Effect of NO Inhibitors on Hypovolemic Shock-Induced Hypotension

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 149, No. 7, pp. 23-27, July, 2010
Original article submitted January 12, 2009

In vivo effect of isothiourea derivatives on NO production was studied by the method of electron paramagnetic resonance spectroscopy with a spin trap. We evaluated the influence of these compounds on hemodynamic parameters in anesthetized rats with hypovolemic shock. A correlation was found between the size of S,N-substituents in isothiourea derivatives (methyl, ethyl, and isopropyl) and NO inhibitory activity of compounds. The antihypotensive effect was more pronounced in compounds with high NO inhibitory activity containing the isopropyl radical.

Key Words: *inhibitors of nitric oxide synthesis; in vivo electron paramagnetic resonance spectroscopy with a NO spin trap; isothiourea derivatives; hypovolemic shock; antihypotensive effect*

Syndrome of arterial hypotension is an urgent medical and social problem. Blood pressure drop contributes to damage to target organs due their impaired perfusion. These changes determine poor prognosis of the disease [14]. A serious problem is arterial hypotension under shock conditions due to sepsis, blood loss, trauma, and some medical manipulations (*e.g.*, hemodialysis and antitumor therapy). There is no general concept of this problem [7]. A small number of pharmaceutical agents with antihypotensive activity are used in medical practice [13]. Our previous studies showed that cyclic and linear isothiourea (ITU) derivatives exhibit antihypotensive properties during experimental septic shock [5,6].

Here we evaluated a relationship between chemical structure, NO inhibitory activity *in vivo*, and effect of new ITU derivatives on blood pressure, HR, and respiratory rate in anesthetized rats with hypovolemic shock.

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MATERIALS AND METHODS

We studied biological activity of N-substituted derivatives of ITU. Experiments were performed with the following compounds (Medical Radiology Research Center): N-acetyl-S-isopropyl ITU hydrobromide (**1**); S-ethyl ITU diethyl phosphate (**2**); isopropyl ITU diisopropyl phosphate (**3**); N-acetyl-S-ethyl ITU hydrobromide (**4**); methyl ITU dimethyl phosphate (**5**); and N-acetyl-S-methyl ITU hydroiodide (**6**).

The compounds were identified by element analysis, nuclear paramagnetic resonance spectroscopy, and chromatography.

NO inhibitory activity of these compounds was studied on male outbred albino mice (parent genotype Swiss). The animals received an intraperitoneal injection of LPS (1.5 mg/kg) in 0.5 ml physiological saline 4 h before euthanasia. The test compounds

0007-4888/10/1501-0018

were administered 3 h after LPS injection. The liver was sampled 1 h after treatment. NO production was measured by the A. F. Vanin method of electron paramagnetic resonance spectroscopy with a spin trap [1].

The data are presented as the relative content of NO in tissue samples [3].

The hemodynamics was studied in male Wistar rats weighing 300-400 g [4,5]. The animals were anesthetized with sodium thiopental (60 mg/kg intraperitoneally). The following parameters were evaluated: respiratory rate (RR), HR, blood pressure (BP) in the left carotid artery, systolic (sBP) and diastolic BP (dBP), and ECG (three standard leads). These parameters were recorded on a Polygraph RM-6000 device and Cardiograph device (Nihon Kohden).

The blood was taken from the jugular vein (1.4-1.9% of body weight) to induce hypovolemic shock [11]. The parameters were repeatedly measured after stabilization. One of the test compounds was injected intraperitoneally. Control animals with blood loss were examined over 90 min.

The data in Figs. 2 and 3 were calculated as follows:

$$\text{AHE (antihypotensive effect)} = \{[BP(tr)-BP(bl)]/[BP(c)-BP(bl)]\}\times 100\%,$$

where BP(tr), BP(bl), and BP(c) are BP in rats receiving the test substances after blood loss, during hypovolemic shock, and under control conditions (intact animals), respectively.

Each experimental group consisted of 7-11 rats and 7 mice. The results were analyzed statistically by analysis of variance, Newman–Keuls test, and Dunnett's test [2].

RESULTS

NO inhibitory activity of compounds was studied *in vivo* in liver samples from mice receiving *E. coli* LPS. This treatment induces significant expression of inducible NO synthase (iNOS) in tissues. NO production increases by 15-30 times and reaches a constant level 4 h after treatment [3,5,6]. *In vitro* studies showed that ITU derivatives do not differ in selectivity to various isoforms of NO synthase [4,9]. Studying NO inhibitory activity during experimental endotoxemia allowed us to evaluate the effect of substances not only on iNOS, but also on constitutive enzymes (neuronal and endothelial NO synthases).

