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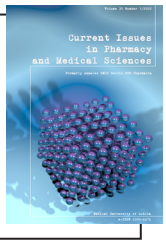

The role of the L-arginine-NO-cGMP pathway in the development of tolerance to mephedrone-induced hyperlocomotion in mice

Gabriela Bielecka-Papierz[1*], Ewa Poleszak[2], Aleksandra Szopa[3], Joanna Listos[4], Jolanta Orzelska-Gorka[5], Małgorzata Jakobczuk[5], Kamila Baluk[5], Sylwia Talarek[5], Anna Serefko[3]

[1] Chair and Department of Applied and Social Pharmacy, Medical University of Lublin, Poland
[2] Laboratory of Preclinical Testing, Chair and Department of Applied and Social Pharmacy, Medical University of Lublin, Poland
[3] Department of Clinical Pharmacy and Pharmaceutical Care, Medical University of Lublin, Poland
[4] Department of Pharmacology and Pharmacodynamics, Medical University of Lublin, Poland
[5] Department of Pharmacology and Pharmacodynamics, Medical University of Lublin, Poland

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ABSTRACT

The tendency of a psychostimulant to increase locomotion in rodents is considered to be associated with its addictive properties. Mephedrone, one of the most popular psychoactive substances used recreationally, is known to enhance locomotor activity in mice, but little is known about the potential development of tolerance to its central effects. In the present study, we decided to evaluate the possible involvement of the L-arginine-NO-cGMP pathway in the development of tolerance to mephedrone-induced hyperlocomotion. Experiments were performed on adult male Albino Swiss mice, and the locomotor activity was measured automatically. Our work indicated that a 5-day administration of L-NAME (25 or 50 mg/kg/day), methylene blue (5 or 10 mg/kg/day), and L-arginine hydrochloride (i.e., 250 mg/kg/day) prevented the development of tolerance to mephedrone-induced (5 mg/kg/day) hyperlocomotion, whereas treatment with L-arginine hydrochloride at a dose of 125 mg/kg/day potentiated the development of tolerance to this central effect of mephedrone. Summarizing, our data revealed that the L-arginine-NO-cGMP pathway contributes to the development of tolerance to mephedrone's central effects since inhibition of this signalling via blocking of NOS or NO-stimulated sGC prevented the development of tolerance to mephedrone-induced hyperlocomotion. As for cGMP-regulated phosphodiesterases, most probably they are not involved in these mechanisms.

INTRODUCTION

Mephedrone (4-methylmethcathinone, 4-MMC), one of the most popular psychoactive substances used recreationally, is a synthetic cathinone, and its chemical structure is quite similar to that of agents from the phenylethylamine family. In fact, mephedrone shares many neuropharmacological and functional properties with methamphetamine (METH) and methylenedioxymethamphetamine (MDMA, 'ecstasy'), but the exact mechanism of mephedrone's action, as well as its neurotoxicity have not been fully described yet. This synthetic cathinone has been reported to cross the blood-brain barrier easily. Moreover, research indicates that it acts as a nonselective substrate for the monoamine plasma membrane transporters and causes a blockage of dopamine, norepinephrine, and serotonin uptake, with a higher affinity to the latter [1-3]. It also has been demonstrated that mephedrone enhances the release of the above-mentioned monoamines [1,4,5], which, in consequence, elevates their levels in the synapses [6].

Eshleman *et al*. [7] showed that mephedrone influences the serotonin receptors, being an agonist of the 5-HT1A receptors and an antagonist of the 5-HT_{2A} and 5-HT_{2C} receptors, but it does not directly affect the dopamine receptors. Generally, mephedrone seems to exert preferential effects on the serotonergic pathway in comparison to the dopaminergic [8]. A high affinity of mephedrone to the α_{1A} and α_{2A} adrenergic

* **Corresponding author**
e-mail: gabriela.bielecka@umlub.pl

receptors was also reported by Simmler *et al*. [9]. Interacting with the monoamine neurotransmission, mephedrone promotes stimulant and sympathomimetic effects, including euphoria, general stimulation, mood elevation and increased sexual desire (for review, see Papaseit *et al*., [10]).

