ALLOPURINOL INTAKE DOES NOT MODIFY THE SLOW COMPONENT OF $\dot{V}O_2$ KINETICS AND OXIDATIVE STRESS INDUCED BY SEVERE INTENSITY EXERCISE

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SUMMARY

The aim of this study was to test the hypothesis that allopurinol ingestion modifies the slow component of $\dot{V}O_2$ kinetics and changes plasma oxidative stress markers during severe intensity exercise.

Six recreationally active male subjects were randomly assigned to receive a single dose of allopurinol (300 mg) or a placebo in a double-blind, placebo-controlled crossover design, with at least 7 days washout period between the two conditions. Two hours following allopurinol or placebo intake, subjects completed a 6-min bout of cycle exercise with the power output corresponding to 75% $\dot{V}O_2$max. Blood samples were taken prior to commencing the exercise and then 5 minutes upon completion.

Allopurinol intake caused increase in resting xanthine and hypoxanthine plasma concentrations, however it did not affect the slow component of oxygen uptake during exercise. Exercise elevated plasma inosine, hypoxanthine, and xanthine. Moreover, exercise induced a decrease in total antioxidant status, and sulfhydryl groups. However, no interaction treatment x time has been observed.

Short term severe intensity exercise induces oxidative stress, but xanthine oxidase inhibition does not modify either the kinetics of oxygen consumption or reactive oxygen species overproduction.

KEY WORDS: xanthine oxidase, total antioxidant status, reactive oxygen species

INTRODUCTION

The increase in reactive oxygen species (ROS) production during skeletal muscle activity is well established. Excess of endogenous oxidants is responsible for lipid peroxidation, protein oxidation and single strand breaks in DNA (Davies *et al*. 1982; Inayama *et al*. 1996; Wierzba *et al*. 2006). Despite its damaging effect, recent evidence suggests that the acute increase in ROS during muscular contraction is directly involved in the up-regulated expression of endogenous antioxidants, the control of redox-sensitive transcription factors, and the stimulation of mitochondrial biogenesis (Ji *et al*. 2004; Kang *et al*. 2009; Silveira *et al*. 2006). The ROS production during exercise can also influence muscle force and fatigue (Andrade *et al*. 2001; Gomez-Cabrera *et al*. 2010)

Despite the initial indications that mitochondria are the predominant site for ROS generation during activity, a number of alternative potential sources are proposed (for review see Powers and Jackson 2008). Strong evidences confirm a great impact of xanthine oxidase (XO) pathway in ROS generation during exercise. Plasma hypoxanthine, xanthine, and uric acid increases dramatically in human subjects after intense exercise (Hellsten-Westing *et al*. 1991; Sahlin *et al*. 1991). Moreover, studies by Gomez-Cabrera and associates (Gomez-Cabrera *et al*. 2003; Gomez-Cabrera *et al*. 2006) indicate that XO inhibition, by allopurinol intake, prevents the exercise-induced muscle damage, and ROS production. Treatment of Tour de France and the Valencia Marathon participants by allopurinol, attenuated the increase of cytosolic enzymes activity, as well the concentration of malondialdehyde (the end product of lipid peroxidation) in plasma (Gomez-Cabrera *et al*. 2003; Gomez-Cabrera *et al*. 2006). However, recent study by Veskoukis *et al*. demonstrated, that allopurinol administration decreases time to exhaustion in swimming rats by 35% (Veskoukis *et al*. 2008). The early fatigue in allopurinol group may be caused by the increased cost of work due to decreased ATP-producing systems efficiency and decrease in muscle contraction efficiency. Both of the

factors may contribute to the slow component of oxygen uptake kinetics during exercise (for review see Zoladz and Korzeniewski 2001). The slow component represents an increasing oxygen (and energy) cost during exercise, despite the rate of external work remaining constant, and may be implicated in the fatigue process. The rising $\dot{V}O_2$ could project to maximal values, curtailing the ability to perform prolonged exercise. Interventions that reduce the $\dot{V}O_2$ slow component amplitude have been reported to improve severe intensity exercise tolerance (Bailey *et al*. 2010; Lansley *et al*. 2011).