NO inhibitory activity of compounds **1** and **3** (10 μmol/kg) was similar to that of the reference compound **2** (30 μmol/kg). Compounds **5** and **6** with S-methyl substituent were less potent in inhibiting the NO production in mouse liver. NO production was 14 and 17% of the control level, respectively, after treatment with these compounds in a dose of 10 μmol/kg. However, these differences were statistically insignificant.

Antihypotensive activity of synthetic derivatives of ITU was studied on the model of hypovolemic shock. Blood loss of 20-25 ml/kg (1.5% of body weight) and 15 mg/kg (0.7% of body weight) is considered to be severe and moderate, respectively [2,3]. In our experiments, blood loss was 1.4-1.9% of body weight (severe injury). This state caused death of some animals due to hypovolemic shock.

The mean BP during experimental blood loss decreased by 95 mm Hg (61% of the baseline level; 156±11 mm Hg). sBP and dBP in rats of the treatment group increased progressively 10-15 min after

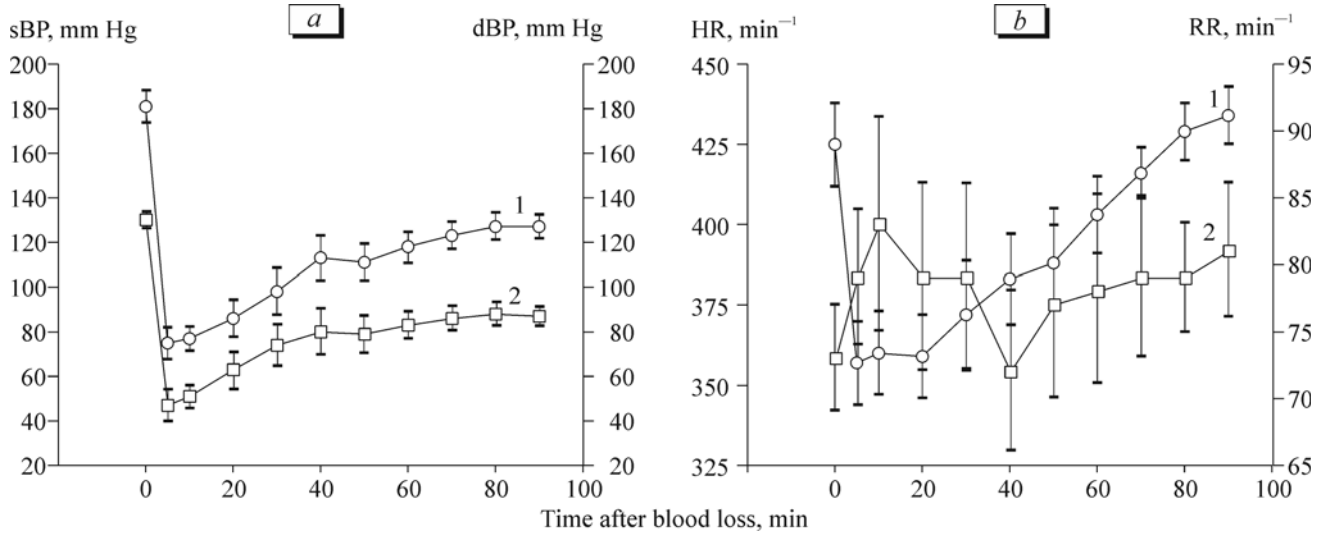

Fig. 1. Effect of blood loss on BP (*a*), HR, and RR in mice (*b*). (*a*) sBP (*1*) and dBP (*2*). (*b*) HR (*1*) and RR (*2*). 0 min, study parameters in intact animals.

blood withdrawal. By the 90th minute, these parameters reached 68-70% of the baseline level (181±7 and 130±3 mm Hg, respectively; Fig. 1). HR decreased after blood loss (up to 300 bpm in some animals), but returned to normal in the follow-up period (Fig. 2). RR remained practically unchanged in various periods of the study (Fig. 2).

Compounds **5** and **6** in doses of 5 and 10 mg/kg had little effect on hemodynamic parameters in rats with blood loss (data not shown). The remaining compounds (**1**-**4**) had a strong modulatory effect on the hemodynamics.

Figure 2 shows the effects of compounds **1**-**4** on sBP.

Compound **1** in a dose of 5 mg/kg did not change in hemodynamic parameters after blood loss. However, treatment with this compound in a dose of 10 mg/kg was followed by a significant and persistent increase in sBP. By the 20th minute, sBP reached 76% of the hypotonic effect of blood loss (Fig. 3). The antihypotensive effect of compound **1** was less pronounced after 40 min, but remained statistically significant in various periods of the study (as compared to animals with hypovolemic shock).

