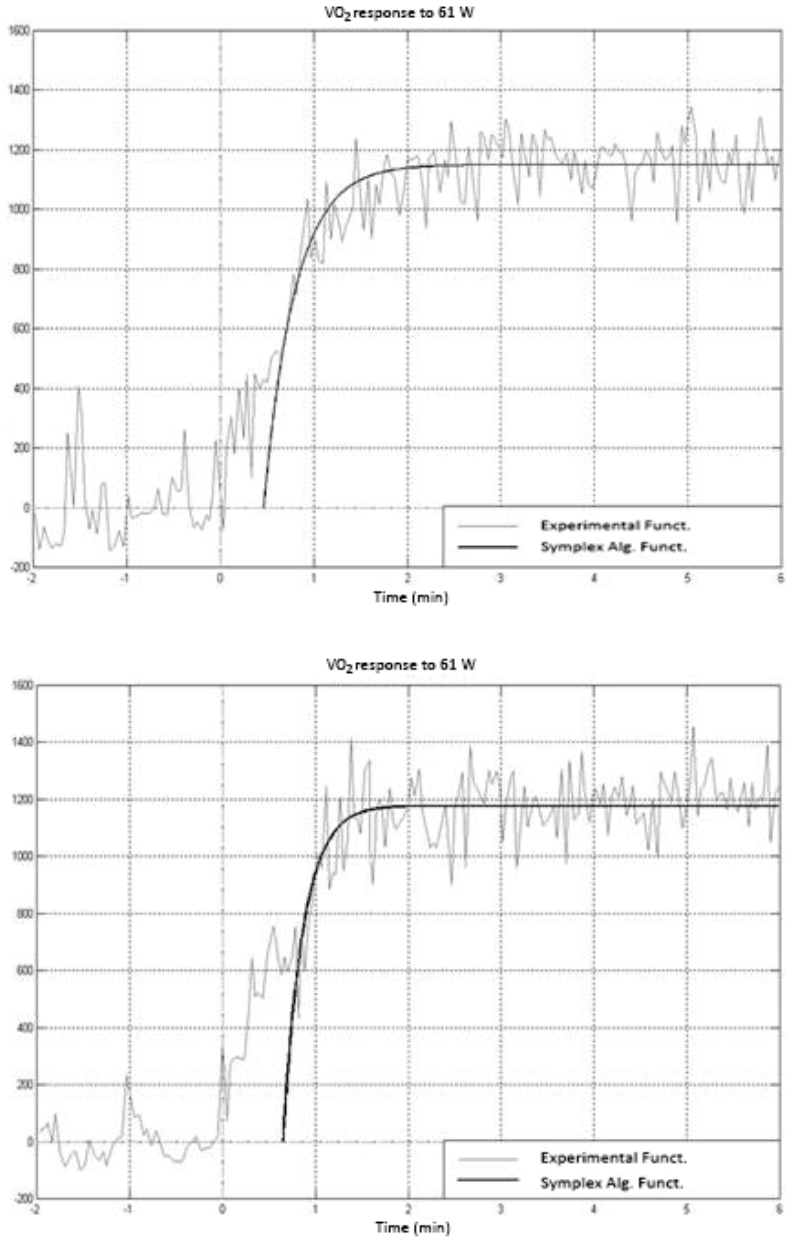

Fig. 3. VO_2 compared to baseline ($ml.min^{-1}$). Above: the first test in a patient (τ=20.08 s); bottom: repeated test after ~ 30 min in same patient (τ=13.06 s); difference of 7.02 s. Raw breath-by breath data.

