Influence of NG-nitro-L-arginine methyl ester on clinical and biochemical effects of methylene blue in pentylenetetrazole-evoked convulsions

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Abstract

Background/Aim. Despite years of research in a number of experimental models the question whether nitric oxide (NO) and methylene blue (MB) have pro- or anticonvulsant effects remains to be fully resolved. Methods. In adult Wistar rats the influence of a non-selective inhibitor of nitric oxide synthase NG-nitro-L-arginine methyl ester (L-NAME, 10 μg) on clinical and biochemical effects of MB (10 μg) given before the in-traperitoneal administration of chemical convulsant pentylenetetrazole (PTZ, 80 mg/kg) was examined. MB and L-NAME were applied intracerebroventricularly. PTZ application was followed by a 4-minute observation time, after which rats were sacrificed and elements of oxidative-reductive balance were measured in a crude mitochondrial fraction of forebrain cortex, hippocampus and striatum. Results. Convulsive responses (forelimb dystonia – FLD, generalised clonic- and clonic-tonic convulsions – GCC and GCTC respectively) were observed in all rats received PTZ, together with significantly decreased lipid peroxidation in the forebrain cortex and striatum and increased superoxide dismutase activity in the hippocampus, in comparison to controls (saline treated). It was registered anticonvulsant effects of L-NAME pretreatment. However, these effects were insignificant. In the hippocampus of these animals there was decrease lipid peroxidation (p < 0.01, p < 0.05 vs saline-treated and PTZ-treated rats, respectively) and reverted PTZ-induced increase of superoxide dismutase activity. But MB individually pretreatment significantly decreased the incidence of CTCS and GCCs (FLD: p = 0.0513), prolonged the convulsive latent time for PTZ, GCTCs and GCCs, in all the examined brain regions increased lipid peroxidation and decreased the level of superoxide anion. Administration of L-NAME 10 minutes before MB reverted all MB-evoked clinical and biochemical effects. Conclusion. Methylene blue applied individually before PTZ has strong anticonvulsant effects that were eliminated by L-NAME pretreatment. These effects and changed biochemical parameters in the brains of animals treated by L-NAME before MB in comparison to MB-treated group suggest involvement of NO in MB’s effects in the animal model of PTZ-evoked convulsions.

Key words: seizures; nitric oxide; methylene blue; pentylenetetrazole; rats; oxidoreductases.

Apstrakt

Uvod/Cilj. I pored višegodišnjeg istraživanja na različitim eksperimentalnim modelima, nije potpuno odgovoreno na pitanje da li azot-oksid (NO) i metilen plavo (MB) imaju pro- ili antikonvulsivne efekte. Metode. Na odraslim pacovima Vistar soja ispitivan je uticaj NG-nitro-L-arhjin metil estra (L-NAME, 10 μg), neselektivnog inhibitora azot oksid sintaze, na kliničke i biohemijske efekte metilene plave (MP, 10 μg) datog intracerebroventrikularno pre hemijijskog konvulziva pentylenetetrazola (PTZ, 80 mg/kg), primenjenog intraperitonealno. Pacovi su posmatrani, u odnosu na kontrolnu grupu (dobila fiziološki rastvor NaCl). Registrovani su antikonvulsivni efekti L-NAME minuta posle davanja PTZ-a, posle čega su žrtvovani i u neprečišćenoj mitohondrijskoj frakciji prednjeg mozga, hipokampusa i strijatuma određeni su parametri oksido-reductivne ravnoteže. Rezultati. Posle primene PTZ-a, konvulzivni odgovor (distanija prednjih nogu – DPN, generalizovane klonične – GKTT, generalizovane klonično-tonične konvulzije – GKTTC) bio je ispoljen kod svih životinja, kao i statistički značajno sniženje lipidne peroksidacije u korit prednjeg mozga i strijatuma, i povećanje aktivnosti superoksid dizmutaze (SOD) u hipokampusu, u poređenju sa kontrolnom grupom (dobila fiziološki rastvor NaCl). Registrovani su antikonvulsivni efekti L-NAME.
Introduction

