



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 μ l of muscle homogenate diluted 1/2 in distilled water was added to 2955 μ l of phosphate buffer (67 mM, pH = 7.4) and the mixture was incubated for 10 min at room temperature. Next, 5 μ l 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 μ mol 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 \pm 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 μ mol/min) compared to the rats administered with resting plasma (i.e., 2.2 ± 0.2 μ mol/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

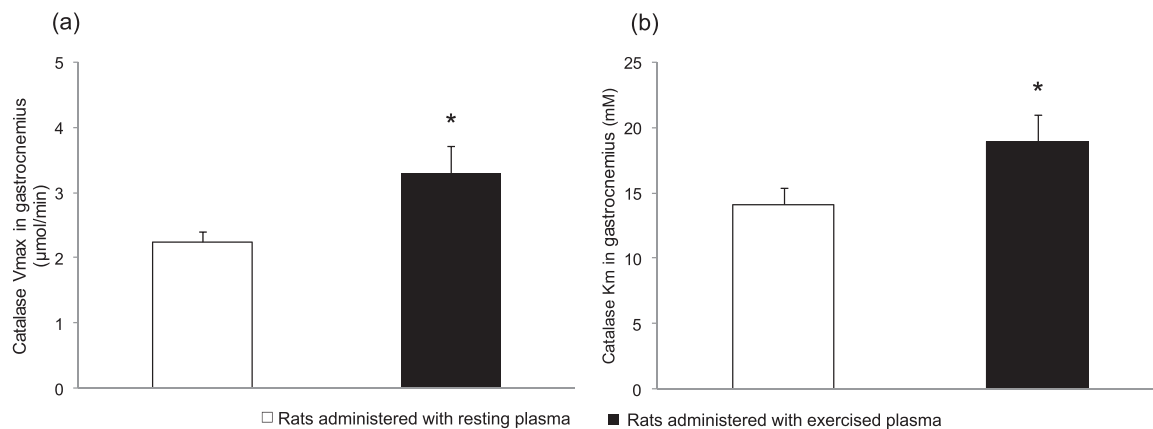


Fig. 1. The effect of either resting (open bars) or exercised (closed bars) plasma administration on catalase V_{max} (1a) and K_m (1b) in gastrocnemius muscle. *Significant difference between the rats administered with resting plasma and the rats administered with exercised plasma.

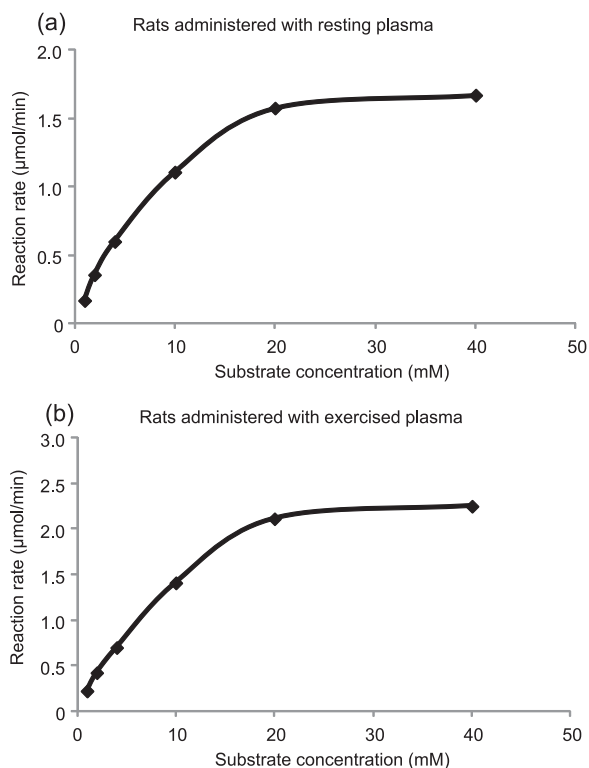


Fig. 2. One representative Michaelis-Menten parabolic plot of the reaction rate (V) versus the substrate (H₂O₂) concentration ([S]) for the rats administered with resting plasma (2a) and the rats administered with exercised plasma (2b).

administered with the resting plasma (Fig. 3a).