Like several other cathinones, mephedrone is known to enhance locomotor activity in mice [2] and rats [11-13] and it is able to potentiate the locomotor-stimulant effects of cocaine [14]. Recently, Gregg *et al*. [15] focused on the fact that mephedrone exists in a form of two enantiomers. The activity of these forms differs significantly. S-mephedrone produces greater serotonergic effects, whereas R-mephedrone displays a dopaminergic action implicated in both rewarding effects and locomotor activation. Of note, tendency of a psychostimulant to increase locomotion in rodents is considered to be strongly associated with its addictive properties [16].

Whereas tolerance to the central effects of other psychostymulants is a widely-described phenomenon, little is known about a potential development of tolerance to effects induced by mephedrone. Tolerance is observed after repeated doses of MDMA [17,18], amphetamine [19], or cocaine [20]. Literature data also gives several examples of the development of tolerance to the effects of different cathinone stereoisomers [21-23] in pre-clinical studies. Furthermore, Schechter [24] revealed the development of tolerance to cathine, a substance chemically similar to cathinone.

For the time being, there are only few reports about the development of tolerance to mephedrone-induced effects in animals [13,25,26]. An increased tolerance to mephedrone effects, for example, has been detected in humans [27,28]. Based on literature data, alterations in the dopaminergic system [25] and endogenous kappa opioid mechanisms [29] most probably contribute to the development of tolerance to some mephedrone activity. However, it would be interesting to know whether other neurotransmissions are engaged in this process as well.

In the present study, we decided to evaluate the potential involvement of the L-arginine-nitric oxide (NO)-cyclic guanosine 3′,5′-monophosphate (cGMP) pathway in the development of tolerance to the central effects of mephedrone. NO is an important messenger molecule in the brain since it takes part in the regulation of neuronal excitability, synaptic plasticity, learning, emotions, seizure activity, drug tolerance, and many other brain processes [30,31]. Previously, our research team demonstrated that this NO-dependent signalling is implicated in the development of tolerance to diazepam [32] and flunitrazepam [33], whereas Mansouri *et al*. [34] and Ozdemir *et al*. [35], confirmed its role in morphine tolerance. NO is synthesized from L-arginine by NO synthase (NOS).

Many physiological effects of NO are mediated *via* its interaction with soluble guanylyl cyclase (sGC) and a subsequent increase of cGMP expression. For its part, cGMP affects the activity of cGMP-dependent kinases, cGMP-gated ion channels, and cGMP-regulated phosphodiesterases (PDE) (Bruckdorfer, 2005). In view of the above, we selected the following substances that differently affect NO pathways for our experiments: (i) Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) – a non-selective inhibitor of NOS; (ii) methylene blue – an inhibitor of NO-stimulated sGC, being also a NOS inhibitor; (iii) L-arginine hydrochloride – an endogenous precursor of NO; and (iv) sildenafil – an inhibitor of PDE type 5 (PDE5; i.e. an enzyme that promotes degradation of cGMP).

MATERIALS AND METHODS

Animals

All experiments were performed on naïve adult male Albino Swiss mice (20-32 g). The animals were kept in rooms with controlled temperature (22±1°C) and a 12-h light/dark cycle. The mice had *ad libitum* access to water and food. Each experimental group consisted of 8-10 subjects. All procedures were approved by the Local Ethics Committee and carried out in accordance with binding European and Polish law.

Drug administration

The following agents were used in the experiments: mephedrone (4-methyl methcathinone, 4-MMC, Toronto Research Chemicals Inc., Canada), Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma-Aldrich, USA), methylene blue (Sigma-Aldrich, USA), L-arginine hydrochloride (Sigma-Aldrich, USA), and sildenafil citrate (Sigma-Aldrich, USA). All were dissolved in saline before use and injected intraperitoneally (*i.p*.). Mice from control groups were given saline (for 6 days) or a higher dose of a respective tested agent (for 5 days). The latter control groups were added in order to confirm that neither L-NAME, methylene blue, L-arginine hydrochloride nor sildenafil citrate influenced *per se* locomotion. The volume of all administered solutions was 10 ml/kg.