Compound **2** in a dose of 5 mg/kg had no effect on hemodynamic parameters in rats. Treatment with this compound in a dose of 10 mg/kg was followed by a significant and persistent increase in sBP. The antihypotensive effect was 54% by the 20th minute (Fig. 2). The antihypotensive effect of compound **2** was less pronounced after 40 min, but remained statistically significant until the 60th minute (as compared to animals with hypovolemic shock).

The antihypotensive effect was observed 1-2 min injection of compound **3** in a dose of 5 mg/kg. By the 5th minute and during the follow-up period, sBP in animals of the treatment group was much higher than in rats not receiving this compound (Fig. 2). A strong hypotensive effect (more than 50% compensation for the decrease in sBP) persisted over 90 min of the study.

Compound **4** (5 mg/kg) caused a significant, but short-term antihypotensive effect. The increase in sBP was observed 1-2 min after injection and reached maximum by the 10th minute (63% of the hypotonic effect of blood loss; Fig. 2). The effect of this compoun†îbecame less significant after 20 min. By the 30th minute, sBP in most rats of this group did not differ from that in untreated animals.

Figure 3 shows the effect of compounds **1**-**4** on dBP.

Administration of compound **1** in a dose of 10 mg/kg was followed by a significant and persistent increase in dBP (20 min postinjection). A 99% compensation for the hypotonic effect of blood loss was

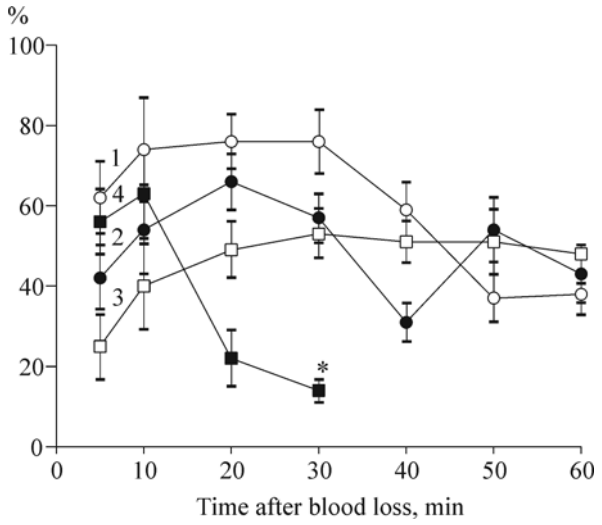

Fig. 2. Effect of ITU derivatives on sBP in rats with hypovolemic shock. Here and in Fig. 3: N-acetyl-S-isopropyl ITU hydrobromide (*1*); S-ethyl ITU diethyl phosphate (*2*); isopropyl ITU diisopropyl phosphate (*3*); N-acetyl-S-ethyl ITU hydrobromide (*4*). *No statistically significant differences. In other observations, the increase in BP after treatment with test compounds was statistically significant ($p<0.05$).

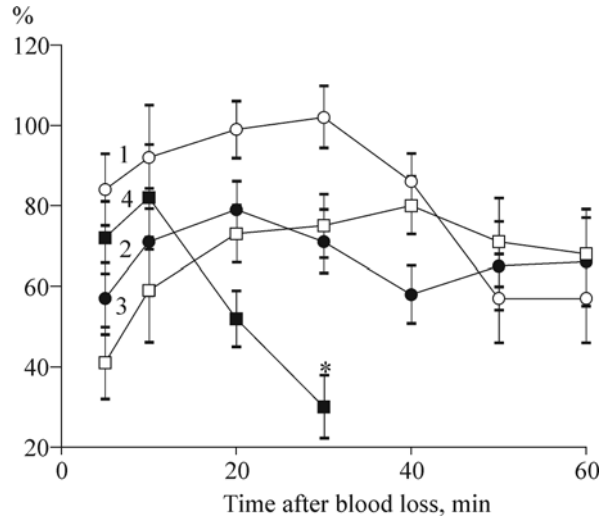

Fig. 3. Effect of ITU derivatives on dBP in rats with hypovolemic shock.

observed in the follow-up period (Fig. 3). The antihypotensive effect of compound **1** decreased progressively after 40 min, but remained statistically significant in various periods of the study (as compared to animals with hypovolemic shock).

Compound **2** in a dose of 10 mg/kg caused a significant and persistent increase in dBP (20 min postinjection, 79% of the hypotonic effect of blood loss; Fig. 3). The antihypotensive effect of compound **2** decreased progressively after 40 min, but remained statistically significant in various periods of the study (as compared to animals with hypovolemic shock).

