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Menthol: a natural analgesic compound

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Abstract

Menthol, after topical application, causes a feeling of coolness due to stimulation of 'cold' receptors by inhibiting Ca^{++} currents of neuronal membranes. Since Ca^{++} channel blockers are endowed with analgesic properties, the aim of the present study was to investigate the potential antinociceptive effect of menthol. (−)-Menthol produced a dose-dependent increase in the pain threshold in the mouse hot-plate (3–10 mg kg^{-1} p.o.) and abdominal constriction (3–10 mg kg^{-1} p.o.; 10 μg per mouse intracerebroventricularly (i.c.v.)) tests. The antinociceptive effect of (−)-menthol was antagonised by the unselective opioid antagonist naloxone and by the selective κ-antagonist nor-NBI. Conversely, CTOP (μ-antagonist), 7-benzylidenenal-trexone (δ_1 antagonist) and naltriben (δ_2 antagonist) did not prevent (−)-menthol antinociception. In both tests, (+)-menthol (10–50 mg kg^{-1} p.o.; 10–30 μg per mouse i.c.v.) was unable to modify the pain threshold. These results indicate that (−)-menthol is endowed with analgesic properties mediated through a selective activation of κ-opioid receptors. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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Menthol is a naturally occurring compound of plant origin, which gives plants of the Mentha species the typical minty smell and flavour. Menthol is present in the volatile oil of several species of mint plants such as peppermint *Mentha piperita* and cornmint oil *Mentha arvensis*. Peppermint and cornmint oil, prepared by steam distillation from the fresh flowering tops of the plant, contains 50% and 70% of (−)-menthol, respectively [2]. Menthol can also be extracted or synthesised from other essential oils such as citronella oil, eucalyptus oil and Indian turpentine oil. Menthol is a cyclic terpene alcohol with three asymmetric carbon atoms. Among the optical isomers, (−)-menthol is the one that occurs most widely in nature and it is endowed with the peculiar property to be a fragrance and flavour compound. For this reason it is widely used as flavouring for toothpaste, other oral hygiene products and chewing-gum [2].

In pharmacy, it is part of topical antipruritic, antiseptic and cooling formulations. Moreover, (−)-menthol is included in eutectic formulations of local anaesthetic agents [8,9]. Peppermint is traditionally used in the symptomatic treatment of digestive disorders; the antispastic, carminative, choleretic and colagogic properties attributed to it are referred to the presence of the essential oil rich of (−)-menthol [20]. Menthol is also employed in external bronchiolytic and secretolytic preparations [9]. Even though their wide use, ethereal oils are endowed with some toxic effects [9].

Applied topically, menthol causes tingling sensation and a feeling of coolness due to stimulation of 'cold' receptors by inhibiting Ca^{++} currents of neuronal membranes [2]. Ca^{++} solutions cause a diffuse sensation of warmth by increasing the frequency of warm-receptor discharge whereas a decrease of external Ca^{++} concentration increases the discharge of cold receptors [16]. It has also been reported that modulation of Ca^{++} currents is involved in the regulation of pain threshold. Inhibition of Ca^{++} currents by administration of voltage-sensitive Ca^{++} channel blockers produce antinociception in laboratory animals [11]. Furthermore, menthol is able to block voltage-gated Ca^{++} channels in human neuroblastoma cells [17]. On these bases, the aim of the present study was to investigate the potential analgesic activity of menthol.

The present investigation was made by using analgesic

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test in which a thermal (hot-plate) or a chemical (abdominal constriction) stimulus was applied. In order to avoid the induction of behavioural side effects, which could lead to a misinterpretation of the obtained results, we also evaluated the motor co-ordination of treated animals (rota rod test). In all behavioural tests male Swiss albino mice (23–30 g) from Morini (San Polo d'Enza, Italy) were used. The mice were housed fifteen per cage. The cages were placed in the experimental room 24-h before the test for adaptation. The animals were fed a standard laboratory diet and tap water ad libitum and kept at 23 ± 1 °C with a 12-h light/dark cycle, light on at 07:00 h. Animals were used a single time. All experiments were carried out according to the guidelines of the European Community Council for experimental animal care.

The hot plate test was performed according to O'Callaghan and Holtzman [13]. Briefly, mice were placed inside a stainless steel container, which was set thermostatically at 52.5 ± 0.1 °C in a precision water-bath from KW Mechanical Workshop, Siena, Italy. Reaction times (s), were measured with a stopwatch before and 15, 30, 45 and 60 min after menthol administration. The endpoint used was the licking of the fore or hind paws. Those mice scoring less than 12 and more than 18 s in the pretest were rejected (30%). To prevent tissue injury, an arbitrary cut-off time of 45 s was adopted.

