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Naloxone Antagonizes Soman-induced Central Respiratory Depression in Rats

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Abstract: The influence of naloxone on respiration impaired by the highly toxic organophosphate nerve agent soman in anaesthetized rats was investigated. Soman, administered in a dose that was ineffective in blocking the electrically induced contractions of the phrenic nerve-diaphragm preparation *in situ*, induced a complete block of the spontaneous respiratory movements of the diaphragm, indicating the domination of central over the peripheral effects. Naloxone dose-dependently antagonized the soman-induced respiratory blockade. Atropine, at a dose that was *per se* ineffective in counteracting soman-induced respiratory depression, potentiated the protective effects of naloxone and completely restored respiration. Naloxone remained completely ineffective in antagonizing respiratory depression induced by the muscarinic receptor agonist the oxotremorine. It is assumed that naloxone antagonizes soman-induced respiratory inhibition by blocking endogenous opioidergic respiratory control pathways that are independent of the stimulation of muscarinic receptors.

It is well known that the toxic doses of cholinesterase inhibitors affect the respiratory function in a complex manner [1] and that respiratory failure precedes cardiovascular collapse [2,3]. The mounting level of acetylcholine in the cholinergic synapses that follows the acetylcholinesterase (AChE) inhibition produces overstimulation of muscarinic and nicotinic cholinoceptors in the central nervous system and elsewhere [4]. Peripheral muscarinic receptor overstimulation results in bronchoconstriction and bronchorrhea [5], while the excessive stimulation of the nicotinic receptors at the neuromuscular junction induces the fasciculations and Wedensky-type depolarization blockade [6]. At the same time, central muscarinic cholinoceptor overstimulation interferes with the different groups of neurons and nuclei involved in the creation of the respiratory drive and rhythm [7]. Acetylcholine seems to be involved in every aspect of respiration, as cholinergic neurons are involved in respiratory rhythm generation, afferent input and efferent output [8].

There is a significant body of evidence that soman and other organophosphorus AChE inhibitors impair the respiratory function predominantly *via* the central muscarinic mechanisms [1,5], although they also affect the neuromuscular transmission *via* the overstimulation of nicotinic receptors [9]. Besides, soman is also known to induce a long-lasting antinociception in mice that can be antagonized by naloxone, a selective competitive antagonist of opioid receptors [10]. As naloxone-reversible respiratory depression and antinociception are otherwise characteristic not only for morphine and other opioid analgesics, but also for endogenous opioids [11], it was assumed that central respiratory effects of soman are, at least partly, mediated by the endogenous opioid pathway.

The aim of this study was to investigate the potential antidotal effects of naloxone against soman-induced central respiratory depression. To explore the mechanisms of the cholinergic–opioidergic interaction in the central control of respiratory function, oxotremorine, a muscarinic receptor agonist, and atropine, a muscarinic receptor antagonist, were used.

Methods

This study was approved by the local Institutional Animal Care and Use Committee. All experiments were performed in male Wistar rats weighing 200–300 g. Animals were housed under the standard conditions and given access to food and water *ad libitum*.

Soman of minimum 95% purity was synthetized at the Military Technical Institute, Belgrade, Serbia. Naloxone hydrochloride, atropine sulphate and oxotremorine sesquifumarate were obtained from the commercial sources. All the substances were dissolved in fresh saline solution and injected into the jugular vein in the volume of 1 ml/kg. The animals were anaesthetized with 25% urethane 0.7 ml/kg intraperitoneally.

Peripheral effects of soman at the level of neuromuscular transmission were investigated in the rat phrenic nerve-diaphragm preparation *in situ* [12]. The amplitude of contractions of diaphragm produced by indirect electrical stimulation *via* phrenic nerve was the parameter measured.

Additionally, combined central and peripheral effects of soman on respiration were tested in the preparation similar to the one described by Schaumann and Job [13]. In this experimental model, anaesthetized rats were thoracotomized and the artificial respiration was established as well, but phrenic nerves were left intact and the spontaneous contractions of diaphragms were driven by relative hypoventilation and hypercapnia. Both amplitude and frequency of the diaphragmal contractions were recorded by means of a mechanoelectrical transducer connected to the falciform ligament of the liver, after the laparotomy was performed by a medial incision between the xiphoid process and the umbilicus. Respiratory Index was

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defined as the sum of the amplitudes of diaphragmal contractions in 10-sec. segments.

