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(−)-Linalool inhibits in vitro NO formation: Probable involvement in the antinociceptive activity of this monoterpene compound

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Abstract

Recent studies performed in our laboratory have shown that (−)-linalool, the natural occurring enantiomer in essential oils, possesses anti-inflammatory, antihyperalgesic and antinociceptive effects in different animal models. The antinociceptive and antihyperalgesic effect of (−)-linalool has been ascribed to the stimulation of the cholinergic, opioidergic and dopaminergic systems, to its local anaesthetic activity and to the blockade of *N*-Methyl-D-aspartate receptors (NMDA). Since nitric oxide (NO) and prostaglandin E_2 (PGE_2) play an important role in oedema formation and hyperalgesia and nociception development, to investigate the mechanism of these actions of the (−)-linalool, we examined the effects of this compound on lipopolysaccharide (LPS)-induced responses in macrophage cell line J774.A1. Exposure of LPS-stimulated cells to (−)-linalool significantly inhibited nitrite accumulation in the culture medium without inhibiting the LPS-stimulated increase of inducible nitric oxide synthase (iNOS) expression, suggesting that the inhibitory activity of (−)-linalool is mainly due to the iNOS enzyme activity. In contrast, exposure of LPS-stimulated cells to (−)-linalool failed, if not at the highest concentration, both in inhibiting PGE_2 release and in inhibiting increase of inducible cyclooxygenase-2 (COX_2) expression in the culture medium. Collectively, these results indicate that the reduction of NO production/release is responsible, at least partially, for the molecular mechanisms of (−)-linalool antinociceptive effect, probably through mechanisms where cholinergic and glutamatergic systems are involved.

Keywords: (−)-Linalool; Inflammation; Hyperalgesia; Nociception; Nitric oxide; iNOS; PGE_2; COX_2

Introduction

(−)-Linalool is the natural occurring enantiomer of the monoterpene compound commonly found as a major volatile component of the essential oils in several aromatic plant species. It has been shown that (−)-linalool possess anti-inflammatory and antinociceptive activity in several experimental models. In fact, (−)-linalool administration in rats inhibits carrageenan-induced oedema (Peana et al., 2002) and reduces pain responses elicited by different stimulus, i.e., acetic acid-induced writhing (Peana et al., 2003), hot plate, formalin injection (Peana et al., 2004a), hyperalgesia induced by carrageenan, L-glutamate and prostaglandin E_2 (PGE_2) (Peana et al., 2004b). Antinociceptive effects of (−)-linalool have been related to the positive interference with muscarinic, opioid and dopaminergic transmission, since it was reduced by pre-treatment with the unselective muscarinic receptor antagonist atropine, the opioid receptor antagonist naloxone or the dopamine D_2 receptor antagonist sulpiride (Peana et al., 2003, 2004a) and negative modulation of glutamate transmission (Elisabetsky et al., 1999; Silva Brum et al., 2001a,b). Furthermore, the antinociceptive effect of (−)-linalool has complicated pathway; indeed, it cannot be excluded an involvement of ATP-sensitive K^+ channels, since glibenclamide, an inhibitor of these channels, has been shown to abolish the antinociceptive effect of (−)-linalool (Peana et al., 2004a). Moreover, this compound possesses local anaesthetic activity (Ghelardini et al., 1999) and antioxidant properties (Celik and Ozkaya, 2002).

The intraplantar injection of carrageenan and formalin cause the production and release of nitric oxide (NO) at the injured side (Omote et al., 2001). In recent years, considerable

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evidence has accumulated suggesting a role for NO as a mediator of inflammation (Lyons, 1995). NO increases the synthesis/release of pro-inflammatory mediators such as cytokines and reactive oxygen species (Marcinkiewicz et al., 1995) and prostanoids (Sautebin et al., 1995), resulting in promotion of inflammatory reaction. In this way, peripherally released NO contributes to the development of oedema and hyperalgesia in tissue injury and inflammation. In the inflammatory process we can observe the expression of several inducible enzymes that contribute to the release of pro-inflammatory mediators like NO and PGE_2 by the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) respectively (Dudhgaonkar et al., 2004). COX-2 is the inducible form of the enzyme, the synthesis of which is triggered by those cytokines that also induce iNOS (Clancy and Abramson, 1995). The two pathways interact closely and NO can stimulate COX-2 activity by combining with its heme component (Dudhgaonkar et al., 2004). Carrageenan and formalin injection induce expression of iNOS and COX-2.

Since NO and/or PGE_2 are related to the transmission of nociceptive stimulus in inflammatory site, in this work we investigate the effect of (−)-linalool on the formation and release of NO and PGE_2 in macrophages cell line J774.A1 stimulated by lipopolysaccharide (LPS), a cellular model of inflammation.