The abdominal constriction test was performed according to Koster et al. [10]. Mice were injected intraperitoneally (i.p.) with a 0.6 % solution of acetic acid (10 ml kg^{-1}) and the number of stretching movements was counted for 10 min, starting 5 min after acetic acid injection.

The motor co-ordination was evaluated by using the rota rod test. The apparatus consists of a base platform and a rotating rod of 3 cm diameter with a non-skid surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into five equal sections by six disks. Thus up to five mice were tested simultaneously on the apparatus, with a rod-rotation speed of 16 rpm. The integrity of motor co-ordination was assessed on the basis of the number of falls from the rod in 30 s. Performance time was measured before and 15, 30 and 45 min after i.p. administration of the investigated compounds.

The following drugs were used: (+)-menthol (Aldrich); (−)-menthol, CTOP (D-phe-cys-tyr-D-trp-orn-thr-pen-thr amide), naltriben methanesulfonate, naloxone hydrochloride, nor-binaltorphimine dihydrochloride; sodium carboxymethylcellulose (Sigma); 7-benzylidenenaltrexone maleate (Tocris).

Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 5 μl per mouse by intracerebroventricularly (i.c.v.) injection and 10 ml kg^{-1} by i.p. or oral (p.o.) administration. All drugs were dissolved in saline solution with the exception of (+)- and (−)-menthol which were solubilised in a vehicle of 1:4 solution of DMSO in H_2O (at the highest concentration whereas successive dilutions were made by using only H_2O) for i.c.v. injections or dispersed in sodium carboxymethylcellulose 1% immediately before use for p.o. injections. I.c.v. administration was performed under ether anaesthesia with isotonic saline as solvent, according to the method described by Haley and McCormick [6]. During anaesthesia, mice were grasped firmly by the loose skin behind the head. A hypodermic needle (0.4 mm external diameter) attached to a 10 μl syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5 μl of solution was then administered. The injection site was 1 mm to the right or left from the midpoint on a line drawn through to the anterior base of the ears. Injections were performed randomly into the right or left ventricle. To ascertain that drugs were administered exactly into the cerebral ventricle, some mice (10%) were injected with 5 μl of diluted 1:10 India ink and their brains were examined macroscopically after sectioning. The accuracy of the injection technique was evaluated with 95% of injections being correct.

All experimental results are given as the means ± SEM. An analysis of variance (ANOVA), followed by Fisher's Protected Least Significant Difference (PLSD) procedure for post-hoc comparison, was used to verify the significance of differences between two means. Data were analysed with the StatView software for the Macintosh (1992).

(−)-Menthol, as shown in Fig. 1, produced a dose-dependent increase in the pain threshold in the mouse hot-plate test (3–10 mg kg^{-1} p.o.). The antinociceptive effect of (−)-menthol peaked 30 min after administration and then diminished. In this test, the antinociception induced by (−)-menthol was comparable to that exhibited by morphine (10 mg kg^{-1} p.o.), used as reference drug (data not shown). (−)-menthol was also able to produce antinocicep-

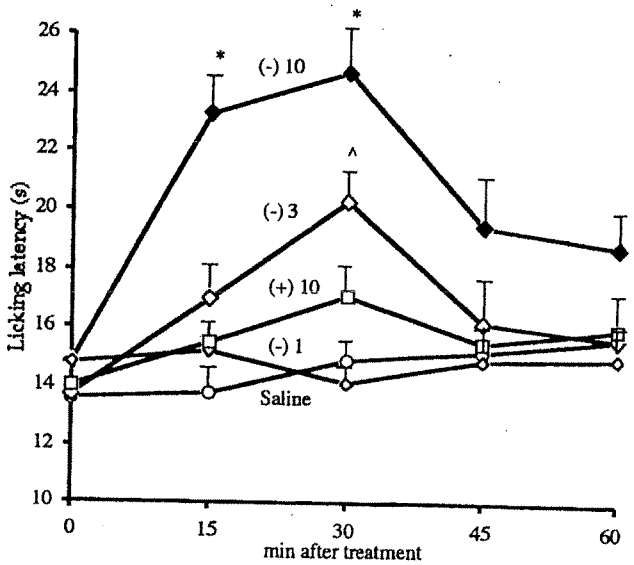

Fig. 1. Effect of (+) and (−)-menthol in the mouse hot-plate test. Doses are expressed as mg kg^{-1} p.o. Each point represents the mean of at least 10 mice. $^P < 0.05$; $*P < 0.01$ in comparison with saline-treated mice.