Statistical analysis was performed with commercially available statistical programmes of ANOVA with Tukey's *post hoc* test.

Results

At the beginning, the dose–effect relationships were studied in two different experimental models: one with intact phrenic nerves reflecting a sum of central and peripheral respiratory components and the other, where contractions of the diaphragm were induced by the electrical stimulation of the phrenic nerve, reflecting the peripheral respiratory component only. The results clearly showed that the increasing doses of soman decreased the amplitude of contractions of rat diaphragm in a dose-dependent manner (fig. 1).

It is obvious that soman acts as a more potent inhibitor of central + peripheral respiration than a purely peripheral one. As an illustration, the ED_{50} value in the preparation reflecting a sum of central and peripheral components of ventilation was only a half of the ED_{50} needed for depression of the contractions of the peripheral preparation (22 ± 2 *versus* 44 ± 5 μg/kg i.v.).

The dose of soman 30 μg/kg i.v. completely abolished the contractions of diaphragm in the central + peripheral model and as such was chosen for further experiments. Moreover, only the central+peripheral model was used in the subsequent experiments, as it was clearly a more sensitive and automatically a more clinically relevant mechanism of soman-induced respiratory depression.

To confirm the known opioidergic depressant effect on respiratory centres, morphine was used. It decreased the respiratory index in the central+peripheral preparation dose-dependently (fig. 2).

The ED_{50} of morphine in this preparation was 56 ± 9 mg/kg i.v., and the respiration was completely blocked by 90 mg/kg i.v.

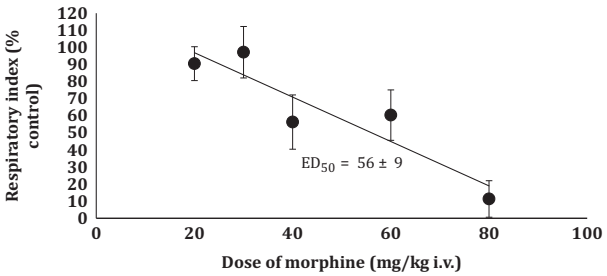

Fig. 2. The effect of morphine on the respiratory index in anaesthetized rats in the hypercapnia-induced model with intact phrenic nerves. Increasing doses of morphine were injected i.v. Each point represents mean value ± S.D. (n = 6).

As in further experiments naloxone and atropine were used as potential antidotes against soman, it was important to study whether each of them has any effect on respiratory index in animals not exposed to soman.

Naloxone, administered in a wide range of doses (0.5–40 mg/kg i.v.) *per se*, did not affect the respiratory index in rats (not shown). The only exception was the dose of 20 mg/kg i.v. that resulted in a 22% increase of respiratory index. Atropine, administered in a dose range of 0.1–50 mg/kg i.v., failed to affect the respiratory index in anaesthetized rats *per se* (not shown).

Further experiments were conducted to determine the quantitative aspects of naloxone- and atropine-induced antagonism of soman respiratory depression.

The main finding was that naloxone, administered in the dose range of 0.5–20 mg/kg i.v., dose-dependently antagonized the soman-induced respiratory index decrease, with the ED_{50} of 9.86 ± 1.1 mg/kg i.v. (fig. 3).

The highest level of the respiratory index recovery was obtained at a dose of 7.5 mg/kg i.v., and therefore, this dose was used for further experiments as the most effective one. The effect of the dose of naloxone of 20 mg/kg was even more potent, but this dose was not used further, as it was

