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DIETHYLAMINE *NONOate* INFLUENCE ON RATS UREA CYCLE ENZYMES ACTIVITY AFTER AND BEFORE HYPOXIA

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ABSTRACT

The relationship between hypoxia and regulation of nitric oxide synthase (NOS) and the urea cycle enzymes activities in different tissues is not thoroughly studied. The mechanisms underlying the cellular response to hypoxia and their consequences are not completely understood. The aim of this study was to investigate the interconnection between hypoxia, NO and urea cycle enzymes (UCE) activity before and after hypoxia through injection of diethylamine NONOate (DEAN, NO donor). Our results indicate that at hypoxia NO influences the urea cycle enzymes activity in a way to ensure the availability of the required substrates for its own biosynthesis. The practical significance of these results are that through influence of NO the mutually agreed changes of UCE activity can indirectly exert influence on the processes of apoptosis, cancer cell metabolism and hypertension.

Key words: *diethylamine NONOate, hypoxia, urea cycle*

INTRODUCTION

The relationship between hypoxia, regulation of nitric oxide synthase (NOS) and the urea cycle enzymes in different tissues is not so well studied [2]. We investigated the effect of diethylamine *NONOate* and hypoxia on urea cycle enzymes activity in liver, brain and kidney of rats. NO is a multifunctional signaling molecule involved in a wide variety of biological processes [6]. Interest to this molecule was increased particularly after the revelation of its vasodilating properties. *NO* is produced from L-arginine by *NO*-synthase [8]. During the syntheses of *NO* the cytrulline formed can transform into arginine by enzymatic action of *argininosuccinate lyase (ASL) and argininosuccinate synthetase* (ASS) identified in brain and kidney, which provide the continuous syntheses of *NO* [3, 8]. It is an integral part of human physiological response to hypoxia [5, 7, 9]. Urea cycle occurs in hepatocytes of liver that consists of 5 enzymatic reactions (carbamoylphosphate synthase (CPS), ornithinecarbamoyl transferase (OTC), ASL, ASS, ureotelic arginase (UA) [3, 4]. It is especially important, that three of five urea cycle enzymes (argininеosuccinate synthase, argininosuccinase and arginase) are found in brain and kidney [3, 8]. With the help of the above mentioned enzymes arginine is synthesized from citrulline *de novo*, which is transformed back again into citrulline by NOS. This process is called citrulline-*NO* cycle. In recent work we have already studied nitric oxide effect on urea cycle enzymes activity *in vitro* [4]. The current study demonstrates inhibition of the activity of all the urea cycle enzymes in liver, brain and kidney after the influence of DEAN (diethylamine NONOate).
The aim of this study was to investigate the interconnection between hypoxia, NO and urea cycle enzymes (UCE) activity before and after hypoxia through diethylamine NONOate (DEAN, NO donor) injection.

MATERIALS AND METHODS

Animals and chemicals. Male adult Wistar rats (200-220 *g*) were used for the experiment. The animals were housed in an air-conditioned room at a temperature of 23±2°*C* with lights on 12 h/day. The rats weighing 200-220 *g* were undergone hypoxia at the Department of Physiology, YSU. Animals were kept in hypoxic chambers for 3 days, twice a day, 20 minutes, in 4500-5000 *m* (PO_2 98-85 *mmHg*). DEAN was injected through lateral vein of rat tail (0.4 *mg/kg*). The animals were killed under ether anesthesia followed by decapitation. The chemicals were obtained from SigmaAldrich Co. Ltd. (Taufkirchen, Germany).
Determination of urea cycle enzymes activity. Carbamoylphosphate synthase I (EC 2.7.2.5) activity was determined by citrulline concentration [1]. During homogenization was used isotonic solution of KCI and ethylene diamine tetraacetic acid of Na. The homogenate was centrifuged at 8000 *g* for 10 min at 4°*C* for 2 times. For the enzymatic assay was used the “washed sediment”. Assay reaction mixture contained 30 μM $NaHCO_3$, 35 μM $MgSO_4$, 25 μM NH_4CI, 40 μM L-ornithine, 120 μM DL-glutamic acid, 10 μM adenosine triphosphate (ATP), pH 7.2; 0.7 *ml* homogenate. The reaction mixture was incubated at 37.5°*C* for 1 h. The generated citrulline was measured by urea

