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# Nitric Oxide – an Activating Factor of Adenosine Deaminase 2 *in vitro*

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**Abstract**—In this study we have investigated the effect of reactive oxygen species produced by some chemicals in aqueous solutions on activity of adenosine deaminase 2 (ADA2) purified from human blood plasma. An activating effect on ADA2 was observed *in vitro* with sodium nitroprusside (SNP), the source of NO (nitrosonium ions $NO^-$ in aqueous solutions). Not SH-groups of cysteine but other amino acid residues sensitive to NO were responsible for ADA2 activation. The SNP-derived activation was more pronounced when purified ADA2 was preincubated with heparin and different proteins as an experimental model of the protein environment *in vivo*. The most effective was heparin, which is known for its ability to regulate enzyme and protein functions in extracellular matrix. We conclude that ADA2 is a protein with flexible conformation that is affected by the protein environment, and it changes its activity under oxidative (nitrosative) stress.

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*Key words*: adenosine deaminase 2, enzyme activation, NO, conformation, allostery, heparin

Adenosine deaminase (ADA, EC 3.5.4.4) is a key enzyme of purine metabolism that regulates the level of adenosine and deoxyadenosine in animal and human tissues, converting them into inosine and deoxyinosine. There are two types of adenosine deaminase: ADA1 and ADA2 [1]. Both enzymes are present in the human organism. In addition, a gene of another enzyme, ADAL (ADA-like, or ADA3), with unknown function has been reported [2]. The main function of ADA1 is elimination of toxic derivates of adenosine and cell protection from apoptosis. Deficiency in ADA1 leads to severe combined immunodeficiency (SCID) [3]. Unlike well-studied ADA1, ADA2 is less studied because of the low level of this enzyme in the organism. Normally ADA2 is observed in the human organism mainly in blood plasma. Its Michaelis constant $K_m$ is ~2 mM, which significantly exceeds adenosine concentration in plasma (<150 nM) [4]. This suggests that the isoenzyme activity under physiological conditions is very low. Recently, it has been shown that ADA2 (product of gene *CECR1* – cat eye syndrome critical region 1 gene) belongs to new family of ADGF (adenosine deaminase growth factors) [5, 6]. The crystal structure of human ADA2 and its complex with an analog of an intermediate state was determined, and it was shown that the ADA2 molecule is a symmetric homodimer containing two domains typical for family ADGF/ADA2 and necessary for enzyme dimerization and its binding to cell surface receptors. Since ADA2 is a molecule rich in glycosyls and has a disulfide bond and signal peptide in its structure, it can be classified as an enzyme functioning in the extracellular space [7, 8]. Unlike ADGF from lower organisms, whose activity is comparable with activity of ADA1, the catalytic ability of human ADA2 is lower ($K_m$ is 100 times higher) and its pH optimum lies in the slightly acidic region at pH 6.0 (for ADA1 at pH 7.4) [5, 9, 10]. This suggests that ADA2 is active under conditions typical for pathological conditions: hypoxia, inflammation, etc. Activity of ADA2 increases considerably during several severe infectious diseases [11-13], which indicates that the enzyme participates in immune response to activity of various pathogens.

Determining which cells produce the enzyme was difficult because of the low concentration of ADA2. It was earlier assumed that it is produced by monocytes/macrophages [14, 15], and only recent advances in ADA2

*Abbreviations*: ADA2, adenosine deaminase 2; ADGF, adenosine deaminase growth factor; GAG, glycosaminoglycans; NEM, N-ethylmaleimide; PG, proteoglycan; ROS, reactive oxygen species; SNP, sodium nitroprusside.

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gene sequencing allowed obtaining its recombinant form and studying its physiological role. It was shown that ADA2 is important for monocyte differentiation and stimulation of macrophage proliferation. Probably, to fulfill this function ADA activity is not essential, but rather ADA2 acts as a growth factor [5, 7, 10]. Studies of causes and mechanisms underlying increase in ADA2 activity in pathology are only beginning.