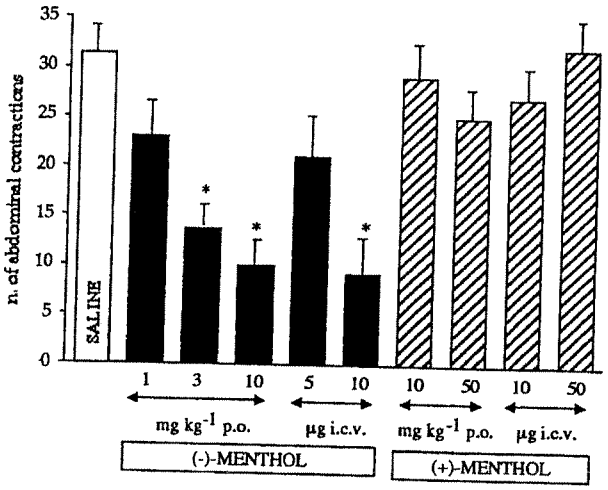

Fig. 2. Effect of (+) and (−)-menthol in the mouse abdominal constriction test. Each column represents the mean of eight mice. Nociceptive response was evaluated 15 min after administration. *$P < 0.01$ in comparison with saline-treated mice.

tion in the mouse acetic acid abdominal constriction test (3–10 mg kg^{-1} p.o.; 10 µg per mouse i.c.v.) (Fig. 2). In both tests, (+)-menthol (10–50 mg kg^{-1} p.o.; 10–30 µg per mouse i.c.v.) was unable to modify the pain threshold (Figs. 1 and 2).

In the mouse abdominal constriction test, the antinociceptive effect of (−)-menthol (10 µg per mouse i.c.v.) was antagonised by the unselective opioid antagonist naloxone (1 mg kg^{-1} i.p.) and by the selective κ-antagonist nor-NBI (735 ng per mouse i.c.v.), administered 15 min before (−)-menthol (Fig. 3). Conversely, the selective µ-antagonist CTOP (50 µg per mouse i.c.v.), the selective δ_1 antagonist 7-benzylidenenal-trexone (BNTX) (3.5 ng per mouse i.c.v.) and the selective δ_2 antagonist naltriben (19 µg per mouse i.c.v.), administered 15 min before test, did not prevent (−)-menthol antinociception in the mouse abdominal constriction test (Fig. 3). All antagonists employed, when injected alone, did not modify the mouse pain threshold (Fig. 3).

Mice pretreated with (−)-menthol and (+)-menthol were evaluated for motor co-ordination by use of the rota-rod test. The number of falls, evaluated 30 min after the beginning of the rota-rod test in correspondence with the maximum analgesic effect of (−)-menthol, showed the lack of any impairment in the motor co-ordination of animals pretreated with (−)-menthol at the doses of 10 mg kg^{-1} p.o. (2.7 ± 0.3) and 10 µg per mouse i.c.v. (2.2 ± 0.4) in comparison with corresponding vehicle-treated mice (2.5 ± 0.4; 2.0 ± 0.3). Conversely, a double dose of (−)-menthol produced an increase in the number of falls from the rotating rod (5.1 ± 0.3 p.o.; 3.9 ± 0.4 i.c.v.) indicating the induction of motor side effects.

(−)-Menthol is a natural compound able to induce analgesia in laboratory animals regardless the noxious stimulus used: thermal (hot-plate) or chemical (abdominal constriction test).

(−)-Menthol antinociception was found to be dependent on activation of the opioid system as this analgesia is antagonised by the unselective opioid antagonist naloxone. In particular, the increase of the pain threshold induced by (−)-menthol is mediated by the stimulation of κ-opioid receptors since this analgesia was prevented by the κ-opioid antagonist nor-NBI. Several literature data have characterised nor-BNI as a long-lasting and selective κ-opioid antagonist after central or peripheral administration [3,7,19]. Selectivity of nor-BNI for κ-opioid receptor-mediated effects in rodents has been reported. These studies demonstrated that subcutaneous or i.c.v. nor-BNI antagonised the antinociceptive effect of κ-opioid agonists U50,488 and U69,593, but, under the same experimental conditions, it did not prevent the analgesia induced by µ agonists, morphine and DAMGO, and that of the selective δ agonist, DPDPE **[3,7,19]**. These findings are in accord with binding data for nor-BNI showing more than 100-fold selectivity for κ over µ receptors [19]. The nor-BNI antagonistic effect cannot be due to a hyperalgesic activity produced by the blockade of κ-opioid receptors since nor-BNI, when injected alone, was unable to modify the pain threshold.

