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EPR INVESTIGATION OF *IN VIVO* INHIBITORY EFFECT OF GUANIDINE COMPOUNDS ON NITRIC OXIDE PRODUCTION IN RAT TISSUES

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The aim of the present study was to evaluate *in vivo* effects on NO production of pharmacologically widely used, commercially available NOS inhibitors, structurally related to guanidine. We compared the NO inhibitory potency and selectivity of L-NAME, aminoguanidine and guanabenz in tissues of normal and LPS-stimulated rats using ex vivo EPR measurements of the NO radical in its complex with dithiocarbamate-Fe(II). The tissues studied were the brain cortex, kidney, liver, heart and testis. Differential inhibitory effects were seen for L-NAME, aminoguanidine and guanabenz when applied during basal or LPS-stimulated conditions. Aminoguanidine exerted inhibition of NO only after stimulation with LPS. Guanabenz had little effect on NO in liver, kidney, testis and heart under normal conditions, while it reduced the basal NO in brain cortex. After stimulation with LPS guanabenz afforded a partial inhibition of the NO formation in all tissues studied. L-NAME was a potent inhibitor of NO synthesis in all tested tissues, both during basal and LPS stimulated conditions. Our results show that compounds containing a guanidine moiety might possess different NOS inhibitory profiles *in vivo*.

Key words: *nitric oxide, EPR, L-NAME, aminoguanidine, guanabenz*

INTRODUCTION

Over the last decades the free radical messenger molecule nitric oxide (NO) has become established as a key mediator in a wide range of biological processes. This includes muscle relaxation, cardiac function, neuronal activity and immune modulation (1, 2). NO is generated from L-arginine in enzymatic reactions catalysed by nitric oxide synthase (NOS) enzymes. Three NOS isoforms exist, which are named according to the cell type or condition in which they were originally identified (3). Thus, eNOS is the constitutively active isoform present in endothelium, nNOS is the neuronal isoform, and iNOS is the inducible isoform, which is expressed in macrophages after their activation with endotoxin, such as bacterial lipopolysacharide (LPS), or inflammatory cytokines.

The NOS isoforms are responsible for forming NO under many physiological and pathological conditions (4-6). It has been recognized that overproduction of NO can disturb cellular signalling, cause severe tissue damage, inflammation and degenerative diseases (7). Since disordered generation of NO is associated with severe pathological conditions, drugs for the pharmacological modulation of NO have been sought (3). However, the therapeutic benefice of agents that decrease NO levels is controversial as NO may exert both positive and negative effects on physiological condition and pathophysiological progressions (8).

A common pharmacological approach to study the role of a biological mediator is to investigate how processes related to it are affected by the administration of specific inhibitors. Thus, guanidines such as L-NAME and aminoguanidine (*Fig. 1*) are extensively used NOS inhibitors in pharmacological tests, where it is believed that their pharmacological effects are exerted by inhibition of NOS isoforms and a presumed lowered NO content in tissues (9, 10). In addition, the clinically used antihypertensive guanidine compound guanabenz (*Fig. 1*) has been shown to have inactivating effects on nNOS (11). However, although there is evidence from in vitro studies for the inhibitory

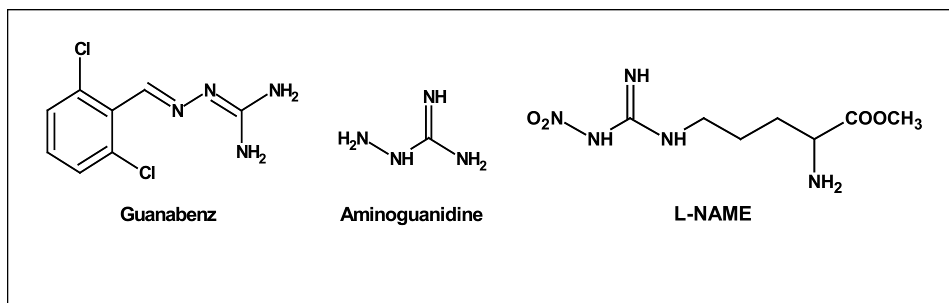

Fig.1. Structural formulas of guanidine compounds

effects of this guanidine, it has not clearly been demonstrated to affect NO contents in tissues *in vivo*.