An accumulated body of evidence supports multiple physiological as well as pathological roles for nitric oxide (NO) in its free radical form. Acting both presynaptically and postsynaptically NO accomplishes its complex participation in a wide range of physiological and pathophysiological phenomena including regulation of vascular tone, inflammation and signalling in the central nervous system (CNS) via poly-synaptic interacting circuits 1, 2. Nitric oxide is synthesised from L-arginine by nitric oxide synthases (NOS) in response to N-methyl-D-aspartate (NMDA) and non-NMDA receptor activation, and it is inhibited by a number of NOS inhibitors, among which is NG-nitro-L-arginine methyl ester (L-NAME). Alternatively, it can be released from NO donors. Besides hemoglobin, NOS and other metalloenzymes which are targets for NO binding, the interaction between NO and heme moiety within the soluble (cytosolic) form of guanylate cyclase (sCG) has particular physiological significance. The consequence of NO binding to sGC is the activation of the latter resulting in an elevation of cyclic guanosine 3',5'-monophosphate (cGMP) and the initiation of a cascade of target cell-specific events 3. Although much effort has been made to elucidate how NO interacts and stimulates sGC the precise mechanism is still unclear to date.

Despite years of research, the role of NO in the pathogenesis of epilepsy and convulsions still remains controversial. NO can provoke convulsions but also it can exhibit anticonvulsant effects 4, 5. In addition, some convulsive patterns are NO-independent 7, 8. Such unbalanced reports may reflect by the use of different experimental conditions, involving not only different animal species and strains, but also other factors including age, sex, convulsive models, the form, the route and doses of the applied substances.

During convulsions the concentration of cGMP increases in specific brain regions. Within the first four minutes of pentylenetetrazole (PTZ) application, which evokes generalised clonic convulsions (GCCs) and generalised clonic-tonic convulsions (GCTCs) in almost all mice, increase in cGMP (3- to 5-fold) were found primarily in the hippocampus and cerebral cortex but also (to a lesser extent) in the cerebellum 7. Within the first minute of PTZ application a rapid and very high level of cGMP was found in ventilated and non-ventilated guinea pigs in all the above-mentioned brain structures and also in the striatum, suggesting that increased cGMP is a pathogenic component of PTZ-evoked convulsions. The above mentioned brain structures are most important in convulsions initiation and propagation.

Methylene blue (MB), a highly active redox compound that readily cycles between oxidised (methylene blue) and reduced (leukomethylene blue) states, is a blue-coloured organic dye. Besides in experimental conditions, MB has importance in human clinical practice too, although data about its effects in humans are not uniform 10, 11.

The effects of MB on cGMP and on NO have already been documented in several studies. Since MB is a nonselective and a weak incomplete inhibitor of sGC, this effect of MB on sGC has been widely studied 12, 13. MB can attenuate cGMP accumulation by inhibiting NO-stimulated cGC without an effect on the basal cGMP level 14. Masaki and Kondo 15 intracerebroventricularly administered MB to rats 30 minutes prior to sacrificing them. In whole brain homogenate the level of cGMP decreased in a MB dose-dependent manner. MB administration can also decrease the NOS activity and NO content in certain brain regions and also prevents experimental seizures 16, 17.

The NO-sGC-cGMP signalling pathway is present in virtually all cells but sGC is expressed at particularly high levels within neurons. This pathway is largely influenced by glutamatergec neurotransmission that is modulated in response to PTZ treatment 19.

According to a number of data, as well as to our own results, it is obvious that elements of oxidative/antioxidative balance are changed in the brains of rats with convulsive response to PTZ and upon L-NAME treatment in such kind of experimental convulsions 19, 20. Because of these facts but also due to MB’s ability as well as L-NAME and PTZ to interfere with synthesis/actions of NO, and due to their convulsing/anticonvulsive properties, we sought to determine their influence on both clinical and brain biochemical changes (parameters of oxidative defence and stress) in a PTZ seizure model in rats 5−7, 12, 16, 17.

