AUGMENTATION OF THE NO-CGMP CASCADE INDUCES ANXIOGENIC-LIKE EFFECT IN MICE

Several studies have reported the anxiolytic-like effects of various nitric oxide synthase inhibitors in distinct animal models. However, in the context of anxiety, the possible involvement of cyclic GMP, believed to be one of the main targets of NO, remains obscure. Cyclic GMP is degraded by the specific phosphodiesterases in the brain. Therefore, we studied the effect of the selective phosphodiesterase type 5 inhibitor sildenafil in the mouse elevated plus-maze test of anxiety and in the open field test of locomotion. We found that sildenafil (0.05-10 mg/kg i.p.) alone did not affect the behavior of animals in the plus-maze or open field tests, but the anxiogenic beta-carboline DMCM given in a subconvulsive dose (2 mg/kg i.p.) decreased the time spent on open arms in the elevated plus-maze. Treatment with the NO precursor L-arginine (200 mg/kg i.p.) did not modify the behavior of animals in the plus-maze, however, when sildenafil (1 mg/kg i.p.) was administered in combination with L-arginine (200 mg/kg i.p.), both the time spent on the open arms and the percentage of open arm visits were significantly decreased. We conclude that augmentation of the NO-cGMP cascade induces anxiogenic-like effect in mice.

Key words: nitric oxide, sildenafil, anxiety, plus-maze

INTRODUCTION

Several studies have implicated nitric oxide (NO) in the regulation of anxiety, but the evidence is still contradictory (1,2). Most of these studies have described the effects of various nitric oxide synthase (NOS) inhibitors in the tests of anxiety. Both anxiolytic- (3) and anxiogenic-like (2,4) effects have been reported
with NOS inhibitors. Much less is known about the consequences of increasing the functioning of NO in the brain. Systemic treatment of animals with the NO precursor molecule L-arginine has been shown to increase the production of NO in the brain (5) but does not modify the behavior in the elevated plus-maze test (1). On the other hand, intracerebral injection of distinct NO-releasing compounds has been shown to induce flight reaction in rats (6), but NO-donor SIN-1 had anxiolytic-like effect in mouse light-dark compartment test (7).

Guanylate cyclase is regarded as a principal target for NO. Whether the effects of NO on the regulation of anxiety are mediated via cyclic GMP (cGMP) is vaguely characterized. The guanylate cyclase inhibitor methylene blue induces a potent anxiolytic-like effect in the plus-maze paradigm, but also acts as a NOS inhibitor in vitro and in vivo (8-10). Sildenafil is a selective phosphodiesterase type 5 (PDE-5) inhibitor increasing the cGMP level in the target tissues, and is widely used in clinical practice to treat erectile dysfunction (11). However, PDE-5 is expressed in different brain regions (12), and inhibition of PDE-5 increases the release of glutamate and aspartate in the nucleus accumbens (13). Animal as well as human studies have suggested that sildenafil may enhance ability to focus attention and improve memory retention (11,14,15).

Elevated plus-maze is a well established method for detecting anxiogenic- or anxiolytic-like effects of drugs (16). We hypothesized that systemic treatment with sildenafil may affect the behavior of mice in the anxiety model, and the aim of the current study was to elucidate the possible effect of sildenafil alone and in combination with the NO precursor L-arginine on the behavior of mice in the elevated plus-maze. Anxiogenic beta-carboline DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate, 2 mg/kg i.p.) was used as a positive control for anxiogenic-like effect. To exclude the non-specific effects of drugs on the locomotor activity the open-field test of locomotor activity was performed.

MATERIAL AND METHODS

Animals

Male NIH mice (National Public Health Institute, University of Kuopio) weighing 20-25g were used. Mice were kept 10 per cage in an animal house under standard conditions. All animal procedures were accepted by the University's Committee for Ethics in Animal Experimentation and complied with "Principles of laboratory animal care" (NIH publication 25-28, 1996).

Mouse elevated plus-maze test

The apparatus and procedure were as described earlier (1). During a 5-minute observation period, the following parameters were measured: number of open arm entries, time spent on open arms and number of closed arm entries. Subsequently, the percentage of the number of entries into the open arms out of the total number of entries into all arms was calculated.
Open field test of locomotor activity

Locomotor activity was measured immediately after plus-maze testing using an automated system with 6 chambers (45x45x45 cm) made from transparent acrylic (MOTI, Technical & Scientific Equipment GMBH, Germany). The apparatus-naive mice were put into the chamber, and vertical and horizontal activity was counted during a 10-minute test period. All the experiments had between subjects design.

Drugs

L-arginine hydrochloride and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) were obtained from RBI (USA). Sildenafil citrate was donated by Pfizer Global Research and Development. L-arginine hydrochloride was dissolved in saline. Sildenafil and DMCM were dissolved in 100μL of 0.1M HCl and made up to final volume with saline. The vehicle also contained appropriate amount of 0.1M HCl. All drugs were freshly prepared and given intraperitoneally (i.p.) in a volume of 0.1 ml per 10 g body weight of mice. Doses of drugs were chosen according to previous studies (1,11,14,15,17).

Sildenafil was administered 30 or 35 min prior to testing and L-arginine 25 min prior to testing in the elvated plus-maze. When sildenafil and L-arginine were combined, the drugs were injected into opposite sides of the peritoneal cavity.

Statistics

Data were statistically treated using one-way or two-way analysis of variance (ANOVA). Post hoc comparisons between individual groups were performed by Newman-Keuls test. Data are expressed as the mean values ± S.E.M. Differences were considered to be statistically significant when p was less than 0.05.

