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Further analyses of mechanisms underlying the antinociceptive effect of the triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene in mice

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ABSTRACT

The present study investigated the mechanisms involved in the antinociception produced by the triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene (TTHL) in mice. TTHL administered by intra-gastric (i.g.) gavage inhibited glutamate-induced nociception with an ID_{50} of 19.0 (13.2–27.5) mg/kg. This action started 60 min (inhibition of: 59 ± 6%) after i.g. administration and remained significant up to 6 h (inhibition of 37 ± 6%). Moreover, TTHL inhibited both phases of formalin induced pain. The antinociception of TTHL was reversed by the pre-administration of naloxone (1 mg/kg; non-selective opioid receptor antagonist), CTOP (1 mg/kg; selective μ-opioid receptor antagonist), nor-binaltorphimine (1 mg/kg; selective κ-opioid receptor antagonist), naltrindol (3 mg/kg; selective δ-opioid receptor antagonist), p-chlorophenylalanine methyl ester (100 mg/kg for 4 consecutive days; inhibitor of serotonin synthesis), WAY100635 (0.5 mg/kg; selective 5-HT_{1A} receptor antagonist) and ketanserin (0.3 mg/kg; selective 5-HT_{2A} receptor antagonist) but not by L-arginine (600 mg/kg; nitric oxide precursor) or ondansetron (0.5 mg/kg; 5-HT_3 receptor antagonist). Furthermore, the TTHL antinociception was prevented by intrathecal (i.t.) pre-treatment with pertussis toxin (0.5 μg/site; inactivator of $G_{i/o}$ protein), charybdotoxin (250 pg/site; blocker of large-conductance calcium-gated K^+ channels), tetraethylammonium (1 μg/site; blocker of voltage-gated K^+ channels) and glibenclamide (80 μg/site; blocker of ATP-gated K^+ channels) but not by apamin (50 ng/site; blocker of small-conductance calcium-gated K^+ channels). The antinociception of TTHL was not it associated with locomotor impairment or sedation. These results showed that TTHL presented a pronounced antinociceptive effect, which is dependent on opioid and serotonergic systems, $G_{i/o}$ protein activation and the opening of specific K^+ channels.

1. Introduction

The sensation of pain is known to be modified by endogenous pain inhibitory systems, predominantly through descending noradrenaline, serotonin, and endogenous opioids such as β-endorphin and dynorphin. The activation of the descending systems markedly modifies not only the release of glutamate from the primary afferent or interneurons but also the release of GABA and glycine (Yoshimura and Furue, 2006).

Evidence has shown that the opening of some K^+ channels plays an important role in the antinociception induced by agonists of many G-protein-coupled receptors (i.e., α_2-adrenoceptors, opioid, $GABA_B$, muscarinic M_2, adenosine A_1, serotonin 5-HT_{1A} and cannabinoid receptors), as well as by other antinociceptive drugs (nonsteroidal anti-inflammatory drugs and tricyclic antidepressants) and natural products (Ocaña et al., 2004).

In recent years, the pharmaceutical industry has shown considerable interest in new drugs originating from natural sources. In this regard, species of *Combretum* are used in traditional medicine to treat various diseases, such as pain. The genus *Combretum* belongs to the *Combretacea* family, and several species are widely distributed around Africa (McGaw et al., 2001). In north and northeastern Brazil, species of this genus are also found, namely *Combretum leprosum* Mart., popularly known as mofumbo, cipoaba or mufumbo. Infusions prepared with the aerial (stems, leaves and flowers) parts and roots of *C. leprosum* are used in folk medicine to heal wounds, in the treatment of hemorrhages or as a sedative (Facundo et al., 2005; Lira et al., 2002). Phytochemical studies performed by Facundo et al. (1993) have revealed some compounds found in this plant, such as flavonols and triterpenes.

We reported that the ethanolic extract obtained from the flowers of *C. leprosum* evoked dose-related antinociception in several models of chemical and thermal pain through mechanisms that involve an interaction with opioid and serotonergic (i.e., through 5-$HT_{1A/1B}$ and 5-HT_{2A} receptors) systems (Pietrovski et al., 2006). Additionally, it was demonstrated that the pentacyclic triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene (TTHL) obtained from this plant presents

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antinociceptive effects that involve the spinal glutamatergic system (Longhi-Balbinot et al., 2009).

However, despite experimental data, the mechanism through which the triterpene evokes systemic antinociception in rodents still remains elusive. In this regard, the aim of the present study was to evaluate the effect of 3β, 6β, 16β-trihydroxylup-20(29)-ene (TTHL) on pathways of pain transmission, other than those described previously for TTHL. Therefore, we investigated the endogenous pain inhibitory systems such as endogenous opioids and serotonin (5-HT), as well as inhibitory signaling pathways, including protein $G_{i/o}$ activation and the opening of potassium channels in mice.

2. Materials and methods

2.1. Animals

All experiments were performed after approval of the protocol by the Ethics Committee for Animal Research of the Federal University of Santa Catarina and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983). Experiments were conducted using male Swiss mice (25–35 g) obtained from the Federal University of Santa Catarina's animal facility that were housed at 22 ± 2 °C under a 12-h light/12-h dark cycle (lights on at 06:00) with access to food and water *ad libitum*. The mice were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments.