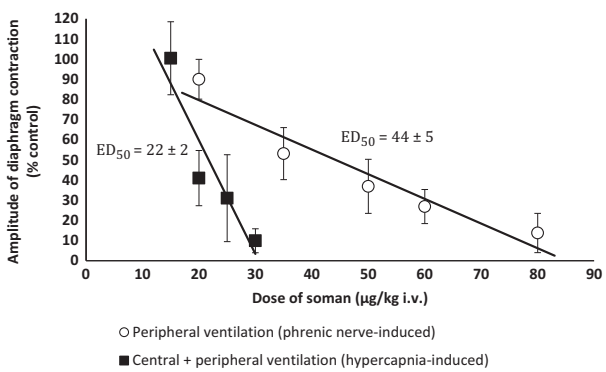

Fig. 1. The effects of soman on the amplitudes of contraction of rat diaphragms in the hypercapnia-induced model with intact phrenic nerves and in the electrically stimulated phrenic nerve-diaphragm preparation *in situ*. Increasing doses of soman were injected intravenously (i.v.). Each point represents mean value ± standard deviation (S.D.) (n = 6).

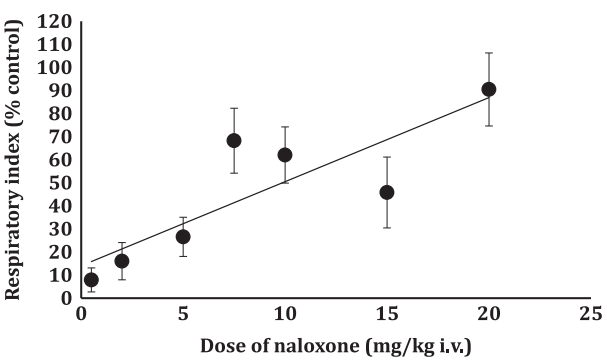

Fig. 3. The effect of naloxone on the respiratory index in anaesthetized rats poisoned with soman in the hypercapnia-induced model with intact phrenic nerves. Increasing doses of naloxone were injected i.v. Each point represents mean value ± S.D. (n = 6). The dose of soman was 30 μg/kg i.v.

previously shown that it increased the respiratory index *per se*, *that is* even in non-poisoned rats.

Atropine produced a quantal effect in antagonizing the soman-induced decrease in respiratory index – doses up to 1.2 mg/kg i.v. were without effect, while higher doses completely restored the respiratory index in rats to and above the control levels (fig. 4).

Once the doses of naloxone and atropine that *per se* do not affect the respiratory index in control animals were defined, it was decided to test them in rats poisoned with soman 30 μg/kg i.v. Naloxone 7.5 mg/kg i.v. effectively antagonized the respiratory index inhibition produced by soman. Addition of atropine 1.2 mg/kg i.v. further potentiated this protective effect of naloxone, leading to the complete restoration of the respiratory index (fig. 5).

In a separate set of experiments, the effect of oxotremorine on respiratory index was evaluated, with the aim to ascertain the influence of the stimulation of central muscarinic receptors on respiratory index (fig. 6).

It was shown that oxotremorine produced a triphasic effect: at low doses (0.05–0.1 mg/kg i.v.) it significantly increased the respiratory index, at middle-range doses (0.2–1.5) it did not significantly alter it, while the highest dose used (2 mg/kg) significantly reduced the respiratory index to 40% of the control.

Our further goal was to investigate whether the protective effect of naloxone in rats poisoned with soman could be obtained in oxotremorine-poisoned rats, as well (fig. 7).

Naloxone had no effect on the oxotremorine-induced respiratory index depression in rats (fig. 7), indicating that no direct interaction between the muscarinic and opioidergic receptors exists in this experimental model.

Discussion

The results presented indicate that soman induces respiratory depression predominantly *via* central mechanism, which is in accordance with other publications [1,5,14,15]. The present

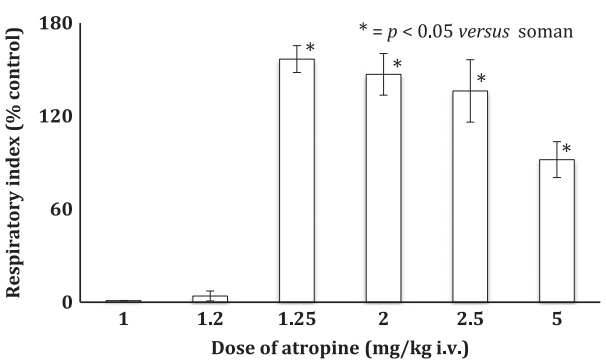

Fig. 4. The effect of atropine on the respiratory index in anaesthetized rats poisoned with soman in the hypercapnia-induced model with intact phrenic nerves. Increasing doses of atropine were injected i.v. Each column represents mean value ± S.D. (n = 6). The dose of soman was 30 μg/kg i.v.

