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Experimental studies of OH° radical/pressure dependence of arginase activity using a molecular chromatography approach

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ABSTRACT

Arginase is an enzyme which plays a role in pathophysiology such as hypertension. Here we demonstrated for the first time the direct implication of pressure and OH° radical formation on the arginase activity via a novel analytical procedure. Pressure increased arginase activity in the range 12–52 bars. Activation by OH° radical showed a hyperbolic response. The OH° radicals produced were significantly inhibited by sulfasalazine (SAZ) and the inhibition of OH° radicals parallels the inhibition of arginase activity.

1. Introduction

In mammalian cells, L-arginine is metabolized by two pathways. Arginase catalyses its hydrolysis to L-ornithine and urea whereas NO synthase (NOS) catalyzes its oxidation to L-citrulline and nitric oxide, NO [1]. Defects of endothelial NO function, referred to as endothelial dysfunctions are associated with major cardiovascular risk factors, such as hyperlipidemia, diabetes, hypertension and severity of artheriosclerosis [2–5]. Since NOS and arginase can be found in similar tissues and cells, and because their expression may be regulated in response to the same stimuli (cytokines, endotoxines), both enzymes are believed to participate in the regulation of NO biosynthesis by competing for the common substrate L-arginine [6]. Conversely, N-hydroxy-L-arginine (NOHA) an intermediate in the reaction catalyzed by NOS is a potent competitive inhibitor of arginase with K_i value of 10–40 μM [7,8]. Recently our group demonstrated a close relationship between arginase activity and blood pressure, making arginase a promising target for antihypertensive therapy [9,10]. As well, it was demonstrated that arginase could be stimulated during oxidative stress [11]. The inhibition of arginase by selective and potent inhibitors thus became the focus of potential therapies for treating several NO-dependent smooth muscle disorders [12–15]. The ex vivo characterization of drug candidates on isolated target enzymes, unfortunately, is often associated with long, labor-intensive assays and a large amount of disposable expensive material. Our group recently developed a novel immobilized arginase reactor for the binding mechanism study of a series of arginase inhibitors with the enzyme and the magnesium effect on this association process [16,17]. This paper describes a novel procedure for studying both the direct role of OH° radical formation and pressure on the arginase activity.

2. Experimental

2.1. Reagents and equipment

Crystalline bovine liver arginase I was obtained from Sigma–Aldrich (Paris, France). The four arginase inhibitors (NOHA, nor-NOHA, BEC, ABH) were obtained from Bachem (Germany). All the other organic solvents were of analytical grade and purchased from Merck (Paris, France). Chromatographic experiments were performed with two HPLC systems and all organic solvents were of analytical grade. One system (system A) was a Hewlett Packard HP 1050 liquid chromatograph (Palo Alto, CA, USA) with a Rheodyne sample valve (20 μL loop) equipped with a Hewlett Packard HP 1050 variable wavelength detector and the enzyme reactor. The preparation of the immobilized enzyme reactor (IMER) using a monolithic support inside a column (25 mm × 4.6 mm i.d.) was given in [16]. The mobile phase A was described below and depended on the developed application.

The second equipment (system B) consisted of a Hewlett Packard HP 1100 liquid chromatograph with a Rheodyne sam-