Therefore the purpose of this study is to examine the effect of allopurinol ingestion on the $\dot{V}O_2$ slow component amplitude and plasma oxidative stress markers in healthy untrained subjects performing severe intensity exercise.

METHODS

Subjects

Six recreationally active, but non-specifically trained male subjects participated in the study (Table 1). The study was approved by the Local Ethics Committee and all subjects gave their informed consent before the start of the study. The subjects were asked to refrain from any physical activity or alcohol consumption for at least 24 hrs prior to testing.

Aerobic and anaerobic power measurement

To determine maximal oxygen uptake ($\dot{V}O_2max$) participants performed a graded cycle ergometry test on an electromagnetically-braked, cycle ergometer (ER 900 Jaeger, Viasys Healthcare GmbH, Germany). The ergometer seat height was individually adjusted and the participants were allowed a 5-min warm-up period at an intensity of 1.5 $W \cdot kg^{-1}$ with a pedaling cadence of 60 rpm. After the warm-up period, work rate was increased by 25 $W \cdot min^{-1}$ until volitional exhaustion. Breath by breath pulmonary gas exchange was measured

by Oxycon-Pro analyzer (Viasys Healthcare GmbH, Germany) and the O_2 and CO_2 analyzers were calibrated prior to each test using standard gases of known concentrations in accordance with manufacturer guidelines (Ziemann *et al*. 2011).

Experimental protocol

The subjects were randomly assigned to receive a single dose of allopurinol or a placebo in a double-blind, placebo-controlled crossover design, with at least 7 days washout period between the two conditions. On the day of the experiment, subjects reported to the laboratory in the morning, consumed a standard breakfast and then ingested either a *placebo* or 300 mg of allopurinol. It has been indicated in a previous study that this dose is sufficient to effectively inhibit XO (Gomez-Cabrera *et al*. 2006). After two hours resting, subjects performed a 6 min bout of exercise with the power output corresponding to 75% $\dot{V}O_2$max (Zoladz *et al*. 1998). Respiratory gas analysis and volume measurements were performed breath by breath with a face-mask connected to the analyzer.

Calculating the O_2 slow component

The difference in $\dot{V}O_2$ between minute 6 and minute 3 of each work bout was chosen to estimate the amplitude of the slow component of $\dot{V}O_2$ kinetics because exponential modeling using a single trial can be too noisy (Bearden and Moffatt 2001).

Blood analysis

Prior to commencing the exercise protocol and then 5 minutes upon completion, blood samples were taken from the antecubital vein. Immediately after collection, the blood samples were divided into two parts. One part was analyzed for hematocrit using an automated hematology analyzer (Sysmex XT 2000, Global Medical Instrumentation, Inc). The other part was centrifuged at 1000g for 10 minutes and separated plasma samples were frozen at –70°C for later analysis.

Biochemical assays

High-performance liquid chromatography (HPLC) was utilized to measure plasma concentrations of inosine, hypoxanthine, xanthine, uric acid, allopurinol, and oxypurinol. 300 μL of plasma was supplemented with 300 μL of 1.3 $mol \cdot L^{-1}$ perchloric acid, vortexed and centrifuged at 20000 g and 4°C for 5 min. 400 μL of the acid supernatant was removed and neutralized with 135 μL of 1 $mol \cdot L^{-1}$ potassium phosphate to pH 5-7. Centrifugation was repeated and the supernatant was withdrawn for HPLC. Analyses were performed with Hewlett-Packard 1050 series chromatography system (Palo Alto, CA) consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Rheodyne 7125 manual injection valve with 20 μL loop, UV-VIS detector, and series 1100 thermostatted column compartment. Separations were achieved on Hypersil BDS 100 x 4.6 mm, 3-μm particle size column (Thermo Scientific Inc.). Modifications were introduced into the original method of Smolenski *et al*. (1990). The mobile phase flowed at a rate of 1.0 $mL \cdot min^{-1}$ and column temperature was 22.0°C. Buffer composition remained unchanged (A: 150 $mmol \cdot L^{-1}$ potassium phosphate buffer, pH 6.0, containing 150 $mmol \cdot L^{-1}$ potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 5.05 min, 100% at 5.35 min, 100% at 7.00 min, 0% at 7.10 min. Samples of 100 μL were injected every 12 min into the injection valve loop. Absorbance was read at 254 nm.