2.2. Glutamate-induced nociception

The procedure used was similar to that described previously (Meotti et al., 2009). First, animals were treated with TTHL (3–100 mg/kg) administered by intra-gastric (i.g.) gavage 1 h before glutamate injection. Control animals received a similar volume of vehicle (10 ml/kg). Then, a volume of 20 μl of glutamate (20 μmol/paw), made up in sterile saline solution with the pH adjusted to 7.0 with NaOH, was injected intraplantarly (i.pl.) under the ventral surface of the right hindpaw. Animals were observed individually for 15 min following glutamate injection. The amount of time they spent licking the injected paw was recorded with a chronometer and was considered indicative of nociception. In separate series of experiments, we investigated the time course of the antinociceptive effect of TTHL (30 mg/kg) given orally at time points 1, 2, 4, 6, 8 and 10 h before glutamate administration. Control animals received a similar volume of vehicle (10 ml/kg) by intra-gastric (i.g.) gavage route and were observed over the same intervals of time.

2.3. Formalin-induced nociception

The procedure used was essentially the same as that previously described (Hunskaar et al., 1985; Tjølsen et al., 1992). Animals received 20 μl of a 2.5% formalin solution (0.92% formaldehyde in saline) in the ventral surface of the right hind paw (i.pl.). Animals were observed from 0 to 5 min (neurogenic phase) and from 15 to 30 min (inflammatory phase) and the time that they spent licking the injected paw was recorded and considered as indicative of nociception. Animals were pre-treated with TTHL (10–300 mg/kg, i.g.) 1 h beforehand. Control animals received the same volume of vehicle (10 ml/kg, i.g.).

2.4. Analysis of possible mechanism of action of TTHL

The mechanisms by which TTHL (30 mg/kg), administered systemically, evokes antinociception were evaluated utilizing the nociception model induced by glutamate. The doses of all substances employed were chosen based on literature data or were selected from preliminary experiments conducted in our laboratory.

2.4.1. Involvement of opioid system

To assess the participation of the opioid system in the antinociceptive effect of TTHL, mice were pre-treated with naloxone (1 mg/kg, intraperitoneally [i.p.], a non-selective opioid receptor antagonist), CTOP (1 mg/kg, subcutaneously [s.c.], a selective μ-opioid receptor antagonist), nor-binaltorphimine (1 mg/kg, i.p., a selective κ-opioid receptor antagonist) or naltrindol (3 mg/kg, s.c., a selective δ-opioid receptor antagonist). After 20 min, the animals received TTHL (30 mg/kg, i.g.), morphine (2.5 mg/kg, s.c.) or vehicle (10 ml/kg, i.g.). Another group of mice was pre-treated with vehicle and after 20 min, received TTHL, morphine or vehicle. The nociceptive response to the i.pl. glutamate injection was recorded 60 min after the administration of TTHL or vehicle and 30 min after the administration of morphine. The doses and scheme of treatment for naltrindole and nor-binaltorphimine were selected based on literature data (Backhouse et al., 2008; Jinsmaa et al., 2006). CTOP was injected subcutaneously based on data showing that it produced effects as a selective μ-opioid antagonist when injected systemically (Banach et al., 2006; Catheline et al., 1999; Varlinskaya and Spear, 2009).

2.4.2. Involvement of serotonergic system

To investigate the possible involvement of endogenous serotonin on the antinociceptive effect of TTHL, animals were pre-treated with p-chlorophenylalanine methyl ester (PCPA, 100 mg/kg, i.p., an inhibitor of serotonin synthesis) or vehicle, once a day, for 4 consecutive days. Twenty minutes after the last administration of PCPA, mice received TTHL (30 mg/kg, i.g.), morphine (2.5 mg/kg, s.c.) or vehicle (10 ml/kg, i.g.). The nociceptive response to i.pl. glutamate was recorded 30 and 60 min after morphine, TTHL or vehicle injection, respectively. In the next series of experiments, we evaluated the contribution of the different subtypes of serotonin (5-HT) receptors. Thus, the animals were pre-treated with WAY100635 (0.5 mg/kg, s.c., a selective 5-HT_{1A} receptor antagonist), ketanserin (1 mg/kg, i.p., a selective 5-HT_{2A} receptor antagonist), ondansetron (0.5 mg/kg, i.p., a 5-HT_3 receptor antagonist) or vehicle (10 ml/kg, i.p.). Twenty minutes after drug administration, the animals were treated with TTHL (30 mg/kg, i.g.) or vehicle (10 ml/kg, i.g.), and as positive controls, buspirone (5 mg/kg, i.p., a 5-HT_{1A} receptor agonist), DOI (1 mg/kg, i.p., a 5-$HT_{2A/2C}$ receptor agonist) or agmatine (3 mg/kg, i.p., which in previous studies in our laboratory had its antinociceptive effects reversed by ondansetron) (Luiz et al., 2007; Santos et al., 2005) was used. Furthermore, a group of mice was pre-treated with vehicle and after 30 min, received TTHL or vehicle. The nociceptive response to i.pl. glutamate injection was recorded 60 min after the administration of TTHL or vehicle and 30 min after the administration of agonists.

2.4.3. Involvement of L-arginine–nitric oxide pathway

To investigate the role played by the L-arginine–nitric oxide pathway in the antinociception caused by TTHL, mice were pre-treated with L-arginine (600 mg/kg, i.p.; a nitric oxide precursor). After 20 min, the mice received TTHL (30 mg/kg, i.g.), L-NAME (20 mg/kg, i.p.; a nitric oxide synthase inhibitor) or vehicle (10 ml/kg, i.g.) (Maia et al., 2006; Santos et al., 2005). Another group of mice was pre-treated with vehicle and after 30 min, received TTHL, L-NAME or vehicle. The nociceptive response to i.pl. glutamate injection was recorded 60 min after the administration of TTHL or vehicle and 30 min after the administration of L-NAME.