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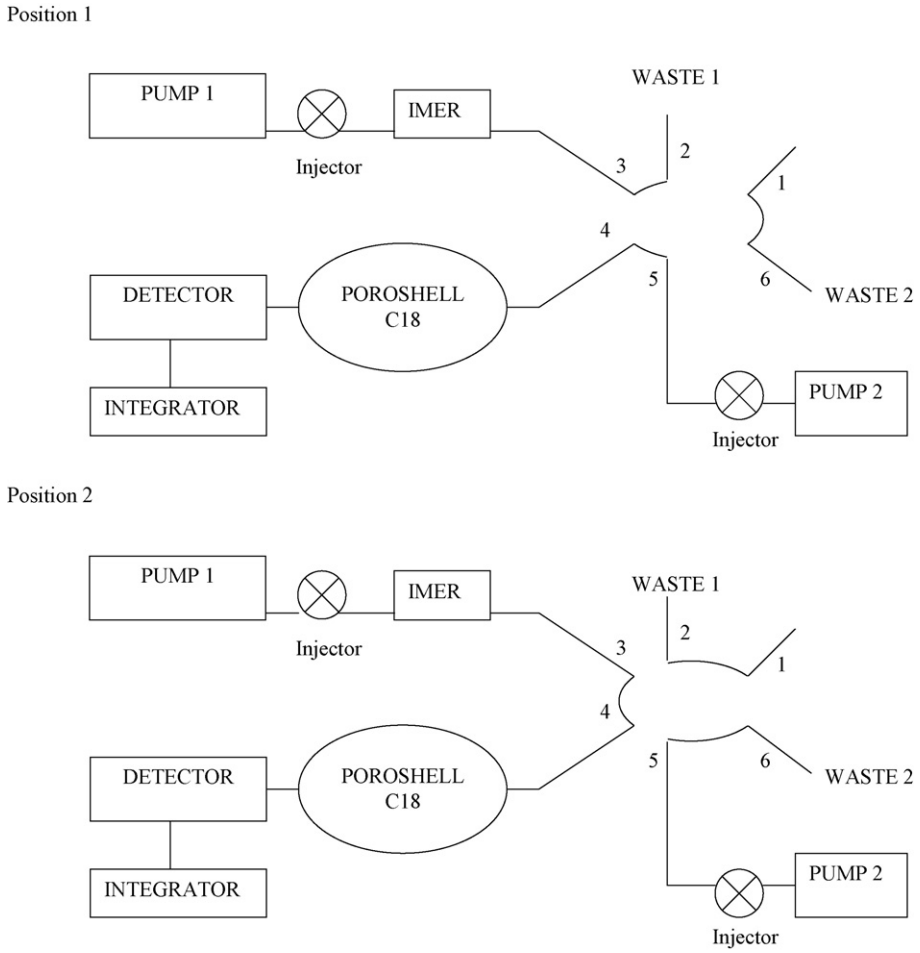

Fig. 1. Chromatographic system coupling the immobilized enzyme reactor (IMER) with the reversed phase analytical column. The substrate is loaded onto the IMER using position 1; the product and the unreacted substrate are switched to the analytical column using position 2.

ple valve (20 μL loop) equipped with a Hewlett Packard HP 1100 variable wavelength detector, a HP 1100 thermostat and an Agilent poroshell C18 column (15 cm × 4.6 mm) (Agilent Technologies, Walbronn, Germany). The mobile phase was a phosphate buffer (pH = 7.00, 50 mM)–methanol (50/50) (v/v). The flow-rate was 3 mL/min and the detection wavelength was 372 nm.

Systems A and B could be used independently or the eluent from system A could be directed onto system B through a HP six-port switching valve as outlined in Fig. 1. Both systems were connected to an HPLC ChemStation (Revision A.04.01).

For analysing the pressure effect on arginase activity, a capillary restrictor (0.0025 in. polyether ether ketone (PEEK) tubing, Upchurch, Oak Harbor, WA, USA; in.= 2.54 cm) was placed downstream the immobilized enzyme reactor, to allow an easy adjustment of the average reactor pressure (ARP). The long term stability of the flow-rate was tested periodically as part of the validation tests equipment recommended by the manufacturer.

2.2. Analytical procedure

2.2.1. Arginase column activity determination

The 1-nitro-3-guanidinobenzene (NGB) was synthesized as described in a previous paper [18]. NGB was used as enzyme substrate yielding products urea plus the chromophore m-nitroaniline (m-NA). 1 mM substrate solutions were injected onto system A at valve position 1 (see Fig. 1). Loading eluent was delivered by the system A pump at a flow-rate of 0.5 mL/min. This eluent (mobile phase A) consisted of 0.1 mM Tris–HCl buffer at pH = 7.4, 10 mM $MnCl_2$. After 0.1 min the valve had been switched and the analytes (products and unreacted substrate) were flushed and focused for 5 min directly to the reversed phase analytical column. The valve was then switched back to its original position for separation with eluent delivered by system B. At 372 nm where the chromatogram was given, the extinction coefficient of NGB (109 M^{-1} cm^{-1}) is much less than of m-NA (1280 M^{-1} cm^{-1}) [18] and this is the great interest of this substrate. As well, the analysis time is very short around 2 min. One unit of enzymatic activity is defined as the amount of enzyme catalyzing the production of 1 μmol of m-NA/min. The Michaelis–Menten trend was found by plotting the rate of enzymatic reaction against the substrate concentration [S]. The kinetic V_m and K_m were obtained via the Lineweaver and Burk plot, which is a linear transformation of the Michaelis–Menten plot. For some experiments it was necessary to measure arginase activity in solution. The description was given in ref [18]. Briefly, to measure the arginase activity in solution (activity of the free enzyme) the concentrations of arginase stock solutions were determined from the absorbance at 280 nm and a stock solution of 200 mM NGB in DMSO was prepared. Assays were performed in 0.1 mM Tris–HCl buffer pH = 7.4 10 mM $MnCl_2$ using a spectrophotometer detector with a detection-wavelength equal to 372 nm corresponding to the liberated product m-NA. In all this analytical procedure each experiment were repeated 5 times (n = 5).