Electron paramagnetic resonance (EPR) is an efficient and powerful method for the direct monitoring of NO radical formation in tissues (12, 13). The aim of the present study was to compare the potency and selectivity of L-NAME, aminoguanidine and guanabenz as inhibitors of NO production using *ex vivo* EPR measurements of NO in its complex with diethyldithiocarbamate-Fe(II) in the brain cortex, kidney, liver, heart and testis of the rat.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250-300 g were housed under standard conditions (21-23°C, 12 h light-dark cycle) with unlimited access to food and water. All experimental procedures were performed in accordance with the regulations of the Animal Ethical Committee of BaltLASA.

Materials

Lipopolisaccharide (LPS, *Escherichia coli* 055:B5), sodium diethyldithiocarbamate (DETC), $FeSO_4$, sodium citrate, Nω-nitro-L-arginine methyl ester (L-NAME), aminoguanidine, and guanabenz were purchased from Sigma (St. Louis, MO, USA). All drugs were dissolved in sterile saline (Grindex, Latvia) just before the experiments.

Experimental protocol

Determination of the content of NO in rat tissues was carried out according to the EPR method developed by Vanin et al., essentially as described previously (14, 15). The experiment was started by *i.p.* administration of NOS inhibitors, i.e., L-NAME (50 mg/kg), aminoguanidine (50 mg/kg) and guanabenz (1 mg/kg). Normal control animals received *i.p.* injections of saline. In LPS treated groups, rats received *i.p.* 20 mg/kg of LPS before NOS inhibitor administration. Spin trap reagents were administered 3.5 hrs after drug administration. Thus, all animals received *i.p.* injections of DETC (400 mg/kg) followed by *s.c.* injections of ferrous citrate, prepared directly in the syringe just before use (40 mg/kg ferrous sulphate + 200 mg/kg sodium citrate), and further 0.5 hrs later animals were decapitated. The tissues (brain, kidney, heart, liver and testis) were dissected and immediately frozen in liquid nitrogen.

Sample preparation and EPR recordings

The frozen tissues were slightly thawed to allow sample preparation by compacting the tissues into plastic tubes to form rods, 20 mm long and 4 mm in diameter, which were then immediately frozen in liquid nitrogen. EPR spectra of the samples were recorded at liquid nitrogen temperature using an EPR spectrometer Radiopan SE/X2544 (Poland) as described previously (15). Measurement parameters were as follows: X-band operation, 25 mW microwave power, 9.24 GHz microwave frequency, 100 kHz modulation frequency, 5 G modulation amplitude, $0.5x10^4$ receiver gain, time constant 1 second. The content of NO was computed from the third component (Ix) at g=2,031 (*Fig 2*). The NO concentration (ng/g of tissue) was calculated according Vanin et al. (16).