concentration. **Ornithinecarbamoyltransferase (EC 2.7.2.5)** activity was determined by concentration of ammonia [3]. Assay reaction mixture contained 1.5 *ml* 100 μM L-citrulline and 500 μM Na_2AsO_4, pH 6.8-7.1, and 0.5 *ml* homogenized material. The reaction mixture was incubated at 37.5°*C* for 40 min. The generated ammonia was measured by the method of E. J. Conway. The activity of **Argininosuccinatesynthetase (EC 6.3.4.5) and Argininosuccinatelyase** (EC 4.3.2.1) was determined by citrulline concentration. Tissues were homogenized in 50 μmol KH_2PO_4, pH 7.2. The incubation mixture contained 20 μM fumaric acid, 20 μM aspartic acid, 20 μM cytrulline, 10 μmol ATP, 5 μmol $MgSO_4$, 20 μM arginase and 1 *ml* homogenate. The reaction mixture was incubated at 37.5°*C* for 40 min. The generated citrulline was measured by using diaceltyl monoxime and read at 487 *nm*.
UA and NUA (EC 3.5.3.1) activity was determined by urea concentration [1, 3]. In the column (2.5×50 *cm*) containing Sephadex G-150 the crude extracts of rat liver, kidney and brain (10%) are added. Enzyme extract was prepared by homogenization using 0.2 *M* Glycine buffer, pH 9,5. The homogenate was centrifuged at 1500 *g* for 10 min at 4°*C*. The column was balanced with phosphate buffer (pH 7.2) and 40 fractions were collected each containing 4 *ml*. After that arginase activity was determined in each fraction. The reaction mixture contained 1.4 *ml* glycil-glycine, pH 9.5, 0.2 *ml* $MnCl_2$, 0.4 *ml* L-arginine, and 1 *ml* enzyme eluate. Reaction mixture was incubated at 37.5°*C* for 1 h. The generated urea was measured by the method of Archibald [1].
Statistical Analysis. Results expressed as means±SD and means±SE enzyme activity. The effect of hypoxia on urea cycle enzymes activity was examined by Student's t-test using Statistica software (StatSoft 7.0).

RESULTS AND DISCUSSION

Male adult Wistar rats weighing 220-250 *g* were undergone hypoxia at the Department of Physiology of Yerevan State University. The rats were divided into 5 groups. First was control group, 2nd was hypoxic group, 3rd group was injected DEAN before hypoxia, 4th was injected DEAN after hypoxia and 5th group was only injected DEAN. Animals were kept in hypoxic chambers for 3 days, twice a day, 20 minutes, in 4500-5000 *m* (98-85 *mm* HgCl). The activity of urea cycle enzymes was determined in liver, brain and kidney homogenates.
Our studies show that after hypoxia the activity of liver Arginase I was increased in 38.9%, kidney Arginase II in 80.3% and brain Arginase I in 5.3 times comparing to the initial activity (*Fig. 1*).

Table 1. The change of OTC and CPS I activity in liver homogenate during normoxia, hypoxia, DEAN injection and DEAN injection befor and after hypoxia. n=5, p<0.05.

Enzyme	Activity±SD (µM citrulline/1minute/1ml)	Inhibition %	Activation %
OTC in norm	1,78±0,05	-	-
Hypoxia	0,56±0,03	68,5	-
DEAN injection	3,125±0,418	-	75,5
Hypoxia → DEAN injection	2,01±0,07	-	12,9
DEAN injection → Hypoxia	1,03±0,06	42,1	-
CPS 1 in norm	2,1±0,048	-	-
Hypoxia	1,28±0,037	39,1	-
DEAN injection	2,07±0,035	1,5	-
Hypoxia → DEAN injection	3,22±0,019	-	53,3
DEAN injection → Hypoxia	2,8±0,032	-	25

At hypoxia the activities of OTC and CPS are decreased in 68.5% and 38.1% respectively, what shows that in hypoxic states the inclusion of L-arginine and L-ornithine in urea synthesis is limited (*Table 1*). The reason of the inhibition is that during hypoxia NOS activity is increased, what leads to growth of NO amounts. Parallel to NO the growth of cytrulline concentration inhibits OTC activity, due to what carbamoyl phosphate in its turn inhibits CPS1 activity. In 3rd group the cumulative activity is increased comparing to the initial activity in liver in 14.6%, in kidney Arginae II in 67.3% and brain Arginase I in 14%. In liver, brain and kidney homogenates of 4th group, the arginase activity is decreased and approaches to the arginase activity in 3rd group. After DEAN injection in 5th group arginase activity in

liver, brain and kidney is inhibited in 4.4%, 15.9% and 14.1% respectively, which reason presumably should be the influence of NOS reaction products. Apparently, the reason of mentioned results should be as direct, as so indirect effect of NO on arginase activity: changing the amounts of arginase and NOS reaction metabolites.