Trial 1

Representative time courses are shown in Figure 3. The time intervals separating two successive tests were: in the 1st group 31.40±4.74 min, in the 2nd group 66.60±10.92 min, in the 3rd group 113.30±10.41 min; in short 30 min, ~1 h, ~ and 2 h. In the 1st group τ average duration in initial test was 28.19±7.56 s and 20.21±9.37 s in repeated tests, with a difference (Δ) of −7.98±2.00 s, (=−28.31 %; d=0.94) ($p<0.0001$); in the 2nd group τ was respectively 23.52±10.91 s and 19.87±8.26 s, with a Δ of −3.65±3.39 s, (=−15.52 %; d=0.23) (p=0.008); in the 3rd group 19.64±7.24 s and 18.44±7.39 s with a Δ of −1.20±1.00 s, (p=0.004), which, expressed as a percentage is −6.11 % (d=0.16). A positive correlation was also found between τ value in 1st test, independent variable, and the difference between τ values of the two successive tests of each subject: in 1st group, r=0.739 (p=0.015) as in 2nd group, r=0.843 (p=0.002). However, this effect did not occur in 3rd group, r=0.072 (p=0.842), likely due to the sample size, that was too limited compared to the small difference in τ duration between the two tests. Thus, τ duration is significantly decreased in repeated exercises and the τ differences between first and repeated tests were reduced significantly from 1st to 3rd group, while remaining still statistically significant in the 3rd. Furthermore, in the first two groups, greater τ duration in the first tests was associated with a greater reduction of τ in the repeated tests.

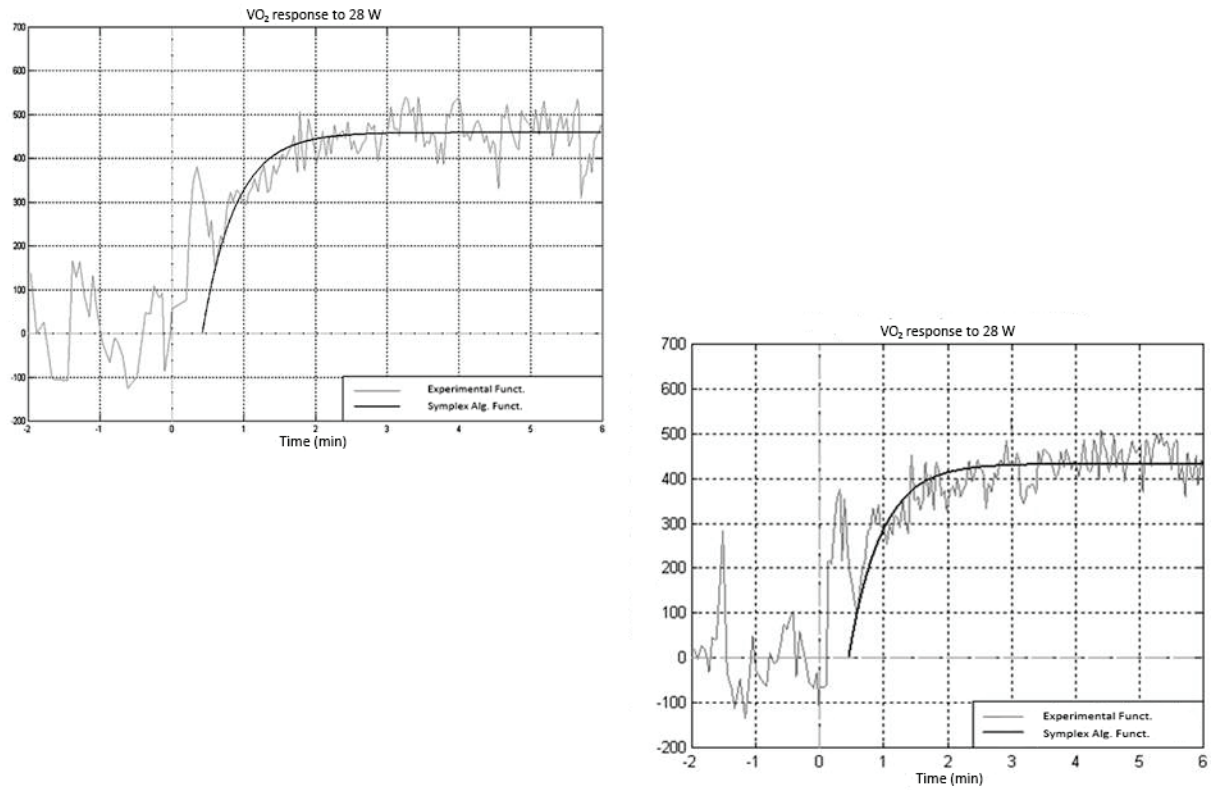

Fig. 4. VO_2 compared to baseline ($ml.min^{-1}$). τ duration in a patient: at left, pre treatment (τ=34.10 s), at right, after taking 2 mg/day for 1 week doxazosin (τ=21.27 s). Raw breath-by breath data.

