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Exogenous gaseous superoxide potentiates the antinociceptive effect of opioid analgesic agents

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Abstract. The study examined the potentiation of the antinociceptive action of opioid analgesics produced by gaseous superoxide (GS) in the rat hind paw withdrawal test (PWT) and by GS or hydrogen peroxide (HP) in the formalin test. In the PWT, inhalation of GS for 50 minutes before i.p. injection of threshold doses of morphine (0.5 mg/kg) and trimeperidine (1.0 mg/kg) increased the threshold of nociception (TN) by a maximum of 43.0% ($p < 0.05$) and 113.4% ($p < 0.01$) respectively. The GS/trimeperidine-dependent increase in TN showed two peaks, the second of which could be suppressed by nialamide. Naloxone abolished the GS/morphine-dependent increased in the TN. In the formalin test, a significant antinociceptive effect developed after GS inhalation or HP administration (intranasally, $2 \times 5\,\mu l$ of 2×10^{-5} mol/l solution in saline) in combination with low doses of Omnopon (0.06–0.75 mg/kg). These results suggest that both GS and HP potentiate the antinociceptive effects of opioid analgesics.

Key words: Superoxide – Nociception – Opioid analgesics – Potentiation of antinociception – Animals

Introduction

The biological action and biochemical characteristics of the metabolic (endogenous) superoxide $O_2^{\bullet -}$ have been widely investigated, particularly in terms of the causal relationship with oxidative stress [1]. Negatively charged oxygen ions are known to be a component of negative atmospheric ions as well [2]. This gaseous superoxide is regarded as the biologically active part of the air ion pool [3].

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Both the CNS and neurohumoral regulation may be involved in the development of the physiological effects of negatively charged atmospheric oxygen ions [4]. Numerous studies have shown an influence of gaseous superoxide on activity of mono-amine oxidase (MAO) and deamination of serotonin in the rat brain [5]. The decrease in the serotonin levels in the brain and blood [6] and tranquillising effects induced by inhaled superoxide becomes more apparent under stress-like conditions [7].

Serotonin as a central transmitter is known to play a role in the central and peripheral mechanisms of nociception [8]. Under different experimental conditions serotonin may facilitate, produce no effect on, or reduce, behaviourally defined analgesia [9]. The serotoninergic pathway in the CNS can mediate antinociception and is involved in the expression of the antinociceptive action of opioids [10]. On the other hand, inhalations of superoxide lead to the activation of the numerous endocrine glands, including the adrenal glands [11]. This could indicate the possible activation of the synthesis and processing of endogenous opioids. Antinociceptive effects may be one of the likely consequences.

Indeed, several studies confirm the impact of inhaled superoxide on nociception. However, these data are sparse and contradictory. Minehart et al. [12] have shown a pain relieving action in patients with post-operative wounds after ion exposure. Olivereau [13] reports that long-term inhalation of negatively charged air increases latency in the hot-plate test in mice. In contrast to this observation, Beardwood and Jordi [14] reported that negative air ion exposure reduces the inhibitory effects of morphine in the tail-flick test.

The effects of metabolic superoxide on the origin and perception of pain are also insufficiently known. At tissue level, superoxide as well as other reactive oxygen species can promote the formation of alkyl radicals and enhance the synthesis of both thromboxane and prostaglandins [15]. These substances may be involved in the development of hyperalgesia and inflammatory pain [16].

However, in recent careful work, Kress et al. [17] found that superoxide, H_2O_2 and $^{\bullet}OH$ do not play a specific role in nociceptor sensitisation.

The afore-mentioned results allow the following general conclusions to be made. Peripherically administered reactive oxygen species are not able to change pain perception. Intranasal administration of negative air ions produces a sedative effect that is developed through central nervous and endocrine mechanisms. Data on the antinociceptive action of negative air ions in animals are contradictory. The analgesic action of negative air ions in humans develops after surgical operation and probably against a background of stress and pre-operative sedation and anaesthesia. Therefore, it can be assumed that these circumstances may be a crucial factor in the onset of the antinociceptive action of gaseous superoxide. It is not yet clear whether the biological activity of gaseous superoxide depends on the charge or on the radical nature of the ion. The free radical properties of superoxide also suggest the involvement of hydrogen peroxide in the physiological response.