Methods

Animals and surgery

Experiments were performed on 13-week old male Wistar rats housed in a temperature-controlled room (23 ± 2°C) with 11-hour light/13-hour dark cycles with free access...
to food and water. Experiments were conducted within a period of two weeks always between the hours of 9:00 am and 2:00 pm. Animals were randomly assigned to different drug treatment regimes. All protocols for handling the rats were approved by the local ethical committee for the use of experimental animals (Military Medical Academy, Belgrade, Serbia).

For intracerebroventricular (icv) application of drugs a polyethylene cannula was stereotaxically implanted into the left lateral ventricle (coordinates: 1.3 mm behind the bregma, 1.8 mm left from the midline suture, 3.7 mm ventral from the durra) under sodium pentobarbital intraperitoneally (ip) anaesthesia (45 mg/kg body weight, Vetanarcol®, WerfH-Cheemie, Vienna). The cannula was fixed to the skull with dental cement and two jeweller screws. The postoperative recovery period lasted six days before experiments were continued.

Treatments

Rats were divided into five groups (Figure 1; in each group there were 7–8 rats). PTZ (Sigma Chemical Co., St Louis) was used as a chemoconvulsant. It was applied ip in a single dose of 80 mg/kg body weight. The control group received sterile isotonic saline icv twice: 30 minutes and 20 minutes before ip application of the same solution. Another group of rats also received sterile isotonic saline icv twice: 30 and 20 minutes before ip application of PTZ (PTZ-treated group). The third group received 10 μg NG-nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Co.) icv 30 minutes before PTZ. Ten minutes after the L-NAME injection the rats also received sterile isotonic saline icv.

MB (10 μg) was administered icv to another two groups of rats 10 minutes after sterile isotonic saline (icv) or L-NAME (10 μg, icv). Twenty minutes after MB (30 minutes after sterile isotonic saline/L-NAME treatment), both groups of rats received PTZ. All drugs were dissolved in sterile isotonic saline. Volume of ip and icv applied solutions were 1 mL/kg body weight and 10 μL, respectively.

Behavioural evaluation

Immediately after PTZ administration the rats were placed individually in transparent perspex cages. The time of observation following PTZ injection was limited to 4-min. During that time convulsive responses were monitored and were graded according to the following scale: fore limb dystonia (FLD), generalised clonic seizures (clonus of the whole body with a loss of righting reflex, GCCs) and generalised clonic-tonic convulsions with tonic hind limb extension (GCTCs). The appearance (incidence) and the time to onset of every convulsive pattern were registered. If the rats did not exhibit convulsive responses, a latent time of 240 sec was assigned.

Biochemical parameters

All the rats were sacrificed by decapitation 4 min after PTZ administration. Their heads were immediately frozen in liquid nitrogen and stored at -70°C.

For biochemical analysis the forebrain cortex, striatum and hippocampus from the contralateral hemisphere relative to cannula insertion were dissected on ice, homogenized in ice-cold buffer, centrifuged and sonicated. Biochemical parameters were determined spectrophotometrically in the crude mitochondrial fraction according to the method of Gurd et al.

The assay for the superoxide radical is based on the reduction of nitroblue tetrazolium (Merck) in an alkaline, nitrogen-saturated buffer with absorbance monitoring at 515 nm.

Total superoxide dismutase (SOD) activity was measured as the inhibition of epinephrine auto-oxidation (Sigma Chemical Co.) in sodium carbonate buffer (Serva) containing 0.1 mM EDTA (Sigma Chemical Co.) at 480 nm.

Lipid peroxidation was measured as a function of malondialdehyde (MDA) production at 533 nm.