2.4.4. Participation of $G_{i/o}$ protein

To determine the involvement of $G_{i/o}$ protein in the antinociceptive action of TTHL, mice were pre-treated intrathecally (i.t.) with pertussis toxin (0.5 μg/site), an inactivator of $G_{i/o}$ protein. A control group was pre-treated with vehicle (5 μl/i.t.). The intrathecal injection was performed as previously described by Hylden and Wilcox (1980). Animals were manually restrained, and a 30-gauge needle connected by polyethylene tubing to a 25 μL Hamilton gas-tight syringe (Hamilton,

Birmingham, UK), was inserted through the skin and between the vertebras into the subdural space of the L5–L6 spinal segments. The tail reflex movement was considered as indicative of success of administration and the injections were given over a period of 5 s. The experiment was carried out as described by Sánchez-Blázquez and Garzón (1991) and Meotti et al. (2007). Seven days after treatment of animals with pertussis toxin or vehicle, mice received vehicle (10 ml/kg, i.g.), TTHL (30 mg/kg, i.g.) or morphine (2.5 mg/kg, s.c.). The nociceptive response to i.pl. glutamate injection was recorded 60 min after the administration of TTHL or vehicle and 30 min after the administration of morphine.

2.4.5. Involvement of K^+ channels

We next investigated the involvement of K^+ channels on the antinociceptive action of TTHL. Mice were pre-treated intrathecally, as described earlier, with K^+ channel blockers tetraethylammonium (1 μg/site, i.t.; a blocker of voltage-gated K^+ channels), apamin (50 ng/site, i.t.; a blocker of small (or low)-conductance calcium-gated K^+ channels), charybdotoxin (250 pg/site, i.t.; a blocker of large (or fast)-conductance calcium-gated K^+ channels) or glibenclamide (80 μg/site, i.t.; a blocker of ATP-gated K^+ channels), and after 15 min, they received TTHL (30 mg/kg, i.g.), morphine (2.5 mg/kg, s.c.) or vehicle (10 ml/kg, i.g.) (Aronson, 1992; Meotti et al., 2007; Santos et al., 1999; Strong, 1990; Welch and Dunlow, 1993). Another group of mice was pre-treated with vehicle and after 15 min, they received TTHL, morphine or vehicle. The nociceptive response to i.pl. glutamate injection was recorded 60 min after the administration of TTHL or vehicle and 30 min after the administration of morphine.

2.5. Measurement of locomotor activity

To evaluate the effect of TTHL on spontaneous locomotor activity, mice were submitted to the open field test (Rodrigues et al., 2002). The open-field apparatus consisted of a wooden box measuring 40 × 60 × 50 cm. The floor of the arena was divided into 12 equal squares, and the number of squares crossed by the animal with all paws was counted in a 6 min session. Mice were treated by intragastric gavage with TTHL (30 and 100 mg/kg) or vehicle (10 ml/kg) 60 min beforehand.

2.6. Drugs

The following substances were employed: formalin and morphine hydrochloride from Merck (Darmstadt, Germany), L-glutamic acid hydrochloride (glutamate), naloxone hydrochloride, (±)-2,5-Dimethoxy-4-iodoamphetamine hydrochloride ((±)-DOI), agmatine sulphate, buspirone hydrochloride, L-arginine, Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), p-chlorophenyl methyl ester hydrochloride (PCPA), N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl] ethyl]-N-2-pyridinylcyclohexanecarboxamide (WAY 100635), pertussis toxin, charybdotoxin, tetraethylammonium and apamin from Sigma Chemical Co. (St. Louis, USA), naloxone hydrochloride, naltrindol hydrochloride, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP), nor-binaltorphimine hydrochloride, glibenclamide and ketanserin tartarate from Tocris Cookson Inc. (Ellisville, USA) and ondansetron hydrochloride from Cristália (São Paulo, Brazil). All drugs were dissolved in saline solution (0.9%), except TTHL and glibenclamide, which were dissolved in saline plus DMSO/Tween 80 or saline plus ethanol/Tween 80, respectively. The final concentration of DMSO, Tween 80 and ethanol did not exceed 10% and did not cause any effect *per se*. The triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene was isolated from the flowers of *C. leprosum* at the Department of Organic Chemistry, Universidade Federal do Ceará, Brazil as previously described and its degree of purity was >98%. (Facundo et al., 1993).

2.7. Statistical analysis

Data are presented as means ± standard error of the mean (S.E.M.), except the ID_{50} values (i.e., the dose of triterpene that reduced the agonists-induced nociceptive response by 50% relative to control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} value was determined by non-linear regression from individual experiments (GraphPad software, San Diego, CA, USA). Comparisons between experimental and control groups were performed by one-way (effect of TTHL in the glutamate- and formalin-induced nociception and open-field behavior) or two-way (experiments dealing with the involvement of the opioidergic and serotonergic system, L-arginine–nitric oxide pathway, participation of $G_{i/o}$ protein, ATP-dependent, voltage- and large (fast)-conductance Ca^{2+}-gated K^+ channels in the effect of TTHL in the glutamate test) Analysis of Variance (ANOVA), followed by Newman Keul's test or Tukey's HSD test when appropriate. *P* values less than 0.05 were considered as indicative of significance.