The involvement of µ and δ opioid receptors can also be ruled out. Pretreatment with the selective µ-opioid antagonist CTOP [5,12] as well as with the δ_1 antagonist BNTX [14] and the δ_2 antagonist naltriben [18] did not modify (−)-menthol-induced analgesia.

(−)-menthol exerts its antinociceptive effect by acting centrally since, after i.c.v. administration, it is able to increase the pain threshold with the same intensity as that obtainable after p.o. administration. The antagonism exerted by i.c.v. injected nor-BNI shows that the site of action of

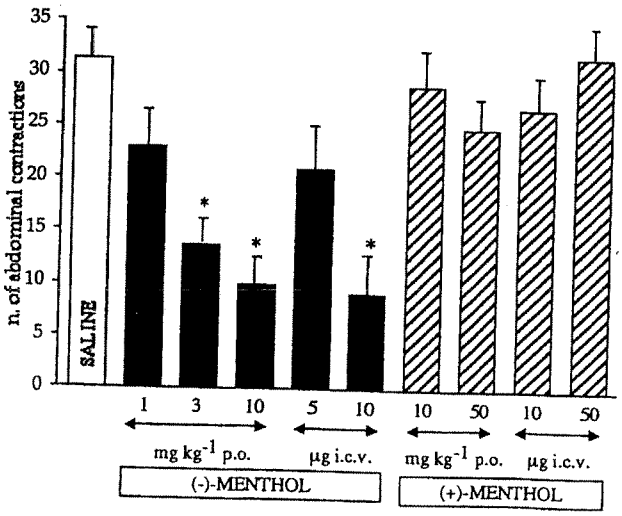

Fig. 3. Effect of naloxone (1 mg kg^{-1} i.p.), CTOP (50 µg per mouse i.c.v.), BNTX (3.5 ng per mouse i.c.v.), naltriben (19 µg per mouse i.c.v.) and nor-binaltorphimine (735 ng per mouse i.c.v.) on (−)-menthol induced antinociception. Each column represents the mean of ten mice. Nociceptive response was evaluated 15 min after administration. *$P < 0.01$ in comparison with (−)-menthol-treated mice.

(−)-menthol is centrally located. The integrity of the central κ-opioid system is, therefore, fundamental for (−)-menthol antinociception. To this end, it is well known that stimulation of central κ-opioid receptors induces an increase of the pain threshold [1,15,18].

Menthol is a natural compound with three asymmetric carbon atoms and, therefore, occurs as four pairs of optical isomers named (+)- and (−)-menthol, (+)- and (−)-neomenthol, (+)- and (−)-isomenthol, (+)- and (−)-neoisomenthol. Among the optical isomers, we investigated the analgesic properties of (+)- and (−)-menthol and we observed the presence of stereoselectivity. (−)-menthol was able to increase the pain threshold whereas (+)-menthol was completely devoid of any analgesic effect.

It is well known that menthol is endowed with local anaesthetic activity. Furthermore, it has been demonstrated that both (+)- and (−)-menthol are equiactive [4]. On the bases of these data, the hypothesis that the analgesic effect is a consequence of the local anaesthetic properties can be excluded. Contrary to that observed for the anaesthetic effect, only (−)-menthol is able to induce analgesia.

Analgesia induced by (−)-menthol in the mouse hot-plate and abdominal constriction tests is obtained without any visible change in the normal behaviour of animals as demonstrated in rota-rod experiments in which no impairment of mouse rota-rod performance is observed. The number of falls progressively decreased since animals learnt how to balance on the rotating rod.

In summary, our results show that (−)-menthol is a cyclic terpene alcohol able to induce analgesia through the activation of the central κ opioid system.

