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Cysteine–iron promotes arginase activity by driving the Fenton reaction

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ABSTRACT

Impairment of nitric oxide bioavailability secondary to increased arginase activity and overproduction of reactive oxygen species (ROS) is thought to be a major cause of vascular complications in sickle cell disease (SCD). However, the role of ROS in the induction of arginase activity is unknown. This study investigated whether the mechanism of arginase activation involves the ROS produced during oxidative stress. Our study reveals that cysteine–iron dose-dependently stimulated arginase activity with a corresponding increase in ·OH radical formation. The ·OH radicals produced were significantly inhibited by salicylic acid derivatives and superoxide dismutase. Surprisingly, the inhibition of ·OH radicals parallels the inhibition of arginase activity, thus suggesting the role of cysteine–iron in the stimulation of arginase via the Fenton reaction. This is the first evidence demonstrating the participation of ·OH radicals in the stimulation of arginase activity, and thus provides novel avenues for therapeutic modalities in hemoglobinopathies and other inflammation-mediated diseases.

In eukaryotic cells, molecular oxygen primarily functions as the terminal acceptor of electrons during mitochondrial electron transport. Most of the oxygen consumed in this process is reduced to water by cytochrome oxidase. Normally, only a small percentage of the total oxygen consumption appears as partially reduced species such as the superoxide ion and hydrogen peroxide. However, in an increasing number of pathological situations such as sickle cell disease (SCD), partially reduced and thereby activated oxygen species are present in greater concentrations and can mediate, in turn, significant cellular injury.

One of the pathologic hallmarks of SCD is vaso-occlusion (VOC), and the etiology is probably multifactorial. However, it is clear that during local vaso-occlusion, areas of ischemia/reperfusion develop that leads to increased production of oxidizing molecules such as $O_2^{-\cdot}$, H_2O_2, ·OH radicals, and $ONOO^-$ [1]. The oxygen-related species (ROS) produced can directly injure the endothelium by peroxidation of the lipid membrane, potentially leading to cellular damage [2,3]. Increased iron absorption associated with intensive erythropoiesis and iron deposition as a result of continuous hemolysis also contribute to cellular injuries in SCD [4]. Osarogiagbon et al. demonstrated that transgenic sickle cell mice had higher baseline levels of oxidative stress markers, such as ethane excretion and ·OH radical generation, than did their normal counterparts [5]. During exposure to hypoxia, these sickle cell mice exhibited evidence of ischemia/reperfusion injury, which is characterized by increased oxygen radical formation and leukocyte adherence and emigration [5,6].

There is an increasing body of evidence indicating that overproduction of ROS associated with SCD reduces the concentration of glutathione, a principal cellular antioxidant. Glutathione depletion in sickle erythrocytes increases their conversion to irreversibly sickle cells that may promote vaso-occlusion [7]. It has however been shown that plasma concentrations of cysteine and other glutathione substrates are elevated in patients with SCD [8]. An increasing number of studies suggest that patients with SCD are subjected to increased intravascular hemolysis and erythrocyte membrane senescence secondary in part to VOC and oxidant damage, respectively. The resultant increased erythrocyte spillage, ROS, and arginase activity in turn scavenge endothelium-derived NO secondary to decreased arginine availability [9,10]. The reduced NO production may then lead to severe pathophysiological derangements and organ failure [11,12]. Furthermore, the etiology of increased arginase activity in patients with SCD is thought to be associated with increased vascular hemolysis [13]. But the role of oxidative stress, induced by cysteine–iron, that leads to the stimulation of arginase activity with the participation of ·OH radicals is unknown.

We have been studying the mechanisms of arginase modulation by antisickling drugs in *in vivo* and in culture systems [14,15]. These studies have been extended in the present report to a consideration of the role of cysteine alone or in combination with molecular iron in the stimulation of arginase activity. The present data documented a requirement for ·OH radicals formed by the

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Fenton reaction and further shed light on the mechanism of arginase stimulation during oxidative stress.

Materials and methods

Reagents

Unless otherwise specified, all reagents and PEG-SOD as well as PEG-Catalase were obtained from Sigma–Aldrich Corp. (St. Louis, MO, USA). Purified Bovine arginase was purchased from AXXORA, LLC, (San Diego, CA, USA).