3. Results

3.1. Glutamate-induced nociception

The results of Fig. 1A show that TTHL (3–100 mg/kg) administered by intra-gastric (i.g.) gavage caused a significant inhibition of glutamate-induced nociception when compared to the control group. The calculated mean ID_{50} was 19.0 (13.2–27.5) mg/kg, and the inhibition was 56 ± 3% at a dose of 30 mg/kg. When glutamate was administered at different time points after TTHL treatment, we verified that TTHL had a peak response after 1 h (inhibition of 59 ± 6%) that lasted up to 6 h (inhibition of 37 ± 6%, Fig. 1B).

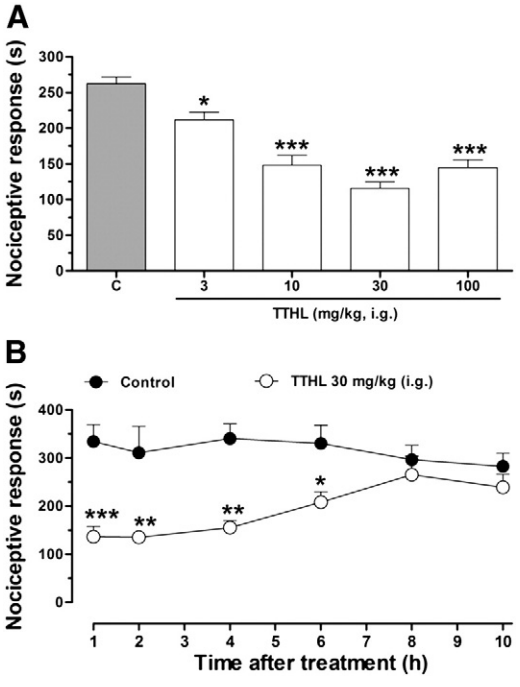

Fig. 1. Dose–response (panel A, open bars) and time-course (panel B, open circle) of the antinociceptive effect of TTHL given by intra-gastric (i.g.) gavage against glutamate-induced nociception in mice. Each group represents the mean of 8 animals and the error lines indicate the S.E.M. The control group (C, grey bar or closed circle) indicates the mice treated by intra-gastric (i.g.) gavage with vehicle (10 ml/kg) before glutamate (20 μmol/paw). The symbols *P<0.05, **P<0.01 and ***P<0.001 denote the significance level when compared to the control group. One-way ANOVA followed by the Newman–Keuls test.

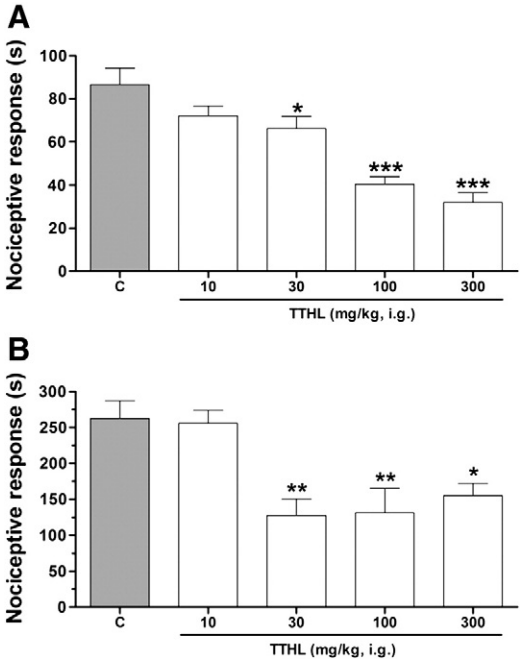

Fig. 2. Effect of TTHL (open bars) administered by intra-gastric (i.g.) gavage against formalin-induced nociception (first phase, panel A, and second phase, panel B) in mice. Each group represents the mean of 6 animals and the error lines indicate the S.E.M. The control group (C, grey bars), indicates the mice treated by intra-gastric (i.g.) gavage with vehicle (10 ml/kg) before formalin. The symbols *$P<0.05$, **$P<0.01$ and ***$P<0.001$ denote the significance level when compared to the control group. One-way ANOVA followed by the Newman–Keuls test.

3.2. Formalin-induced nociception

The results depicted in Fig. 2A and B show that TTHL (10–300 mg/kg) administered by intra-gastric (i.g.) gavage caused significant inhibition of both neurogenic (0 to 5 min) and inflammatory phases (15 to 30 min) of formalin-induced licking. The calculated mean ID_{50} values for these effects were 108.0 (72.6–160.8) mg/kg and ~30 mg/kg and the inhibitions observed were 63 ± 5% and 51 ± 9% at a dose of 100 mg/kg and 30 mg/kg, for the first and second phases, respectively.

3.3. Analysis of possible mechanism of action of TTHL

3.3.1. Involvement of opioidergic system in the antinociceptive effect of TTHL

The results depicted in Fig. 3A show the effect of naloxone (1 mg/kg, i.p.; a non-selective opioid receptor antagonist) on the antinociceptive effect promoted by TTHL (30 mg/kg, i.g.) against glutamate-induced nociception. The two-way ANOVA revealed significant main effects of naloxone pre-treatment [$F(1,16)=6.81$; $P<0.05$] and TTHL [$F(1,16)=16.09$; $P<0.001$], and a naloxone pre-treatment × TTHL interaction [$F(1,16)=7.10$; $P<0.05$]. Post hoc analyses (Tukey HSD) indicated that the pre-administration of naloxone significantly prevented the anti-nociceptive effect elicited by TTHL ($P<0.01$).