The incidence of convulsive patterns was expressed as the percentage of convulsing rats of the total number of rats in a group and was analysed using Kolmogorov-Smirnov test. The latent time of convulsions was calculated in seconds and expressed as the mean ± standard deviation (SD) and was analysed using the Kruskal-Wallis test. Biochemical parameters were expressed as the mean ± SD and were analysed using the ANOVA, followed by Tuckey test. Differences between experimental groups were considered significant when p < 0.05.
Results

Behavioural effects

In the control group of rats (sterile isotonic saline treatment, icv and ip) no convulsions were observed. In contrast, convulsive responses were observed in all PTZ-treated rats (Figure 2).

Pretreatment with L-NAME led to some anticonvulsant, however insignificant effects on PTZ-induced convulsions.

MB pretreatment was very effective against PTZ-evoked convulsions. GCTCs were completely prevented \((p < 0.01)\) and GCCs appeared only in 25% of animals \((p < 0.01)\). FLD was apparent in 50% of the rats \((p = 0.0513)\).

When L-NAME was administered before MB, the anti-convulsant effects of MB were lost, in particular GCTCs and GCCs; their incidence increased from 0 to 83% \((p < 0.01)\), and from 25 to 83% \((p < 0.05)\). In addition the incidence of FLD was doubled, from 50 to 100% \((p = 0.0513)\).

The latent time of PTZ-evoked convulsions was not influenced by L-NAME pretreatment (Table 1). MB very strongly influenced the latent time of all three convulsive patterns \((p < 0.01)\) for FLD and GCTC, \(p < 0.05\) for GCC, compared with the PTZ-treated group and groups pretreated with L-NAME). But MB’s ability to extend the latent time of PTZ-induced convulsions was completely blocked by L-NAME.

Biochemical effects

In the PTZ-treated group of rats decreased lipid peroxidation in the forebrain cortex and striatum compared with the control group was found \((p < 0.05 \text{ and } p < 0.01, \text{ respectively})\) (Figure 3). In addition, increased SOD activity in the hippocampus compared with the control group was noted \((p < 0.05)\) (Figure 4).

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Latent time (seconds)</th>
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</thead>
<tbody>
<tr>
<td>L-NAME and MB (µg)</td>
<td>FLD</td>
</tr>
<tr>
<td>NaCl + PTZ</td>
<td>49.5 ± 11.3***</td>
</tr>
<tr>
<td>L-NAME (10) + PTZ</td>
<td>57.7 ± 11.9***</td>
</tr>
<tr>
<td>MB (10) + PTZ</td>
<td>159.2 ± 88.7***</td>
</tr>
<tr>
<td>L-NAME (10) + MB (10) + PTZ</td>
<td>53.3 ± 8.8***</td>
</tr>
</tbody>
</table>

\(p < 0.05, 0.01\) is the level of significance when compared with the corresponding values (mean ± SD) of 0.9% NaCl \((*, **)\) and PTZ-treated rats \((*, **; ANOVA test)\). PTZ was applied intraperitoneally, NaCl intracerebroventricularly, L-NAME and MB intracerebroventricularly.
Treatment with L-NAME prior to PTZ administration decreased lipid peroxidation in the hippocampus \((p < 0.01,\) compared with the control group; \(p < 0.05,\) compared with the PTZ-treated group) (Figure 3). SOD activity was decreased in the forebrain cortex \((p < 0.05,\) compared with the control group) (Figure 4). Superoxide anion content of the hippocampus was also decreased \((p < 0.05,\) compared with the control group) (Figure 5).

MB administered before PTZ resulted in increased lipid peroxidation compared with the control group \((p < 0.05\) in the forebrain cortex, \(p < 0.01\) both in the striatum and hippocampus) and compared with the PTZ-treated group of rats \((p < 0.01\) for all brain regions) (Figure 3). The level of superoxide anion was decreased in the MB+PTZ-treated group of rats compared with both the control and PTZ-treated groups \((p < 0.01\) in all brain regions) (Figure 5).