Patients/blood samples

Preparation of arginase enzyme and erythrocyte hemolysate. Blood samples were collected in heparinized tubes from pediatrics patients with homozygous sickle cell (Hb S) disease. To be eligible for blood donation for this study, SCD patients must have had no blood transfusions for at least 6 months before the initiation of the study. All procedures involving the use of human subjects were approved by the University of Missouri Pediatric Institutional Review Board, and informed consent was obtained from all subjects prior to blood collection. Within 24 h of venipuncture, RBC portion was separated from whole blood in several steps as outlined below. First, whole blood was washed three times with 0.15 M NaCl. RBCs were filtered over cellulose to remove granulocytes [16]. Following this procedure, microscopic injection and monitoring by hemocytometer confirmed the absence of leukocytes (WBC) and platelets. One volume of washed RBCs was added to two volumes of hemolysing solution [15] and the hemolysate was then immediately stored at −80 °C until ready for use. Purified arginase-1 from bovine liver (AXXORA, LLC, (San Diego, CA, USA) was also employed for the study. One unit of enzyme activity was defined as the amount of the enzyme that produces 1 μmol of ornithine/min at 37 °C. Specific activity was expressed in enzyme units per mg of protein. Protein concentration was measured at A280 using bovine serum albumin as a standard. To ensure lack of interference of other Hb variants, Hb S levels were determined using 50 μL of heparinized blood by high-performance liquid chromatography (HPLC).

Determination of hematological parameters

The concentrations of hemoglobin in 50 μL of heparinised blood were measured by high-performance liquid chromatography. Also, aliquots (10 μL) of whole blood were collected and the complete blood cell count was determined with a HEMAVET counter (CDC Technologies Inc., Oxford, CT, USA).

Measurement of arginase activity

The enzyme activity was determined in the absence or presence of cysteine alone or in combination with iron (II) sulfate. All reactions were run in 96-well microplates. The level of arginase activity was determined using a modified spectrophotometric assay that was originally developed by Chinard [17,18]. Briefly, 16.67 μL of purified enzyme or erythrocyte hemolysate was added to a reaction buffer (0.1 mM Tris–HCl buffer) at pH 7.4 containing 10 mM $MnCl_2$. Arginine was added to the sample to initiate the reaction, followed by incubation at 37 °C in a shaking water bath for 1 h. The reaction was stopped by the addition of 166.67 μL of 0.72 M HCl. Further, 0.67 ml of 6% ninhydrin dissolved in 2-methoxyethanol was added and the sample was boiled at 100 °C for 25 min. Thereafter, the sample reaction mixture was allowed to cool to room temperature and the absorbance was measured at 505 nm using the SpectraMax® spectrophotometer (Molecular Device, Sunnyvale, CA 94089, USA). The level of arginase activity was expressed as μmol of ornithine produced per min. Inhibition was monitored by measuring rates of ornithine produced in the presence of radical scavengers at defined concentrations.

Measurement of hydroxyl radical formation

To investigate whether a combination of cysteine–iron has an effect on ·OH radical formation, we determined its levels in a cell free system measured by the deoxyribose degradation method as described previously [19]. Briefly, 10 μL sample in the presence or absence of 2 mg/ml cysteine–10 μM iron (II) sulfate was mixed with 10 μL of 300 mM 2-deoxy-D-ribose and made up to 1 ml of total volume with 10 mM phosphate buffer (pH 7.4). The solution was incubated at 37 °C for 60 min. After incubation, the reagents were mixed with 1 ml of 1% (w/v) solution of thiobarbituric acid in 50 mM NaOH and 1 ml of 2.8% (w/v) aqueous trichloroacetic acid, heated at 100 °C for 15 min, cooled, and then read at 532 nm. Using predetermined concentrations, the effects of various radical scavengers on the release of ·OH radicals were also determined.

Statistical analyses

Data are expressed as means ± standard error of the mean (SEM). The Student's *t*-test was used for comparison of the arginase activities or rate of ·OH radical production between studied pairs. For multiple comparisons, the significance of differences was evaluated by the modified *t*-test according to Dunnett by use of the computer program, Graphpad Prism Instat (GraphPad Software, San Diego, CA, USA). The level of significance was set at $p < 0.05$.