Procedures

In order to develop tolerance to the mephedrone-induced hyperlocomotion, 5 mg/kg of this agent was given once a day for 6 consecutive days. A substance affecting the L-arginine:NO:cGMP pathway, that is L-NAME (25 or 50 mg/kg), methylene blue (5 or 10 mg/kg), L-arginine hydrochloride (125 or 250 mg/kg) or sildenafil citrate (5 or 10 mg/kg), was administered for 5 consecutive days 10 min before the mephedrone injection. On the 6th day of the experiment, test mice received only mephedrone. The behavioural test was performed 20 min after administration of mephedrone. All doses and pretreatment schedules were selected on the basis of literature data and the results of previous experiments performed in our lab [11,22,36].

Locomotor activity of animals was measured on the 1st and the 6th day of the experiment, and was conducted in actimeter cages (Multiserv, Lublin, Poland; 32 cm in diameter, two light beams). Herein, mice movements were detected by photocell beams and recorded automatically. Animals were placed in cages individually and results were assessed after 10 and 30 min. All experiments were carried out according to the National Institute of Health Guidelines for the care and use of laboratory animals and the European Council Directive on 24 November 1986 for Care and Use of Laboratory Animals (86/609/EEC), and were approved by the Local Ethics Committee

Statistical analysis

The obtained data was evaluated either by one-way or two-way analysis of variance (ANOVA) followed by the Tukey's *post hoc* test, depending on the behavioural study. Results were regarded as statistically significant when $p<0.05$.

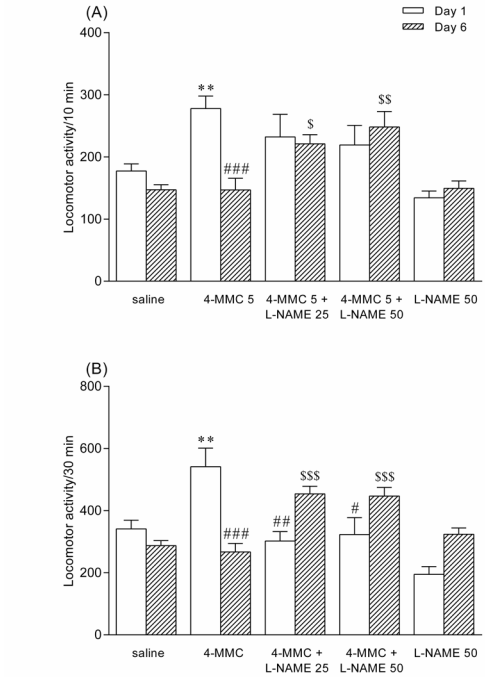
RESULTS

Influence of a 5-day administration of L-NAME or methylene blue on the development of tolerance to the mephedrone-induced hyperlocomotion

An acute administration of mephedrone (5 mg/kg) on the 1st day of our experiment increased locomotor activity of the tested animals, as compared to the saline-treated group. After 6 consecutive days of mephedrone treatment at a dose of 5 mg/kg/day, the mice were also less active, as compared to the outcomes obtained on the 1st day. These findings confirmed the development of tolerance to the mephedrone-induced hyperlocomotion (Fig. 1 and 2). The statistical analysis of the obtained results supported these observations. Two-way ANOVA indicated a significant treatment-time period interaction [$F(1,28)=10.68$; $p=0.0029$] with a significant effect of the introduced treatment [$F(1,28)=10.63$, $p=0.0029$] and a significant effect of time period [$F(1,28)=27.39$, $p<0.0001$] for a 10-min measurement, as well as a significant treatment-time period interaction [$F(1,28)=8.97$; $p=0.0057$] with a significant effect of the introduced treatment [$F(1,28)=5.99$, $p=0.0209$] and a significant effect of time period [$F(1,28)=19.98$, $p=0.0001$] for a 30-min measurement.

On the 6th day of the experiment, we found out that a 5-day administration of L-NAME (25 or 50 mg/kg/day) prevented the development of tolerance to the mephedrone-induced hyperlocomotion, independently of the fact whether the assessment was carried out for 10 or for 30 min (Fig. 1). According to one-way ANOVA, differences between the tested groups were significant: $F(4,32)=8.581$; $p=0.0003$ (when the locomotor activity was measured for 10 min) and $F(4,32)=17.08$; $p<0.0001$ (when the locomotor activity was measured for 30 min). Moreover, the statistical analysis also revealed that on the 1st day of the experiment an acute co-injection of L-NAME (25 or 50 mg/kg) and mephedrone (5 mg/kg) led to a significant decrease in locomotion when the 30-min period of time was taken into consideration (one-way ANOVA: $F(4,32)=5.952$; $p=0.0028$). Both an acute and a 5-day administration of L-NAME (50 mg/kg) did not affect locomotion of the tested mice ($p>0.05$).