Macrophages play a significant role in functioning of innate immunity and during inflammation. Activated by pathogens and proinflammatory cytokines, they produce a large amount of reactive oxygen species (ROS) – e.g. NO, superoxide ions, and hydrogen peroxide – that have strong cytotoxicity toward microorganisms and other cells including macrophages. Abundant or uncontrolled production of NO results in formation of reactive nitrogen species – nitrosyl-metals, S-nitrosothiols, $N_2O_3$, peroxynitrite ($ONOO^-$) – that lead to NO-dependent (nitrosative) stress. NO can also have cytoprotective function in low nontoxic concentrations [14, 16]. Oxidation-reduction mechanisms in signal functioning are now considered in cell biology as fundamental regulatory mechanisms. Many cell proteins (transcription factors, receptors, and enzymes) are sensitive to ROS. Oxygen radicals can affect enzyme activity and interact with SH-groups of proteins forming disulfide bonds or dimers. This mechanism has been intensively studied for many enzymes and proteins regulated by NO [17-20].

Since ADA2 is an enzyme active under pathological conditions, it is logical to suppose that it can be resistant to ROS and/or can interact with it. The goal of this work was to study the influence of ROS produced by several chemical compounds in aqueous solutions on the activity of ADA2 purified from human blood plasma.

## MATERIALS AND METHODS

**Materials.** Donor blood plasma from the R. O. Eolyan Hematology Center was used in this study. Sephadexes DEAE A-50, CM, SP C-50, G-200 superfine, and G-25 superfine (Pharmacia, Sweden), protein G-Sepharose (Hi Trap protein G-Sepharose; GE Healthcare, USA), adenosine, BSA, N-ethylmaleimide, and glutathione (Sigma-Aldrich Ltd, USA), sodium nitroprusside (Poland), and heparin sodium salt (Belmedpreparaty, Belarus) were used in the study. Proteoglycan was collected as described earlier [21]. Other chemicals were of high purity degree.

**Collection and purification of ADA2 from donor blood plasma.** For ADA2 isolation and purification the approach described earlier [22] was used supplemented with chromatography on SP-Sephadex C-50 (in 0.02 M K-phosphate buffer, pH 6.0). The supplementary stage provided 10-fold increase in purification degree of the protein. The final sample was purified approximately 5600-fold with 10% yield and specific activity equal to 500 nmol $NH_3$/min per mg.

Before using ADA2 in experiments with ROS, it was additionally purified from accompanied immunoglobulin G by passing the sample through an affinity column with protein G-Sepharose pre-equilibrated with 0.02 M phosphate buffer, pH 7.0, containing 0.1 M NaCl. After concentration, fractions were analyzed by SDS-PAGE in a 7.5% polyacrylamide gel [23]. However, these stages of purification freeing the enzyme from most of the IgG resulted in loss of enzyme activity by approximately 50%. Protein samples with specific activity ~250 nmol $NH_3$/min per mg were used in experiments.

**Determination of ADA2 activity and kinetic parameters.** ADA activity was determined as described in study [24] according to amount (nmol) of formed ammonia per mg protein during 1 min. To determine the catalytic parameters, 10-20 μl of ADA2 sample (1 mg/ml) was incubated with 0.1-10 mM adenosine in 0.5 ml 0.04 M K-phosphate buffer, pH 6.0. The values of $V_{max}$ and $K_m$ were estimated in Lineweaver–Burk coordinates. Protein was measured by Bradford's technique [25] using BSA as a standard.

**Influence of radicals on ADA2 activity.** To estimate impact of sodium nitroprusside (SNP) and potassium ferricyanide on ADA2 activity, aliquots of purified enzyme (20 μg in 50 μl of 0.04 M K-phosphate buffer, pH 6.0) were incubated with different concentrations of reagents (0.1-10 mM) at 37°C for 10 min. Then 10 μl from each sample was collected to determine enzyme activity. To study participation of sulfhydryl groups from cysteine residues in enzyme activation, ADA2 aliquots (20 μg in 50 μl of 0.04 M K-phosphate buffer, pH 6.0) were incubated with N-ethylmaleimide (NEM) (0.1-10 mM), which was used as an SH-blocking substance, or with glutathione as a reducing agent of thiol groups at 37°C for 60 min. Then 5-10 μl of the sample was collected to determine activity. ADA2 (0.2 mg in 200 μl of 0.04 M K-phosphate buffer, pH 6.0) preliminarily treated with NEM or glutathione (final concentration 1 mM) was passed through a column with Sephadex G-25 superfine (0.5 × 12 cm) to remove excess of the SH-reagent, then 10 μg aliquots were incubated with SNP as described earlier and 10 μl from each sample was collected to determine enzymatic activity. To determine the influence of heparin, proteoglycan, or BSA on ADA2 activity, 100 μg of the enzyme was preincubated in 0.04 M K-phosphate buffer, pH 6.0, with heparin, proteoglycan, and BSA (final concentration 0.02 mM) at 37°C for 20 min. Then the sample was incubated with SNP (0.1-10 mM) in the same buffer at 37°C for 10 min, and 10 μl of each sample was collected to determine enzymatic activity.