[1] Dykstra, L.A., Preston, K.L. and Bigelow, G.E., Discriminative stimulus and subjective effects of opioids with mu and kappa activity: data from laboratory animals and human subjects, Psychopharmacology (Berl.), 130 (1997) 14–27.
[2] Eccles, R., Menthol and related cooling compounds, J. Pharm. Pharmacol., 46 (1994) 18–630.
[3] Endoh, T., Koike, H., Matsuura, H. and Nagase, H., Norbinaltorphimine (nor-BNI): a potent and selective κ opioid receptor antagonists with ultra-long-lasting activity in vivo, In J.M. Van Ree, A.H. Mulder, V.M. Wiegant and T.B. Van Wimersma (Eds.), Greidanus 4EDS7. New leads in opioid research, Elsevier, Amsterdam, 1990, pp. 82–83.
[4] Galeotti, N., Ghelardini, C., Di Cesare Mannelli, L., Mazzanti, G., Baghiroli, L. and Bartolini, A., Local anaesthetic activity of (+) and (−)-menthol, Planta Med., 66 (2000) 1–2.
[5] Gulya, K., Krivan, M., Nyolczas, N., Sarnyai, Z. and Kovacs, G.L., Central effects of the potent and highly selective μ opioid antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) in mice, Eur. J. Pharmacol., 150 (1988) 355–360.
[6] Haley, T.J. and McCormick, W.G., Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse, Br. J. Pharmacol. Chemother., 12 (1957) 12–15.
[7] Horan, P., Taylor, J., Yamamura, H.I. and Porreca, F., Extremely long-lasting antagonistic actions of nor-binaltorphimine (nor-BNI) in the mouse tail flick test, J. Pharmacol. Exp. Ther., 260 (1992) 1237–1243.
[8] Jyvakorpi, M.A., Comparison of topical Emla cream with Bonain's solution for anesthesia of the tympanic membrane during tympanocentesis, Eur. Arch. Otorhinol., 253 (1996) 234–236.
[9] Korting, G.W. and Weigand, U.A., New case of reticular hyperplasia connected with volatile oils, Hautarzt, 26 (1975) 352–356.
[10] Koster, R., Anderson, M. and De Beer, E.J., Acetic acid for analgesic screening, Fed. Proc., 18 (1959) 412.
[11] Malmberg, A.B. and Yaksh, T.L., Voltage-sensitive calcium channels in spinal nociceptive processing: blockade of N- and P-type channels inhibits formalin-induced nociception, J. Neurosci., 41 (1994) 4882–4890.
[12] Miaskowski, C. and Levine, J.D., Inhibition of spinal opioid analgesia by supraspinal administration of selective opioid antagonists, Brain Res., 596 (1992) 41–45.
[13] O'Callaghan, J.P. and Holtzman, S.G., Quantification of the analgesic activity of narcotic antagonists by a modified hot-plate procedure, J. Pharmacol. Exp. Ther., 192 (1975) 497–505.
[14] Portoghese, P.S., Sultana, M., Nagase, H. and Takemori, A.E., A highly selective δ_1-opiod receptor antagonist: 7-benzylidenenaltrexone, Eur. J. Pharmacol., 218 (1992) 195–196.
[15] Rady, J.J., Takemori, A.E., Portoghese, A.S. and Fujimoto, J.M., Supraspinal δ receptor subtype activity of heroin and 6-monoacetyl morphine in Swiss Webster mice, Life Sci., 55 (1994) 603–609.
[16] Schäfer, K., Braun, H.A. and Hensel, H., Static and dynamic activity of cold receptors at various calcium levels, J. Neurophysiol., 47 (1982) 1017–1028.
[17] Sidell, N., Verity, M.A. and Nord, E.P., Menthol blocks dihydropyridine-insensitive Ca^{2+} channels and induces neurite outgrowth in human neuroblastoma cells, J. Cell. Physiol., 142 (1990) 410–419.
[18] Sofuoglu, M., Portoghese, P.S. and Takemori, A.E., Differential antagonism of δ opioid agonists by naltrindone and its benzofuran analog (NTB) in mice. Evidence for δ opioid receptor subtypes, J. Pharmacol. Exp. Ther., 257 (1991) 676–680.
[19] Takemori, A.E., Ho, B.Y., Naeseth, J.S. and Portoghese, P.S., Nor-binaltorphimine, a highly selective κ opioid antagonist in analgesic and receptor binding assays, J. Pharmacol. Exp. Ther., 246 (1988) 255–258.
[20] Van Hellemont, J., Mentha piperita, Compendium de Phytotherapie, Association Pharmaceutique Belge, Bruxelles, 1985, pp. 253–256.