Effect of NO Synthase Inhibitor 2-Amino-5,6-Dihydro-4*H*-1,3-Thiazine on Endotoxin-Induced Changes in Hemodynamic Parameters and Respiration in Rats

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We studied the effect of NO synthase inhibitor 2-amino-5,6-dihydro-4*H*-1,3-thiazine (2-ADT) on the cardiovascular system in rats with endotoxic shock. Blood pressure, heart rate, and respiratory rate were recorded. *E. coli* lipopolysaccharide decreased blood pressure and heart rate. 2-ADT in a dose of 5 mg/kg normalized these hemodynamic parameters. The normalizing effect of 2-ADT decreased with increasing the dose of this preparation. 2-ADT in high doses (10, 20, and 30 mg/kg) and repeated injections of the preparation caused death of experimental animals.

Key Words: *NO synthase inhibitor; septic shock; blood pressure; heart rate; respiratory frequency*

The role of NO• in hypotension accompanying septic shock was studied on animals and humans [10]. The content of nitrite formed during NO• metabolism increases during infectious diseases [9,15] and cytokine chemotherapy for cancer [11]. Endotoxin-induced hypotonia is less pronounced in mice with inducible NO synthase (iNOS) gene knockout, while the survival rate in these mice is higher than in animals with intact gene [12]. The results of experimental studies were not confirmed by clinical observations with the NO synthase inhibitor N-monomethyl-L-arginine [7].

The search for new agents and methods to normalize hemodynamic parameters during septic shock and other pathological conditions accompanied by a sharp decrease in blood pressure (BP) is an urgent problem. Our previous studies showed that 2-ADT, a parent compound for many potential NO synthase inhibitors (6-membered cyclic derivatives of isothiourea), *in vivo* inhibits endotoxin-induced NO production in mice [3].

Here we studied the effect of 2-ADT on the cardiovascular system in rats with septic shock produced by bacterial endotoxin.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats weighing 290-400 g. The animals were randomly selected and divided into groups (3-7 rats per group). Septic shock was modeled using *E. coli* lipopolysaccharide (LPS, Sigma). 2-ADT was synthesized at the Department of Radiochemistry (Chemical Department, M. V. Lomonosov Moscow State University). Both preparations were dissolved in physiological saline. Systolic and diastolic BP were measured in the ostium of the left carotid artery. Heart rate (HR) was estimated by *RR* intervals. The respiratory rate (RR) was recorded [3]. LPS in a dose of 20 mg/kg was infused into the jugular vein for 3-5 min immediately before measuring baseline parameters. 2-ADT was

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intraperitoneally injected to rats with persistent hypotonia (20 min after LPS treatment). The test parameters and general state of animals were monitored for 30-110 min.

Some rats intraperitoneally received 18 mg/kg LPS 3 h before narcosis. In this series 2-ADT in a dose of 5 mg/kg was injected immediately after recording of baseline parameters.

Control animals received an equivalent volume of physiological saline.

The results were analyzed by Student's *t* test.

RESULTS

Under normal conditions constitutive endothelial NO synthase (eNOS) is involved in the maintenance of vascular tone. Therefore vasoconstrictor effect of preparations characterizes their eNOS-inhibitory activity. Administration of 2-ADT in doses of 5-100 mg/kg significantly increased BP in healthy narcotized rats, which remained high over 5 min after treatment ($p<0.05$). Increasing the dose of 2-ADT prolonged the hypotensive effect from 30 to 90 min, but made it less pronounced (Fig. 1, *a*). Other physiological parameters rapidly reached a constant level and remained practically unchanged over the first 30 min after injection of 2-ADT. Therefore, most studies were performed in this period.

HR significantly decreased in rats receiving 2-ADT. It should be emphasized that increasing the dose of 2-ADT above 10 mg/kg did not produce further decrease in HR (Fig. 1, *b*). 2-ADT in doses of 5 and 10 mg/kg had no effect on RR. However, RR increased by 70% in animals treated with 2-ADT in a dose of 100 mg/kg (Fig. 1, *c*).