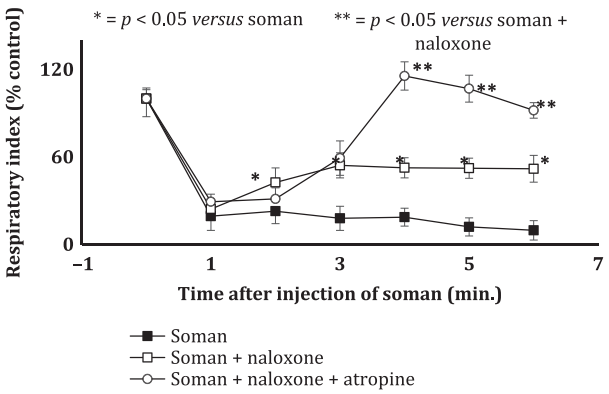

Fig. 5. The effects of naloxone and the combination of naloxone and atropine on the respiratory index in anaesthetized rats poisoned with soman in the hypercapnia-induced model with intact phrenic nerves. The administered doses were as follows: soman 30 μg/kg i.v., naloxone 7.5 mg/kg i.v. and atropine 1.2 mg/kg i.v. Each point in the diagram represents results obtained in groups of six rats (n = 6).

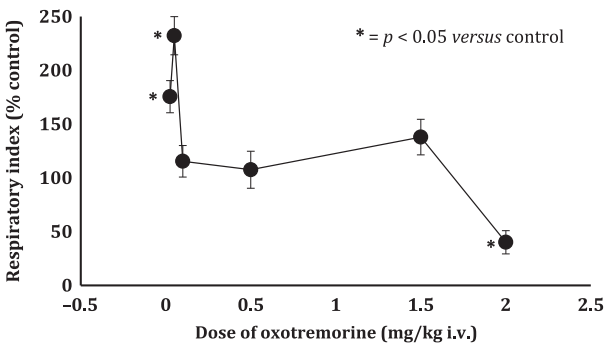

Fig. 6. The effect of oxotremorine on the respiratory index in anaesthetized rats poisoned with soman in the hypercapnia-induced model with intact phrenic nerves. Increasing doses of oxotremorine were injected i.v. Each point represents mean value ± S.D. (n = 6).

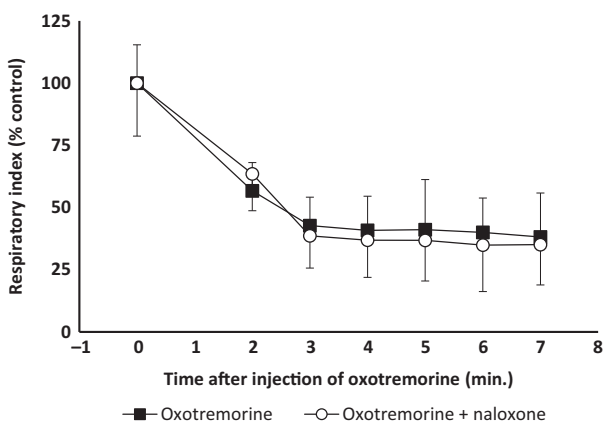

Fig. 7. The effects of oxotremorine and the combination of oxotremorine and naloxone on the respiratory index in anaesthetized rats in the hypercapnia-induced model with intact phrenic nerves. The administered doses were as follows: oxotremorine 2 mg/kg i.v. and naloxone 7.5 mg/kg i.v. Each point in the diagram represents mean value ± S.D. (n = 6).

finding was in accordance with Bajgar *et al.* [16], who compared the level of AChE inhibition in various brain regions of rats poisoned with 0.5 LD_{50} of soman and found the strongest inhibition in the pontomedullary region, where the respiratory centres are located.