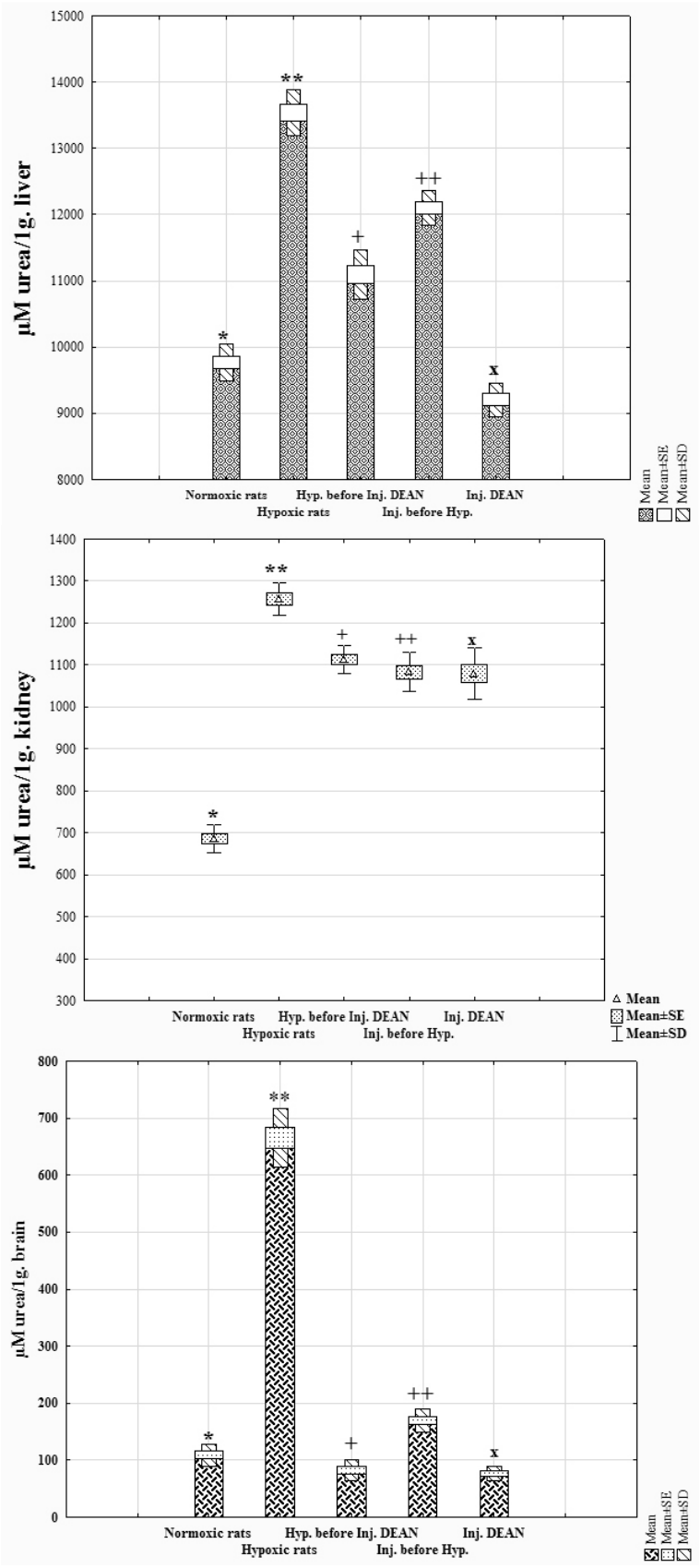

Fig. 1. The change of arginase activity in liver, kidney and brain homogenates during normoxia, hypoxia, DEAN injection and DEAN injection befor and after hypoxia. n=5, $p<0.05$.
* - normoxia, ** - hypoxia, + - DEAN injection after hypoxia, ++ - DEAN injection before hypoxia, x – DEAN injection.