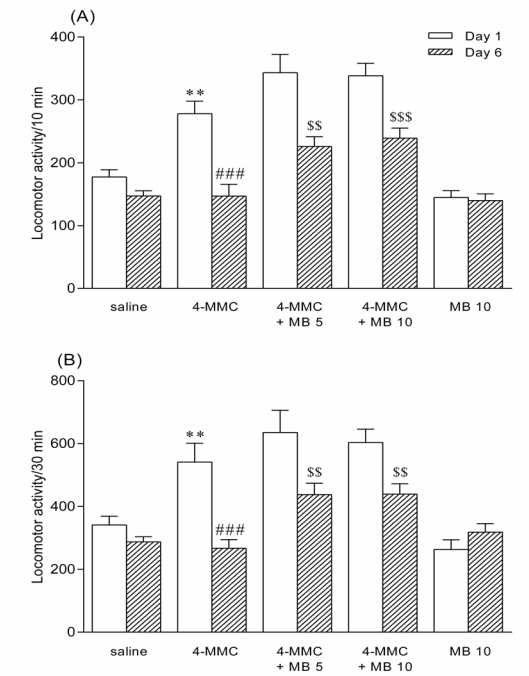
Co-administration of methylene blue (5 or 10 mg/kg/day) with mephedrone (5 mg/kg/day) prevented the development of tolerance to the mephedrone-induced hyperlocomotion when assessed on the 6th day of the experiment. Following one-way ANOVA analysis, significant differences between



The values represent mean + SEM (n = 8 animals per group).
**p < 0.01 versus saline on the Day 1; #p < 0.05, ##p < 0.01, ###p < 0.001 versus 4-MMC on the Day 1; $p < 0.05, $$p < 0.01, $$$p < 0.001 versus 4-MMC on the Day 6 (Tukey's post-hoc test)

L-NAME (25 or 50 mg/kg/day) was administered intraperitoneally (i.p.) for 5 consecutive days 10 min before an injection of mephedrone (4-MMC, 5 mg/kg/day, i.p.). On the 6th day of the experiment the mice received only 4-MMC, and locomotor activity was measured 20 min after injection for (A) 10 min or (B) 30 min. Control groups received saline or L-NAME (50 mg/kg/day) for 6 or 5 consecutive days, respectively

Figure 1. Influence of a 5-day administration of L-NAME on the development of tolerance to the mephedrone-induced hyperlocomotion in mice



The values represent mean + SEM (n = 8 animals per group).
**p < 0.01 versus saline on the Day 1; ###p < 0.001 versus 4-MMC on the Day 1; $$p < 0.01, $$$p < 0.001 versus 4-MMC on the Day 6 (Tukey's post-hoc test)

Methylene blue (MB, 5 or 10 mg/kg/day) was administered intraperitoneally (i.p.) for 5 consecutive days 10 min before an injection of mephedrone (4-MMC, 5 mg/kg/day, i.p.). On the 6th day of the experiment the mice received only 4-MMC, and locomotor activity was measured 20 min after injection for (A) 10 min or (B) 30 min. Control groups received saline or MB (10 mg/kg/day) for 6 or 5 consecutive days, respectively

Figure 2. Influence of a 5-day administration of methylene blue on the development of tolerance to the mephedrone-induced hyperlocomotion in mice

the tested groups were recorded when the measurements were carried out for 10 min [$F(4,32)=10.81$; $p<0.0001$] and for 30 min [$F(4,32)=10.22$; $p=0.0001$]. Neither an acute nor a 5-day administration of methylene blue (10 mg/kg or 10 mg/kg/day, respectively) when given alone changed animal mobility.