Despite the use of a relatively large challenge dose of soman and *iv* route of administration in our experiments, even the doses of atropine as low as 1.25 mg/kg *iv* were sufficient to abolish the soman-induced respiratory block, implying that muscarinic receptors have a dominant role in the respiratory failure exerted by large doses of soman. In accordance with this report was the finding that 0.5 mg/kg of atropine can eliminate the respiratory block induced by 2 LD_{50}s of soman in monkeys [17]. Other authors, as early as in 1950s and thereafter, reported that atropine, scopolamine and other centrally acting antimuscarinic agents antagonized the seizures and respiratory depression produced by soman or other nerve agents [14,18–22]. This antidotal effect of atropine depends on its own dose but also on the dose of soman [3].

The importance of central muscarinic receptors for the organophosphorus compound-induced respiratory depression was documented when even the 100-fold higher equimolar doses of N-methylatropine, a quaternary muscarinic antagonist that cannot pass the blood–brain barrier, could not mimic the respiration-restoring effects of atropine in rats challenged with low-dose paraoxon [23]. Besides, local administration of soman into the intermediate part of the ventral surface of cat medulla oblongata produced AChE inhibition, hypotension and respiratory depression; the latter two being mimicked by local application of muscarinic receptor agonist oxotremorine and blocked by local administration of atropine [24].

The lethal sequence of events after the massive soman intoxication can be fully traced only in non-anaesthetized animals, and it involves first the occurrence of hyperpnoea, then loss of consciousness, which coincided with the occurrence of seizures, followed by dyspnoea, hypopnoea and, finally, with respiratory failure [5]. The nature of the present experiment required use of anaesthesia, and it is therefore necessary to address these conditions in animals poisoned with soman. Urethane was chosen due to a lack of known specific receptor interactions in the central nervous system (CNS) of the anaesthetized rodents, although it was proven to block both clinical and electroencephalographic seizures induced by the electrical stimulation of the amygdala and by pentylenetetrazol [25]. Other anaesthetic agents, such as pentobarbital and ketamine, were avoided to eliminate the negative cardiovascular effects of the former [3] and the interference with the glutamate receptors of the latter one [4], as both the N-methyl-D-aspartate (NMDA) and non-NMDA receptor were shown to stimulate phrenic motoneurons in rats [26]. Although it was previously said that organophosphate-induced seizures and central respiratory depression coincide, it is obvious that in the present experiments, soman induces central respiratory depression also in urethane-anaesthetized rats. As the dose of urethane used was the same in all groups of rats, we decided to consider its influence on rat CNS irrelevant for the problem studied.

The major finding of this research was that the soman-induced respiratory failure in anaesthetized rats is, at least partly, mediated by opioid receptors, as it can be antagonized by naloxone in a dose-dependent manner. We could not find a similar report in the literature. In fact, when it comes to the soman–naloxone antagonism in general, it was documented only regarding the antinociceptive effects of soman, which were also antagonized by naloxone [10,27]. Other authors hypothesized that soman and other organophosphorus compounds in fact accentuate the effects of endogenous opioids by inhibiting the enzymes responsible for their destruction [10,28,29], although other authors opposed that finding [30].

As this analgesic effect of soman was speculated to be mediated by the endogenous opioids, it was reasonable to assume that it is the case with its effect on respiration, as well. This assumption seems even more probable based on the findings that centrally acting anticholinesterase agent physostigmine, but not the peripherally acting AChE inhibitor neostigmine, liberates beta-endorphin in human beings [31]. Indeed, in a hypercapnia rebreathing model, somewhat similar to ours, Eager *et al.* [32] demonstrated that μ-receptor agonist dermorphin attenuated and opioid antagonist naloxone (0.4 mg/kg) increased the slope of the pCO_2/respiration dose–response curve, implying that the endogenous opioids may exert a modulatory influence on respiration by altering the sensitivity of the central respiratory control system to CO_2. Additionally, local application of physostigmine, dissolved in mock cerebrospinal fluid, on caudal ventral medulla of anaesthetized cats produced a biphasic effect on cholinergic CO_2-sensitive neurons – it first stimulated and then depressed the neuronal activity; the latter effect being always abolished by naloxone [7]. This report is in accordance with the findings of the present experiment.