The objective of this work was to investigate the antinociceptive action of inhaled gaseous superoxide and intranasally administered hydrogen peroxide *per se* and in combination with opioid analgesic agents, and to prove the involvement of MAO-dependent processes in this interaction.

Materials and methods

Animals

All experiments were performed on 150–250 g male white mongrel rats. The animals were housed in standard sawdust-lined cages in groups of 5–7 under natural light and with free access to food and water. Room temperature and humidity were 21–24°C and 65–70% respectively. All tests were conducted between 11:00 h and 18:00 h. There was no adaptation procedure performed for PWT. Independent groups were used for each experiment. The total number of animals was 296.

Drugs and reagents

The opioid analgesics used were morphine (Merck KGaA, Darm-Stadt, Germany) and trimeperidine (syn. Promedol, 1,2,5-trimethyl-4-phenyl-4-propionoxypiperidine), as well Omnopon (syn. Pantopon, containing 48–50% morphine, 29.9–34.2% other opium alkaloids and 15% papaverine, both were obtained from Medbioprom, Ukraine). Further drugs and reagents used were naloxone and the MAO inhibitors pheniprazine and nialamide as well superoxide dismutase (SOD, EC 1.15.1.1; from bovine erythrocytes), hydrogen peroxide (puriss. p.a.) and formalin (all were obtained from Sigma Chemie GmbH, Deisenhofen, Germany).

Nociception tests

Hind paw withdrawal reflex. A Randall and Selitto apparatus was used to evaluate the response to noxious pressure applied to the hind paw (Ugo Basile, Varese, Italy). This applies a linearly increasing force to the dorsum of the rat hind paw via a blunt wedge-shaped piston with a surface area of 1.75 mm^2. The value of the critical pressure (VCP, in g) required to elicit withdrawal was determined. To estimate the baseline VCP, three measurements were taken at 10-min intervals. The VCP measurements were taken at 30 min intervals.

Formalin test. The formalin test was originally described by Dubuisson and Dennis [18] and found increasing use as a model of prolonged pain. The analgesic effect of drugs is established by the behavioural expression of pain (favouring, lifting, licking and flinching/shaking of the injured paw) and a combination of these [19]. To evaluate nociceptive reflexes in rats, 50 µl of 3.0 % formalin (diluted in saline) was injected subcutaneously into the plantar surface of the fore paw. The interval between the start of the noxious stimulus and the first contact of the paw with the cage floor was determined. In all experiments, each animal was examined only once. Saline was administered to the control animals.

Superoxide production. We used a superoxide producing device with an oxygen flow rate of 150–180 ml/min directed from a cylinder of medical oxygen through a silent corona discharge region [3]. The DC voltage applied to the discharge electrode was -4 kV. The biochemically detectable gaseous superoxide was measured at a distance of 10 mm from the carbon fibre sigrafil-D2® (Sigri GmbH, Germany) electrode. The reduction in nitroblue tetrazolium and cytochrome *c* were used to determine the rate of superoxide production. It was approximately 0.25 µmol/min at a room temperature of 21–24°C and a relative humidity of 65–70 %. The specificity of superoxide production was confirmed by inhibition of these reactions with SOD. The formation rate of ozone did not exceed 0.05 ppm/min (Dräger tubes 6733181, Germany).

Experimental design. The oxygen/superoxide gas mixture was blown through a nozzle at the top of the cages of animals. The cages were equipped with vents. The cage dimensions were 35 × 30 × 30 cm; 3–4 rats were treated simultaneously. The duration of inhalation was 50 min in the PWT and 60 min in the formalin test. The control animals were placed in identical cages with a similar supply of pure oxygen. Both the formalin test and the hind paw withdrawal test were performed after gas inhalation and injection of the drugs.