Plasma lactate was determined using a standard kit (Randox Laboratories Ltd.) based on the lactic acid oxidase method (LC2389).

Plasma total antioxidant status (TAS) was determined using a standard test kit (Randox Laboratories Ltd.). In this assay, ABTS (2,2′-azinobis(3- ethylbenzothiazoline-6-sulfonate)) was incubated with metmyoglobin and hydrogen peroxide to produce $ABTS^{*+}$. The change in absorbance of this species was measured at 600 nm. Antioxidants present in

the sample caused a reduction in absorption proportional to their concentration. TAS values of the samples tested were expressed as an equivalent of the millimolar concentration of standard Trolox solution (Miller *et al*. 1993). Concentration of sulfhydryl groups (SH) was determined according to Ellman's method (Ellman 1959). Briefly, plasma samples were incubated with 100 $\mu mol \cdot L^{-1}$ DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) (Wako Pure Chemicals) at room temperature for 60 min. Absorbance was determined at 412 nm.

The spectrophotometric measurements were performed using Super Aquarius CE9200 (Cecil Instruments Ltd.).

Correction for plasma volume shifts

Changes in plasma volume were calculated from measurements of haemoglobin and haematocrit and the concentration of all measured compounds was corrected according to the method described previously (Dill and Costill 1974).

Statistical analysis

To determine the existence of significant differences in the oxygen uptake, slow component amplitude and blood lactate between *placebo* and allopurinol trials paired t-tests were used. Statistical significance was accepted at $P < 0.05$. Two-way analysis of variance (ANOVA) with repeated measures was used to determine whether there were statistical differences in the blood data for time, treatment, and time x treatment variables. All data are expressed as means ± SEM (standard error of mean).

RESULTS

A single oral allopurinol ingestion did not affect the pulmonary $\dot{V}O_2$ uptake and blood lactate during the exercise (Figure 1 & Table 2).

Two hours following allopurinol administration, plasma allopurinol and oxypurinol reached 5.56 ± 0.35 and 19.92 ± 1.17 $\mu mol \cdot L^{-1}$ respectively, whereas after the *placebo* intake

they were not detectable. Allopurinol intake caused ten-fold increase of xanthine ($p < 0.001$) and almost doubled hypoxanthine plasma concentrations. Exercising muscles elevated plasma inosine ($p < 0.01$), hypoxanthine ($p < 0.02$), and xanthine ($p = 0.14$), however no interaction treatment x time was observed in the purine metabolites (Table 3).

Exercise induced changes in ROS production (Table 4). TAS decreased from 1.540 ± 0.043 to 1.367 ± 0.060 mmol·L^{-1}, whereas sulfhydryl groups from 544.7 ± 3.9 to 507.7 ± 16.3 μmol·L^{-1} in the *placebo* trial. Allopurinol intake did not affect these changes either in TAS ($p = 0.415$) nor in –SH groups ($p = 0.671$). Moreover no interaction treatment x time has been observed ($p = 0.157$ and $p = 0.868$ in TAS and –SH groups respectively).

DISCUSSION

The main finding of this study is that a single oral allopurinol intake does not modify energy metabolism during 6 minutes of severe intensity exercise (Tables 2 & 3), and does not affect plasma oxidative stress markers (Table 4).

No effect of allopurinol on time performance in cycling or running humans has been reported in the previous studies (Gomez-Cabrera *et al*. 2003; Gomez-Cabrera *et al*. 2006). However, these studies were performed on longer duration (Tour de France stage and Valencia Marathon), and thus lower intensity exercises compared to our study. On the contrary, Veskoukis *et al.* reported that a single intraperitoneal dose of allopurinol given 1.5h before the exercise decreased swimming performance in rats (Veskoukis *et al*. 2008). Reported time to exhaustion (36 ± 5 minutes) suggests that the exercise was performed at higher intensity. Differences in the studies may be due to the use of animal and human models, but it seems more plausible that the amount of allopurinol administered may be responsible for this discrepancy (50 mg kg^{-1} body mass in rats (Veskoukis *et al*. 2008) vs. approximately 4 mg kg^{-1} body mass in our experiment). Moreover, increased level of

oxidative stress markers suggests, that such high dose of allopurinol may induce oxidative stress before commencing the exercise, which may influence performance (Veskoukis *et al*. 2008).