Results

Cysteine–iron exerts stimulatory effect on erythrocyte arginase activity

To explore the effect of cysteine on arginase activity, we examined the effect of increasing concentrations of cysteine alone or in combination with iron (II) sulfate on the enhancement of arginase activity in human sickle erythrocytes or purified arginase-1 from bovine liver. With an increasing concentration of cysteine in the presence of 10 μM iron (II) sulfate, arginase activity increased in a dose-dependent manner (Fig. 1A and B). However, the treatment of arginase with iron alone did not stimulate arginase activity (results not shown). Having established the cysteine–iron-mediated stimulation of arginase activity, we proceeded to determine the role of the highly potent ·OH radical in the stimulation of arginase activity. We treated cell hemolysate or purified arginase-1 with predetermined concentrations of 3-nitro salicylic acid (3-NSA), 5-nitro salicylic acid (5-NSA), sulfasalazine (SAZ), or 5-aminosalicylic acid (5-ASA), since salicylic acid and its derivatives are known specific ·OH radical scavengers [20]. Our results indicate that salicylic acid derivatives potently inhibited arginase activity. Maximal inhibitory effect of SAZ on purified arginase activity was observed at a concentration of 100 μM (Fig. 2A and B). Overall there was more significant inhibition of arginase activity in purified arginase-1 than in erythrocyte hemolysate, possibly due to the presence of increased extraneous macromolecular interactions in cell hemolysate than in purified enzyme.

Incubation of the hemolysate or purified arginase-1 with a highly stable PEG-SOD significantly inhibited the arginase activity induced by cysteine–iron, indicating that oxygen species might be responsible in part for the observed increase in arginase activity. It is interesting to know that upon pretreatment of arginase-1 with

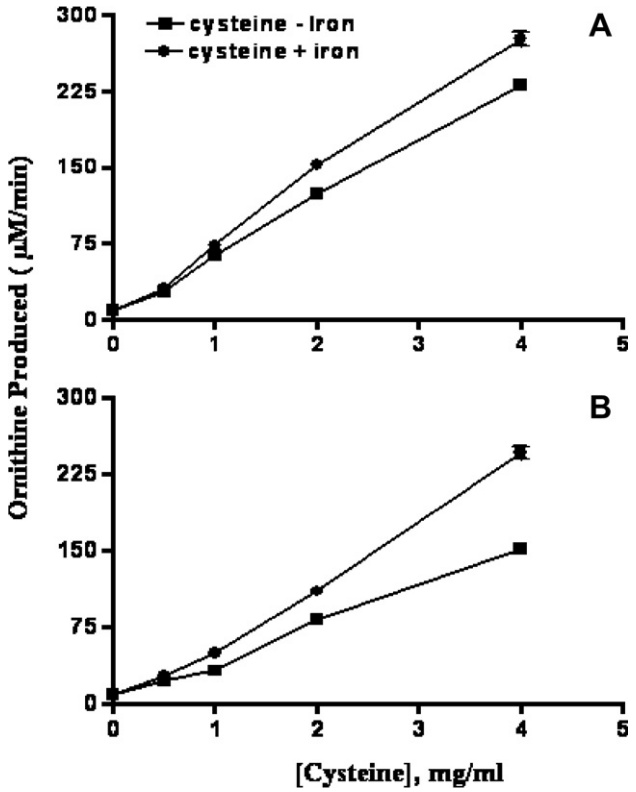

Fig. 1. Effect of increasing concentrations of cysteine in the absence or presence of 10 μM Iron (II) sulfate on arginase activity at pH 7.4, 37 °C. (A) Arginase activity in erythrocyte hemolysate (closed circle, plus iron and close square, minus iron). (B) Purified arginase-1 activity from bovine liver. The enzyme activity was monitored with 1.0 mM arginine substrate under standard assay conditions as described in Materials and methods. Results are expressed as micromolar ornithine produced per unit time. Each point represents the average ± SEM of triplicate determinations of two independent experiments.