RESULTS

Effects of DMCM and sildenafil on behavior in the elevated plus-maze and open field test of locomotor activity

Treatment with sildenafil or DMCM had a significant effect upon time on open arms (F_{5,70}=7.8, p<0.05), and number of total arm entries (F_{5,70}=5.4, p<0.001) in the elevated plus-maze. The anxiogenic beta-carboline DMCM given in a subconvulsive dose (2 mg/kg) but not sildenafil significantly decreased the time spent on open arms (P<0.01, Newman-Keuls test, Fig 1a) and total number of arm entries (p< 0.01). Sildenafil did not modify the behavior of animals in the open field test of locomotor activity (Fig 1b), but DMCM decreased both horizontal (p<0.001) and vertical (p<0.001) activity of animals.

Effect of co-treatment with L-arginine and sildenafil on behavior in the elevated plus-maze and open field tests

In a separate experiment the effect of co-treatment with sildenafil (1 mg/kg) and L-arginine (200 mg/kg) was studied in the elevated plus-maze (Fig 2a). Two-way ANOVA of time spent on open arms indicated a significant effect of L-
arginine (F \(_{1,35}=8.2\), p<0.01), sildenafil (F \(_{1,35}=1.47\), p=0.23), L-arginine x sildenafil interaction (F \(_{1,35}=2.37\), p=0.13). Post-hoc comparisons revealed that sildenafil and L-arginine given alone were without effect but in combination induced decrease in time spent on open arms (p<0.05 compared to all other groups). Two-way ANOVA of percentage of open arm entries indicated a significant effect of L-arginine (F \(_{1,35}=6.57\), p<0.05), sildenafil (F \(_{1,35}=1.63\), p=0.21), L-arginine x sildenafil interaction (F \(_{1,35}=3.80\), p=0.059). Post-hoc comparisons revealed that sildenafil and L-arginine given alone were without effect but in combination decreased percentage of open arm entries (p<0.05 compared to control and sildenafil groups, p=0.059 compared to L-arginine group). Treatment did not influence the activity of animals in the open field (Fig 2b).

**DISCUSSION**

The main finding of the current study is that sildenafil alone had no effect on the behavior of mice in the elevated plus-maze test, but induced a robust...
anxiogenic-like action when given in combination with NO precursor L-arginine. This effect was even more pronounced than that of the anxiogenic beta-carboline DMCM in the elevated plus-maze. The finding is unlikely to be due to the unspecific effect of drug combination on locomotion, since it did not modify the behavior of animals in the open field. There are no studies characterizing the effect of systemic treatment with sildenafil on the brain cGMP level, but sildenafil (3 mg/kg i.p.) has been shown to facilitate retention of an inhibitory avoidance response (15). We cannot, however, totally exclude the possibility of cGMP-independent effects of sildenafil on behavior as it has been shown in vitro that the drug may have other effects besides inhibiting PDE 5 (18). As the NO precursor L-arginine (150 and 300 mg/kg) has been shown to increase NO synthesis in the brain after systemic administration (5), it appears that only the simultaneous increase in NO synthesis and inhibition of cGMP degradation in the brain results in anxiogenic-like effect.

Our results are in accordance with the suggested role of NO in the regulation of anxiety as an anxiogenic mediator (1,3,19) and also demonstrate that cGMP

Figure 2
a. Effect of co-treatment with sildenafil (1 mg/kg) and L-arginine (200 mg/kg) in the elevated plus-maze test (n=9-10). Sildenafil was injected 35 and L-arginine 25 min prior to testing. Results are expressed as mean ± S.E.M. *P< 0.05, versus all other groups. †P< 0.05, versus vehicle and sildenafil groups.

b. Effect of co-treatment with sildenafil (1 mg/kg) and L-arginine (200 mg/kg) in the open field test (n=9-10). Sildenafil was injected 40 and L-arginine 30 min prior to testing. Results are expressed as mean ± S.E.M.
is involved as a mediator of NO induced anxiety. Lack of the effect of L-arginine given alone on behavior of animals replicates previous findings. Thus we and other groups have shown that L-arginine (16-600 mg/kg i.p) does not affect the behavior of animals in the plus-maze paradigm (1,19-21). Interestingly, L-arginine (100 mg/kg) was able to block the anxiolytic-like effect of diazepam in the plus-maze test (20). Why sildenafil or L-arginine alone had no effect on the behavior in the elevated plus-maze test remains to be determined, but it may reflect the fact that the activity of the NO-cGMP cascade is under physiological conditions limited by strict negative feedback mechanisms. Further studies will demonstrate whether systemically given sildenafil is capable of decreasing NOS activity in the brain. The reason why some NOS inhibitors induced anxiogenic- (2,4) and NO-donor anxiolytic-like effect in animal studies (7) remains unclear. It is possible that the distinct NOS inhibitors possess different selectivity towards NOS isoforms. Thus, we have hypothesized that nNOS may be the principal target for anxiolytic- and eNOS for anxiogenic-like effect (22).

Interestingly, possible CNS side effects of sildenafil have been suggested also in patients (for review see (11). In a recent report, the appearance of tonic-clonic seizures in 2 patients after taking sildenafil was described (23). This anecdotal report is intriguing, since many anxiogenic substances also induce seizures in high doses. We speculate that anxiogenic or panicogenic side effects of sildenafil may appear in patients with anxiety disorders or in susceptible persons.

We conclude that the augmentation of the NO-cGMP cascade induces anxiogenic-like effect in mice, further supporting the role of NO in the regulation of anxiety.

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