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Administration of exercise-conditioned plasma alters muscle catalase kinetics in rat: An argument for *in vivo*-like K_m instead of *in vitro*-like V_{max}

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ABSTRACT

Maximal velocity (V_{max}) is a well established biomarker for the assessment of tissue redox status. There is scarce evidence, though, that it does not probably reflect sufficiently *in vivo* tissue redox profile. Instead, the Michaelis constant (K_m) could more adequately image tissue oxidative stress and, thus, be a more physiologically relevant redox biomarker. Therefore, the aim of the present study was to side-by-side compare V_{max} and K_m of an antioxidant enzyme after implementing an *in vivo* set up that induces alterations in tissue redox status. Forty rats were divided into two groups including rats injected with blood plasma originating from rats that had previously swam until exhaustion and rats injected with blood plasma originating from sedentary rats. Tail-vein injections were performed daily for 21 days. Catalase V_{max} and K_m measured in gastrocnemius muscle were increased after administration of the exercise-conditioned plasma, denoting enhancement of the enzyme activity but impairment of its affinity for the substrate, respectively. These alterations are potential adaptations stimulated by the administered plasma pointing out that blood is an active fluid capable of regulating tissue homeostasis. Our findings suggest that K_m adequately reflects *in vivo* modifications of skeletal muscle catalase and seems to surpass V_{max} regarding its physiological relevance and biological interpretation. In conclusion, K_m can be regarded as an *in vivo*-like biomarker that satisfactorily images the intracellular environment, as compared to V_{max} that could be aptly parallelized with a biomarker that describes tissue oxidative stress in an *in vitro* manner.

1. Introduction

During the last five years, several high prolific studies have challenged the common belief that blood is an inert tissue, which passively accepts molecules (including reactive oxygen and nitrogen species and metabolic products) generated by skeletal muscle and other tissues. Indeed, there is much accumulative *in vitro* and *in vivo* evidence suggesting that blood is not just an inactive tissue but, on the contrary, a body fluid that can regulate tissue homeostasis. In this sense, a recent *in vivo* experimental study from our group, probably the first relevant attempt in the field of redox biology, demonstrated that plasma administration from exercised rats to sedentary rats induced blood and tissue inflammation [1]. Thus, this article saliently highlights the ability of plasma molecules to stimulate tissue redox adaptations. In line with this study, *in vitro* incubation of cells with sera originating from athletes, who participate in different sports resulted in distinguishable redox responses [2]. Relevant *in vivo* parabiosis studies have also verified the active role of blood as demonstrated by the reversal of age-related cardiac hypertrophy [3], restoration of synaptic plasticity [4] and vascular, and neuronal rejuvenation in old mice surgically joined to young mice [5]. Thus, these sophisticated experiments document clearly that blood is capable of inducing alterations and adaptations in tissues in diverse biochemical and physiological processes, such as inflammation, aging and oxidative stress.

A common practice in the assessment of tissue oxidative stress is the measurement of the maximal velocity (V_{max}) of antioxidant enzymes. Catalase V_{max} is, in fact, an established redox biomarker for assessing oxidative stress in blood and tissues [6–11]. Nevertheless, this tenet has been sporadically challenged. Interestingly, the Michaelis constant (K_m) has been suggested to be a more reliable index, compared to V_{max}, for monitoring structural and functional modifications of redox related enzymes. Indeed, Ji et al. [12] described that metabolic adaptations to exercise training were clearly denoted by changes in the K_m values of lactate dehydrogenase (LDH) in rat muscles. In addition, Somani and Husain [13] reported differential alterations of the K_m values in several tissues of trained rats, suggesting that exercise training aids in coping

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with oxidative stress in old age. Favero et al. [14] demonstrated that the functional alterations of skeletal muscle LDH after endurance training in rats are best reflected by the K_m values of the enzyme and not by the modifications of LDH isozyme pattern. The above studies support the hypothesis already proposed by Gollnick and Saltin since 1982 [15], that K_m is indicative of the energy metabolism enhancement by training. To our knowledge, these are the only studies suggesting an important potential for K_m in the assessment of redox-induced alterations. However, they have offered only circumstantial evidence on the potential superiority of K_m over V_{max} to adequately reflect responses and adaptations in metabolic pathways *in vivo*. This is because no study to date has side-by-side compared the V_{max} and K_m of an antioxidant enzyme using an *in vivo* redox-altering experimental set up.

In light of the above, the main aim of the present study was to directly compare V_{max} and K_m of an antioxidant enzyme in an *in vivo* setting. Furthermore, we intended to investigate whether a usually overlooked *in vitro* kinetic parameter (i.e., K_m) of skeletal muscle catalase could describe in an *in vivo*-like manner the manifestation of a complicated phenomenon, such as oxidative stress. The redox status-altering stimulus used was administration of plasma from exercised rats to sedentary rats, which is an experimental approach capable of inducing blood-borne effects in blood and tissues. We have hypothesized that K_m might be more physiologically relevant and, thus, a more useful redox biomarker compared to V_{max}, considering that it represents one fundamental biological property of catalase, namely the affinity for its substrate.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (380 ± 27 g) were housed under a 12 h light: 12 h dark cycle, controlled temperature (21–23 °C) and humidity (50–70%). Commercial rat chow and tap water were provided *ad libitum*. All procedures were in accordance with the European Union guidelines for the care and use of laboratory animals, as well as the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985). The project was reviewed and approved by the institutional review board and the appropriate state authority (#359888/3612).