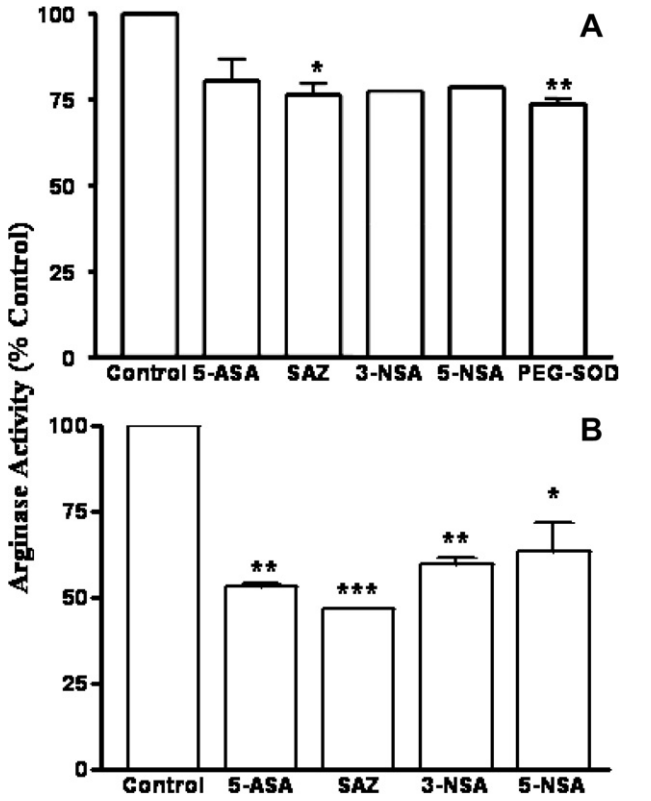

Fig. 2. Effect of various agents on arginase activity. The reaction was initiated by addition of 1.0 mM arginine as substrate. Arginase activity was stimulated with 2 mg/ml cysteine and 10 μM iron (II) sulfate, followed by addition of 100 μM 5-ASA, 100 μM SAZ, 500 μM 3-NSA, 500 μM 5-NSA or 100 μM PEG-SOD. The reaction mixture was then incubated for 20 min at 37 °C. (A) Erythrocyte arginase activity, (B) Purified arginase-1 activity. Results are expressed in percent arginase activity vs control. Values are means ± SEM of triplicate determinations of two independent experiments. $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$ compared with control.

10 μM PEG-SOD, the inhibition of arginase activity in comparison with the cysteine–iron treated control was non-significant. We then reasoned that the possible partial accumulation of hydrogen peroxide generated as well as inadequate consumption of the available superoxide by the low concentration of PEG-SOD might be responsible. Against this background, we combined PEG-SOD with a highly stable PEG-catalase. Surprisingly, a further decrease of arginase activity was observed in comparison with the PEG-SOD-treated samples alone (Fig. 3). The further decrease in arginase activity is possibly due to the fact that PEG-catalase neutralizes the available H_2O_2 to produce water and oxygen, thus supporting the involvement of the Fenton reaction.

Highly potent hydroxyl radical participates in the stimulation of arginase activity

In view of the elevated iron levels in sickle erythrocytes along with the corresponding increased formation of ·OH radicals possibly due to the Fenton reaction [9], we sought to determine whether increased ·OH radical formation stimulates arginase activity. The formation of ·OH radicals in the presence of cysteine–iron was measured by the deoxyribose degradation assay. As shown in Fig. 4, the formation of ·OH radicals in a cell free system induced by cysteine–iron was inhibited by salicylic acid derivatives 3-NSA and 5-NSA, as well as SAZ and its intermediate, 5-ASA. Furthermore, ·OH radical formation was also inhibited by PEG-SOD, thus indicating that the ·OH radical production induced by cysteine in the presence of iron was via the Fenton reaction. The presence of