Septic shock was accompanied by a sharp decrease in BP, which reached a constant level after 20 min. In further experiments 2-ADT was injected during this period. HR in all rats significantly decreased 15-20 min after treatment. Extrasystoles were revealed in some animals. RR did not differ from the control.

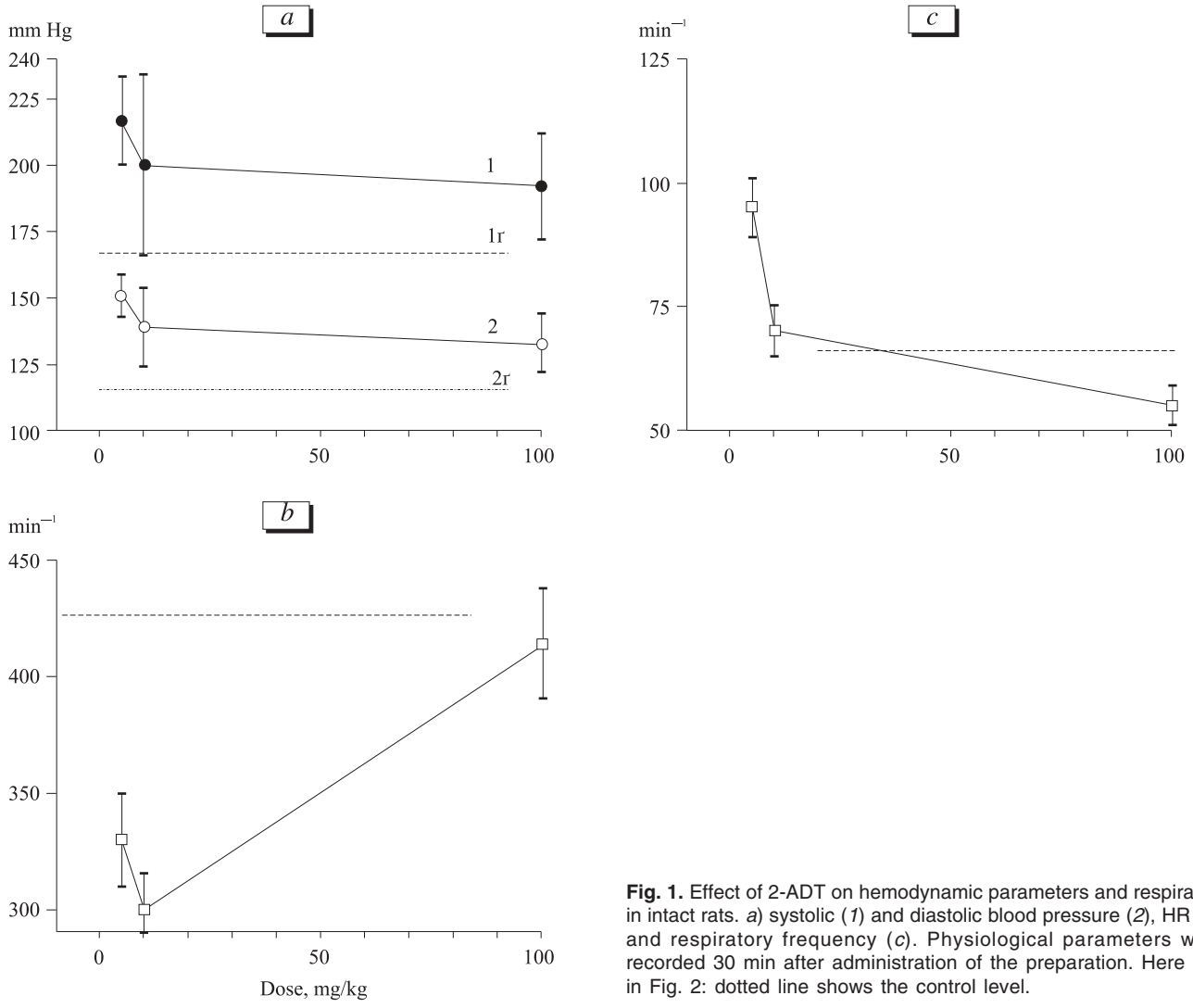

Fig. 1. Effect of 2-ADT on hemodynamic parameters and respiration in intact rats. *a*) systolic (*1*) and diastolic blood pressure (*2*), HR (*b*), and respiratory frequency (*c*). Physiological parameters were recorded 30 min after administration of the preparation. Here and in Fig. 2: dotted line shows the control level.