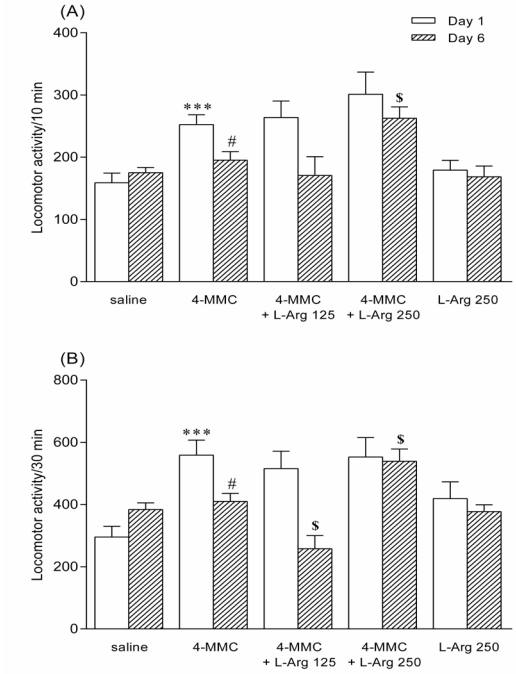
Influence of a 5-day administration of L-arginine hydrochloride or sildenafil citrate on the development of tolerance to the mephedrone-induced hyperlocomotion

As presented in Fig. 3 and 4, a 6-day administration of mephedrone (5 mg/kg/day) resulted in the development of tolerance to the effect of increased locomotion in animals which was recorded on the 1st day. On the 6th day of the experiment, the experiment revealed that the concurrent 5-day administration of L-arginine hydrochloride (125 mg/kg/day) with mephedrone (5 mg/kg/day) potentiated the development of tolerance to the mephedrone-induced hyperlocomotion, and that the animals that received the L-arginine hydrochloride-mephedrone combination were less active than animals that were treated with only mephedrone for 6 days. This effect was more pronounced (i.e. statistically significant) when locomotion was measured for 30 min. In contrast, the higher tested dose of L-arginine hydrochloride (i.e., 250 mg/kg/day) attenuated the development of tolerance to the mephedrone-induced hyperlocomotion. The same trend was observed when the outcomes were analysed for 10 and 30 min (Fig. 3A and 3B). One-way ANOVA revealed significant differences between the analysed groups: $F(4,34)=3.916$; $p=0.018$ (when the locomotor activity was measured for 10 min) and $F(4,35)=11.80$; $p<0.0001$ (when the locomotor activity was measured for 30 min). L-arginine hydrochloride when given once at a dose of 250 mg/kg or for 5 days at a dose of 250 mg/kg/day did not influence locomotion.

We found that a 5-day administration of sildenafil citrate at a dose of 5 or 10 mg/kg/day did not influence the development of tolerance to the mephedrone-induced hyperlocomotion when analysed on the 6th day of our experiment (one-way ANOVA: $F(4,32)=0.9164$; $p=0.4457$ for the 10-min schedule and $F(4,34)=0.5811$; $p=0.6320$ for the 30-min schedule). The obtained results are presented in Fig. 4. Sildenafil citrate did not *per se* affect locomotion of mice when given acutely (10 mg/kg) and for 5 days (10 mg/kg/day).

DISCUSSION

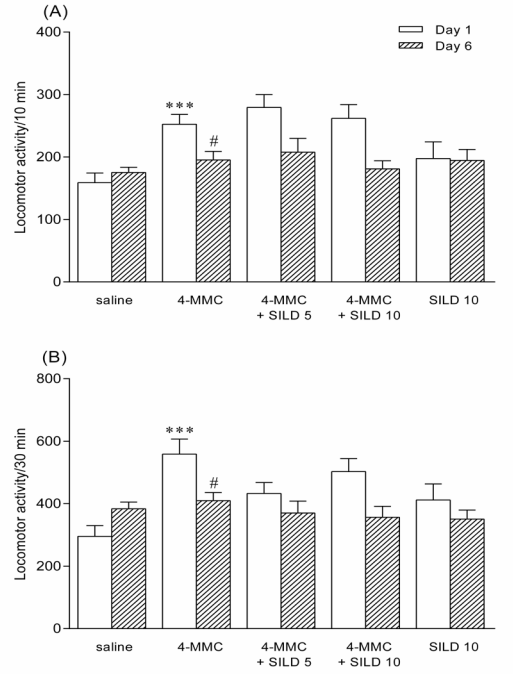
The results of our study are in line with outcomes obtained by other authors who demonstrated that an acute administration of mephedrone increases the locomotor activity of rodents [2,11,12]. According to literature data, the mephedrone-induced ambulatory hyperactivity is