The efficiency of the muscles to produce work has been related to reactive oxygen and nitrogen species (Ferreira and Reid 2008; Lamb and Westerblad 2011). Recent studies indicated that a diet rich in the amino acid L-arginine and/or nitrate, which increases nitric oxide (NO) synthesis, reduces oxygen cost of exercise during low intensity work (Lansley *et al*. 2011; Larsen *et al*. 2007), and increases exercise tolerance during severe intensity exercise in healthy humans (Bailey *et al*. 2010; Larsen *et al*. 2010). NO is synthesized by nitric oxide synthase, but can also be generated by XO catalyzing the reduction of nitrate to nitrite and nitrite to NO in the presence of NADH as electron donor (Zhang *et al*. 1998). The XO reactions can be completely blocked by allopurinol. We have not determined the nitrite / nitrate level in the blood, but obtained results suggests that XO plays a minor role in modification of energy metabolism during short term severe intensity exercise in healthy humans (Tables 2 & 3).

The muscle fatigue accompanied by muscle metabolites accumulation is the most likely cause of an increase in oxygen cost of work i.e. a decrease in muscle efficiency, as demonstrated recently by Zoladz *et al.* (2008) and Cannon *et al.* (2011). When during the work, expenditure of ATP exceeds the rate of ATP generation, part of the adenine nucleotide pool is deaminated to inosine monophosphate (IMP) and ammonia (NH_3) by AMP deaminase (Parnas 1929). IMP can either be reaminated back to AMP or degraded further to hypoxanthine, xanthine and urate. The cellular membrane is permeable to NH_3 and hypoxanthine but impermeable to phosphorylated compounds, which will remain in the cellular compartment. Increases in the degradation products of ATP can be detected in blood (hypoxanthine and NH_3) or muscle (IMP and NH_3) and may be used as markers of energy

deficiency (Sahlin *et al*. 1998). The direct correlation between plasma NH_3 and the slow component has been recently shown (Malek *et al*. 2008; Sabapathy *et al*. 2005). Moreover, Zhang *et al*. (1993) found that the slow component is related to a net increase in plasma hypoxanthine concentration.

Two hours following allopurinol intake we noted the presence of allopurinol and oxypurinol, the major metabolite of allopurinol, in the plasma. Both are structural analogs of hypoxanthine and xanthine, respectively and competitively bind to XO. Thereby, they inhibit the conversion of hypoxanthine to xanthine and xanthine to uric acid. Allopurinol induced an increase in resting xanthine but had no effect on plasma inosine or hypoxanthine concentrations (Table 3), which is consistent with previous studies (Heunks *et al*. 1999; Stathis *et al*. 2005). Similar changes were observed in post-exercise plasma concentrations (Table 3). Since allopurinol ingestion did not affect energy metabolism, no modification in $\dot{V}O_2$ slow component was noted (Figure 1).

The change in plasma oxidative stress markers following exercise noted in our study (Table 4), is consistent with the previous studies indicating that ROS generation is observed after exercise with similar workloads (Ji *et al*. 1992; Lamprecht *et al*. 2009; Lovlin *et al*. 1987; Wang and Huang 2005). Recent studies, have suggested that XO is a relevant source of ROS during exercise (Gomez-Cabrera *et al*. 2003; Gomez-Cabrera *et al*. 2006; Vina *et al*. 2000). Allopurinol administration attenuates ROS production and tissue damage induced by prolonged aerobic exercise (Gomez-Cabrera *et al*. 2003; Gomez-Cabrera *et al*. 2006). It has also been reported that plasma XO activity increased 10-fold after a single bout of exhaustive exercise (Radak *et al*. 1995). However, in that study, Radak *et al*. (1995) demonstrated that superoxide dismutase derivative administration before the start of exercise, effectively inhibits the increase of XO activity. Since xanthine dehydrogenase can be converted to xanthine oxidase by reversible sulfhydryl oxidation (Enroth *et al*. 2000), it seems plausible that another

source of ROS is necessary to activate XO. In our study we observed decreased post-exercise concentration of plasma sulfhydryl groups, possibly caused by their increased oxidation, which is consistent with ROS-mediated XO activation induced by exercise.