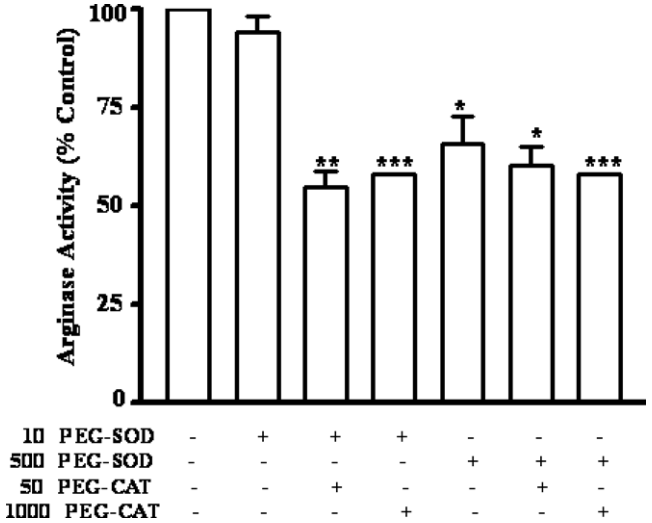

Fig. 3. Effect of PEG-SOD alone or in combination with PEG-catalase on purified arginase-1 activity. The reaction was initiated by addition of 1.0 mM arginine. Arginase-1 activity was stimulated with 4 mg/ml cysteine and 10 μM iron (II) sulfate, followed by addition of various concentrations of PEG-SOD alone or in the presence of 50 U/ml or 1000 U/ml. PEG-catalase. The reaction mixture was then incubated for 1 h at 37 °C. Results are expressed in percent arginase activity vs control. Values are means ± SEM of triplicate determinations of two independent experiments. $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$ compared with control.

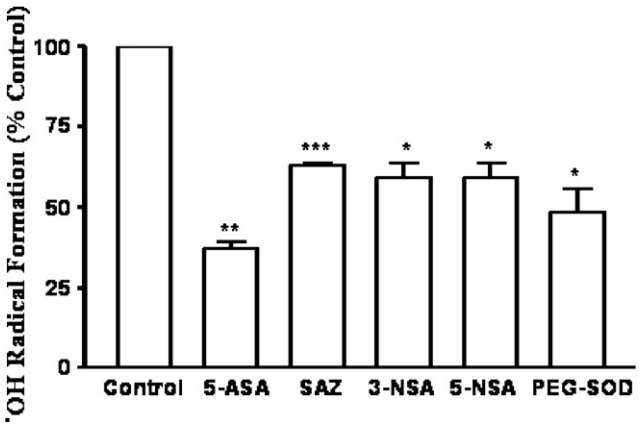

Fig. 4. Effect of various agents on hydroxyl radical formation as measured by deoxyribose degradation. The reaction was initiated by addition of 2 mg/ml cysteine and 10 μM iron (II) sulfate, in the presence of 3 mM deoxyribose(control), followed by addition of 100 μM 5-ASA, 100 μM SAZ, 500 μM 3-NSA, 500 μM 5-NSA or 100 μM PEG-SOD. Results are expressed in percent hydroxyl radical formation vs control. Values are means ± SEM of duplicate determinations. $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$ compared with control.

PEG-catalase in the cell free system also inhibited ·OH radicals formation (results not shown).

Discussion

The data presented in this study demonstrate that cysteine in the presence of molecular iron stimulates arginase activity in sickle erythrocytes and purified arginase-1 from bovine liver via induction of ROS, and that this enhancement of arginase activity was reduced in the presence of radical scavengers. Increased erythrocyte arginase activity has been implicated in several pathological conditions in SCD and other inflammatory disorders [21]. Indeed, recent evidence suggests that a dysregulation of the nitric oxide (NO) synthase–arginase pathway may contribute to the pathology of NO-disorders such as pulmonary hypertension [13]. It has been suggested that arginase may contribute to the induction of inflammation and leucocytosis that constitute a striking pathological hallmark of SCD and the sequelae that occur with SCD [22]. There is substantial evidence that the expression of arginase is elevated by proinflammatory cytokines [23]. We recently demonstrated that chloroquine, an immunomodulating drug, inhibits arginase activity in sickle erythrocytes via a competitive mechanism [15]. However, information on the stimulation of arginase activity via cysteine–iron-induced oxidative stress with the participation of ·OH radicals is scarce.

Our data have shown that cysteine alone or in the presence of iron (II) sulfate stimulates arginase activity in sickle erythrocytes and purified arginase-1 due to ·OH radical formation. Inhibition of arginase was equally effective whether the reaction mixture was first pretreated with radical scavengers and subsequently exposed to cysteine–iron or simply treated with radical scavengers at the time of exposure to cysteine–iron. However, the treatment with iron (II) sulfate alone did not have any effect on arginase activity, demonstrating the role of cysteine in iron oxido-reduction cycling that eventually involves the Fenton reaction as shown in the scheme below:

$$Cys^{red} + Fe^{3+} \rightarrow Cys^{ox} + Fe^{2+} \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^{-} \text{ (the Fenton reaction)} \quad (2)$$