The values represent mean + SEM (n = 8-10 animals per group)
***$p<0.001$ versus saline on the Day 1; #$p<0.05$ versus 4-MMC on the Day 1; $$p<0.05$, versus 4-MMC on the Day 6 (Tukey's post-hoc test)

L-arginine hydrochloride (L-arg, 125 or 250 mg/kg/day) was administered intraperitoneally (i.p.) for 5 consecutive days 10 min before an injection of mephedrone (4-MMC, 5 mg/kg/day, i.p.). On the 6th day of the experiment the mice received only 4-MMC, and locomotor activity was measured 20 min after injection for (A) 10 min or (B) 30 min. Control groups received saline or L-arg (250 mg/kg/day) for 6 or 5 consecutive days, respectively

Figure 3. Influence of a 5-day administration of L-arginine hydrochloride on the development of tolerance to the mephedrone-induced hyperlocomotion in mice



The values represent mean + SEM (n = 8 animals per group)
***$p<0.001$ versus saline on the Day 1; #$p<0.05$ versus 4-MMC on the Day 1 (Tukey's post-hoc test)

Sildenafil citrate (SILD, 5 or 10 mg/kg/day) was administered intraperitoneally (i.p.) for 5 consecutive days 10 min before an injection of mephedrone (4-MMC, 5 mg/kg/day, i.p.). On the 6th day of the experiment the mice received only 4-MMC, and locomotor activity was measured 20 min after injection for (A) 10 min or (B) 30 min. Control groups received saline or SILD (10 mg/kg/day) for 6 or 5 consecutive days, respectively

Figure 4. Influence of a 5-day administration of sildenafil citrate on the development of tolerance to the mephedrone-induced hyperlocomotion in mice

dose-dependent, appears fairly quickly after an injection [2], and lasts for a shorter time than hyperactivity detected after MDMA administration [37]. Most probably, this effect is associated with alterations in the serotonergic and dopaminergic neurotransmissions since antagonism of the serotonin 5-HT_{1A}, 5-HT_{1B}, and 5-HT_2 receptors [2,13], inhibition of serotonin synthesis [2], serotonin depletion [13] and blockage of the D_1 receptors [2,38] prevented mephedrone-induced hyperlocomotion.

After 6 consecutive days of mephedrone administration (5 mg/kg/day), we recorded the development of tolerance to the mephedrone-induced hyperlocomotion. Medical literature defines drug tolerance as a gradual decrease in the effect of a given agent when it is administered repeatedly [39]. In fact, development of tolerance to the mephedrone-induced effects had been reported before. Shortall *et al.* [13] demonstrated a weakening of the mephedrone-induced hypothermia after repeated mephedrone dosing. When the tested animals received 3 *i.p*. injections of mephedrone (10 mg/kg at 2-hour intervals), their body temperature decreased rapidly after the first dose, but the magnitude of this decrease following the second and the third dose was attenuated. The authors did not detect a parallel development of tolerance to the locomotor (and dopamine) response(s) following mephedrone treatment, which was in contrary to outcomes of the present study. However, such a discrepancy between the results of our experiments and the outcomes obtained by Shortall *et al.* [13] could have appeared since designs of these two studies were different, as were the introduced schemes of mephedrone administration (our experiment lasted much longer).

The molecular mechanism underlying tolerance to mephedrone-induced effects is not fully understood. Mephedrone is known to affect both serotonergic and dopaminergic neurotransmissions, having preferential effects on the serotonin [8], however, neurotoxic effects after a repeated administration of mephedrone are controversial. Most of the toxicological studies do not reveal any changes in brain levels of dopamine in animal studies (for review, see Pantano *et al*., [40]), but several authors pointed out persistent serotonergic deficits in animal cortex and striatum [4,6]. Furthermore, it has been reported that repeated high doses of mephedrone can cause a rapid decrease in functioning of the dopamine and serotonin transporters [6,25,41], reduced expression of enzymes involved in monoamine biosynthesis [25,41], and decreased concentration of the D_2 and 5-HT_{2A} receptors in the brain [41].