Spectra were measured using Specord M-40 and Specoll-211 spectrophotometers (Germany).

Results were analyzed using the Enzyme Kinetics and Non-Linear Fit matrices of GraFit program, v.5.0.3 for Windows (Erithacus Software, USA) [26].

Statistical analysis was conducted using InStat v.3 for Windows (GraphPad Software, Inc., USA), specific differences being estimated in Student's two-tailed *t*-test. Data are presented as average ± standard error.

## RESULTS

**Influence of ROS on adenosine deaminase activity.** Electrophoretic separation of an ADA2 sample in SDS-polyacrylamide gel is shown in Fig. 1. Except for the wanted enzyme, the sample also contains immunoglobulin G that accompanies ADA2 at all steps of purification as shown earlier [5]. The presence of additional IgG in the sample keeps ADA2 in active conformation, while the highly purified protein rapidly loses its activity. In experiments with ROS, when samples of ADA2 with high amounts of IgG were used, the activating effect was less evident and was from 15 to 25% of activity, while after additional purification on protein G-Sepharose the activation was 50-60%. For this reason we made further experiments using enzyme sample that passed this purification stage.

Sodium nitrite, hydrogen peroxide, peroxynitrite, $Fe^{2+}$ + ascorbate, and Fenton reagent ($Fe^{2+}$ + $H_2O_2$) used as ROS sources did not activate ADA2 (data not shown). Increase in enzymatic activity was observed only under the influence of SNP. The curve is bell-shaped with maximum at 1-1.5 mM SNP. Curves of ADA2 activity as functions of increasing SNP concentration (dose dependence) incubated with enzyme are presented in Fig. 2. Increase in ADA2 activity was observed. Since SNP is used as a source of NO (nitrosonium ions in aqueous solutions, $NO^-$), we supposed that they are responsible for change in activity. To prove this fact, the enzyme was also incubated with potassium ferricyanide, which differs from sodium nitroprusside by the absence of the NO-group. The relationship between ADA2 activity and potassium ferricyanide concentration is presented in Fig. 2a; change in activity was not observed.

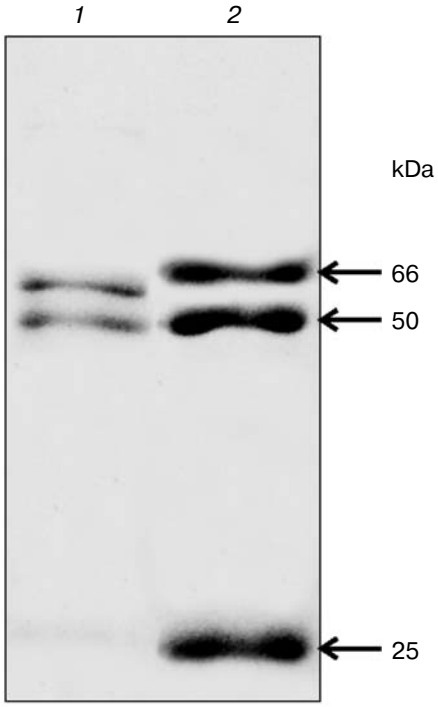


**Fig. 1.** Electrophoretic separation of an ADA2 sample that had been passed through a column with protein G-Sepharose (30 μg of protein was applied): *1*) upper band (~60 kDa) corresponding to ADA2; *2*) marker proteins: 66 kDa BSA (30 μg was applied); heavy (50 kDa) and light (25 kDa) rabbit IgG chains (50 μg was applied). Molecular weights of marker proteins are shown to the right.

Lineweaver–Burk curves for control (without additional substances) and for sample treated with 1.5 mM SNP (concentration when maximal effect was observed) are presented in Fig. 2b. After incubation with the reagent, $K_m$ decreased from 1.93 ± 0.62 to 0.53 ± 0.1 mM but changes of $V_{max}$ values (from 327.6 ± 68 to 268 ± 9.9 nmol $NH_3$/min per mg) were statistically unreliable ($p > 0.05$).