All analgesics and MAO inhibitors were injected intraperitoneally at a volume of 0.2 ml. The control animals received saline only. SOD administration (intranasally, 2 × 25 µl of solution in saline contained 100 un./ml of enzyme) was performed before inhalation of superoxide. Hydrogen peroxide administration (intranasally, 2 × 25 µl of 2×10^{-5} mol/l solution in saline) was performed simultaneously with injection of formalin and Omnopon and the start in the formalin test, and 15 min before injection of trimeperidine and the start of the PWT. The conditions of treatment are shown in each figure (see Results).

Statistics

The initial VCP of each animal in the hind paw withdrawal test was defined as the mean value of three measurements. The mean values were determined for every control and experimental group and for every time point. All statistical comparisons between VCP values for control and experimental groups were conducted using the Mann-Whitney rank sum test for unmatched random samples. For clarity of data presentation and to facilitate inter-group comparisons, all data were expressed as a percentage of the corresponding control values, which were defined as 100%, using the formula:

$$\frac{[\mathrm{VCPexp.,i} - \mathrm{VCPexp.,0}] - [\mathrm{VCPcontr.,i} - \mathrm{VCPcontr.,0}]}{\mathrm{VCPcontr.,\ i}} \times 100\%$$

where VCPexp.,0 and VCPcontr.,0 data is defined as the VCP values in each experimental and control group for the baseline, and VCPexp.,i and VCPcontr.,i as the values for successive measurement points respectively.

In the formalin test, the statistical significance of the differences

in the values of the duration of pain was verified using Student's *t*-test for independent groups. All p values are based on two-tailed comparisons. In both test's p values less than 0.05 were considered as significant.

Results

Hind paw withdrawal test

Lack of effect of the gaseous superoxide and hydrogen peroxide. Figures 1 and 2 present a nonsignificant slight alteration in the hind paw withdrawal response in rats after inhalation of superoxide. Intranasally administered hydrogen peroxide did not change nociception either (data not shown).

Gaseous superoxide potentiates the antinociceptive action of trimeperidine and morphine. Three doses of trimeperidine (5.0, 2.0, and 1.0 mg/kg) and 2 doses of morphine (1.0 and 0.5 mg/kg) were tested. The antinociceptive action of effective doses of both trimeperidine (Fig. 1) and morphine (not shown) developed during the first 30 min after drug injection. The threshold dosage of trimeperidine and morphine without superoxide inhalation was 1.0 and 0.5 mg/kg respectively (Fig. 1 and 2).

In animals treated with threshold doses of analgesics and after inhalation of superoxide the VCP in both experimental groups (e.g. GS/trimeperidine and GS/morphine) was significantly higher than each of the controls. In the rats treated with GS/trimeperidine (1.0 mg/kg, Fig. 1), the VCP increased significantly between 90 and 120 min and between 210 and 240 min. In the rats treated with GS/morphine (0.5 mg/kg), a significant decrease in nociception was observed between 120 and 180 min (Fig. 2 and 4). These results demonstrate the potentiation of the antinociceptive activity of trimeperidine and morphine after inhalation of gaseous superoxide. Such action does not develop after administration of hydrogen peroxide in combination with trimeperidine (data not shown). The effect of hydrogen peroxide in combination with morphine was not investigated.

Nialamide and naloxone alter the potentiation effect of superoxide. Nialamide (1.0 mg/kg, i.p.) was injected 1 h before superoxide inhalation. It suppressed the second (between 210 and 240 min) peak of antinociception developed in the rats treated with the combination GS/trimeperidine (Fig. 3). The first antinociception peak was insensitive to nialamide. Naloxone (0.5 mg/kg, i.p.) injected 15 min before superoxide inhalation abolishes the antinociception induced by GS/morphine (Fig. 4).

Formalin test

Gaseous superoxide potentiates the antinociceptive action of Omnopon. The nociceptive responses after subcutaneous injection of formalin develop in the first 60 sec and include behavioural manifestations such as favouring, lifting, licking and flinching/shaking of the injured paw. The first contact of the paw with the cage floor was used

