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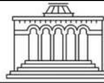
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THE INFLUENCE OF N^G-HYDROXY-L-ARGININE ON THE ACTIVITY OF THE UREA CYCLE ENZYMES

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Between the relationship of NOS (NO-synthase) and arginase in complicated and peculiar metabolic pathway of L-arginine there are many undiscovered points up till now. The aim of our study was to investigate the influence of N^G-hydroxy-L-arginine (NOHA) on the activity of urea cycle enzymes in vitro. Studies have shown that NOHA decreases the activity of all the urea cycle enzymes in liver, except ureotelic arginase, and increases the activity of argininosuccinate synthase and argininosuccinatelyase in brain and kidney. Our results show that the influence of NOHA on various organs in a different way is a new revelation in the pathway of L-arginine. We show that NOHA performs an important role in the metabolism of urea cycle metabolites (ornithine, citrulline and arginine) in different tissues.

Urea cycle – N^G-hydroxy-L-arginine – nitric oxide – arginase

L-արգինինի բարդ և յուրօրինակ մետաբոլիկ ուղում գործող արգինազ և NO-սինթազ ֆերմենտների փոխհարաբերություններում կան չբացահայտված կողմեր: Աշխատանքի նպատակն է in vitro պայմաններում ուսումնասիրել N^G-հիդրօքսի-L-արգինինի (ՀՕԼԱ) ազդեցությունը օրնիթինային ցիկլի ֆերմենտների ակտիվության վրա: Հետազոտությունները ցույց են տվել ՀՕԼԱ-ի արգելակող ազդեցությունը օրնիթինային ցիկլի բոլոր ֆերմենտների վրա լյարդում (բացառությամբ ուրեոթելիկ արգինազի), ակտիվացնող ազդեցությունը արգինինոսուկցինատսինթազ և արգինինոսուկցինատլիազ ֆերմենտների վրա երիկամներում և գլխուղեղում: ՀՕԼԱ-ի ազդեցության տարբերության հայտնաբերումը տարբեր օրգաններում նոր բացահայտում է L-արգինինի նյութափոխանակությունում: Ստացված արդյունքները ցույց են տալիս տարբեր հյուսվածքներում ՀՕԼԱ-ի կարևորագույն դերը միզանյութի սինթեզի մետաբոլիտների (օրնիթին, ցիտրուլին, արգինին) նյութափոխանակությունում:

Միզանյութի սինթեզ – N^G-հիդրօքսի-L-արգինին – ազոտի մոնօքսիդ – արգինազ

В сложном и своеобразном метаболическом пути L-аргинина между отношениями NOS и аргиназы до сих пор есть много неисследованных вопросов. Нашей целью было изучение влияния N^G-гидрокси-L-аргинина (ГОЛА) на активность ферментов цикла мочевины in vitro. Исследования показали, что ГОЛА ингибирует активность всех ферментов цикла мочевины, кроме уреотелической изоформы аргиназы в печени, повышает активность аргининосукцинатсинтазы и аргининосукцинатлиазы в разных тканьях. Наши результаты показали, что влияние ГОЛА на ферменты различных органов по-разному – это новое открытие в метаболическом пути L-аргинина. Нами показано, что ГОЛА играет важную роль в метаболизме метаболитов цикла мочевины (орнитин, аргинин и цитрулин) в различных тканях.

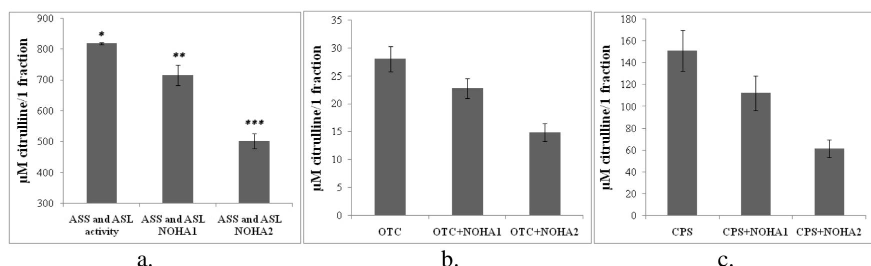
Синтез мочевины – N^G - гидрокси -L- аргинин – моноксид азота – аргиназа

Arginase (EC 3.5.3.1) hydrolyses L-arginine into urea and L-ornithine, and NO-synthase (NOS) (EC 1.14.13.39) hydrolyses L-arginine into NO and L-citrulline [3, 6].