Outcomes of the present study give evidence that the L-arginine-NO-cGMP pathway must be partially involved in the development of tolerance to the mephedrone-induced behavioural effects. This is not surprising, since NO modulates brain levels of several neurotransmitters (such as dopamine, serotonin and glutamic acid) that are responsible for the development of neuroadaptive changes when an addictive substance is administered chronically [42]. Accordingly, the L-arginine-NO-cGMP pathway is implicated in the development of tolerance to benzodiazepins [32,33] and morphine [34,35], for example. It should be underlined that the obtained results of our study were not falsified by a possible influence of the tested NO modulators on the spontaneous locomotor activity of mice since none of the applied agents (i.e., L-NAME, methylene blue, L-arginine hydrochloride, and sildenafil citrate) given *per se* significantly changed animal motility.

In the presented experiments, both tested doses of a non-selective inhibitor of NOS – L-NAME (i.e., 25 or 50 mg/kg/day), and both tested doses of an inhibitor of NO-stimulated sGC – methylene blue (i.e., 5 or 10 mg/kg/day) prevented the development of tolerance to the mephedrone-induced hyperlocomotion in mice. It is worth mentioning, that L-NAME (at both tested doses) when co-injected acutely with mephedrone decreased the level of stimulatory effects induced by the latter, though the detected differences reached statistical significance only in the 30-min assessment. According to literature data, concurrent administration of NOS inhibitors and amphetamine derivatives reduces hyperlocomotion exerted by psychostimulants. Such an effect was observed for amphetamine [43], metamphetamine [44], cocaine [45], and methylphenidate [46].

We demonstrated that effects of an endogenous precursor of NO, L-arginine hydrochloride, were dose-dependent. When given at a dose of 250 mg/kg/day, L-arginine hydrochloride prevented the development of tolerance to the mephedrone-induced hyperlocomotion, but its lower tested dose (*i.e.*, 125 mg/kg/day) potentiated the development of tolerance to the mephedrone-induced hyperlocomotion. This dual effect was independent of whether the evaluation was carried out for 10 or for 30 min. As of today, it is difficult to explain this phenomenon, but it is well-known that the synthesis of NO in the brain under normal conditions is unsaturated with respect to L-arginine, so administration of L-arginine at high doses may trigger some compensative mechanisms in the L-arginine-NO-cGMP pathway [47]. Furthermore, L-arginine may be converted to agmatine which in turn, as an inhibitor of NOS, can abolish effects exerted by L-arginine [48]. Consequently, two different doses of L-arginine hydrochloride can produce inverse effects – as was observed in our experiments. Of note, it was previously demonstrated that L-arginine hydrochloride facilitated the development of tolerance to the diazepam-induced motor-impairment [32] and enhanced the development of tolerance to the morphine-induced antinociceptive effects [49]. Based on these findings, it seems that inhibition of NO production results in attenuation of tolerance to the mephedrone effects and that the cGMP system is also implicated in mephedrone tolerance. Similar observations were made by Ozdemir *et al*. [35] in relation to the development of morphine antinociceptive tolerance.

In the presented experiments, co-administration of sildenafil citrate (5 or 10 mg/kg/day) and mephedrone did not influence the development of tolerance to the mephedrone-induced increase in locomotion, indicating that cGMP-regulated phosphodiesterases are probably not involved in the above-mentioned mechanisms. In previous work, we revealed a similar lack of effect of sildenafil citrate on the development of tolerance to the central effects of flunitrazepam, despite the fact that the L-arginine-NO-cGMP pathway seems to be implicated in this process [33].

CONCLUSIONS

In conclusion, our data indicate that the L-arginine-NO-cGMP pathway contributes to the development of tolerance to the central effects of mephedrone since inhibition of this signalling via blocking of NOS (by L-NAME) or NO-stimulated sGC (by methylene blue) prevented the development of tolerance to the mephedrone-induced hyperlocomotion. As for cGMP-regulated phosphodiesterases, most probably they are not involved in these mechanisms, since a potent PDE5 inhibitor (*i.e.*, sildenafil citrate) did not influence tolerance to mephedrone effects.

ORCID iDs

Ewa Poleszak https://orcid.org/0000-0003-4359-3953
Aleksandra Szopa https://orcid.org/0000-0002-7756-2904
Joanna Listos https://orcid.org/0000-0002-5103-1830
Jolanta Orzelska-Górka https://orcid.org/0000-0001-6132-6934
Anna Serefko https://orcid.org/0000-0002-5732-8950

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