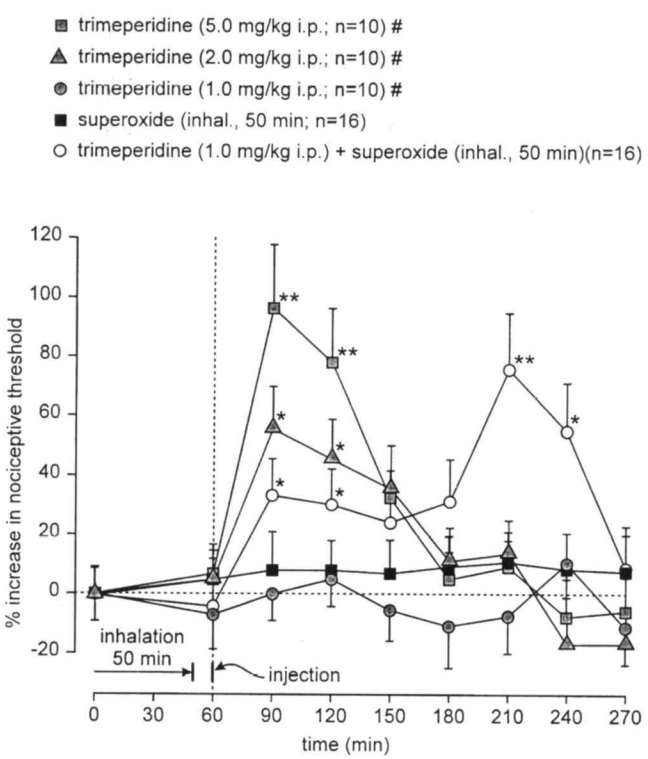

Fig. 1. Inhaled gaseous superoxide potentiates the antinociceptive action of threshold doses of trimeperidine in the rat PWT. Basic pressure values were (mean ± SD) 136.6 ± 11.8 g. VCP values were expressed as percentage response of the control (oxygen-inhaled, saline-treated rats) group. Control: n = 10, * $p < 0.05$, ** $p < 0.01$ compared to control, # = treatment with trimeperidine without inhalation.

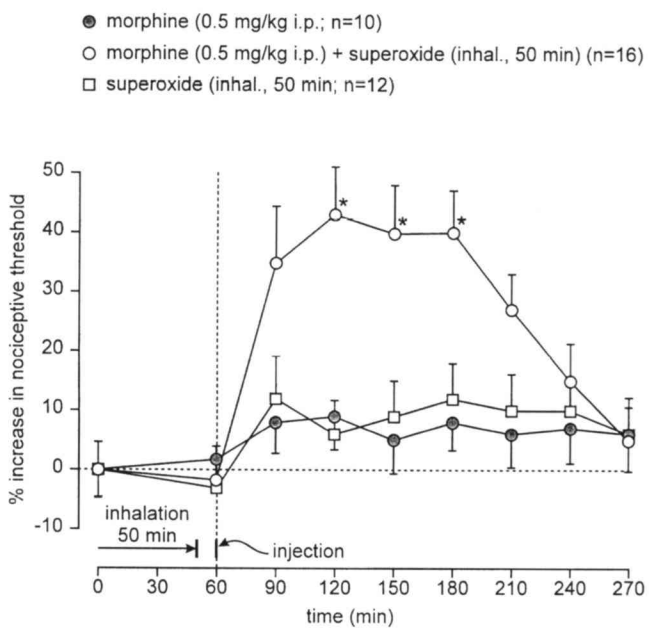

Fig. 2. Inhaled gaseous superoxide potentiates the antinociceptive action of threshold doses of morphine in the rat PWT. Basic pressure values were (mean ± SD), 129.8 ± 12.1 g. Data are expressed as described in the legend of Fig. 1. Control: n = 10, * $p < 0.05$ compared to control.