TABLE 1. NO Inhibitory Activity of ITU Derivatives (M±m)

Compound, No.	Name	Dose, μmol/kg (mg/kg)	Relative content of NO	Percentage inhibition[1] (time of administration[2])
	LPS (control)	1.5 mg/kg	91±37	100
1	N-acetyl-S-isopropyl ITU hydrobromide	10 (2.4)	4±3	5*
2	S-ethyl ITU diethyl phosphate	31 (7.9)	4±3	4*
3	Isopropyl ITU diisopropyl phosphate	10 (3.0)	7±4	8*
4	N-acetyl-S-ethyl ITU hydrobromide	10 (2.3)	20±11	22*
5	Methyl ITU dimethyl phosphate	10 (2.2)	15±7	17*
6	N-acetyl-S-methyl ITU hydroiodide	10 (2.6)	13±4	14*

Note. *p<0.05 compared to the control. [1]Ratio of NO content in the liver of treated mice to NO content in the liver of LPS-receiving mice. [2]Compounds were administered 1 h before euthanasia of animals. Compounds were tested in 2-3 independent series. Each group consisted of 7 specimens.

The antihypotensive effect was revealed 1-2 min after injection of compound **3** in a dose of 5 mg/kg. By the 5th minute, dBP in rats of this group was much higher than in untreated animals (Fig. 3). A strong antihypotensive effect (70% compensation for the decrease in dBP due to hypovolemic shock) was observed for more than 90 min.

Compound **4** in a dose of 5 mg/kg caused a significant, but short-term antihypotensive effect (Fig. 3). The increase in dBP was observed 1-2 min after injection and reached maximum by the 10th minute (63% of the hypotonic effect of blood loss). The effect of compound **4** became less significant after 20 min. By the 30th minute, sBP in most rats of the treatment group did not differ from that in non-treated animals with hypovolemic shock.

Analysis of the effect of hypovolemic shock on HR showed that HR in intact animals (413 bpm) 5 min after blood withdrawal decreased to a level observed during hypovolemic shock (360 bpm). HR remained unchanged up to the 30th minute, but increased progressively and reached the control level by the 70th minute after treatment. Administration of test compounds after blood loss had little effect on study parameters. In various periods of the study (5-60 min), HR in rats of the treatment group differed from that in animals with hypovolemic shock only by 8-12%.

Similar results were obtained in studying the effect of hypovolemic shock on RR. In various periods of the study (5-90 min), only nimor changes were found in RR of animals receiving these compounds after blood loss (72-81 breaths per minute). This process was modified only by 10% after treatment with test compounds (statistically insignificant).

Analysis of S-alkyl derivatives of ITU showed that NO inhibitory activity of S-substituents increases in the following order: methyl<ethyl<isopropyl. Similar results were obtained in *in vitro* experiments with analogues of compounds **2**, **3**, and **5** in the system of enzyme release [9]. We found that the structure-activity dependence is observed for N-acetylated ITU under *in vivo* conditions.

Inhibitory activity was lowest for compounds **5** and **6** (Table 1). These compounds in various doses (5 and 10 mg/kg) were ineffective as antihypotensive drugs. However, compounds **1** and **3** with high NO inhibitory activity had greater antihypotensive effect.

We conclude that overproduction of NO and secretion of NO-induced hypotensive agents play a role in the decrease of BP during hypovolemic shock. Published data show that transcriptional expression of iNOS occurs over several hours [8]. Constitutive NOS (endothelial [NOS3] and neuronal [NOS1] forms) probably serve as a source of NO in the early period after blood loss.

Selective iNOS inhibitor (1400W) was shown to prevent liver injury in rats with experimental shock/resuscitation. However, the inhibition of a signal pathway (eNOS/protein kinase G (PKG)/vasodilation-stimulating phosphoprotein (VASP) induced by the antishock agent estrogen increases the severity of inflammation and lung injury [11]. Soluble guanylate cyclase (sGC) inhibitor methylene blue had little effect on the consequences of hemorrhage [10]. The other mechanisms mediated by NO are probably realized via transcriptional activator HIF-1α [15] and sympathetic nervous system [12].

The role of NO in adverse consequences of hemorrhagic shock is poorly understood, which makes it difficult to select the targets for pharmacological agents. The search for antihypotensive products of NO inhibitors (ITU derivatives) holds much promise

for the development of antishock agents that improve tissue perfusion and prevent the development of polyorgan insufficiency.

We are grateful to I. M. Fedosov for electron paramagnetic resonance spectroscopy with a spin trap. Information was provided by www.molbiol.ru.

This work was supported by the State Budget (issue No. 0120.0 602187), Russian Foundation for Basic Research, and Administration of the Kaluzhskaya oblast (project No. 07-04-97607).

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