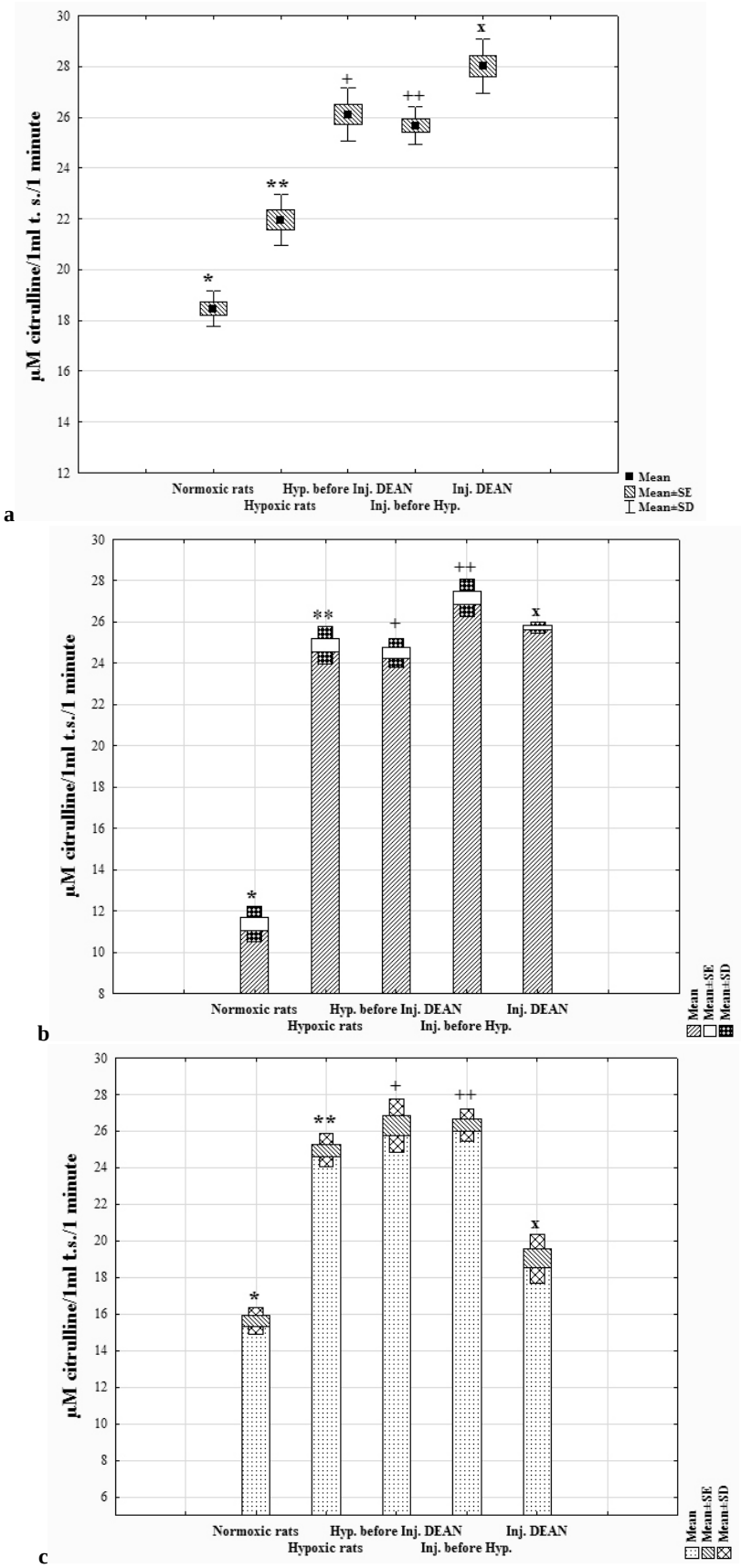

Fig. 2. The change of ASS and ASL activity in liver (a), kidney (b) and brain (c) homogenates during normoxia, hypoxia, DEAN injection and DEAN injection befor and after hypoxia. n=5, $p<0.05$.
* - normoxia, ** - hypoxia, + - DEAN injection after hypoxia, ++ - DEAN injection before hypoxia, x – DEAN injection.

The inhibition of OTC and CPS1 and increase of NUA activity indicates that during hypoxia ornithine is involved in biosynthesis of many metabolites that are necessary for the adaptive reactions at hypoxia. According to the literature

data after hypoxia are activated different mechanism for preservation of homeostasis, with participation of GABA, creatine, polyamines etc..

The injection of DEAN after and before hypoxia increases the activity of OTC and CPS1 in 12.9-53.2% due to the necessity of cytrulline, because at the same time are increased the activities of Arginase, ASS and ASL. In hypoxic conditions OTC and CPS1 participate not only in urea cycle but also prevent necessary precursors for NO synthesis.

In all groups (except control) the activities of ASL and ASS are increased in 15.2-115.4% (*Fig. 2*). Hypoxia stimulates the activity of ASS and ASL, which is necessary to provide the essential amounts of L-arginine. And the increase of NO quantity after DEAN injection is an additional signal for the stimulation of ASS and ASL activities. The increase of activation of these enzymes is needed to supply arginase for the biosynthesis of ornithine and NO, because during NOS activation the amounts of arginine are fast consumed.

These results indicate that there is no direct interconnection between NO and upper mentioned enzymes, otherwise there will be noticed activity inhibition. We can assume that NO increase the activity of ASS and ASL through product-substrate and substrate-enzyme interactions.

CONCLUSION

The enzymes of the urea cycle in liver and especially in brain and kidney act as a united system for the preservation of *NO* homeostasis. Experimental results confirm, that for the changec of *NO* amounts during hypoxia are responsible not only NOS and cytochrom *C* oxidase, but also ASS, ASL and NUA of the brain and kidney.

The practical significance of these results is that through influence of NO the mutually agreed changes of urea cycle enzymes activity can indirectly exert influence on the processes of apoptosis, cancer metabolism and hypertension.

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