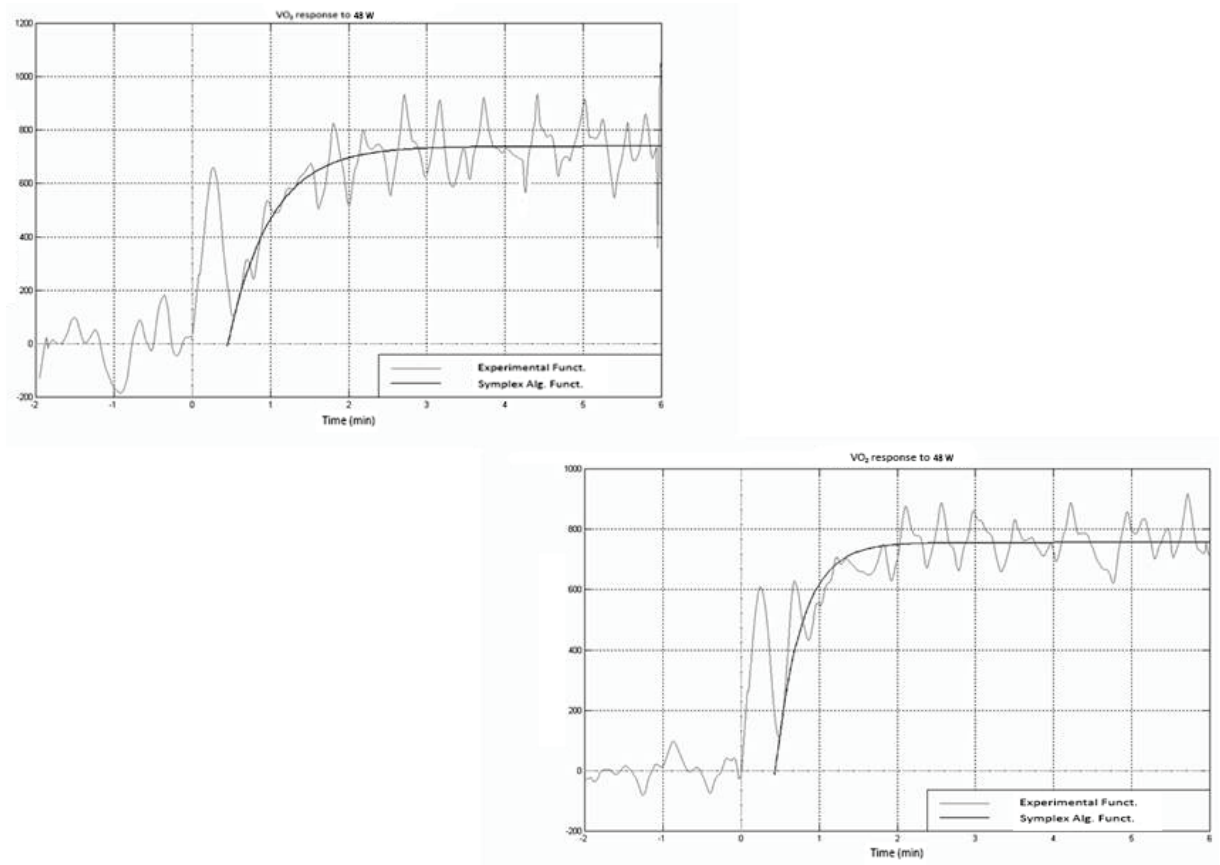

Fig. 5. τ duration in a patient: at left, pre treatment (τ=38.52 s), at right, after taking 20 mg tadalafil (τ=24.71 s); averaged data over 5 of 7 breaths.