Fig. 3B shows the effect of the pre-administration of CTOP (1 mg/kg, s.c.; a selective μ-opioid receptor antagonist) on the antinociceptive effect promoted by TTHL (30 mg/kg, i.g.) against glutamate-induced nociception. The two-way ANOVA revealed significant main effects of CTOP pre-treatment [$F(1,32)=24.72$; $P<0.001$] and TTHL [$F(1,32)=99.24$; $P<0.001$], and a CTOP pre-treatment × TTHL interaction [$F(1,32)=38.27$; $P<0.001$]. Post hoc analyses indicated that the pre-treatment of mice with CTOP significantly reversed the antinociceptive effect evoked by TTHL ($P<0.001$).

Fig. 3C shows the effect of pre-administration of nor-binaltorphimine (1 mg/kg, i.p.; a selective κ-opioid receptor antagonist) on the antinociceptive effect caused by TTHL (30 mg/kg, i.g.) against glutamate-induced nociception. The two-way ANOVA revealed significant main effects of nor-binaltorphimine pre-treatment [$F(1,32)=17.0$; $P<0.001$] and TTHL $F(1,32)=108.51$; $P<0.001$], and a nor-binaltorphimine pre-treatment × TTHL interaction [$F(1,32)=23.10$; $P<0.001$]. Post hoc analyses indicated that the pre-administration of nor-binaltorphimine prevented the antinociceptive effect elicited by TTHL ($P<0.01$).

Fig. 3D shows the effect of the pre-treatment of mice with naltrindole (3 mg/kg, s.c.; a selective δ-opioid receptor antagonist) on the antinociceptive effect promoted by TTHL (30 mg/kg, i.g.) against glutamate-induced nociception. The two-way ANOVA revealed

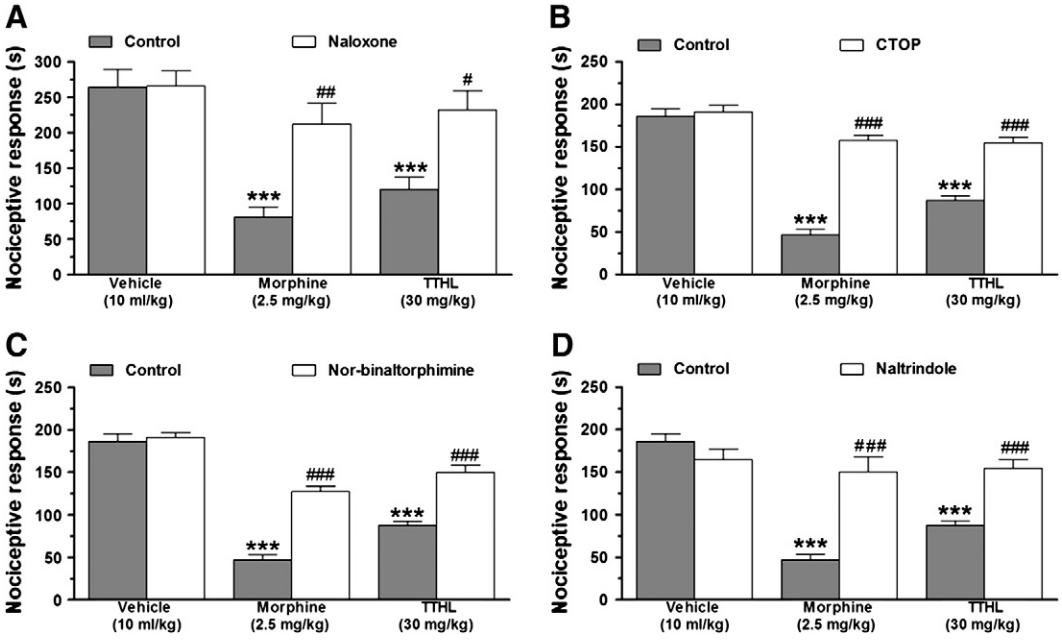

Fig. 3. Effect of pre-treatment with naloxone (1 mg/kg, i.p., panel A), CTOP (1 mg/kg, s.c., panel B), nor-binaltorphimine (1 mg/kg, i.p., panel C) and naltrindole (3 mg/kg, s.c., panel D) on the antinociceptive effect of TTHL (30 mg/kg, i.g.) against glutamate-induced nociception in mice. Each column represents the mean of 9 animals and the error lines indicate the S.E.M. in the absence (grey bars) or presence (open bars) of opioid antagonists. The symbols denote significance levels: ***$P<0.001$ compared to the control group (animals injected with vehicle alone); ##$P<0.01$ and ###$P<0.001$ compared to the TTHL group Two-way ANOVA followed by the Tukey HSD test.

significant main effects of naltrindole pre-treatment [$F(1,32)=4.47$; $P<0.05$] and TTHL [$F(1,32)=47.05$; $P<0.001$], and a naltrindole pre-treatment × TTHL interaction [$F(1,32)=34.09$; $P<0.001$]. Post hoc analyses indicated that the pre-administration of naltrindole prevented the antinociceptive effect elicited by TTHL ($P<0.001$).

The pre-administration of naloxone, CTOP, nor-binaltorphimine or naltrindole reversed the antinociceptive effect caused by morphine (2.5 mg/kg, s.c., used as a positive control) [$F(1,20)=24.6$; $P<0.001$], [$F(1,32)=170.97$; $P<0.001$], [$F(1,32)=254.32$; $P<0.01$] and [$F(1,32)=80.86$; $P<0.01$] respectively, when analyzed against glutamate test (data not shown).