**Influence of SH-reagents on ADA2 activation.** Mechanisms described in the literature of the influence of oxygen radicals on proteins are predominantly connected with modification of thiol groups in the protein molecule: S-nitrosylation in the case of NO, which can lead to changes in structure and enzyme activity [27, 28]. There are four cysteine residues in the ADA2 monomer. Two of them forms disulfide bond essential for maintaining protein dimensional structure. The active center of the enzyme does not contain cysteine residues [5, 7]. To determine whether they participate in ADA2 activation under the influence of SNP, we incubated protein aliquots with SH-group modifiers: NEM, *p*-chloromercuribenzoate, iodoacetamide. The SH-blockers had no impact on ADA2 activity, and in contrast, their high concentrations rather inhibited enzyme activity (data not shown). Results of experiments using NEM and glutathione are presented in Fig. 2a. Preliminary incubation of the enzyme with 1 mM NEM did not prevent subsequent influence of SNP on its activity. Adding glutathione (1 mM) to the incubation medium also had no effect on enzyme activity and did not prevent activation of the enzyme by nitroprusside. These results suggest that cysteine residues do not participate in this reaction, and other amino acids sensitive to NO must be responsible for the change in ADA2 activity.

**Effect of heparin, proteoglycan, and BSA on ADA2 activation under the influence of SNP.** Previously it has been shown that ADA2 can specifically bind to heparin. This property was used for enzyme purification [5]. We tested the ability of ADA2 to be activated in the presence of heparin. When heparin was added to the medium, sig-

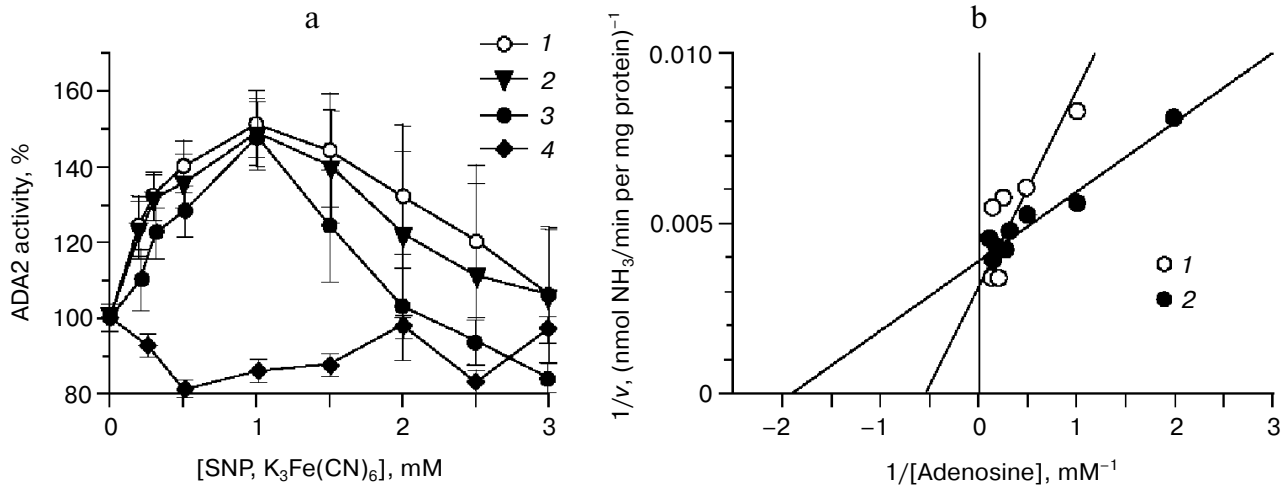


**Fig. 2.** Effect of SNP on ADA2 activity. a) Activity dependence on SNP concentration without additional substances (*1*) and after preliminary treatment with 1 mM glutathione (*2*) or 1 mM NEM (*3*) and on concentration of $K_3Fe(CN)_6$ (*4*). Control (100%) activity values are the result of enzyme incubation with buffer only. Here and in Figs. 3 and 4 each point is an average of three independent experiments. Differences between control and activity values under the influence of ferricyanide are unreliable ($p > 0.05$). b) Lineweaver–Burk relationships for control (*1*) and SNP-activated ADA2 (*2*).