2.2.2. Free radical production

To investigate the effect of OH° radical formation on the arginase activity, the following system for the production of free radicals was

Table 1
Evaluation of K_m and V_m for IMER to IMER, reproducibility (i.e., arginase column). Mobile phase A: 0.1 mM Tris–HCl buffer pH = 7.4–10 mM $MnCl_2$–Fenton reagent ($FeSO_4$ (x mM) + H_2O_2 (x mM))/flow-rate: 0.5 mL/min. Mobile phase B: phosphate buffer (pH = 7.00, 50 mM)–methanol (50/50) (v/v)/flow-rate: 3 mL/min. Detection wavelength: 372 nm. IMER temperature: 298 K.

IMER	K_m (mM)	V_m (μmol/min)
1	13.8 (0.1)	138.5 (0.4)
2	13.9 (0.1)	138.3 (0.5)
3	13.5 (0.2)	138.6 (0.3)

Standard deviations were in parentheses.

used; $FeSO_4$ (x mM) and H_2O_2 (x mM) were added in the mobile phase A, i.e., 0.1 mM Tris–HCl buffer pH = 7.4–10 mM $MnCl_2$. In the presence of iron a Fenton reaction will occur between Fe^{2+} and H_2O_2 giving rise to the OH° radical.

3. Results and discussion

The sensitivity of the immobilized enzyme in the reactor to the arginase inhibitors was examined. Four inhibitors were examined. The mobile phase A was 0.1 mM Tris–HCl buffer pH = 7.4–10 mM $MnCl_2$, the IMER was maintained equal to 25 °C at a flow-rate of 0.5 mL/min. Their IC50 were determined by the conventional spectrophotometric method described briefly above [18]. Then the IC50 of the four known inhibitors (NOHA, nor-NOHA, BEC, ABH) was assessed by using the IMER by extrapolation from the inhibition curves. The inhibition curves were obtained by injecting in system A simultaneously both the substrate at a fixed saturating concentration, as determined by the Michaelis–Menten plot, and inhibitors at increasing concentration. Increasing reduction of the m-NA peak area when compared to the area obtained by the sole substrate, was observed for increasing inhibitor concentration. The percent inhibition was plotted against the logarithm of inhibitor concentration to obtain the inhibition curves. The PIC50 (i.e., −log(IC50)) values obtained on the IMER were compared with the values obtained for the free enzyme and a valid correlation was obtained ($r^2 = 0.999$). This allowed a direct comparison between on line determined inhibition potencies and PIC50 values determined with the classical spectrophotometric method. For example, the IC50 values obtained with the IMER and the free enzyme were respectively equal to 10.6 μmol/L, 10.2 μmol/L for NOHA, 0.56 μmol/L, 0.59 μmol/L for nor-NOHA and 0.57 μmol/L, 0.58 μmol/L for BEC. The values obtained were similar as those obtained for the biological substrate arginine [19,20]. These results indicated that the IMER could be used to on line screen for new inhibitors and the enzyme immobilization on the chromatographic support did not alter its biological properties. To evaluate the IMER to IMER reproducibility, three IMERs were prepared under identical conditions [16]. The mobile phase A was 0.1 mM Tris–HCl buffer pH = 7.4–10 mM $MnCl_2$, the IMER was maintained equal to 25 °C at a flow-rate of 0.5 mL/min. The K_m and V_m values for NGB were calculated using these three IMERs (Table 1). The results showed that the technique was reliable and reproducible. To investigate the flow-rate effect on the arginase activity, the kinetic parameters (V_m and K_m) of the IMER were determined at different flow-rates. As shown in Figs. 2 and 3 the effect of flow-rate on the K_m value is very small while maximum velocities V_m decreased. Since only the product formation was affected, it might be speculated that the increasing friction due to higher flow-rate could negatively influence the enzyme catalytic efficiency. From Fig. 2, the K_m value was around 13.8 ± 0.3 mM. This value was determined in a 0.1 mM Tris buffer pH = 7.4. An experiment was carried out in a 0.1 mM Tris buffer pH = 8.6 (mobile phase A). The value obtained was 3.1 ± 0.2 mM. This variation agrees with previous study which demonstrated that the K_m value decreased with the pH value. For human liver arginase