3.3.2. Involvement of serotonergic system in the antinociception afforded by TTHL

Fig. 4A shows the effect of the pre-administration of PCPA (100 mg/kg, i.p., an inhibitor of serotonin synthesis, for 4 consecutive days) on the antinociception promoted by TTHL (30 mg/kg, i.g.) against glutamate-induced nociception. The Two-way ANOVA revealed significant main effects of PCPA pre-treatment [$F(1,28)=5.93$; $P<0.05$] and TTHL [$F(1,28)=12.22$; $P<0.001$], and a PCPA pre-treatment × TTHL interaction [$F(1,28)=18.12$; $P<0.001$]. Post hoc analyses indicated that the pre-treatment of mice with PCPA significantly reversed the antinociceptive effect elicited by TTHL ($P<0.001$).

Fig. 4B shows the effect of the pre-treatment of mice with WAY100635 (0.5 mg/kg, s.c.; a selective 5-HT_{1A} receptor antagonist) on the antinociceptive effect promoted by TTHL (30 mg/kg, i.g.) against glutamate-induced nociception. The two-way ANOVA revealed significant main effects of WAY100635 pre-treatment [$F(1,24)=5.62$; $P<0.05$] and TTHL [$F(1,24)=64.96$; $P<0.001$], and a WAY100635 pre-treatment × TTHL interaction [$F(1,24)=16.45$; $P<0.001$]. Post hoc analyses indicated that the pre-treatment of mice with WAY100635 prevented the antinociceptive effect elicited by TTHL ($P<0.001$).

Fig. 4C shows the effect of the pre-treatment of mice with ketanserin (0.3 mg/kg, i.p.; a selective 5-HT_{2A} receptor antagonist) on the antinociceptive effect promoted by TTHL (30 mg/kg, i.g.) against glutamate-induced nociception. The Two-way ANOVA revealed significant main effects of ketanserin pre-treatment [$F(1,20)=7.80$; $P<0.05$] and TTHL $F(1,20)=25.82$; $P<0.001$], and a ketanserin pre-treatment × TTHL interaction [$F(1,20)=7.07$; $P<0.05$]. Post hoc analyses indicated that the pre-administration of ketanserin prevented the antinociceptive effect elicited by TTHL ($P<0.01$).

Fig. 4D shows the effect of the pre-administration of ondansetron (0.5 mg/kg, i.p.; a selective 5-HT_3 receptor antagonist) on the antinociceptive effect promoted by TTHL (30 mg/kg, i.g.) against glutamate-induced nociception. The two-way ANOVA revealed significant differences of TTHL treatment [$F(1,24)=56.93$, $P<0.001$], but not of ondansetron pre-treatment [$F(1,24)=0.56$, $P=0.45$] and ondansetron pre-treatment × TTHL treatment interaction [$F(1,24)=1.98$, $P=0.17$].

The pretreatment of mice with PCPA (100 mg/kg, i.p. for 4 consecutive days) reversed the antinociceptive effect caused by morphine (2.5 mg/kg, s.c., used as a positive control) [$F(1,28)=74.84$; $P<0.001$] when analyzed against glutamate test (data not shown). Furthermore, WAY100635 (a selective 5-HT_{1A} receptor antagonist), ketanserin (a selective 5-HT_{2A} receptor antagonist) and ondansetron (a selective 5-HT_3 receptor antagonist), prevented the antinociceptive effect of their respective agonists, used as a positive control, buspirone (5 mg/kg, i.p.; a 5-HT_{1A} receptor agonist) [$F(1,24)=51.60$; $P<0.001$], DOI (1 mg/kg, i.p.; a 5-$HT_{2A/2C}$ receptor agonist) [$F(1,20)=17.89$; $P<0.001$] and agmatine (3 mg/kg, i.p.) [$F(1,24)=15.52$; $P<0.001$] when analyzed against glutamate test, respectively (data not shown).

3.3.3. Involvement of L-arginine–nitric oxide pathway

The results in Fig. 5 illustrate the involvement of L-arginine–nitric oxide pathway in the antinociceptive effect of TTHL (30 mg/kg, i.g.) in the glutamate test. Fig. 5 shows that the pre-treatment of mice with L-arginine (600 mg/kg, i.p.; a nitric oxide precursor) was not able to prevent the antinociceptive effect of TTHL in the glutamate test. The two-way ANOVA revealed significant differences of TTHL treatment [$F(1,24)=73.86$; $P<0.001$], but not of L-arginine pre-treatment [$F(1,24)=1.85$; $P=0.18$] and L-arginine pre-treatment × TTHL treatment interaction [$F(1,24)=0.24$, $P=0.62$].

The pre-administration of L-arginine, given 20 min beforehand, completely reversed the antinociceptive effect caused by L-NAME (20 mg/kg, i.p.; a nitric oxide synthase inhibitor, used as a positive control) [$F(1,24)=32.60$; $P<0.001$] (data not shown).