4. Discussion

The present study examined whether an overlooked *in vitro* kinetic parameter, such as K_m, is more physiologically relevant than the widely used V_{max} in assessing the *in vivo* behavior of catalase and reflecting tissue oxidative stress, after the implementation of a stimulus disrupting redox equilibrium and inducing adaptations. The levels of catalase V_{max} and K_m were both increased after administration of exercised plasma in rats, indicating that skeletal muscle adaptations were induced by blood-borne factors. Our findings clearly demonstrate that K_m adequately reflects *in vivo* redox-related modifications of skeletal muscle catalase.

Catalase is a significant enzyme of the antioxidant defence present in almost all aerobes. It catalyzes the dismutation of two H₂O₂

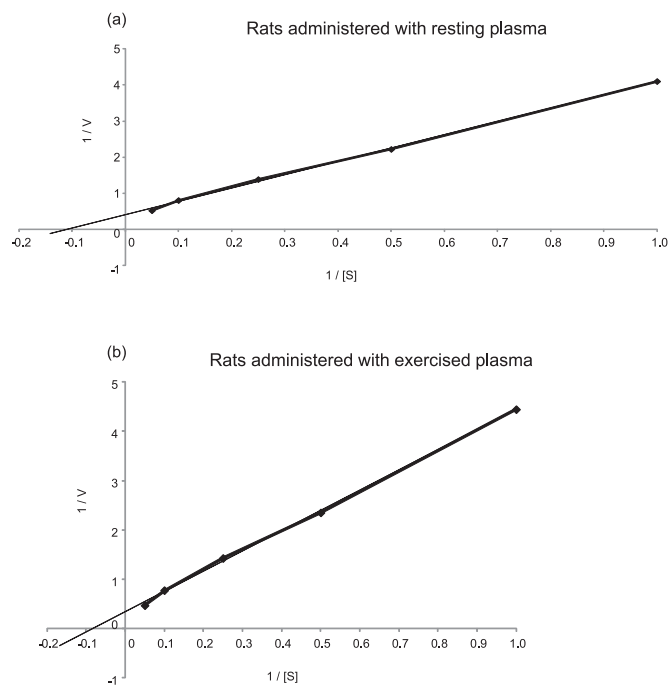


Fig. 3. Two representative Lineweaver-Burk plots for reciprocal values of the velocity (1/V) versus the substrate (H₂O₂) concentration (1/[S]) for the rats administered with resting plasma (3a) and the rats administered with exercised plasma (3b).

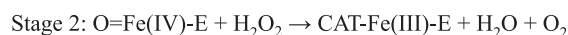
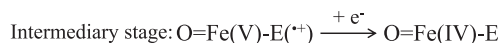


Fig. 4. The reaction mechanism of catalase. CAT: catalase; Fe-E: heme and its iron center attached to the enzyme; O=Fe(V)-E⁽⁺⁾: compound I [oxoporphyrin-cation radical (haem⁺)]; O=Fe(IV)-E: compound II.

molecules, that is the reduction of one H₂O₂ molecule to H₂O and the oxidation of the second to O₂ [16]. While the exact catalase mechanism is still unresolved, it is currently accepted that it operates in two stages (Fig. 4). During the first stage, H₂O₂ interacts with asparagine 148 and histidine 75 and the ferric form [Fe(III)] of catalase heme is converted to a nominal valency of Fe(V) oxoporphyrin-cation radical (i.e.,

compound I). Compound I is subsequently reduced by one electron producing Fe(IV) heme (compound II), which, at the second stage oxidizes a second H_2O_2 molecule to form O_2 [17]. The electron in question may be generated by amino acids present in catalase molecule that consequently are converted to free radicals. Nicotinamide adenine dinucleotide phosphate (NADPH), however, that is bound to all four subunits of catalase usually offers an electron to compound I hindering amino acid oxidation. Furthermore, NADPH recycles catalase regenerating its active ferric form from compound II and, thus, considerably contributes to the regulation of tissue redox equilibrium [16,18,19].