In conclusion, the results from this study have established that XO inhibition does not modify energy metabolism during short term severe intensity exercise in healthy humans. Moreover, short term exercise at 75% $\dot{V}O_2$max induces oxidative stress, whereas allopurinol intake does not influence ROS overproduction.

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CONFLICT OF INTEREST

There is no conflict of interest.

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TABLE 1 Subjects characteristics.

Variable	Mean ± SEM
Age (yr)	22.5 ± 0.2
Height (cm)	185.7 ± 2.8
Weight (kg)	79.7 ± 2.6
VO_2 max ($mlO_2 \cdot min^{-1}$)	3957 ± 171
Power max. (W)	335.8 ± 13.1

TABLE 2 Oxygen uptake and blood lactate in placebo and allopurinol trials. Values are means ± SEM.

	Placebo	Allopurinol	Paired t-test p-value
Oxygen uptake			
Pre-exercise ($mlO_2 \cdot min^{-1}$)	319 ± 26	335 ± 22	0.741
End of exercise ($mlO_2 \cdot min^{-1}$)	2933 ± 79	3064 ± 95	0.231
Slow component amplitude ($mlO_2 \cdot min^{-1}$)	315 ± 36	322 ± 18	0.733
Blood lactate			
Rest **(**$mmol \cdot L^{-1}$**)**	1.3 ± 0.2	1.1 ± 0.2	0.311
End of exercise **(**$mmol \cdot L^{-1}$**)**	6.4 ± 0.9	6.4 ± 0.6	0.912
Δ **(**$mmol \cdot L^{-1}$**)**	5.0 ± 0.8	5.4 ± 0.4	0.628

TABLE 3 Plasma concentrations of purines. Values are means ± SEM.

	Placebo		Allopurinol		ANOVA p-value		
	prior exercise	post exercise	prior exercise	post exercise	treatment	time	treatment x time
Inosine ($\mu mol \cdot L^{-1}$)	0.044 ± 0.012	0.106 ± 0.020	0.047 ± 0.009	0.099 ± 0.011	0.869	0.008*	0.615
Hypoxanthine ($\mu mol \cdot L^{-1}$)	0.247 ± 0.040	3.246 ± 1.018	0.404 ± 0.083	3.453 ± 0.630	0.643	0.013*	0.936
Xanthine ($\mu mol \cdot L^{-1}$)	0.260 ± 0.020	0.318 ± 0.027	2.703 ± 0.208	2.834 ± 0.189	<0.001*	0.142	0.463
Uric acid ($\mu mol \cdot L^{-1}$)	253.6 ± 12.3	220.5 ± 9.7	246.1 ± 14.9	218.7 ± 15.0	0.653	0.001*	0.474

* statistically significant

TABLE 4 Plasma oxidative stress markers. Values are means ± SEM.

	Placebo		Allopurinol		ANOVA p-value		
	prior exercise	post exercise	prior exercise	post exercise	treatment	time	treatment x time
TAS ($mmol \cdot L^{-1}$)	1.540 ± 0.043	1.367 ± 0.060	1.537 ± 0.027	1.458 ± 0.029	0.415	0.011*	0.157
SH ($\mu mol \cdot L^{-1}$)	544.7 ± 3.9	507.7 ± 16.3	538.5 ± 12.7	495.9 ± 19.2	0.671	0.003*	0.868