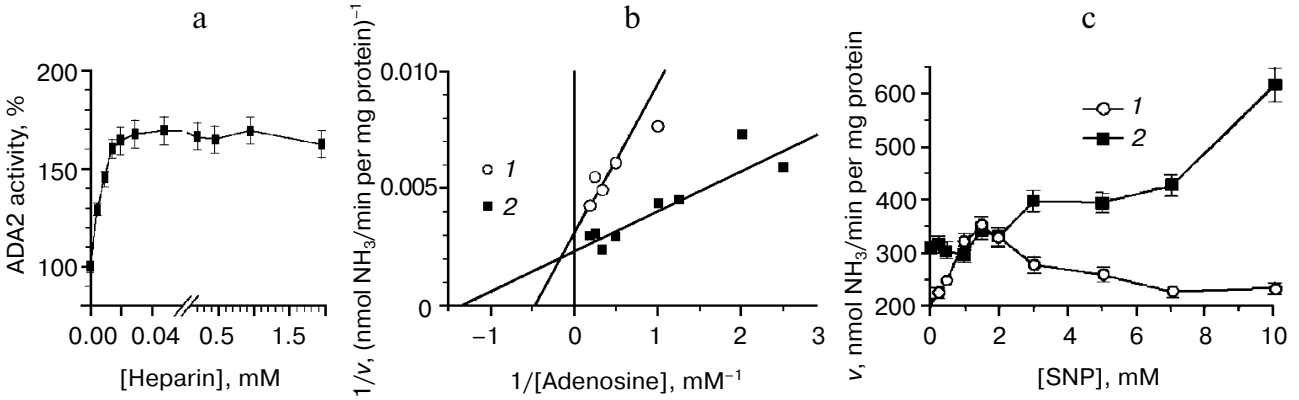


**Fig. 3.** Effect of heparin on ADA2 activity. a) Relationship between activity and heparin concentration. Control (100%) values of activity are represented by the result of enzyme incubation with buffer only. All ADA2 activity values under the influence of SNP significantly differed from control ($p < 0.005$). b) Lineweaver–Burk plots of the relationship between activity and substrate concentration for control (*1*) and previously incubated with heparin (*2*) enzyme. c) Relationship between activity and SNP concentration without heparin (*1*) and with 0.02 mM heparin (*2*) added into the incubation medium.

nificant and stable increase in ADA2 activity was observed (Fig. 3a). Plots of the relationship between activity and substrate concentration with and without heparin are presented in Lineweaver–Burk coordinates in Fig. 3b. In the presence of heparin $V_{max}$ increases from 355.14 ± 28.5 to 429.3 ± 28.6 nmol $NH_3$/min per mg, and $K_m$ decreases in comparison with control (from 1.98 ± 0.35 to 0.73 ± 0.19 mM).

To simulate the extracellular environment of the enzyme, BSA and proteoglycan (PG) were used. When they were added to the incubation medium the ADA2 activity increased significantly. The relationships between ADA2 activity and SNP concentration in the presence of BSA are presented in Fig. 4. An activating effect was observed in the presence of PG and BSA at concentrations comparable to heparin concentrations, but the relationship between ADA2 activity and heparin concentration had more stable effect. Reaching a maximum level, the enzyme activity did not change further, while in experiments with PG and BSA the ADA2 activity returned to the initial level and lower (in the case of BSA) after reaching a maximum (Fig. 4, a and b). However, plots of the relationship between ADA2 activity and SNP concentration continued to increase with increasing SNP concentration when heparin (Fig. 3c) or BSA (Fig. 4c) was added to the incubation medium.