Materials and methods

Pharmacological methods

Reagents

Escherichia coli lipopolysaccharide (LPS) was obtained from Fluka (Milan, Italy). 3-(4,5-Dimethyl-thiazolyl-2yl) 2,5 diphenyl tetrazolium bromide (MTT), PBS, NaCl, KCl, Na_2HPO_4, K_2HPO_4, Tris–HCl, sodium orthovanadate, phenylmethylsulfonylfluoride, bovine serum albumin (BSA) and 6-mercaptopurine (6-MP) were obtained from Sigma Chemicals Co. (Milan, Italy). Kodak X-Omat film, ECL detection system, Hybond polyvinyldene difluoride membrane were from Millipore (USA). Leupeptin, trypsin inhibitor, Nonidet P40, Biorad reactive, Laemmli's sample buffer, polyacrylamide, non-fat milk, Tween 20, horseradish peroxidase conjugated goat anti-mouse, mouse monoclonal antibody for iNOS and COX-2 was obtained from Transduction Laboratories (Lexington, KY, UK). The peroxidase secondary antibody was purchased from Jackson (West Grove, PA). Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, HEPES, glutamine, foetal calf serum (FCS) and horse serum were from Hy Clone (Euroclone-Cellbio, Pero, Milan, Italy).

Cell treatment

Monolayers of J774.A1 cells were routinely harvested by gentle scraping with a teflon cell scraper, diluted in fresh medium and cultured to confluency at 37 °C. Prior to each experiment cells were harvested, plated to a seeding density of 1.5×10^6 in P60 well plates. After cell adhesion, (−)-linalool (Sigma) (0.0001–0.01–1 mM) was added to the culture medium 1 h before and always simultaneously to LPS (6×10^3 u/ml/24 h).

Analysis of nitrite

Nitrite accumulation, an indicator of NO release, was measured in the culture medium by Griess reaction (Green et al., 1982) 24 h after LPS challenge. Briefly, 100 μl of cell culture medium were mixed with 100 μl of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid 0.1% (w/v) naphthylethylenediamine-HCl] and incubated at room temperature for 10 min, and then the absorbance at 550 nm was measured in a Titertek microplate reader (DASIT). Fresh culture medium was used as blank in all the experiments. The amount of nitrite in the samples was calculated from a sodium nitrite standard curve freshly prepared in culture medium. Results are expressed as percentage of inhibition calculated versus cells treated only with LPS.

Analysis of PGE_2 production

PGE_2 levels in macrophages' medium were quantified by EIA kit according to the manufacturer's instructions.

Western blot analysis for iNOS and COX-2 expression

After 24 h of incubation with LPS medium was removed and cells were washed twice with ice cold PBS and lysed in Tris–HCl (20 mM pH 7.5) containing 10 mM NaF, 150 mM NaCl, 1% Nonidet P40, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadate, leupeptin (10 μg/ml) and trypsin inhibitor (10 μg/ml). After 1 h, cell lysates were obtained by centrifugation at 100,000×*g* for 15 min at 4 °C. Protein concentrations were estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Equal amounts of protein (70 μg) of cell lysates were dissolved in Laemmli's sample buffer, boiled for 5 min, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% polyacrylamide). Western blot was performed by transferring protein from a slab gel to a sheet of polyvinyldene difluoride membrane at 5 mA/min for 40 min at room temperature. Filters were blocked with PBS, 5% (w/v) non-fat dry milk for 40 min at room temperature and then skinned overnight at 4 °C with the monoclonal antibody for iNOS or COX-2, diluted 1:10,000 in PBS, 5% w/v non-fat dry milk and 1% tween-20. Blots were then skinned and incubated, after four washes in PBS containing 5% w/v non-fat dry milk and 1% tween-20, with horseradish peroxidase conjugated goat anti-mouse IgG (1:5000) for 1 h at room temperature. Immunoreactive bands were visualized using ECL detection system according to the manufacturer's instructions and exposed to Kodak X-Omat film. The protein bands of iNOS or COX-2 on X Omat films were quantified by scanning densitometry (Imaging Densitometer GS-700 BIO-RAD, USA).

Statistics

All data were expressed as the mean ± S.E.M. from each group and were analysed by analysis of variance Student's *t*-test (ANOVA *t*-test).

Results

Effect of (−)-linalool on NO release and iNOS expression

Stimulation of macrophages J774.A1 with LPS (10 μg/ml) induced the expression of iNOS measurable as protein expression or as NO release. (−)-Linalool added to LPS-stimulated macrophages J774.A1 at concentrations of 10^{-7}, 10^{-5} or 10^{-3} M, significantly reduced NO release evaluated as nitrites with respect to the control LPS group (Fig. 1). This inhibition was not very high but it was however concentration related.

The Western blotting analysis for the iNOS expression did not reveal any change in the enzyme formation induced by (−)-linalool treatment to tested concentrations (Fig. 2).