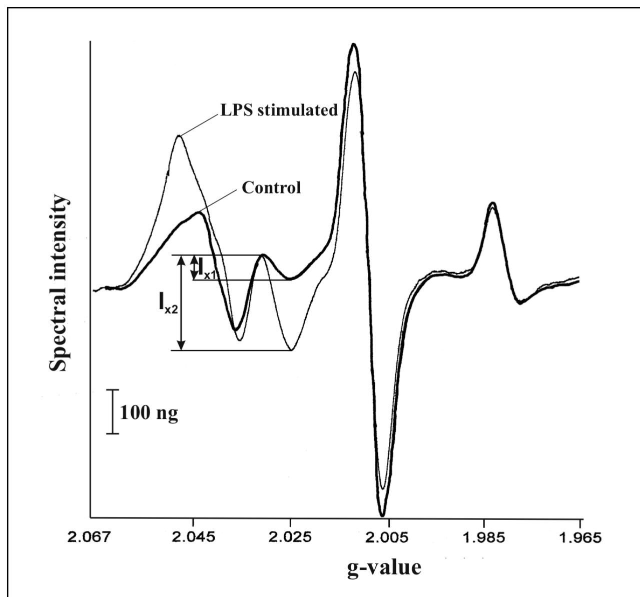

Fig.2. Representative EPR spectra of brain cortex tissue Cu-DETC complex with a superposed NO peak

RESULTS

After injection of spin trapping reagents in accordance with established techniques we recorded characteristic NO radical EPR spectra (*Fig. 2*). Thus, in all studied rat tissues the NO radical trapped with scavenger DETC and ferrous citrate, was seen as an intense and persistent EPR signal. The brain cortex, heart, kidney, liver and testis samples were prepared 30 min after injection of spin trapping reagents and yielded the typical shape of the Cu-DETC spectrum, with a superposed NO peak (*Fig. 2*) (17).

As can be seen from Fig. 3, the highest NO contents of normal control rat tissues were found in the cortex and liver (41.5 ± 3.2 and 21.4 ± 3.4 ng/mg tissue, respectively). Testis, kidney and heart tissues contained less NO (*Fig. 3*). After injection of guanidines the basal NO contents of tissues changed to different extent depending on which compound had been administered. The non-specific inhibitor of NOS, L-NAME, significantly reduced NO in the cortex, liver and kidney. After treatment with aminoguanidine we found significantly lowered NO contents only in the liver. Guanabenz, on the other hand, inhibited basal NO formation only in the cerebral cortex (*Fig. 3*).

LPS is known to stimulate the production of NO (18). Here we obtained a 5-81-fold increase of the NO content in all tissues after administration of LPS 20 mg/kg *i.p.* compared to the basal control levels (*Table I*). The highest stimulation level of NO was observed in the liver, 1442 ± 128 ng/kg (*Fig. 4*). In heart and kidney LPS increased the NO concentration 55 and 81-fold, respectively. Comparatively weak stimulation (5-fold) was seen in the brain cortex.

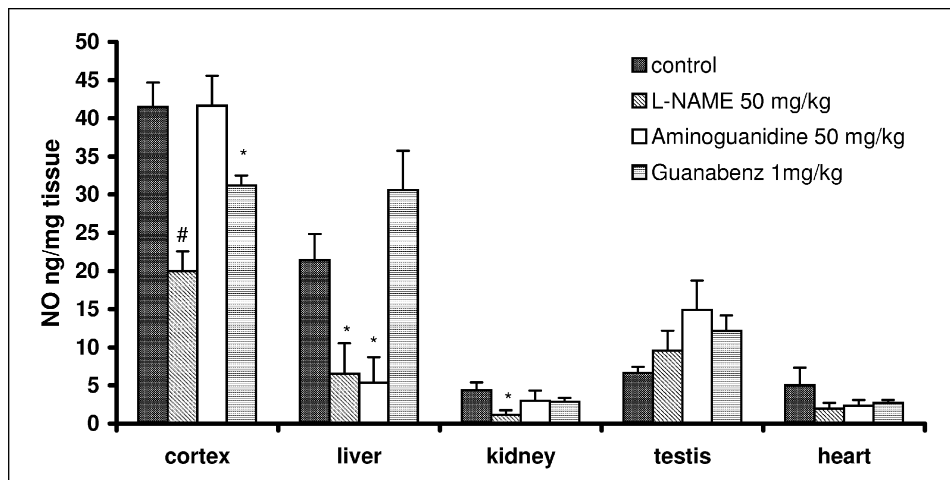

Fig.3. Nitric oxide concentration in tissues of normal rats. Data are present as mean ± SEM. *Significantly different from control, p<0,05. # Significantly different from control, p<0,01