2.2. Study design

Phase 1: Rats were randomly divided in two groups, as described previously [1]. Briefly, the animals of the exercise group underwent swimming until exhaustion [1,6,9], whilst the animals of the sedentary group were placed in the water tanks for a mere ten minutes and remained in their cages without any treatment. The rats were familiarized with water according to the protocol previously presented by our group [1,6,9]. Whole blood was collected from the rats of both groups, plasma samples were isolated, homogenized into two containers, separated into aliquots of 0.8 ml each and stored at −80 °C for use in phase 2. Plasma from the rats of the exercise group is hereafter considered as the "exercised plasma" and from the rats of the sedentary group as the "resting plasma".

Phase 2: Experimental rats were randomly divided into two groups (20 animals per group) as follows: the first group involved rats that were injected intravenously with resting plasma and the second group consisted of rats that were injected intravenously with exercised plasma. Tail vein injections at the dose of 2 ml/per kg of body weight were performed daily for 21 consecutive days by using 1 ml insulin syringes (Terrumo, Tokyo, Japan). Twenty-four hours after the last injection, rats of both groups were killed and gastrocnemius muscle was collected and stored at −80 °C for further analysis.

2.3. Blood and tissue collection and preparation

Rats were deeply anesthetized by exposure to ether. The depth of anesthesia was assured by the constriction of the pupils as well as simple sensory tests, such as the absence of eye blinking when the eyelid was touched and the absence of foot withdrawal when it was pinched. Subsequently, the thoracic cavity was opened and the rats were killed by blood collection via cardiac puncture in the right ventricle using a 10 ml syringe during both phases 1 and 2. During phase 2, immediately after blood collection, gastrocnemius muscle was quickly excised, snapped frozen in liquid nitrogen and stored at −80 °C. In preparation for analysis, muscle samples were initially ground using a mortar and a pestle under liquid nitrogen and homogenized as previously demonstrated [1].

2.4. Analysis of kinetic parameters

The V_{max} and K_m values were calculated using the protocol for catalase activity as it has been previously described [11]. Specifically, 40 μl of muscle homogenate diluted 1/2 in distilled water was added to 2955 μl of phosphate buffer (67 mM, pH = 7.4) and the mixture was incubated for 10 min at room temperature. Next, 5 μl of 30% hydrogen peroxide (H_2O_2) (to a final concentration of 20 mM) was added and the absorbance was monitored at 240 nm for 2 min. This protocol was performed in six different concentrations of the substrate (H_2O_2), namely 1 mM, 2 mM, 4 mM, 10 mM, 20 mM and 40 mM for each of the 40 muscle samples. Both the Michaelis-Menten parabolic plot of the catalytic rate, corresponding to the μmol of H_2O_2 decomposed per minute by catalase (i.e., the reaction velocity, V) for all six substrate concentrations versus the substrate concentration [S] and the reciprocal Lineweaver-Burk plot (i.e., the 1/V vs. 1/[S] plot) were built. The straight line Lineweaver-Burk plot was used to calculate K_m (x intercept = $-1/K_m$) and V_{max} (y intercept = $1/V_{max}$) values. Each assay was performed in triplicate. All reagents were purchased from Sigma-Aldrich (St. Louis, Mo.).

2.5. Statistical analysis

The distributions of the dependent variables were examined using the Shapiro-Wilk test and were found not to differ significantly from normality. The values of V_{max} and K_m were analyzed using Student's *t*-test for independent samples (Excel 2010, Microsoft Corporation, Redmond, WA, USA). Data are presented as mean ± SEM and the significance level was set at $P < 0.05$.

3. Results

The effect of administration of exercised plasma on catalase V_{max} and K_m of the gastrocnemius muscle is demonstrated in Fig. 1. Catalase V_{max} value was significantly increased ($P = 0.02$) in the rats administered with exercised plasma (i.e., 3.3 ± 0.4 μmol/min) compared to the rats administered with resting plasma (i.e., 2.2 ± 0.2 μmol/min) (Fig. 1a). Similarly, catalase K_m value was also significantly elevated ($P = 0.05$) in the rats administered with exercised plasma (i.e., 18.9 ± 2.1 mM) compared to the rats administered with resting plasma (i.e., 14.1 ± 1.3 mM) (Fig. 1b). Fig. 2 illustrates two representative plots of the reaction rate (i.e., Michaelis-Menten plots) for the examined groups of rats. Fig. 2a and b depict the catalase reaction rate (i.e., the rate of product generation) in the rats administered with resting plasma and exercised plasma, respectively. The reaction rate of the group administered with exercised plasma is higher as demonstrated by the increase of the V_{max} value. Two representative Lineweaver-Burk, double reciprocal plots for the aforementioned groups of rats are shown in Fig. 3. The intersection of the line of the plot with x and y axis is representative of the increase in K_m and V_{max} values, respectively, in the rats administered with exercised plasma (Fig. 3b) compared to the rats