Effect of (−)-linalool on PGE_2 release and COX-2 expression

The LPS (10 μg/ml) stimulation on macrophages J774.A1 induced a significant ($P<0.0001$) increase in the PGE_2 formation. (−)-Linalool treatment (10^{-7}, 10^{-5} or 10^{-3} M) on macrophages J774.A1 1 h previously and simultaneously to LPS stimulation revealed a significant ($P<0.005$) reduction of PGE_2 formation only with the highest used dose (10^{-3} M) compared to control LPS group (Fig. 3).

Similar results were obtained with Western blot analysis for COX-2 expression. Indeed only the highest concentration (10^{-3} M) significantly ($P<0.05$) inhibited the expression of COX-2 (Fig. 4).

Discussion

Recent studies have suggested that NO peripherally produced by different NOS isoforms contributes to oedema formation and development of nociception and hyperalgesia (Omote et al., 2001; Rivot et al., 2002). Results of present study show that the antinociceptive activity of (−)-linalool could be related to the inhibition of NO formation/release, but not to the interference in the PGs pathway. Exposure of LPS-stimulated J774.A1 macrophages to (−)-linalool, significantly and in concentration-dependent manner, inhibited nitrite accumulation in the culture medium. This effect is due to the inhibitory interaction of (−)-linalool on the iNOS enzyme; indeed Western blotting analysis revealed no reduction of protein expression of this enzyme. In contrast, exposure of LPS-stimulated J774.A1 macrophages to (−)-linalool (10^{-7} or 10^{-5} M) did not modify the PGE_2 formation and COX_2 expression. The reduction of PGE_2 formation obtained with higher concentration (10^{-3} M) of this monoterpene could be addressed to a reduction of COX-2 expression operated by (−)-linalool as revealed by Western blotting analyses. This effect could be due to an aspecific action on transduction factors.

These in vitro effects of (−)-linalool might contribute in the anti-inflammatory, antinociceptive and antihyperalgesic activity of (−)-linalool. In fact, our previous in vivo studies showed that systemic administration of (−)-linalool attenuated the

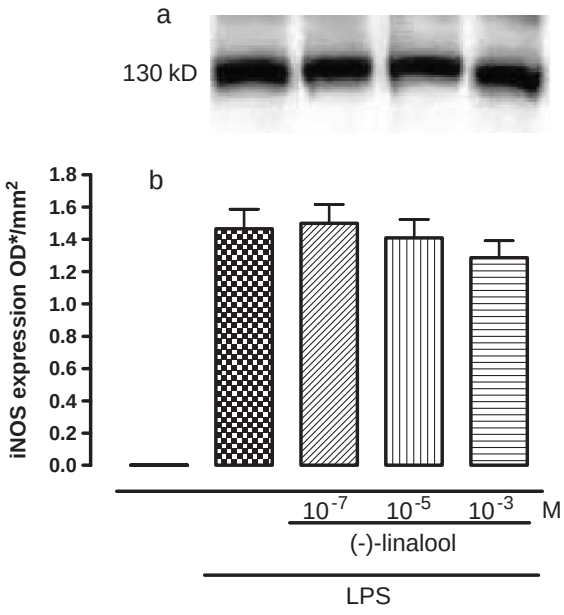

Fig. 2. Effect of (−)-linalool added to LPS-stimulated macrophages J774.A1 on iNOS expression. Data represent mean values ± S.E.M.

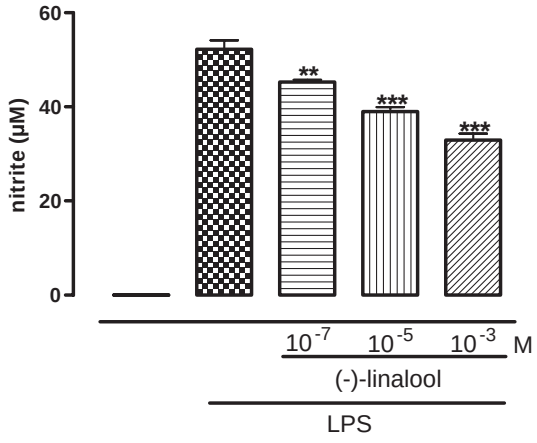

Fig. 1. Effect of (−)-linalool added to LPS-stimulated macrophages J774.A1 on NO release evaluated as nitrites (NO_2^-) in incubation medium. Data represent mean values ± S.E.M. Significant differences from LPS control group are indicated by asterisks (**$P<0.01$; ***$P<0.005$; ANOVA t-test).