All guanidines reduced significantly the NO contents in the LPS-stimulated rat tissues (*Fig. 4*). L-NAME and aminoguanidine showed here similar potency. In some cases the guanidines inhibited the NO levels so that they became not significantly different from the non-stimulated control levels of respective tissue. This was seen in the cerebral cortex and heart in case of NAME, and in the cortex and testis in the case of aminoguanidine (*Table I*). In liver and kidney the NO contents were lowered about 4 times by both L-NAME and aminoguanidine

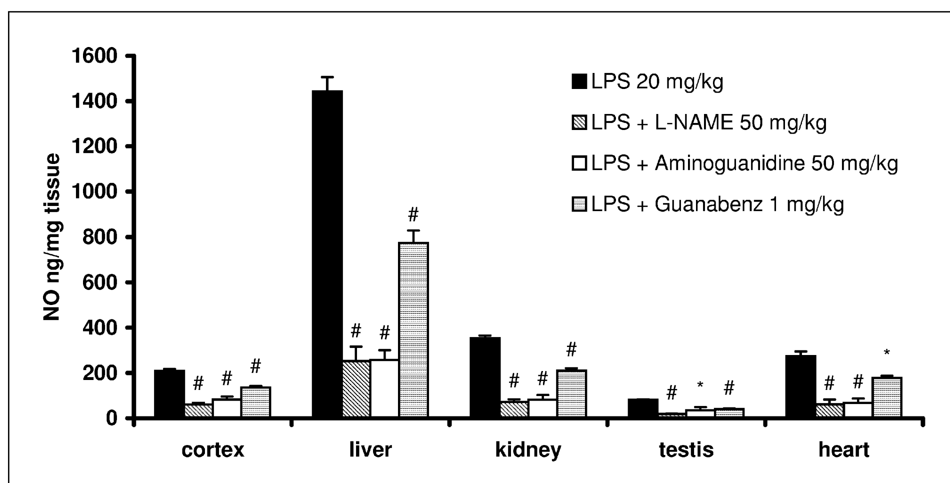

Fig.4. Nitric oxide concentration in tissues of LPS treated rats. Data are present as mean ± SEM. *Significantly different from LPS, p<0,05. # Significantly different from LPS, p<0,01.

Table I. Elevated NO levels in LPS treated rats (n-fold stimulation of normal control)

	Control	LPS	L-NAME	Aminoguanidine	Guanabenz
cortex	1	5	1*	2*	3
liver	1	67	12	12	36
kidney	1	81	17	19	48
testis	1	12	3	5*	6
heart	1	55	12*	14	36

* $p>0.05$ versus normal control

compared to the LPS control levels (*Fig. 4, Table I*). Guanabenz also inhibited the LPS-stimulated NO production in all tissues, though to a somewhat lesser extent than the other guanidines (*Fig. 4*). We observed a slight increase of the NO content in non-stimulated rat testis after administration of inhibitors; however it was found to be non-significant due to the notable variations of NO content in testis of intact animals (*Fig. 3*).

DISCUSSION

Of the methods available for measuring the free radical formation, EPR spin trapping is one of the most appropriate and sensitive. Its greatest advantage is that the measurements are performed directly on the trapped radical, rather than by the indirect measurements of its non-radical product(s), which route of formation may be uncertain. It must be mentioned that in animal tissues the accumulation of the trapped NO that can be detected by EPR has a dynamic character, which is determined by the rate of complex formation and its transformation into EPR silent forms after reaction with superoxide and peroxynitrite (20, 21). However, the method can still be used to compare the effect of different compounds on NO generation under identical experimental set-ups, i.e., using constant LPS, DETC and iron concentration for NO trapping.