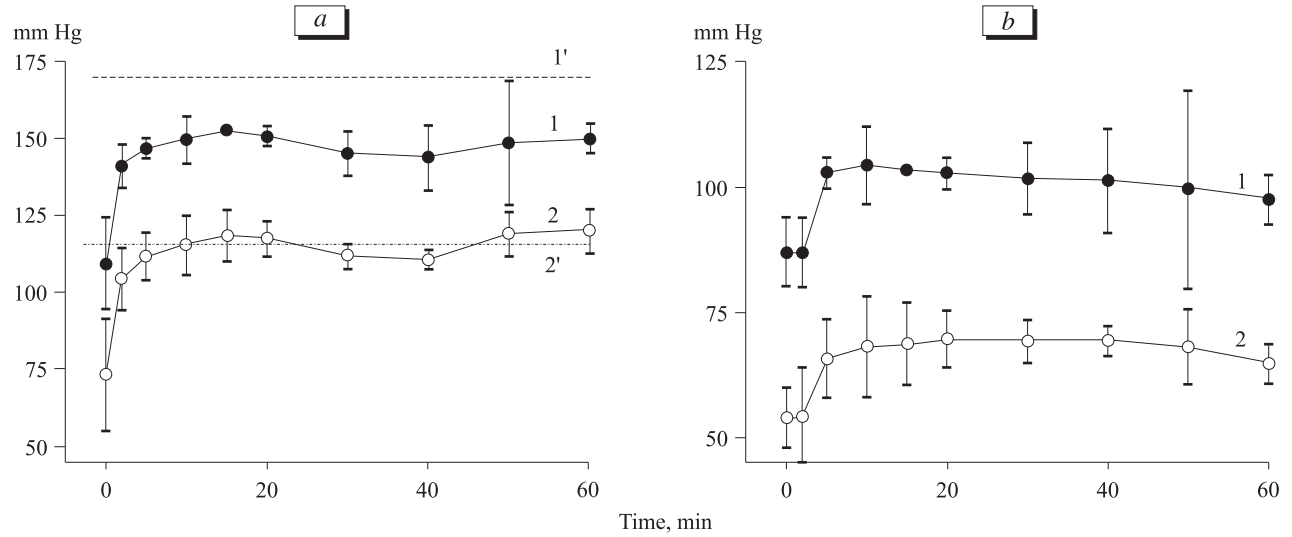

Fig. 2. Systolic (*1*) and diastolic blood pressure (*2*) in rats receiving 2-ADT in a dose of 5 mg/kg 20 min (*a*) and 3 h (*b*) after LPS injection.

Administration of 2-ADT in a dose of 5 mg/kg to LPS-treated rats produced a significant increase in BP (by 20-30%), which persisted for 70 min (Fig. 2, *a*). Repeated treatment with the preparation after 100 min had no hypertensive effect in animals with developed hypotonia. Repeated administration of 2-ADT in high doses (10, 20, and 30 mg/kg) caused respiratory arrest and animal death. It should be emphasized that these doses of 2-ADT are less than $^1/_8$ LD_{50}. Other NO synthase inhibitors, *e.g.* aminoguanidine and methyls esters of N-nitro-L-arginine and N-methyl-L-arginine in high doses also produce toxic effects on endotoxemic rodents [6,14]. However, selective iNOS inhibitor L-canavanine protects mice from endotoxemic death [8].

Since LPS-induced synthesis of iNOS and overproduction of NO occur over 3-6 h, it was interesting to evaluate the vasoconstrictor effect of 2-ADT in this period. Administration of 2-ADT 3 h after LPS treatment was followed by a significant and long-term increase in BP (60 min, Fig. 2, *b*). However, the degree of hypertensive changes was 2-fold lower compared to animals receiving 2-ADT 20 min after LPS treatment.