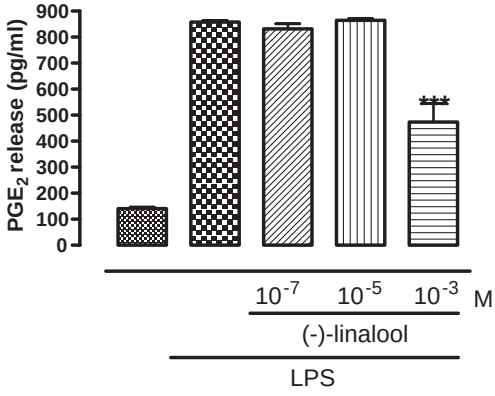

Fig. 3. Effect of (−)-linalool added to LPS-stimulated macrophages J774.A1 on PGE_2 release in the incubation medium. Data represent mean values ± S.E.M. Significant differences from LPS control group are indicated by asterisks (ANOVA t-test).

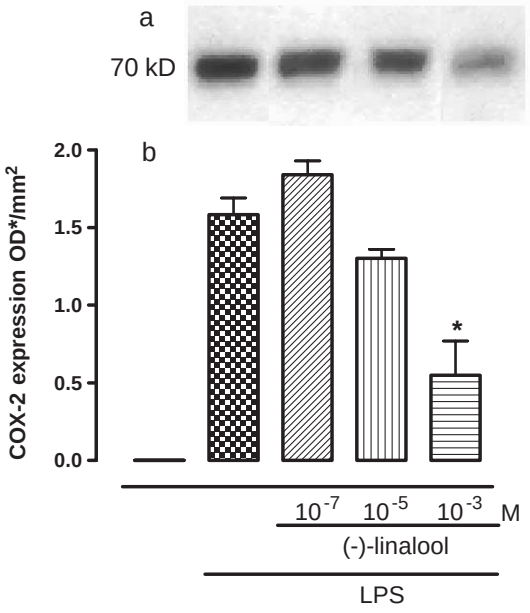

Fig. 4. Effect of (−)-linalool added to macrophages J774.A1 1 h before and simultaneously to LPS on COX_2 expression. Data represent mean values±S.E.M. Significant differences from LPS control group are indicated by asterisk (ANOVA t-test).

development of carrageenan-induced oedema and hyperalgesia in rat (Peana et al., 2002, 2004b) as well as antagonized different pain responses in different animal models (Peana et al., 2003, 2004a). (−)-Linalool did not interfere on PGE_2 release and on COX_2 expression, thus the efficacy in reducing oedema induced by carrageenan or by formalin as well as the hyperalgesia induced by carrageenan, L-glutamate or by prostaglandin E_2 (Peana et al., 2004b) might be related not only to the inhibitory effect of this monoterpene on nitrite accumulation but also to its inhibitory effect on different transmission systems such as glutamate, acetylcholine, K^+ channels, dopamine and opioids (Peana et al., 2004a,b). (+/−)-Linalool is able to block NMDA receptors activity (Silva Brum et al., 2001a,b); it is generally accepted that NMDA transmission is involved in the nociceptive responses (Haley et al., 1990; Coderre and Van Empel, 1994; Chizh et al., 2001) and that the activation of peripheral NMDA receptors contributes to the development of thermal hyperalgesia (Eide et al., 1995; Jackson et al., 1995; Carlton and Coggeshall, 1999). Because activation of the NO cascade is known to occur secondary to NMDA receptor activation, evidence have been accumulated implicating a role for spinal NO in models of thermal hyperalgesia (Coutinho et al., 2001; Kawabata et al., 2002). Likewise with our observation, ketamine, an NMDA receptor antagonist, has been reported to significantly reverse the formalin and carrageenan-induced increase of NO release (Rivot et al., 2002).

NO is implicated also, in cholinergic and opioid analgesia, in cholinergic function and muscarinic M_2 receptors as well as in brain levels of glutamate and dopamine (Smith and Ogonowski, 2003).

Previous study demonstrated that systemic administration of NO synthase inhibitors increased the antinociception induced by the administration of the muscarinic receptor agonist oxotremorine (Pavone et al., 1997) indicating an involvement of L-arginine–NO pathway in antinociceptive effects of cholinergic stimulation (Machelska et al., 1999). Thus the muscarinic M_2 properties of (−)-linalool-induced antinociceptive effect (Peana et al., 2004a) might also concur in determining its effect on iNOS enzyme activity. The increase of potassium conductance in cholinomimetic spinal dorsal horn neurons results in a reduced activity of nociceptive neurons and in a diminished neurotransmitter release (Iwamoto and Marion, 1993, 1994). Some evidence indicated localization of these muscarinic receptors in nerve terminals of the primary afferent where glutamate is one of the main neurotransmitters involved in nociceptive transmission (Machelska et al., 1999).

Collectively, these results indicate that the reduction of NO production/release is responsible, at least partially, for the molecular mechanisms of (−)-linalool antinociceptive effect, probably through mechanisms where cholinergic and glutamatergic systems are involved.

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