The aim of the present study was to evaluate the inhibitory effect *in vivo* for pharmacologically widely used, commercially available NOS inhibitors structurally related to guanidine. Many studies have been described using aminoguanidine as an iNOS specific inhibitor, and L-NAME (being a false substrate to NOS) as a NOS isoform non-specific inhibitor (10, 19). The mechanism of inhibition of NOS by guanidines is thought to be due to their binding to heme iron at the catalytic site or competitive binding with L-arginine (22). It is presumed that at certain concentrations the substances would possess distinct NOS inhibitory profiles. However, *in vivo* effects of aminoguanidine and L-NAME have not been compared and reported using the accurate EPR approach for radical measurements, as far as we know. Moreover, we wanted to evaluate

the effect of another guanidine compound, clinically used antihypertensive, guanabenz, in the same experimental set-up.

Aminoguanidine is known as a potent inhibitor of iNOS, while the eNOS responsible for the vascular control is only weakly inhibited by aminoguanidine (23). This pattern of selectivity of NOS isoforms is in line with its reported *in vivo* effects showing that 10 mg/kg aminoguanidine has no effect on mean arterial blood pressure, while a similar dose of L-NAME elevates blood pressure (24). In the present study the aminoguanidine treatment inhibited significantly LPS-induced NO production in all tissues. In control animals, aminoguanidine did not influence significantly the NO content in rat brain cortex, kidney, testis and heart. However, in the liver it exerted a similar inhibitory effect as L-NAME. These patterns can be explained by the presence of some expression of iNOS in the liver tissues due to natural exposure of the rats to some inflammatory agent (25). Thus, we show that aminoguanidine may be used as a selective inhibitor of LPS-induced NO formation *in vivo*. L-NAME, which is a non-selective inhibitor of NOS isoforms, inhibited the formation of NO during basal conditions in the cortex, liver and kidney (*Fig. 3*). However, in rat testis and heart its effect was not significant.

Guanabenz is known as a classical centrally acting antihypertensive (26, 27). In our hands guanabenz possessed an activity that resembled that of aminoguanidine, i.e., it inhibited the LPS-stimulated NO production. Even though it has been claimed that administration guanabenz at a dose of 0.5 mg/kg daily for 4 days may inhibit the activity of penile NOS tested in tissue homogenates (11), we show here that guanabenz does not significantly inhibit NO formation in peripheral tissues after its single dose administration at 1 mg/kg. Guanabenz has been shown to act as a metabolism-based irreversible inactivator of neuronal NOS, enhancing the proteolytic turnover of the enzyme *in vitro* (28). Here we showed that guanabenz inhibited NO in rat brain cortex. This inhibition was the only statistically significant effect observed by guanabenz during the basal conditions of LPS-non-treated control animals (*Fig. 3*).

Numerous NO inhibitors have been developed and used to study diverse sets of NO functions using various pharmacological and biochemical test set-ups. Although these studies give valuable information it has to be kept in mind that the *in vivo* and *in vitro* effect of a compound can differ substantially. Compounds may be subjected to metabolism, and an apparently specific inhibitor *in vitro* may exhibit additional effects when applied *in vivo*. To prove that the effect of certain drug is in fact directed onto NO it is required that one investigates both its effect on the NOS enzymes *in vitro*, as well its effects on NO production *in vivo*. Our data here show that some guanidine derivatives reduce the levels of NO *in vivo* during stimulated and non-stimulated condition.

In conclusion, our results confirm the selective inhibition of the LPS-inducible NO formation by aminoguanidine *in vivo* in the rat. Under normal conditions guanabenz has little effect on NO in liver, kidney, testis and heart, while it inhibits

NO in brain cortex. After stimulation with LPS, guanabenz affords a partial inhibition of NO formation in the tissues tested. L-NAME was a potent inhibitor of NO production in all tested tissues, both during basal and stimulated conditions. Our results indicate that compounds containing guanidine moiety might have different NOS inhibitory profiles *in vivo*.

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