It is known that in SCD, reduced glutathione leads to a transient increase in cysteine to meet the demand for glutathione production [8]. It is plausible that available free iron then catalyzes electron transfer from excess cysteine to H_2O_2, generating ·OH radicals. The requirement for ·OH radicals in the stimulation of arginase activity was demonstrated by the ability of the specific ·OH radical scavengers (3-NSA, 5-NSA, 5-ASA, SAZ) to inhibit the activity of arginase, which paralleled the reduction of ·OH radical levels in the reaction medium. Other radical scavengers, including PEG-SOD, also inhibited arginase activity, possibly implicating other ROS. It is interesting to know that the combination of PEG-SOD with PEG-Catalase further inhibited arginase-1 activity in comparison with PEG-SOD-treated samples alone. This observation could be due in part to PEG-SOD- induced generation of H_2O_2 from its action on superoxide generated by reduced cysteine and ferric iron (see Eq. (1) above). The available H_2O_2 subsequently reacts with ferrous iron to produced more ·OH radicals as shown in Eq. (2) above. By adding PEG-Catalase, this enzyme eventually utilizes the residual H_2O_2 as substrate to produce molecular oxygen and water. This observation further supports the involvement of the Fenton reaction.

On the stimulation of arginase activity, we initially reasoned that specific stimulators of arginase, such as cytokines [23] probably present in the erythrocyte hemolysate, might be responsible. However, the demonstration in this study of ROS-induced activation of purified arginase-1 activity allowed us to hypothesized that ·OH radicals are in part directly responsible for arginase activation. Under these experimental conditions, purified arginase-1 represented the only macromolecular target present in the incubation mixture, and no possible interferences by eventual low molecular weight modulators might have occurred.

Although further studies will be necessary for elucidating the mechanism of ROS-mediated activation of arginase activity, as well as the specific amino acid residues implicated and the structural changes driving this activation, we have demonstrated, for the first time, the direct interaction of ROS with an enzyme involved in the regulation of nitric oxide homeostasis and inflammation. The mechanism of cysteine-mediated direct stimulation of arginase via the Fenton reaction, as shown in this study, may

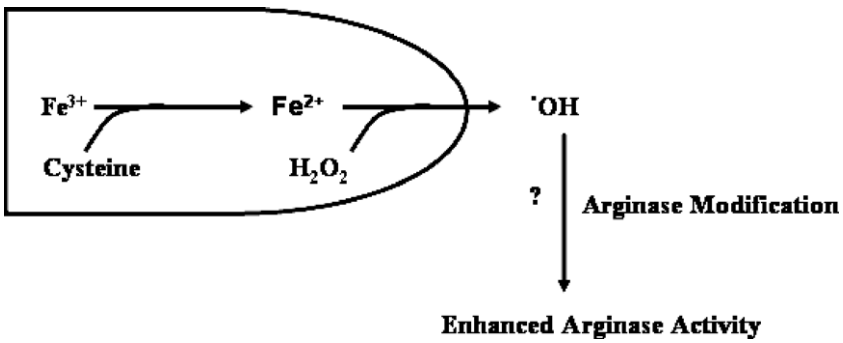

Fig. 5. Proposed mechanism of cysteine-mediated stimulation of arginase activity.

involve the thiol (SH) modification of the enzyme, as documented in the erythrocyte enzyme AMP-deaminase and other proteins [24,25]. Fig. 5 outlines the proposed sequence of event. In this study, we have not excluded the possibility that other reactive oxygen species may also play a role in the stimulation of arginase activity.

The importance of the results reported in the present study lies in the key role played by arginase in regulating nitric oxide homeostasis, especially in the pathologic state. As has been reviewed elsewhere, a state of nitric oxide resistance leads to SCD vasculopathy [11]. From the pathophysiological point of view, this finding implies that ROS activation via oxidative stress might represent one of the main negative events leading to profound activation of arginase in SCD, physiological (aging) and other pathological conditions.

In conclusion, we believe that our results represent a new and important steps towards understanding the role of ROS-induced activation of arginase activity, with specific reference to the participation of the highly potent ·OH radical. Incorporating this template into structure-based enzyme design studies could lead to the production of new forms of efficient arginase inhibitors, which could be useful as therapeutic regimen in hemoglobinopathies and other related inflammation-mediated diseases.

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