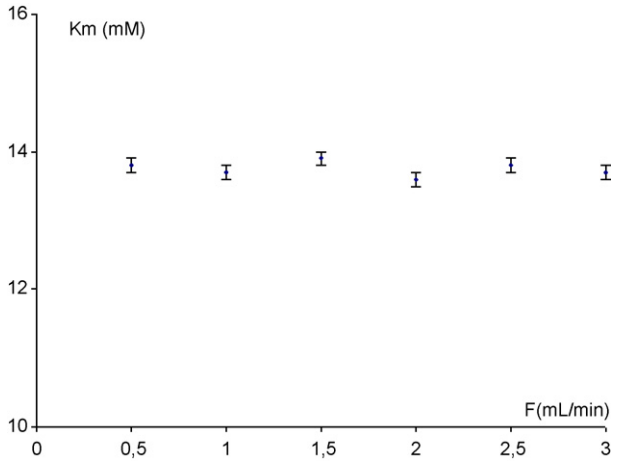

Fig. 2. K_m (mM) vs. the flow-rate A (mL/min). Mobile phase A: 0.1 mM Tris–HCl buffer pH = 7.4–10 mM $MnCl_2$. Mobile phase B: phosphate buffer (pH = 7.00, 50 mM)–methanol (50/50) (v/v)/flow-rate: 3 mL/min. Detection wavelength: 372 nm. IMER temperature: 298 K.

enzyme, the K_m for arginine declined from 15.4 to 1.6 mM over the pH range 6.5–9.5 [21]. As well, the K_m value should be treated with caution, because of the significant buffer effect. The K_m value for arginase catalyzed NGB hydrolysis in a 50 mM Bicine–NaOH (pH 8.6) (mobile phase A) was determined 1.8 ± 0.4 mM. This value is nearly identical to that obtained in a previous paper [18] but lower than in Tris. For analysing the pressure effect all the experimental data were measured with a 1.5 mL/min mobile phase flow-rate A. The corresponding natural pressure drop was 52 bars at 25 °C (the IMER was 25 mm length and 4.6 mm i.d.). The ARP was gradually decreased by cutting short section of the restrictor tubing from the natural column back pressure (52 bars at 25 °C). The ARP was monitored within the interval 12–52 bars with an accuracy of 1 bar. In this pressure domain, in chromatography it is usually assumed that the IMER permeability is independent of the position and that the liquid compressibility is negligible. Fig. 4 reports all the data acquired on the evolution of the arginase activity when pressure increased from 12 to 52 bars. Looking at the experimental data, it is evident that the trend is not linear but in the domain of studied pressure, the activity increased when pressure increased. These results demonstrated clearly and for the first time the direct implication of pressure for arginase activation. Fig. 5A shows that arginase activation responded hyperbolically to change of OH° concentration. To interpret this variation two further aspects are illustrated in Fig. 5B and C. First, the removal of H_2O_2 demon-

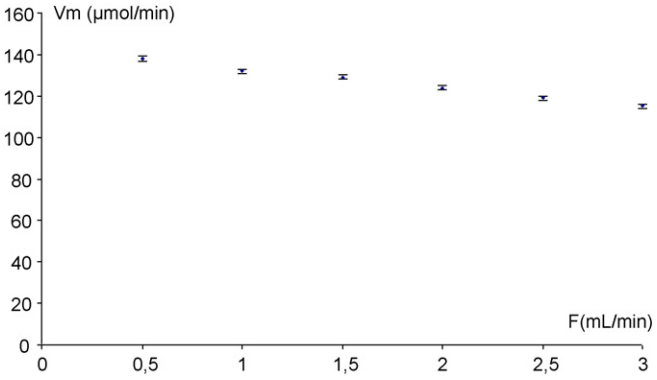

Fig. 3. V_m (μmol/min) vs. the flow-rate A (mL/min). Mobile phase A: 0.1 mM Tris–HCl buffer pH = 7.4–10 mM $MnCl_2$. Mobile phase B: phosphate buffer (pH = 7.00, 50 mM)–methanol (50/50) (v/v)/flow-rate: 3 mL/min. Detection wavelength: 372 nm. IMER temperature: 298 K.