Pretreatment with L-NAME reverted the MB+PTZ-induced increase in lipid peroxidation (Figure 3) and decrease in superoxide anion (Figure 5) in all brain regions back to the control values, except in the forebrain cortex where the superoxide content was increased, while SOD activity was decreased \((p < 0.05\) in comparison with the control and PTZ-treated groups of rats, and the control group, respectively).

**Discussion**

We found that both L-NAME and MB affected PTZ-evoked convulsions (insignificant and almost completely/completely prevented, respectively). However, co-administration of L-NAME and MB did not result in an additive/synergistic effects. Instead the effects (clinical and biochemical) of MB were severely abrogated by L-NAME.

The convulsant effects of PTZ are quite complex and are not yet completely understood. Reduced \(\gamma\)-aminobutyric (GABA) activity (GABA suppresses NOS), and enhanced excitatory amino acids release/transmission is believed to be the underlying mechanism. Thus, PTZ’s effects on both the GABA-ergic and glutamatergic system lead to overproduction of NO and potentiation of sGC activity.

NO preferentially reacts with other radicals despite being a free radical itself. Its reactivity with biological molecules is low but it readily reacts with the hydroxyl radical. In this way it can act as an antioxidant by scavenging free radicals thereby inhibiting lipid peroxidation. Chiueh proposed at least four mechanisms by which NO acts as an antioxidant, including inhibition of lipid peroxidation. Thus, within the first few minutes of PTZ administration when cGMP production is rapidly increased, which is an indirect measure of NO production, a defence reaction against PTZ’s toxic effects (via the antioxidative activity of NO) may take place. Under very strong production of NO and at later times this antioxidative defence is overcome and lipid peroxidation ensues, as described by a number of studies. For example, Patsoukis et al. found that the intensity of lipid peroxidation was increased in the mouse hippocampus and the striatum 15 min after PTZ was applied ip at a dose of 60 mg/kg. Furthermore, increased lipid peroxidation was within control values 30 min and 24 h post PTZ administration. However, lipid peroxidation was not increased in the mouse cerebral cortex in each monitoring time. A lower dose of PTZ (40 mg/kg) did not in any way influence lipid peroxidation, meaning that lipid peroxidation is PTZ dose- and time-dependent.

In the research of Bashkatova et al., PTZ (120 mg/kg administered subcutaneously) caused convulsions in all animals within 2–3 minutes. Moreover, one hour later the level of NO was increased 5-fold and lipid peroxidation 2-fold in the frontal cortex. Despite the fact that PTZ was used as a chemoconvulsant in both our and the above-mentioned studies, to some degree different results concerning lipid peroxidation are most likely explainable due to different experimental procedures.

The anticonvulsant effects of MB described in our studies are in accordance with those of Furian et al. In addition to MB’s ability to increase the latency of methylmalonate-induced convulsions in adult male Wistar rats, abrogation of the methylmalonate-induced striatal NO level elevation was also observed. Furthermore, in the case of catalepsy induction Echeverry et al. demonstrated similar effects of L-NAME and MB, both applied icv. Also, the level of NO products was decreased in the striatum.

However, discrepancies between our results and the results obtained by Deutsch et al. who did not record the anticonvulsant effects of MB in electrically precipitated tonic hind limb extension in mice, could be explained by the use of different experimental models of convulsions, routes of drug administration, time points and dose of MB application, since pharmacokinetics and organ distribution of MB depends on the way of its application.