Catalase K_m represents the affinity of the enzyme for its substrate (i.e., H_2O_2). Specifically, the larger the K_m value, the weaker the binding of catalase with H_2O_2 . Maximal velocity corresponds to the maximum rate at which catalase works at saturating concentrations of H_2O_2 and, basically, illustrates the enzyme efficacy. Catalase affinity for H_2O_2 was compromised in the gastrocnemius muscle of the rats administered with exercised plasma (i.e., K_m augmentation) probably due to post-translational modifications of the enzyme caused by irreversible alterations (e.g., carbonyl formation or thiol residue oxidation) [20]. It is plausible to assume that this is a negative adaptation stimulated by the administered exercised plasma pointing out that blood, contrary to the prevailing muscle-centric redox beliefs, is an active body fluid that regulates tissue homeostasis [21]. This finding is in accordance with our previous evidence that exercised plasma induced blood and systemic inflammation [1]. Simultaneously, the enzyme activity was reinforced (i.e., increased V_{max}), a fact that could possibly be attributed to the increase in the number of catalase molecules leading to the induction of the enzyme. This could be considered as an adaptive response to inflammation induced oxidative stress, since the antioxidant defence mechanism of skeletal muscle is enhanced to cope with increased H_2O_2 generation.

Interestingly, apart from the enhancement in the maximal rate of catalase catalysis, exercised plasma administration negatively affected catalase function but this modification could only be reflected by K_m and not V_{max} . It has been reported that the extracellular and intracellular concentrations of H_2O_2 in contracting skeletal muscle equal to 15–20 μM and 0.1–0.2 μM , respectively [22]. These values are 1000–10,000 fold lower than the gastrocnemius catalase K_m value found in the present study (i.e., approximately 20 mM) in the rats administered with exercised plasma. An enzyme with a high K_m value relative to the concentration of its substrate is not normally saturated, thus, its activity will vary because the rate of the product formation depends on the availability of the substrate [23]. Therefore, although the volume of the administered exercised plasma equals to approximately a mere 3% of total blood volume of the rats, there is a possibility that the increased H_2O_2 levels contained in it have affected catalase kinetics. This is in line with our hypothesis that the exercised plasma is an active fluid capable of altering tissue homeostasis and, specifically, inducing inflammation as we have previously reported [1] and, thus, oxidative stress. However, even though an increase in H_2O_2 levels may "prime" the enzyme reactivity as indicated by the augmented V_{max} levels, the concomitant enhancement of K_m levels imply that this is not the case. Specifically, the increased V_{max} value indicates a positive effect as the enzyme activity is reinforced, but the augmentation of K_m value denotes that the exercise conditioned plasma has negative impact on the enzyme since its affinity for H_2O_2 is impaired. This is a highly important biological trait of K_m that permits a prediction of whether or not the rate of product formation will be affected by the availability and the physiological concentrations of the substrate. Given the fact that exercised plasma administration increased catalase K_m value 1000–10,000 times above the normal levels of H_2O_2 in contracting muscle, it seems that K_m is more physiologically relevant (i.e., it reflects the H_2O_2 -related variations of catalase efficacy that are induced by muscle physiology-modifying treatments) and sufficient, than V_{max} , in addressing biological questions in oxidative stress context.

One of the major goals of biology is to create comprehensive and quantitative *in vitro* models that promote the understanding of cellular behavior [24]. A basic question rises here: can *in vitro* kinetics describe the *in vivo* function of complex biochemical pathways? [25,26]. According to the literature, several factors including endogenous metabolite localization and metabolite channelling may modulate enzyme kinetics and improve its translational potential [24,27,28]. Noteworthy, with regards to yeast glycolysis, kinetics did describe satisfactorily *in vivo* function [25]. Presumably, K_m reflects more adequately the adaptations induced by administration of exercised plasma, thus could be considered as *in vivo*-like oxidative stress biomarker. This promotes the comprehension of *in vivo* redox status in terms of *in vitro* biochemistry offering the chance to solve *in vitro*–*in vivo* discrepancies. Alternatively, *in vivo*-like kinetics improves the predictive value of kinetic models concerning fundamental biochemical processes [29].

5. Conclusion

This article bears a commentary sentiment intending to investigate whether V_{max} is a mechanistically appropriate index adequately reflecting tissue oxidative stress. According to the present as well as previous findings, V_{max} could be aptly parallelized with a biomarker that describes a biochemical phenomenon in *in vitro* terms unlike to K_m , which can be regarded as *in vivo*-like biomarker that more satisfactorily images the intracellular environment. Apparently, V_{max} is a well established and reliable redox biomarker. Still, the negative adaptations induced by the exercised plasma administration in sedentary rats could only be reflected by K_m , which seems to surpass V_{max} as regards its physiological relevance and biological interpretation.

Conflict of interest

The authors declare no conflict of interest.

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