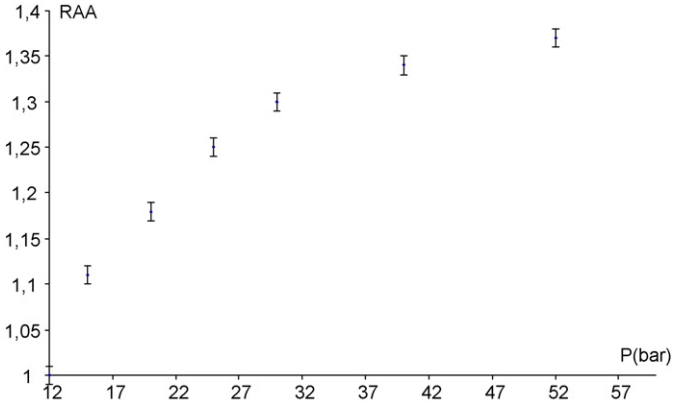

Fig. 4. Relative arginase activity (RAA) (i.e., arginase activity relative to the value at $P = 12$ bars) vs. the IMER pressure P (bar). Mobile phase A: 0.1 mM Tris–HCl buffer pH = 7.4–10 mM $MnCl_2$/flow-rate: 1.5 mL/min. Mobile phase B: phosphate buffer (pH = 7.00, 50 mM)–methanol (50/50) (v/v)/flow-rate: 3 mL/min. Detection wavelength: 372 nm. IMER temperature: 298 K.

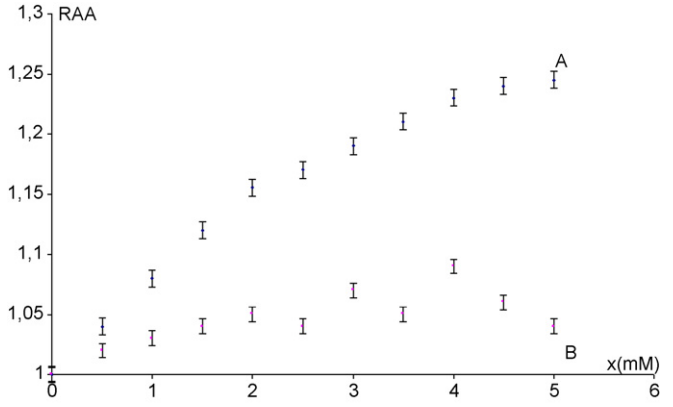

Fig. 6. Relative arginase activity (RAA) (i.e., arginase activity relative to the value at $x = 0$) vs. x (mM): A corresponding to OH°; B corresponding to (OH° + SAZ). Mobile phase A: 0.1 mM Tris–HCl buffer pH = 7.4–10 mM $MnCl_2$–Fenton reagent ($FeSO_4$ (x mM) + H_2O_2 (x mM))/flow-rate: 0.5 mL/min. Mobile phase B: phosphate buffer (pH = 7.00, 50 mM)–methanol (50/50) (v/v)/flow-rate: 3 mL/min. Detection wavelength: 372 nm. IMER temperature: 298 K.

strated no activation given by Fe^{2+} (Fig. 5B) and the removal of $FeSO_4$ showed that H_2O_2 deactivated arginase in the entire H_2O_2 concentration studied (Fig. 5C). Therefore, it can be deduced that the OH° radical production lead to an increase of the arginase activity for $x \leq 5$ mM. This is an important finding as it was the first time that the direct implication of the OH° radical on the arginase activity was clearly visualized. The requirement for OH° radicals in the stimulation of arginase activity was confirmed by the ability of a specific OH° radical scavenger (sulfasalazine (SAZ) [22]) to inhibit the activity of arginase, which paralleled the reduction of OH° radical levels in the medium. For this, the following solution (1 mM (NGB) + 1 mM (SAZ) in 0.1 mM Tris–HCl buffer pH = 7.4) was injected onto system A at valve position 1 (Fig. 1) (mobile phase A was $FeSO_4$ (x mM) + H_2O_2 (x mM) + 0.1 mM Tris–HCl buffer pH = 7.4–10 mM $MnCl_2$). For these experiments, the mobile phase flow-rate A was 0.5 mL/min. Fig. 6 indicates clearly that SAZ alone did not change arginase activity but potently inhibited arginase activity in the presence of OH° radicals in the medium.