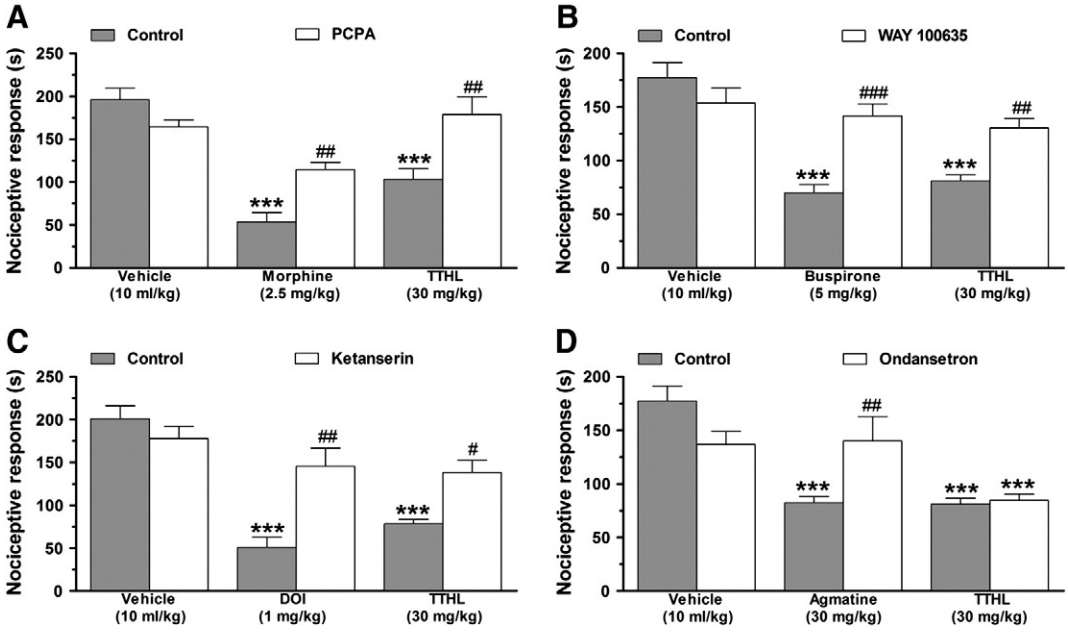

Fig. 4. Effect of pre-treatment with PCPA (100 mg/kg, i.p., for 4 consecutive days, panel A), WAY 100635 (0.5 mg/kg, s.c., panel B), ketanserin (0.3 mg/kg, i.p., panel C) and ondansetron (0.5 mg/kg, i.p., panel D) on the antinociceptive effect of TTHL (30 mg/kg, i.g.) against glutamate-induced nociception in mice. Each column represents the mean of 7 animals and the error lines indicate the S.E.M. in the absence (grey bars) or presence (open bars) of PCPA or serotonergic antagonists. The symbols denote significance levels: ***$P<0.001$ compared to the control group (animals injected with vehicle alone); $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared to the TTHL group. Two-way ANOVA followed by the Tukey HSD test.

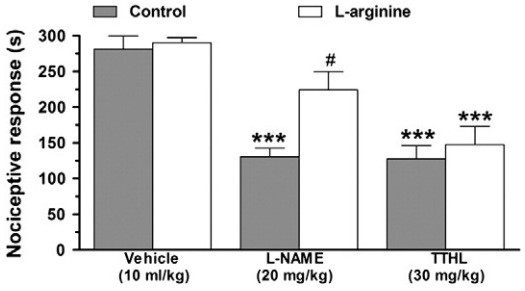

Fig. 5. Effect of pre-treatment with L-arginine (600 mg/kg, i.p.) on the antinociceptive effect of TTHL (30 mg/kg, i.g.) against glutamate-induced nociception in mice. Each column represents the mean of 8 animals and the error lines indicate the S.E.M. in the absence (grey bars) or presence (open bars) of L-arginine. The symbols denote significance levels: ***$P<0.001$ compared to the control group (animals injected with vehicle alone). Two-way ANOVA followed by the Tukey HSD test.

3.3.4. Participation of $G_{i/o}$ protein

Fig. 6 shows the effect of the pre-administration of pertussis toxin (0.5 μg/site, i.t. for 7 consecutive days) on the antinociceptive effect promoted by TTHL (30 mg/kg, i.g.) against glutamate-induced nociception. The two-way ANOVA revealed significant main effects of pertussis toxin pre-treatment [$F(1,36)=41.01$; $P<0.001$] and TTHL [$F(1,36)=46.70$; $P<0.001$], and a pertussis toxin pre-treatment × TTHL interaction [$F(1,36)=10.22$; $P<0.001$]. Post hoc analyses indicated that the pre-treatment of mice with pertussis toxin completely abrogated the antinociceptive effect elicited by TTHL ($P<0.001$).

The pre-treatment of mice with pertussis toxin (0.5 μg/site, i.t. for 7 consecutive days) completely reversed the antinociceptive effect caused by morphine (2.5 mg/kg, s.c., used as a positive control) [$F(1,36)=52.33$; $P<0.001$] (data not shown).

3.3.5. Involvement of ATP-dependent, voltage- and large (fast)-conductance Ca^{2+}-gated K^+ channels in the antinociceptive effect of TTHL

Fig. 7A shows the effect of the intrathecal pre-treatment of mice with tetraethylammonium (1 μg/site, i.t.; a blocker of voltage-gated K^+ channels) on the antinociception promoted by TTHL (30 mg/kg, i.g.) against glutamate test. The two-way ANOVA revealed significant main effects of tetraethylammonium pre-treatment [$F(1,28)=9.07$; $P<0.001$] and TTHL [$F(1,28)=12.92$; $P<0.001$], and a tetraethylammonium pre-treatment × TTHL interaction [$F(1,28)=7.61$; $P<0.05$]. Post hoc analyses indicated that the pre-administration of tetraethylammonium significantly reversed the antinociceptive effect elicited by TTHL ($P<0.001$).