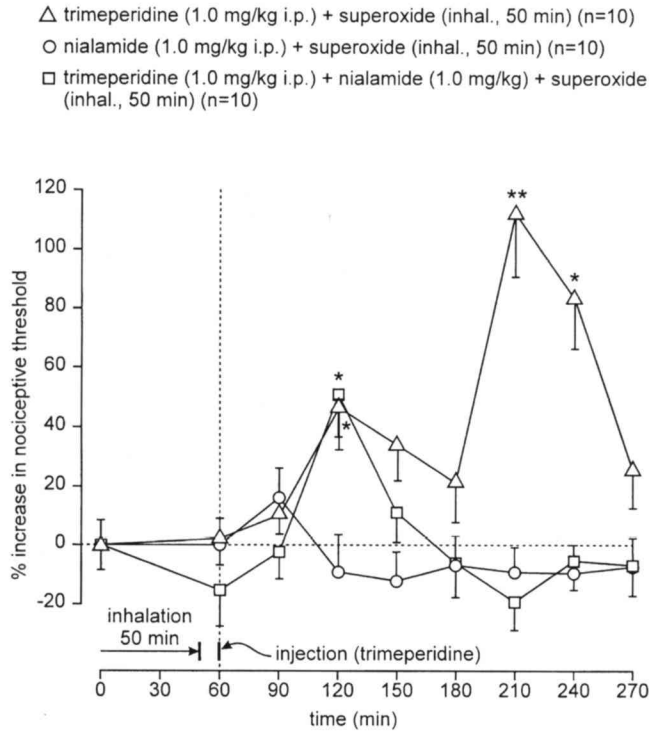

Fig. 3. Inhibitory action of nialamide (1 h before superoxide inhalation) on the antinociceptive action of superoxide/trimeperidine in the rat PWT. Basic pressure values were (mean ± SD), 143.9 ± 12.2 g. Data are expressed as described in the legend of Fig. 1. Control, n = 10. * $p < 0.05$, ** $p < 0.01$ compared to control.

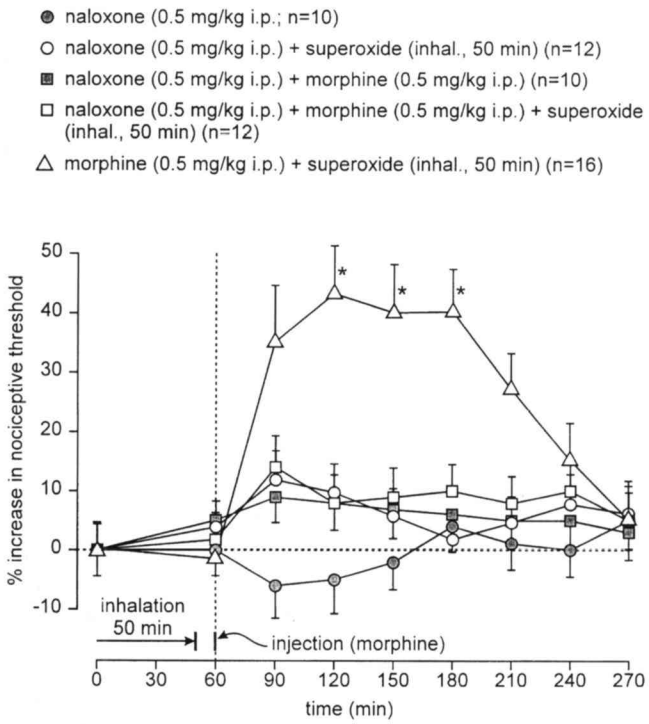

Fig. 4. Inhibitory action of naloxone (15 min before superoxide inhalation) on antinociception in the rat PWT caused by superoxide and superoxide/morphine. Basic pressure values were (mean ± SD), 147.9 ± 13.0 g. Data are expressed as described in the legend of Fig. 1. Control: n = 12, * $p < 0.05$ compared to control. The group "morphine + superoxide" conform to Fig. 2.