The Michaelis–Menten approach assumed that a rapid equilibrium was established between the free reactants (enzyme (E) + substrate (S)) and the transition state complex (ES), followed by slower conversion of the ES complex back to free enzyme (E) and product (P). However there were a series of rapid chemical events following ES complex formation. For simplicity, the overall rate for these collective chemical steps can be described by a single first-order rate constant k_{cat} proportional to the V_m values (i.e., $k_{cat} = [E]_0 V_m$ where $[E]_0$ is the arginase concentration immobilized) [23,24]. As well, the ratio k_{cat}/K_m can be related to the free energy difference ΔG_{ES} between the free reactants and the transition state complex (ES). For a Fenton reagent in the medium x less than 5 mM, the V_m value increased with x. Beyond this value, V_m remained relatively constant (Fig. 7). K_m did not change its value in the entire x value domain (Fig. 8). Therefore, the difference in the transition state energies $\Delta\Delta G_{ES}$ with and without OH° radical in the medium was given by the following expression [25]:

$$\Delta\Delta G_{ES} = -RT \ln\left(\frac{V_{m,x}}{V_{m,x=0}}\right)$$

where R was the gas constant and T the temperature in Kelvin (Table 2). The K_m values did not change significantly with the OH° radical production. This was because there was no change on the arginase affinity towards the substrate. These results indicated classical activation kinetic without a significant influence on the rate of substrate binding [26]. From sequential Monte Carlo/DFT calculations, the dipole moment and the hydration enthalpy of the OH° radical in water were respectively around 2.2D and −39.1 kJ/mol [27]. Thus, the effect of chemical modifications with OH° on the increased of V_m value is assumed due to the fact that OH° radical

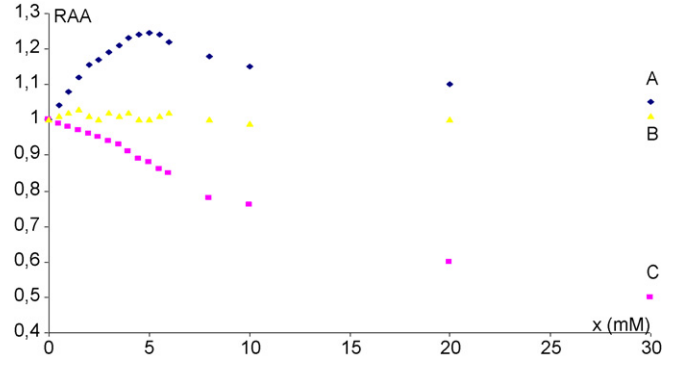

Fig. 5. Relative arginase activity (RAA) (i.e., arginase activity relative to the value at $x = 0$) vs. x (mM): A corresponding to OH°; B corresponding to Fe^{2+}; C corresponding to H_2O_2. Mobile phase A: 0.1 mM Tris–HCl buffer pH = 7.4–10 mM $MnCl_2$–Fenton reagent ($FeSO_4$ (x mM) + H_2O_2 (x mM))/flow-rate: 0.5 mL/min. Mobile phase B: phosphate buffer (pH = 7.00, 50 mM)–methanol (50/50) (v/v)/flow-rate: 3 mL/min. Detection wavelength: 372 nm. IMER temperature: 298 K.

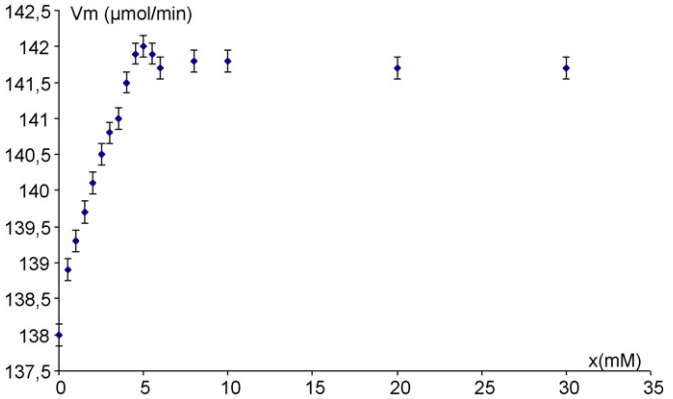

Fig. 7. V_m (µmol/L min) vs. x (mM). Mobile phase A: 0.1 mM Tris–HCl buffer pH = 7.4–10 mM $MnCl_2$–Fenton reagent ($FeSO_4$ (x mM) + H_2O_2 (x mM))/flow-rate: 0.5 mL/min. Mobile phase B: phosphate buffer (pH = 7.00, 50 mM)–methanol (50/50) (v/v)/flow-rate: 3 mL/min. Detection wavelength: 372 nm. IMER temperature: 298 K.