Trial 2

The reduction of load from 90 % of AT (τ=11.90 s) to 80 % (τ=11.10 s) in healthy subjects did not produce significant changes in τ (Δ=−0.80±1.20 s or −6.72 %, d=0.16, p=0.212), while in heart patients the reduction from 90 % AT (τ=24.87 s) to 70 % AT (τ=20.50 s) has significantly shortened τ (Δ=−4.38±1.65 s or =−17.57 %, d=0.40, p=0.013).

Trial 3

No direct relation, by linear correlation, was found between NYHA class, EF, and the two variables derived from incremental cycle CPX, or between EF and τ (r=0.34, p=0.78); while NYHA II and III classes groups were well correlated with τ duration (r=0.92, p=0.0001).

Trial 4

Representative time courses are shown in Figure 4. The effect in treated heart patients of the α_1-blocker, 2 mg/day doxazosin, was to reduce τ from 34.10±6.26 s before treatment to 21.27±3.89 s one week after treatment, with a mean Δ between before and after treatment of −12.83±3.36 s, or −37.5±5.03 %, d=2.46, p=0.005.

Trial 5

Representative time courses are shown in Figure 5. The effect of 20 mg tadalafil 28 h after administration was to decrease τ, from 31.15±16.51 s to 22.90±15.26 s with a mean Δ between before and after treatment of −8.25±4.06 s, or −26±12 %, d=0.52, p=0.027.

Discussion

Trial 1 – the effect of the retest on τ (Fig. 3)

Initially, we subjected each subject to at least four tests at constant load separated by 30-40 min, following the example of previous researchers, who performed up to eight or nine repetitions (Casaburi *et al.* 1989, Sietsema *et al.* 1989, Whipp *et al.* 1982). The tests were then interpolated once per second, time aligned to exercise start, superimposed and averaged, for subsequent curve fitting. For later tests, including those presented here, only a single exercise at constant load was performed, for two reasons: to prevent the inevitable time lag, increased with the number of repetitions requested, between the verbal command to start a test and the actual onset of test, with repercussions on the data; but, more particularly, because in the repetitions of the same load we found that the τ duration was much shorter, the lower was the interval between the two tests. Besides, the greater was the τ duration in the first tests the greater was the reduction in repeated tests. It is well-known that in healthy subjects endothelial NO release is significant even at rest, but increasingly compromised with greater degrees of endothelial dysfunction (Afanas'ev 2009, Förstermann 2010, Halcox *et al.* 2009, Hughson 1990, Li and Förstermann 2009, Münzel *et al.* 2008). Thus, both arteriolar vasodilation at the beginning of dynamic effort, which represents a powerful physiological stimulus to increase NO bioavailability, and inertia in the mitochondrial oxidative metabolic machinery, can be improved during the repeated exercise of subjects with increased endothelial dysfunction compared to normal, physically active, subjects, in which these effects are more evident and already present at rest (Green *et al.* 2004): i.e. it is more difficult for an already normal endothelial function to improve. Several mechanisms to explain the persistence of vasodilation in repeated tests have been proposed (Camley *et al.* 2007, Francis *et al.* 2008, Smith *et al.* 1996), but as yet there is no consensus on the matter. Consequently, in our protocol, subjects performed the tests after 2 h of rest to avoid the influence of walking, prior to their arrival at our laboratory, on the results of the incremental and constant load tests and remained at rest between tests and subsequent. For the same reason, all tests were performed without warm-up. In the third group of the first trial, the difference in τ average duration between initial and repeated tests was of −6.11 %; the small difference can be taken as an indication of the repeatability of the test.