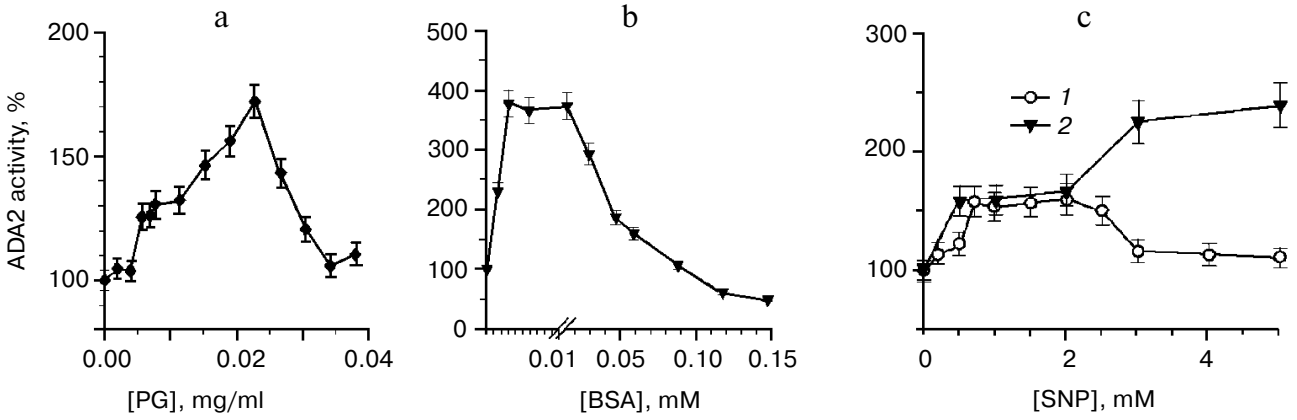


**Fig. 4.** Effect of PG and BSA on ADA2 activity. a, b) Relationships between ADA2 activity and PG and BSA concentrations, respectively. c) Relationship between ADA2 activity and SNP concentration without BSA (*1*) and after incubation with 0.02 mM BSA (*2*). Control (100%) values of activity were obtained when the enzyme was incubated without additional substances. All ADA2 activity values in the presence of PG and BSA significantly differed from control ($p < 0.0001$).

## DISCUSSION

Our results reveal a new aspect in the mechanism of ADA2 activity regulation. There are a few examples of enzyme activation under the influence of ROS that are mentioned in the literature [27, 28]. ROS sometimes have inhibiting effects on enzymes. ADA2 with detectable and even significant activity in certain body fluids in severe infectious diseases accompanied by inflammatory processes appeared to be sensitive to NO. This activation *in vitro* reached 60% of the initial activity of the purified enzyme. The activation is "non-essential" and reversed to the initial value with increasing reagent concentration (Fig. 2a).

Previously it has been shown that ADA2 is able to bind heparin and its analogs *in vitro*; the enzyme can be eluted from a heparin-Sepharose column by concentrated solution of sodium chloride (0.5 M), which indicates electrostatic nature of this binding [5, 7]. In our experiments incubation with heparin also resulted in significant increase in ADA2 activity (Fig. 3b).

There is increasing evidence appearing in the literature confirming the fact that components of extracellular matrix previously considered only as structural elements can possess signal function and directly regulate inflammatory processes [29-32]. Ability to bind a great number of different proteins, cytokines, and growth factors distinguishes heparin and heparan sulfates from other glycosaminoglycans (GAG). Proteoglycans play the same role in processes of intracellular chemical communications. They consist of protein and GAG chains bound to it. There are a few facts concerning mechanisms of regulation of enzyme activity. Cysteine proteinases are the most studied: heparin induces strong increase in the affinity of papain to its substrate [33], sulfated GAG intensify endopeptidase activity of cathepsin B [34], and they are natural allosteric modifiers of cathepsin K [35]. Collagenolytic activity of cathepsin K *in vitro* depends on its complex with chondroitin sulfate. Enzyme activity and stability increase at physiological blood pH [36], and cathepsin K appears in multiple functionally different conformational states. All sulfated GAG behave as non-essential activators. When chondroitin sulfate binds to one enzyme region, heparin has an additional binding region and has stabilizing effect on the enzyme [35].

Based on our results, it seems that ADA2 also is quite flexible, the extracellular environment of the enzyme seemingly stabilizing it *in vivo* in its active (or in one of its active) conformation(s). And under oxidative (nitrosative) stress ADA2 can display high activity on changing its conformation. Binding to heparin is notable for its stable activation, which does not weaken even at heparin high concentrations, while increase in BSA and proteoglycans concentrations results in loss of activating effect. However, both heparin and BSA added at concentrations maximally activating the enzyme led to further significant increase in ADA2 activity under NO stress *in vitro*.

Therefore, the presented results highlight a new aspect in the ADA2 regulation mechanism. It was shown for the first time that at high NO concentrations the enzyme has high activity. Not SH-groups of cysteine, but other sensitive to NO amino acid residues are responsible for this activity. In a specific environment *in vivo* and during pathologies this effect can by significantly intensified.

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