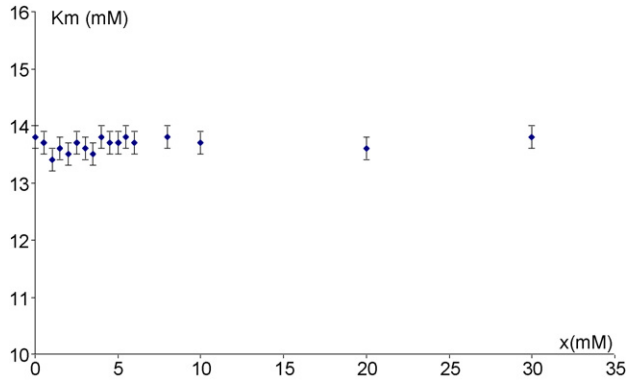

Fig. 8. K_m (mM) vs. x (mM). Mobile phase A: 0.1 mM Tris–HCl buffer pH = 7.4–10 mM $MnCl_2$–Fenton reagent ($FeSO_4$ (x mM) + H_2O_2 (x mM))/flow-rate: 0.5 mL/min. Mobile phase B: phosphate buffer (pH = 7.00, 50 mM)–methanol (50/50) (v/v)/flow-rate: 3 mL/min. Detection wavelength: 372 nm. IMER temperature: 298 K.

Table 2
$\Delta\Delta G_{ES}$ (J/mol) vs. x (mM). Mobile phase A: 0.1 mM Tris–HCl buffer pH = 7.4–10 mM $MnCl_2$–Fenton reagent ($FeSO_4$ (x mM) + H_2O_2 (x mM))/flow-rate: 0.5 mL/min. Mobile phase B: phosphate buffer (pH = 7.00, 50 mM)–methanol (50/50) (v/v)/flow-rate: 3 mL/min. Detection wavelength: 372 nm. IMER temperature: 298 K.

x (mM)	$\Delta\Delta G_{ES}$ (J/mol)
0.5	−16.1 (1.2)
1	−23.1 (2.1)
1.5	−30.3 (2.3)
2	−37.4 (1.2)
2.5	−44.5 (1.7)
3	−49.8 (1.7)
3.5	−53.3 (2.1)
4	−62.0 (1.1)
4.5	−69.5 (1.2)
5	−70.8 (1.3)
5.5	−69.6 (1.6)
6	−65.6 (1.2)
8	−67.3 (1.4)
10	−67.3 (1.2)
20	−65.6 (1.4)
30	−65.6 (1.3)

Standard deviations were in parentheses.

is likely to interact with the surface of arginase active site through dipole–dipole interactions and can therefore affect proximity and arginase active site orientation [28,29]. A variation in the micropolarity of the environment surrounding some side chains of certain active site residues of the arginase enzyme due to the strong role played by OH° radical as a proton donor in water [27] is a possibility of explanation for this activation. As well, for $x \leq 5$ mM, the higher the OH° radical production was, the lower $\Delta\Delta G_{ES}$ value obtained, this indicated that the enzyme was more flexible in the presence of OH° radical and it tends to be more easily activated (Table 2). The fact that low concentrations of OH° radical enhanced arginase activity and yet have no effects at higher concentration can be explained by the fact that for $x > 5$ mM the enzyme flexibility is reduced (Table 2).

4. Conclusion

For the first time, the direct implication of pressure and OH° radical formation on the arginase activity was clearly visualized. Both OH° radical production and pressure increased the arginase activity. An activation kinetic by OH° radicals without a significant influence on the rate of substrate binding was indicated. As well, our results demonstrated the great interest to synthesize new arginase inhibitors with radical scavenger characteristics for the treatment of several NO-dependent smooth muscle disorders.

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