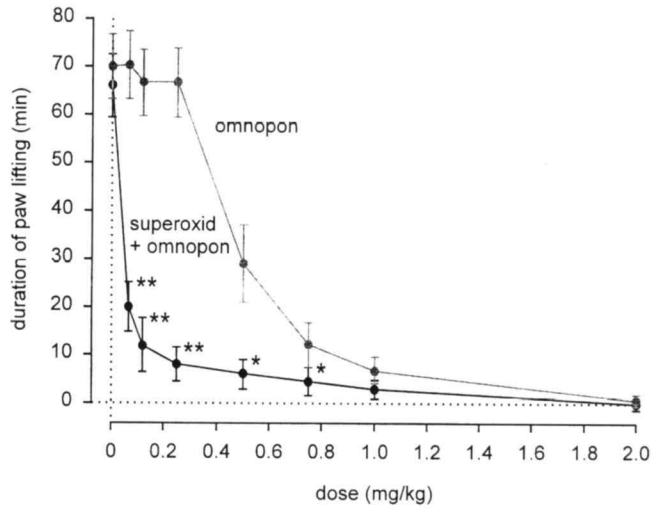

Fig. 5. Omnopon- and superoxide/Omnopon-dependent antinociception in the formalin test: Gaseous superoxide potentiates the antinociceptive action of low doses of Omnopon (0.06 - 0.75 mg/kg). Mean ± SD. * $p < 0.05$, ** $p < 0.01$ (Student's *t*-test) compared to oxygen-inhaled, Omnopon-treated rats. n = 6 in each point.

as a sign of the suppression of the nociceptive response. In control animals, the time interval between the injection of formalin and the earliest step of the injured paw was 70.1 ± 6.8 min. Inhalations of gaseous superoxide alone did not suppress nociception. Superoxide potentiated Omnopon-related antinociception at an Omnopon dosage of 0.06 to 1.0 mg/kg was observed. Dosages from 1.0 mg/kg to 2.0 mg/kg were not investigated; the effect of 2.0 mg/kg was identical in both groups (Fig. 5). The Omnopon dosage in all following tests was 0.75 mg/kg. Without inhalation of gaseous superoxide, this dosage of Omnopon decreased the duration of nociception to 12.1 ± 4.7 min ($p < 0.05$, Fig. 5, 6-2). The duration of lifting of the injured paw in GS/Omnopon-treated rats was less than in Omnopon-treated animals. In Omnopon-treated animals, an aggravation of the licking and flinching/shaking of the injured paw, and vocalisation, developed between the 15th and the 20th min. In GS/Omnopon-treated rats, these reactions were very rare.

Effect of pheniprazine. In pheniprazine-treated rats (10.0 mg/kg, i.p., 1 h before injection of formalin and Omnopon), the duration of lifting of the injured paw increased significantly in comparison with animals treated with Omnopon or GS/Omnopon. Superoxide inhaled for 60 min after injection of pheniprazine partially abolishes this effect (Fig. 6-4 and 6-5).

Effect of SOD. The administration of SOD, but not thermoinactivated SOD, before inhalation of superoxide significantly decreased the duration of the paw lifting response (Fig. 6-6). It is known that the hydrogen peroxide H_2O_2 is produced by the enzymatic dismutation of superoxide [1]. Considering these results, the involvement of hydrogen peroxide in the potentiation of analgesics may be assumed. Indeed, the duration of paw lifting after administration of hydrogen peroxide and Omnopon was comparable with the response on administration of "SOD + GS/Omnopon" (Fig. 6-7). Hydrogen