N^G-hydroxy-L-arginine (NOHA) occurs during L-arginine conversion into NO, where NOHA is the principal intermediate in the reaction and a strong inhibitor for arginase [6]. As arginase and nitric oxide synthase (NOS) share a common substrate, the regulation of arginase is linked with nitric oxide (NO) production, and it has been suggested that the balance of L-arginine metabolism between these two pathways has important pathophysiological effects [7, 9]. The regulation of L-arginine metabolism in tissues that possess both arginase and NOS activities is poorly understood [3, 10]. Urea cycle in hepatocytes of liver consists of 5 enzymatic reactions (carbamoylphosphate synthase (CPS), ornithinecarbamoyltransferase (OTC), argininosuccinate synthase, argininosuccinaseand ureotelic arginase (UA) [1, 11]. In nature existed 3 isoforms of NOS: neuronal (nNOS or NOS I), inducible (iNOS or NOS II), endothelial (eNOS or NOS III) [3]. It is especially important, that three of five urea cycle enzymes (ASL, ASS and arginase) are found in brain and kidney [2, 8]. In brain and kidney the mentioned 2 enzymes of the urea cycle and NUA and NOS are enzymes of one united system that provide unobstructed synthesis of NO 10, 11]. In our previous investigations was revealed the inhibiting influence of NO on the activity of the urea cycle enzymes both in liver, kidney and brain [10]. Now our aim is to investtigate and reveal the new points of the biological role of NOHA the metabolic pathway of L-arginine.

Materials and methods. Male adult Wistar rats (200-220 g) were fed a standard chow diet. The animals were killed under ether anesthesia followed by decapitation. The chemicals were obtained from Sigma-Aldrich Co. Ltd. (Taufkirchen,Germany). Carbamoylphosphate synthase I (EC 2.7.2.5) activity was determined by citrulline concentration [1, 4]. Assay reaction mixture contained 30 μM $NaHCO_3$, 35 μM $MgSO_4$, 25 μmol NH_4CI, 40 μM L-ornithine, 120 μM DL-glutamic acid, 10μM ATP, pH 7.2; 0.7 ml homogenate. Ornithinecarbamoyltransferase (EC 2.7.2.5) activity was determined by concentration of ammonia [1]. Assay reaction mixture contained 1.5 ml 100 μM L-citrulline and 500 μM Na2ASO4, pH 7.2, and 0.5ml homogenized thick material. The activity of argininosuccinate synthetase (EC 6.3.4.5) and argininosuccinatelyase (EC 4.3.2.1) activity were determined by citrulline concentration [8]. The incubation mixture contained 20 μM fumaric acid, 20 μM aspartic acid, 20 μM citrulline, 10 μM ATP, 5 μM $MgSO_4$, 20 μM arginase and 1ml homogenate. The citrulline formed was measured by using diaceltylmonoxime and read at 487 nm [5]. UA and NUA activity were determined by urea concentration [2, 4]. In the column (2,5×50 cm) containing Sephadex G-150 are added the crude extracts. The homogenate was centrifuged at 1500 g for 10 min at 4°C. The column was balanced with phosphate buffer (pH 7,2) and was collected 40 fractions each one of for 4 ml. The reaction mixture contained 1.4 ml glycil-glycine, pH 9.5, 0.2 ml $MnCI_2$, 0.4 ml L-arginine, and 1ml enzyme eluate. Results expressed as means ± SD. NOHA effect on urea cycle enzymes activity was examined by Student's t-test using StatSoft 7.0.

Results and Discussion. To reveal the mechanism and nature of the NOHA influence on the urea cycle enzymes we use 2 concentration of NOHA – 1 and 2 μM in potassium phosphate buffer (0.2 M, pH 7.4). The mentioned concentrations of NOHA were added to the incubation mixture of urea cycle enzymes. In fig. 1 is shown the NOHA influence on the activity of ASS and ASL (based on the used method, mentioned enzymes activities are presented in a united way), OTC and CPS in the hepatocytes of the liver. Liver ASL and ASS united activity is inhibited by NOHA in 14,7 % adding 1 μM and in 39,1% adding 2μM of NOHA (fig. 1, a). OTC activity is inhibited by 18,5 and 40,75%, adding in the incubation mixture 1 and 2μM of NOHA corresponding (fig. 1, b). CPS activity is inhibited by 22,9 and 58,8%, adding in the incubation mixture 1 and 2 μM of NOHA corresponding (fig. 1, c). Mentioned facts show the inverse correlation relationship between the activty of NOS and urea cycle enzymes. The results noted above and below indicate the competitive relationship between NOS and urea cycle enzymes not only for L-arginine [5, 10], but also towards to other important biological metabolites as are L-citrulline and L-ornithine.



a. b. c.