* statistically significant

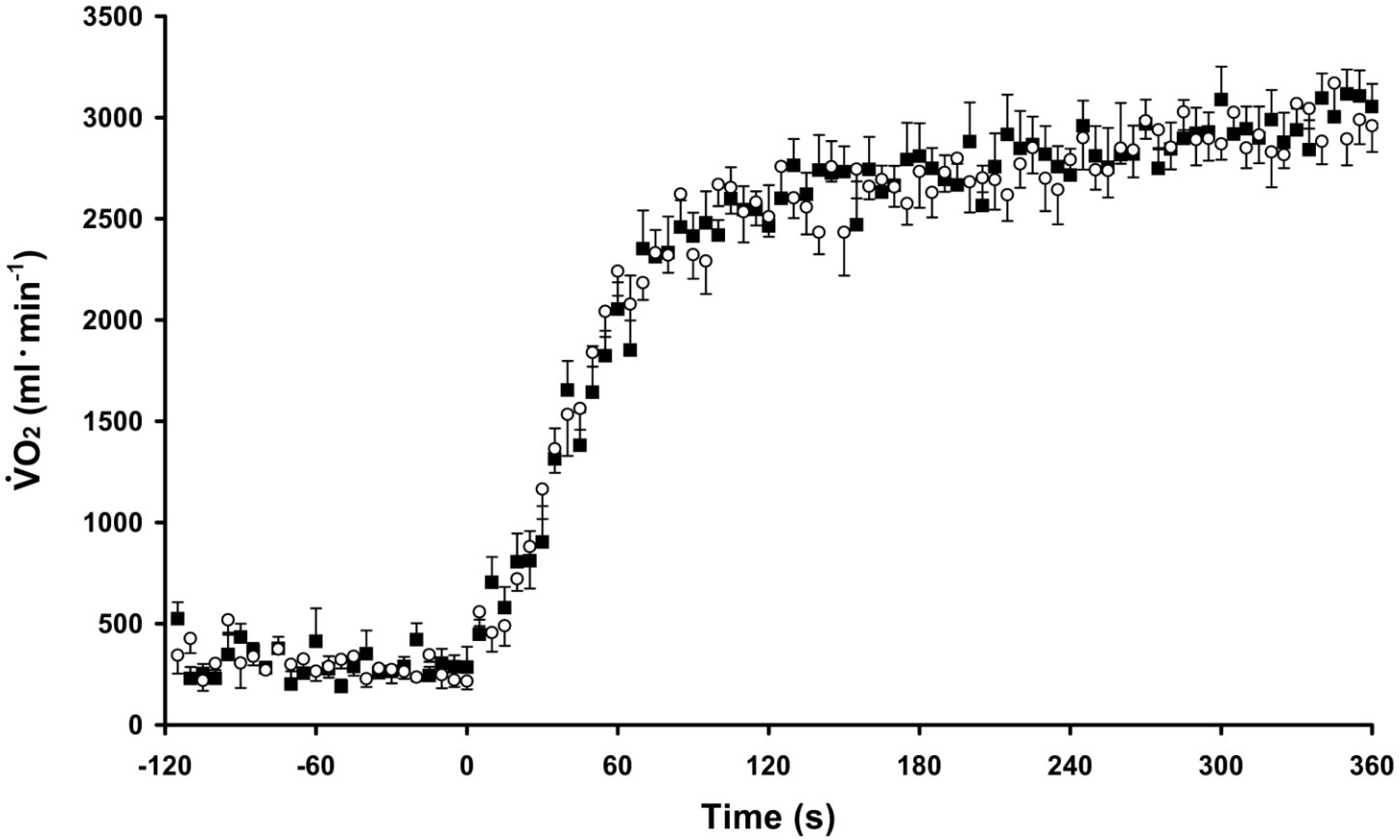
3500
3000
2500
2000
1500
1000
500
0
V̇O2 (ml · min-1)
-120
-60
0
60
120
180
240
300
360
Time (s)

FIGURE LEGEND

FIGURE 1 Pulmonary $\dot{V}O_2$ during cycling at the power output corresponding to ~75% $\dot{V}O_2$ max. after ingestion of allopurinol (■) or *placebo* (○). Data are averaged every 5 seconds (mean ± SEM).