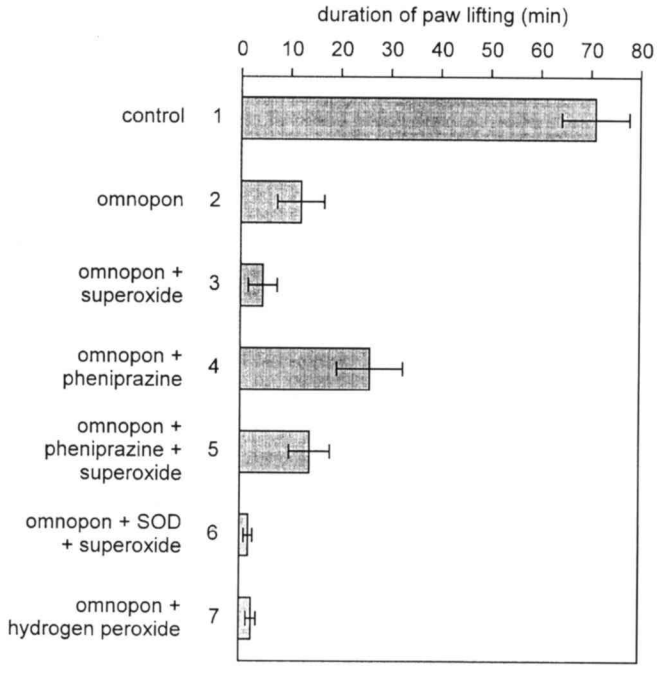

Fig. 6. Omnopon- and superoxide/Omnopon-dependent antinociception in the formalin test: Effects of pheniprazine, SOD and hydrogen peroxide. In all cases, Omnopon dose = 0.75 mg/kg. $n_{(1\text{-}6)} = 6$, $n_7 = 8$. Student's *t*-test: $p_{1 \text{ vs. } 2} < 0.001$, $p_{2 \text{ vs. } 3,4,6,7} < 0.05$, $p_{4 \text{ vs. } 5} < 0.1$ (not significant).

peroxide alone does not influence the response investigated (data not shown).

Discussion

Potentiation effects of exogenous gaseous superoxide depend on its free radical properties. In the work presented, we have obtained evidence that both inhaled gaseous superoxide and intranasally administered hydrogen peroxide are involved in the central mechanisms of nociception. Because of the physical and chemical nature of superoxide, its biological activity may depend either on the electrical charge of the ion or on free-radical properties. The overwhelming majority of authors regard the electrical charge of negative air ions as the basis of their biological activity [2]. Only three reports connect the biological effects of negative air ions with the free radical activity of superoxide [3, 20, 21]. In the present paper, we have shown that free radical properties of gaseous superoxide may play a favourable role in the potentiation of antinociception. General arguments to corroborate this theory are that 1) in the formalin test, intranasally administered native SOD modifies the physiological effect of superoxide; it is well known that SOD removes $O_2^{\bullet -}$ by converting it into hydrogen peroxide [1]:

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2,$$

that 2) SOD increases but does not suppress GS-dependent antinociception, and that 3) "SOD + GS"-like activity can be reproduced by intranasal administration of hydrogen peroxide at a very low concentration (Fig. 6-7).

Potentiation effect of gaseous superoxide involves different physiological mechanisms. Exogenous gaseous superoxide potentiates the antinociceptive activity of the narcotic analgesics investigated. In both tests, antinociception was potentiated by a combination of superoxide with threshold or low doses of analgesics. This effect resembles the potentiation of threshold doses of analgesics by low dosages of sedatives [22]. The tranquillising action of negative air ions is well known [5, 13].

In the hind paw withdrawal test, naloxone abolishes the antinociception caused by superoxide/morphine (Fig. 4). This demonstrates the involvement of morphine receptors. The biochemical mechanisms of the potentiation are a matter of speculation. In the antinociception caused by superoxide/trimeperidine, two peaks were observed (Fig. 1) which are due to different causes. Only the second peak was nialamide-sensitive, i.e. MAO-dependent (Fig. 3). It is possible that the pheniprazine-induced increase in duration of paw lifting in the formalin test (Fig. 6–4) is of the same origin. In accordance to the data that gaseous superoxide increase MAO-dependent metabolism of serotonin [5], the functional antagonism between superoxide and MAO-inhibitors seems to be natural enough.

The effects of time-factor may also be different. The afore-mentioned results observed by Beardwood and Jordi [14] may be caused by extremely long-term (144 h) treatment of the animals. Morphine is also known to suppress the tail-flick response in spinal rats [23]. However, the effects observed with inhaled superoxide develop primarily at brain level. Both catecholamines and serotonin are necessary for the development of the antinociceptive action of morphine [24]. An alteration in the metabolic rate of these transmitters caused by long-term action of gaseous superoxide may also influence the function of morphine receptors. Beside that, the transmission of thermal and non-thermal nociception may involve different classes of opioid receptors [25].

At the endocrine level, the inhalation of gaseous superoxide leads in rats to marked morphological and physiological activation of the endocrine glands, and primarily the pituitary and adrenal glands [13]. We have observed a two-fold increase in the number of ACTH-producing cells in the rat pituitary after repeated inhalation of gaseous superoxide (Goldstein, unpublished). This may serve as evidence of the increased synthesis of endogenous opioids, in particular beta-endorphins. In the development of antinociception in the formalin test, the double stimulation of the endogenous opioid system during exogenous superoxide and stress can be assumed.

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