Fig. 1. The influence of NOHA on urea cycle enzymes activity in rat liver. a – NOHA influence on ASS and ASL activity, b – NOHA effect on OTC activity, c – NOHA effect on CPS activity, CPS – Carbamoylphosphatesynthase, ASS - *Argininosuccinate synthetase, ASL - Argininosuccinatelyase,* OTC – Ornithinecarbamoyltransferase, NOHA – N^G-hydroxy-L-arginine, * – enzyme activity in norm, ** – enzyme activity + 1 μM NOHA, *** – enzyme activity + 2 μM NOHA (n=7, p<0.05).

The results of NOHA influence on the activity change of the enzymes in brain and kidney are completely different from the results in the liver hepatocytes (fig. 2). NOHA increases the activity of ASS and ASL in brain and kidney. In kidney the united activity of ASS and ASL is increased in 32,3 and 87,1%, adding 1 and 2 μM of NOHA in the incubating mixturecorresponding (fig. 2, a). Brain ASS and ASL united activity is increased by 9,8 and 19,8%, adding 1 and 2μM of NOHA in the incubation mixture corresponding (fig. 2, b). The unsimilar response of ASS and ASL activity change in brain, kidney and liver to the NOHA influence, can be because of the possible existance of ASS and ASL isoforms in different organs, which have different functions.

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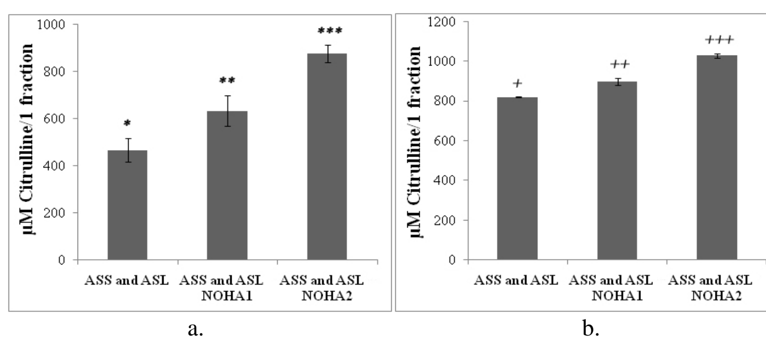

a. b.

Fig. 2. The influence of NOHA on ASS and ASL activity in kidney (a) and brain (b).* – ASS and ASL activity in norm in kidney, ** –Σenzymes activity + 1 μM NOHA, *** – Σenzymes activity + 2 μM NOHA, + – enzyme activity and NOHA influence in brain(n=7, p<0.05).

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The most probable biological reason of the highly mentioned activity increase should be the provision of the essential quantities of L-arginine for the synthesis of NO. Further investigations about the above mentioned facts are being confirmed, but the results already let us to make similar conclusions. In this work also is presented the influence of NOHA on the activity of NUA and UA of the liver, and NUA of the brain and kidney. With the assistance of Lineweaver-Burk's curve was revealed the mechanism of the inhibition of nonureotelic arginase. Beforehand with the assistance of gel-filtration (Sephadex G-150) and ion-exchange chromatography (CM-cellulose) was organized the separation and partially purification of the 2 isoforms of arginase from the investigated organs.

It was shown simultaneously, that with different properties (K_m, effect of Mn^{2+}, molecular weight, intracellular localization, hormonal induction) high-molecular-weight isoform of liver is ureotelic and other isoforms are nonureotelic [1, 5]. Datas show that brain and kidney arginases are significantly inhibited by N^G-hydroxy-L-arginine (tab. 1).

Table 1. Effect of N^G-hydroxy-L-arginine on different isoforms of arginases in rats different organs (gel-filtration + ion-exchange chromatography, n=7, p<0.05)

Tissues	Arginase activity		Arginase activity+N^6-hydroxyl-L-arginine			
	I apex (NUA)	II apex (UA)	I apex (NUA)	Inhibition, %	II apex (UA)	Inhibition, %
Liver	129,2±0,13	360±0,1	51,6±0,17	60	358±0,12	0
Brain	12±0,17	–	2,6±0,13	78,3	–	–
Kidney	5,28±0,5	5,48±0,18	3,12±0,1	41	2,1±0,56	62

In liver tissue low-molecular-weight isoform is inhibited significantly, but high-molecular-weight isoform is inhibited only in 9.3%. Since high-molecular fraction is not uniform, we assume that inhibition is related to NUA activity. Therefore was performed fractionation on high-molecular-weight isoform with CM-cellulose. Data in tab.1 confirm, that N^G-hydroxy-L-arginine inhibits only nonureotelic isoform of arginase while as ureotelic isoform do not respond to inhibitor's effect. These results demonstrate that NOHA influences exactly on the special stereospecific regulatory center of the enzyme. The Lineweaver-Burk's curve is presented only for nonureotelic arginase-1 of the liver (K_m=26,3×10^{-3}M).