A shorter φII τ was also found by Rossiter *et al.* (2001) in seven well-trained males in the second of two consecutive 6 min bouts of high-intensity square-wave knee-extensor exercise in prone position with 6 min rest interval (Rossiter *et al.* 2001). Besides, several authors invoked the NO-Cox interaction to explain the O_2 cost reduction in moderate-intensity exercise, 80 % AT, in well-trained healthy subjects after consumption of nitrate supplementation or of nitrate-rich vegetables (Bailey *et al.* 2009, Lansley *et al.* 2011, Larsen *et al.* 2007, 2010), and of a beverage containing L-arginine (Bailey *et al.* 2010), an effect that is generally observed in high-intensity, supra-AT exercise (Maione *et al.* 2013). If this effect were present in our tests it could have affected the τ duration, but even in our patients treated with a PDE5 inhibitor (trial 5, Fig. 5), and hence with increased NO bio-availability, the O_2 cost was unchanged. Literature

data (Brown and Cooper 1994, Erusalimsky and Moncada 2007, Flammer and Lüscher 2010, Palacios-Callender *et al.* 2007, Sarti *et al.* 2000) support the belief that this mechanism, which reduces O_2 cost in supra-AT exercises, is primed (and it can be supported by a possible and not necessary reduction of O_2 concentration) by an increase of NO concentration which exceeds some definite but unpredictable level (Cooper and Giulivi 2007), but also by AMP/ATP-dependent AMPK activation, when metabolism becomes primarily aerobic (Hardie and Sakamoto 2006). Our subjects were either heart patients or healthy, sedentary or moderately active but otherwise untrained, volunteers; and their exercise load, namely 90 % AT, was considerably less than that employed by Rossiter *et al.* (2001) to challenge the well-trained subjects, at 80 % AT because these subjects reached AT at a significant higher load. Thus we conclude that the O_2 cost reduction, always present in supra-AT exercise, occurs in sub-AT only if the load is sufficiently high to both inhibit mitochondrial respiration by NO over-expression, and to activate AMPK, consequences naturally more apparent in trained individuals. Besides, it is unlikely, with the loads used in our protocol, that NO inhibited mitochondrial cytochrome c oxidase (CcO), during φII and φIII, as is observed at high concentrations of NO, with consequent O_2 savings (Brown and Cooper 1994, Erusalimsky and Moncada 2007, Flammer and Lüscher 2010, Sarti *et al.* 2000), a mechanism that characteristically depends just on the NO/O_2 ratio (Palacios-Callender *et al.* 2007). Also, sGC, the enzymatic target of NO, is approximately 50 times more sensitive to NO than to CcO (Bellamy *et al.* 2002) so it is unlikely that NO would be able both to maximally activate sGC and to inhibit cellular respiration (Bellamy *et al.* 2002, Rodríguez-Juárez *et al.* 2007), not least because CcO can also be partially inhibited by NO without an effect on cellular respiration (O_2 consumption) (Palacios-Callender *et al.* 2007).

Trial 2 – the dependence of τ on load intensity

In this trial, the τ of healthy subjects was not significantly changed by varying the load, provided that the exercise was always performed below the AT; in contrast, in cardiac diseases a lower load was associated with a significantly shorter τ. Consequently, the patients' responses to effort depend not only on the clinical conditions but also on the load. A pathologic response is observed at a higher load, while normal responses are maintained with a lower load. Therefore, at least in subjects without severe impairment, it is not possible to talk of myopathy caused by heart failure, but of non-permanent metabolic adaptations to stress in conditions of impaired local perfusion.

Trial 3 – the correlation between τ, NYHA class, and some heart efficiency ratios

In heart failure patients, by linear correlation, a direct relation was demonstrated between the τ duration and the NYHA class while no correlation was found with echocardiographically obtained EF. These results showed that τ duration enables the severity of heart failure to be classified in manners parallel to the NYHA classification.

Trial 4 – the effect of the addition of doxazosin on τ (Fig. 4)

In treated heart patients, standard pharmaceutical treatment β-blockers inclusive lengthens the τ duration by bradycardia and increase in peripheral resistance induced, as a result of the α tone escape. This β-block effect is also present with the specific β-blocker bisoprolol, while does not occur with the latest β-blockers with intrinsic vasodilating action. Treatment with the α_1-blocker doxazosin has speeded up τ in our study compared to before values observed prior to administration due to its vasodilator activity. Therefore, the association between β-blockers and doxazosin is favorable because it may partially limit the not always useful effects of β-blockade.