Opioids – both endogenous and exogenous – act mainly on μ- and δ-receptors on the respiratory-related neurons in the ventral part of the medulla and dorsal regions of pons to depress the breathing depth and rate, blunt respiratory responsiveness to CO_2 and hypoxia, increase the upper airway resistance and reduce pulmonary compliance [33]. It was shown that naloxone increased the sensitivity of neurons in the caudal respiratory chemosensitive area of cats to hypercapnia, which again implies the significant depressant role of endogenous opioids [34,35]. Moreover, it was shown that beta-endorphin causes respiratory depression [36]. At the same time, it was reported that μ-receptor agonists [D-Ala^2, N-$MePhe^4$, Gly-ol]-enkephalin (DAMGO) or [Met(5)]encephalin injected into Kölliker–Fuse neurons in the dorsal pons of rats *in situ* induced hyperpolarization of these neurons and irresponsiveness to excitatory transmitters and cause a strong apneusis that can be reversed by naloxone [37].

It was demonstrated in mice that a small group of proopiomelanocortin (POMC) neurons of the nucleus of the solitary tract (NTS) has significant projections throughout the brainstem, including the areas responsible for respiration, such as Kölliker–Fuse nucleus, Bötzinger complex, pre-Bötzinger complex, rostral ventral respiratory group and lateral reticular nucleus [11]. Moreover, the same authors showed that opto-

genetic activation of the POMC neurons of NTS in the mouse working heart–brainstem preparation leads to respiratory inhibition that is believed to be mediated by the local release from these neurons of beta-endorphin, as this effect can be blocked by systemic naloxone [11].

The fact that naloxone was without any effect on respiratory failure induced by oxotremorine suggests that it is not the activation of muscarinic receptors that induces the opioidergic influence of respiratory centre in the pontomedullar region of the rat brain. Similar lack of interaction between oxotremorine and naloxone was described in the tail-immersion nociceptive test in mice [38].

An alternative explanation would be that the major part of this effect is played by nicotinic receptors that, of course, cannot be blocked by atropine [39]. Some other experiments have shown that use of high doses of central nicotinic antagonist mecamylamine prolonged survival and eliminated some signs of respiratory irregularities, thus underlying the generally undervalued importance of nicotinic receptors in soman neurotoxicity [40]. This paper clearly indicates that the hyperstimulation of the post-synaptic nicotinic receptors by the excess of acetylcholine induces release of the excitotoxic amino acid glutamate that can be responsible for the adverse respiratory effects of soman [40]. Some authors interpret the fact that atropine cannot completely restore the activity pattern of the respiratory centre in animals poisoned by even lower doses of soman with the fact that there are not only muscarinic, but also nicotinic receptors in the respiratory centre neurons [3].

Indeed, it seems that, in the presence of soman and atropine, it is the stimulation of nicotinic presynaptic receptors that induces the release of glutamate [41]. There is some evidence that this pathway can also represent a link between cholinergic and glutamatergic neurons that in turn exerts control over release of endogenous opioids in the brain [42].

Bonham [43] showed that the opioids decreased the excitatory glutamatergic effects of respiratory neurons. Both NMDA- and non-NMDA-glutamate receptors were found to mediate the excitatory control on phrenic nucleus motoneurons discharge [26]. Moreover, acetylcholine can directly release endogenous opioids in the brain *via* the stimulation of nicotinic receptors [44].

In conclusion, the results of this study show that soman induces a central respiratory depression in anaesthetized rats that can be blocked individually by the muscarinic receptor antagonist atropine, but also by the opioid receptor antagonist naloxone. Administered together, naloxone and atropine produced a synergistic respiration-restoring effect. As naloxone was unable to block the respiratory depression exerted by muscarinic receptor agonist oxotremorine, it seems that soman induces central respiratory depression via a complex mechanism that includes at least two pathways – cholinergic and opioidergic, although the possible role of glutamatergic transmission cannot be ruled out.

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