Apart from inhibition of sGC, MB has several other nonspecific effects on NO including inhibition of NOS. In cultured endothelial cells Shimizu et al. found that MB, which is known to inactivate iron-containing enzymes, reacts with the heme moiety of NOS. Vallo et al. found similar effects on hippocampal brain NOS activity in vivo. Further-
more, MB-mediated inhibition of hippocampal NOS was dose-dependent and the degree of inhibition was similar to that obtained with an unselective NOS inhibitor N-G-nitro-L-arginine (L-NNA). In comparison with sGC inhibition, MB-mediated NOS inhibition appeared more potent. The susceptibility of different NOS isoforms to MB remains unclear. Because of NOS inhibition by MB, in our research it was expected that L-NAME, a competitive non-selective NOS inhibitor, enhances MB’s anticonvulsant effects. However, the opposite was apparent: MB’s anti-convulsant effects were reverted back to control values. In other words, combined pretreatment with L-NAME and MB exerted deleterious effects on MB-preventable PTZ-evoked convulsions. It is difficult to propose the reason for the obtained events. In addition to nitricergic system a number of other effects of both substances have to be taken into consideration before any conclusion, such as their influence on cholinergic, dopaminergic, serotonergic and other transmissions that are important in the pathogenesis of convulsions.

Despite much more evidence pointing towards its antioxidant effects, MB can also be prooxidative causing singlet oxygen and superoxide formation, weak peroxide generation and direct glutathione oxidation. Inactivation of NO by superoxide is ranked as one of the most rapid nonenzymatic reactions in biology. In other words one of MB effect could be related to the removal of NO already present. The chemical reaction between NO and superoxide, which could be generated in the presence of MB, may lead to the formation of peroxynitrite anion. The latter is a source of the hydroxyl radical which can intensify lipid peroxidation. In our previous study it was found increased NO levels in the hippocampus, forebrain cortex, striatum and some other brains structures within 30 minutes of PTZ application in the PTZ-evoked convulsions in rats. Therefore, abovementioned effects of PTZ are in agreement with our observation regarding increased lipid peroxidation and a decreased level of superoxide anion in all the examined brain structures isolated from rats pre-treated with MB. Reestablishing level of lipid peroxidation in all brain regions and superoxide anion (except in the forebrain cortex) without increased SOD activity when L-NAME was coadministered with MB coincide with the suggested explanation of increased lipid peroxidation upon MB treatment; it could indicate decreased interaction between NO and superoxide anion. However, increased lipid peroxidation that was associated with anticonvulsant effects of MB is undesired for any antiepileptic drug and substance and needs scientific evidence reliable of benefit/risk ratio with the aim to study whether benefits of such treatments (anticonvulsant effects) overwhelm risks of their potential harmful effects (increased lipid peroxidation).

A cascade of prooxidative events associated with MB pretreatment was not at all disrupt by coadministration of L-NAME. That was demonstrated in increased level of superoxide anion in the forebrain cortex. It could be the result of L-NAME effects on SOD activity, which was decreased in this structure when L-NAME was individually applied before PTZ. Thus, L-NAME did not only prevent anticonvulsant and lipid peroxidation effects of MB, but also it made conditions for forebrain cortex oxidative damage. In other words, all three structures in which most of the complex events of epileptic seizures are generated were very sensitive and prone to PTZ, MB and L-NAME influence.

In our PTZ-mediated convulsing rat model it was registered very early and rapid events (within the first 4 minutes after PTZ application): MB, when applied individually, prevented convulsive responses, especially GCTCs and GCCs; modulation of the NO system by MB in the settings of PTZ-induced convulsions may elicit anticonvulsant effects, but only if MB was applied on its own, not in combination with L-NAME, indicating that NO plays important role into anticonvulsant effects of MB under applied conditions; convulsive responses to PTZ was associated with a decreased and/or unchanged level of lipid peroxidation in the forebrain cortex, striatum and hippocampus. In contrast, anticonvulsant effects of MB coincide with increased lipid peroxidation. These clinical and biochemical effects of MB were abandon by L-NAME treatment.

Conclusion

MB’s strong anticonvulsant effects in PTZ-evoked convulsions, especially against generalised clonic and clonic-tonic convulsions, and changes of the examined biochemical parameters in brain structures, as well as prevention of all these effects by L-NAME applied before MB could be partly the result of the nitricergic system modulation. Our results have far reaching therapeutic implications, ie translation into clinical practice benefits, particularly the anticonvulsant effects of MB. Future investigations will hopefully shed some light on the intricacies of MB’s effects.

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