Trial 5 – the effect of the addition of tadalafil on τ (Fig. 5)

Tadalafil, a long lasting phosphodiesterase inhibitor (PDE-5) that prolongs NO and cGMP bioavailability, used in men with erectile dysfunction, was administered to four patients, inducing, even 28 h after its administration, a significant reduction of τ. The persistence after so many hours of arterial (and venous) vasodilation by tadalafil is still not entirely clear (Camley *et al.* 2007, Francis *et al.* 2008, Smith *et al.* 1996).

The results of the trials suggest that the pharmacological and non-pharmacological treatment of heart disease, which reduces both shear-stress and oxidative stress (Nediani *et al.* 2011, Versari *et al.* 2009), quickly improves endothelial function and can also normalize the τ duration even without changing, initially, the severity of myocardial damage, which is persistently detected by echocardiography. In treated heart patients, in which the τ duration is not yet completely in the normal range, we speculate that the test could be used to optimize the treatment, by add in new drugs and/or increasing the dosage of those already administered, up to achieve the

normalization of τ and probably the recovery of a proper endothelial function.

An alternative, and currently more popular, technique to indirectly assess endothelial function, "flow mediated dilation", uses the shear stress induced in the endothelium of peripheral conductance arteries (normally the brachial artery) by a non-physiological stimulus, namely post ischemic hyperemia, which involves the application of a blood pressure cuff for 5 min, causing limb ischemia, and its abrupt deflation to restart a hyperemic limb blood flow. Ischemia of such duration can cause the formation of reactive O_2 species (ROS), such as superoxide anion (O_2^-) and H_2O_2, which, apart from increasing vascular tone, readily react with vascular NO to form peroxynitrite ($ONOO^-$), a powerful oxidant. Tetrahydrobiopterin (BH4), an eNOS-dependent cofactor to NO synthesis, is highly sensitive to oxidation by $ONOO^-$. Excessive oxidation and BH4 depletion promote eNOS uncoupling, the production of O_2^- by eNOS rather than NO (Milstien and Katusic 1999), and interfere with the test sensitivity. Even organic nitrate administration is not infallible as a means to show a possible ROS effect. Hemoprotein sGC, the first enzyme in the NO signal cascade, is sensitive to the gas only if the iron in its prosthetic heme group is in a reduced or bivalent form. In pathological conditions, the iron may be in an oxidized or trivalent form due to oxidative stress, thus rendering sGC insensitive to NO. The prosthetic group may even be removed, thereby blocking the downstream signal (Miller *et al.* 2009, Stash *et al.* 2006). Thus unchanged test results in patients after nitrate administration may signify an NO insensitive catalytic sGC center, rather than ROS production.

On the other hand, the physiological stimulus outlined in the current work, namely dynamic cycloergometer exercise at mild to moderate intensity, altering VO_2 kinetics sub-AT, allows the sensitive evaluation of endothelium-dependent vasodilation at onset exercise within muscular microcirculation where gas exchange occurs. Obviously the method has limitations, as well as advantages: the procedure is relatively complex and unable to provide correct results in cases of major reduction of lower limb blood flow due to arterial stenosis, in severe bronchopulmonary disease with serious obstacle to air flow and/or pulmonary gas exchange or significant pulmonary hypertension. Additionally, in athletes the φII time constant is very often less than 7 s, too short to be properly estimated by exponential fitting of breath-by-breath data. However, with the method described we obtained a fitting curve which was closer to the actual breath-by-breath data collected during this type of exercise. In this operation we followed the path suggested by Whipp *et al.* (1982).

Conclusion

In conclusion the kinetic of O_2 consumption during the on-transition of constant load exercise below the anaerobic threshold appears to be highly sensitive to endothelial function in muscular microcirculation, and may represent a marker for the evaluation of endothelial dysfunction.

Conflict of Interest

There is no conflict of interest.

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