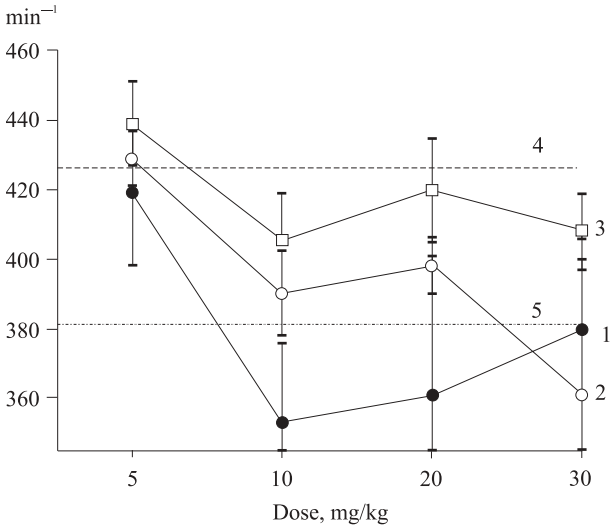

Fig. 3. HR in rats receiving 2-ADT 10 (*1*), 30 (*2*), and 60 (*3*) min after LPS injection. HR in intact animals (*4*) and 20 min after LPS injection (*5*).

In vitro NO-inhibitory activity of NO synthase inhibitors increases with increasing the dose of preparations. Therefore, we studied the effect of 2-ADT in various doses on physiological parameters. Increasing the dose of 2-ADT above 10 mg/kg reduced the hypertensive effect (Fig. 3). In rats receiving 2-ADT in dose of 5 mg/kg HR returned to normal over the observation period. HR in animals receiving 2-ADT in higher doses did not differ from that in rats treated with endotoxin alone.

No differences were revealed in HR of control animals (66±4 bpm) and rats receiving LPS alone (68±3 bpm) or in combination with 2-ADT in doses of 5, 10, 20, and 30 mg/kg.

These data suggest that 2-ADT *in vivo* retains NO synthase-inhibitory activity.

In vitro experiments with enzymes from human cells showed that 2-ADT is as potent as N-nitro-L-arginine in inhibiting iNOS [4]. The concentration of the inhibitor decreasing the rate of L-arginine conversion into L-citrulline with iNOS was ≈3 μM (IC_{50}). In these experiments 2-ADT did not exhibit selectivity for specific NOS isoform. The study with cultured RAW mouse macrophages showed that inhibitory activity of 2-ADT in relation to NO synthesis with the LPS-induced enzyme is 5 times lower compared to isolated enzyme (IC_{50} 14 μM) [13].

In our experiments 2-ADT was administered immediately after the development of persistent septic hypotonia (20 min after LPS injection). Under these

conditions relaxation of vessels results from stimulation of NO production by the constitutive enzyme (eNOS). Considerable amounts of iNOS could not be formed during this period [14]. Therefore, the vasoconstrictor effect and stimulation of the cardiovascular function reflect NO-inhibitory activity of 2-ADT in relation to the constitutive enzyme.

To evaluate the potency of 2-ADT in inhibiting iNOS, the preparation was injected 3 h after the development of endotoxemic hypotonia. During this period the inducible enzyme is the major source of NO in the organism, whose content increases by 20-50 times [1,2,14]. 2-ADT in a dose of 5 mg/kg normalized hemodynamic parameters in rats treated immediately after LPS injection, but was less potent under these conditions. It was probably related to low inhibitory activity of the preparation in relation to iNOS and activation of other neurohormonal mechanisms for endotoxin-induced vasorelaxation. Our assumption is confirmed by low vasoconstrictor activity of 2-ADT in intact animals with the normal system for regulation of cardiovascular function (Figs. 1 and 2). Probably, endotoxemic shock is accompanied by impairment of this regulatory system. NO-inhibiting preparations can act as potent antishock agents during this period. It should be emphasized that 2-ADT in effective doses had no effect on respiratory function in animals.

Our results indicate that 2-ADT-related compounds hold promise for the therapy of septic shock.

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