To confirm this we study the dependence of isoform activity from the concentration of the substrate (10-300 μM) in the presence and absence of N^G-hydroxy-L-arginine (fig. 3). In all Lineweaver-Burk curves for nonureotelic isoforms with and without the inhibitor, intersection is in one point of abscissa, what speaks about noncompetitive nature of the inhibition. It has allosteric nature.

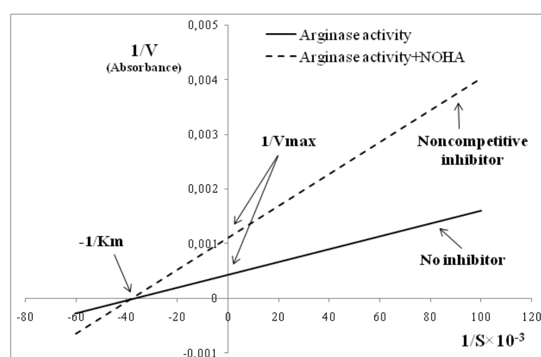

Fig. 3. Lineweaver-Burk substrate-velocity curve for liver nonureotelicarginase (n=5, p<0.05).

The influence of NOHA on the activity of 5 enzymes of the urea cycle in liver, and ASS, ASL and nonureotelicarginase (NUA) activity in brain and kidney was investigated for the first time.The results show the inverse correlation relationship between the activities of NOS and urea cycle enzymes. The results noted previously indicate the competitive relationship between NOS and urea cycle enzymes not only for L-arginine but also towards to other important biological metabolites as are L-citrulline and L-ornithine. The ASS and ASL activity differently respond to the influence of NOHA in liver hepatocytes and in brain and kidney cells. The probable reason of the above mentioned facts, should be the possible existance of different isoenzymes of ASS and ASL in various organs, which have dissimilar function. The last points that NOS compete for L-arginine only with nonureotelic isoforms of arginase and it has allosteric nature.

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REFERENCES

1. *Давтян М.А.* Эволюционные аспекты образования и нейтрализаций аммиака. III Сисакяновские чтения, с. 106-154, 2005.
2. *Давтян М.А. Буниатян Г.Х.* Очистка и свойства аргиназы головного мозга крыс. Биохимия, *35*, 2, с. 412-417, 1970.
3. *Alderton W, Cooper C, Knowles R.* Nitric oxide synthase: structure, function and inhibition. Biochem. J., 357, 593-615, 2001.
4. *Archibald R.M.* The colorimetric determination of urea. J. Biol. Chem., *167*, 507, 1945.
5. *Avtandilyan N.V., Karapetyan S.A., Davtyan M.A.,* Polyamines are noncompetitive inhibitors for nonureotelicarginases in the different tissues of rats. National Academy of Sciences of RA, Electronic Journal of Natural Sciences, *20*, 1, pp. 7-10, 2013.
6. *Boucher, J.-L., J. Custot, S. Vadon, M. Delaforge, M. Lepoivre, J.-P. Tenu, A. Yapo, and D. Mansuy.* NG-hydroxy-Larginine, an intermediate in the L-arginine to nitric oxide pathway, is a strong inhibitor of liver and macrophage arginase. Biochem. Biophys. Res. Commun. *203*, 1614-1621, 1994.
7. *Buga, G.M., R. Singh, S. Pervin, N.E. Rogers, D.A. Schmitz, C.P. Jenkinson, S.D. Cederbaum, and L.J. Ignarro.* Arginase activity in endothelial cells: inhibition by N^{G}-hydroxy-L-arginine during high-output NO production. Am. J.Physiol. 271 (Heart Circ. Physiol. 40): H1988–H1998, 1996.
8. *Buniatian, H.Ch. and Davtian, M.A.* Urea synthesis in brain. Journal of Neurochemistry, *13*, 743-753, 1966.
9. *Daghigh, F., J.M. Fukuto, and D.E.* Ash. Inhibition of rat liver arginase by an intermediate in NO biosynthesis, N-hydroxyarginine: implications for the regulation of nitric oxide biosynthesis by arginase. Biochem. Biophys. Res. Commun., *202*, 174-180,1994.
10. *Javrushyan H.G., Khachatryan M.A., Davtyan M.A.* No effect on urea cycle enzymes activity in different tissues of rats. Perspectives for development of molecular and cellular biology-4. Biolog. Journal of Armenia, Supplement, *65,* 1, pp. 80-81, 2013.
11. *Sidney M. Morris Jr.* Enzymes of arginine metabolism. J. Nutr., *134,* 2743S-2747S. 2004.

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