Fig. 7B shows that the intrathecal pre-administration of apamin (50 ng/site, i.t.; a blocker of small (or low)-conductance calcium-gated K^+ channels) was not able to reverse the antinociceptive effect of TTHL in the glutamate test. The two-way ANOVA revealed significant differences of TTHL treatment [$F(1,24)=23.04$; $P<0.001$], but not of apamin pre-treatment [$F(1,24)=0.02$; $P=0.88$] and apamin pre-treatment × TTHL treatment interaction [$F(1,24)=2.43$; $P=0.13$].

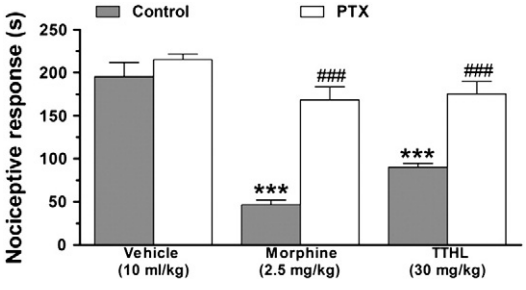

Fig. 6. Effect of pre-treatment (prior seven days) with pertussis toxin (PTX) (0.5 μg/site, i.t.) on the antinociceptive effect of TTHL (30 mg/kg, i.g.) against glutamate-induced nociception in mice. Each column represents the mean of 10 animals and the error lines indicate the S.E.M. in the absence (grey bars) or presence (open bars) of pertussis toxin. The symbols denote significance levels: ***$P<0.001$ compared to the control group (animals injected with vehicle alone); ###$P<0.001$ compared to the TTHL group. Two-way ANOVA followed by the Tukey HSD test.

Fig. 7C shows the effect of the intrathecal pre-treatment of mice with charybdotoxin (250 pg/site, i.t.; a blocker of large (or fast)-conductance calcium-gated K^+ channels) on the antinociception promoted by TTHL (30 mg/kg, i.g.) against glutamate test. The two-way ANOVA revealed significant main effects of charybdotoxin pre-treatment [$F(1,28)=4,57$; $P<0.05$] and TTHL [$F(1,28)=14.89$; $P<0.001$], and a charybdotoxin pre-treatment × TTHL interaction [$F(1,28)=10.37$; $P<0.05$]. Post hoc analyses indicated that the pre-administration of charybdotoxin prevented the antinociceptive effect elicited by TTHL ($P<0.01$).

Fig. 7D shows the effect of intrathecal pre-administration of glibenclamide (80 μg/site, i.t.; a blocker of ATP-gated K^+ channels) on the antinociception promoted by TTHL (30 mg/kg, i.g.) against glutamate test. The two-way ANOVA revealed significant main effects of glibenclamide pre-treatment [$F(1,24)=30.83$; $P<0.001$] and TTHL [$F(1,24)=10.88$; $P<0.001$], and a glibenclamide pretreatment × TTHL interaction [$F(1,24)=26.83$; $P<0.001$]. Post hoc analyses indicated that the pre-treatment of mice with glibenclamide prevented the antinociceptive effect elicited by TTHL ($P<0.001$).

The pre-administration of tetraethylammonium, apamin, charybdotoxin or glibenclamide reversed the antinociceptive effect caused by morphine (2.5 mg/kg, s.c., used as a positive control) [$F(1,28)=28.36$; $P<0.001$], [$F(1,24)=27.43$; $P<0.001$], [$F(1,28)=45.07$; $P<0.001$] and [$F(1,24)=136.27$; $P<0.001$] respectively, when analyzed against glutamate test (data not shown).

It is important to point out, that the pre-administration of all antagonists/blockers employed did not produce any effect *per se* on the glutamate-induced nociception (Figs. 3, 4, 5, 6 and 7).

3.4. Effect of TTHL on the locomotor activity (open-field test)

TTHL (30 and 100 mg/kg), given 60 min beforehand, did not affect the locomotor activity in the open-field test when compared to mice that received vehicle. The means ± S.E.M. of crossing numbers were 96.8 ± 8.0 for vehicle, 111.0 ± 9.0 for TTHL 30 mg/kg, 104.8 ± 6.5 for TTHL 100 mg/kg.

4. Discussion

In the present work, we confirm and largely extend previous data from the literature and demonstrate that the systemic (intra-gastric gavage) administration of TTHL, a pentacyclic triterpene isolated from *C. leprosum,* significantly inhibited the nociception induced by peripherally (intraplantar)-injected glutamate that lasts up to 6 h after treatment.

Studies in both humans (Gazerani et al., 2006; Nordlind et al., 1993; Warncke et al., 1997) and laboratory animals (Cairns et al., 1998; Davidson et al., 1997; Davidson and Carlton, 1998) have shown that excitatory amino acids, such as glutamate, in the peripheral endings of small diameter afferent fibers might contribute to the development and/or maintenance of pain. It has been demonstrated that intraplantar (i.pl.) injection of L-glutamate into the hind paw evokes hyperalgesia in rats (Carlton et al., 1995). Furthermore, Beirith et al. (2002) showed that nociception caused by i.pl. glutamate seems to involve peripheral, spinal and supra-spinal sites, and that its action is mediated by NMDA and non-NMDA receptors, as well as by nitric oxide (NO) release. Thus, the suppression of glutamate-induced nociception by TTHL can be associated with its interaction with the glutamatergic system or inhibition of NO production (Beirith et al., 2002; Ferreira et al., 1999).

Another interesting finding of the present study was the demonstration that TTHL caused significant antinociception when administered orally against both neurogenic (early phase) and inflammatory

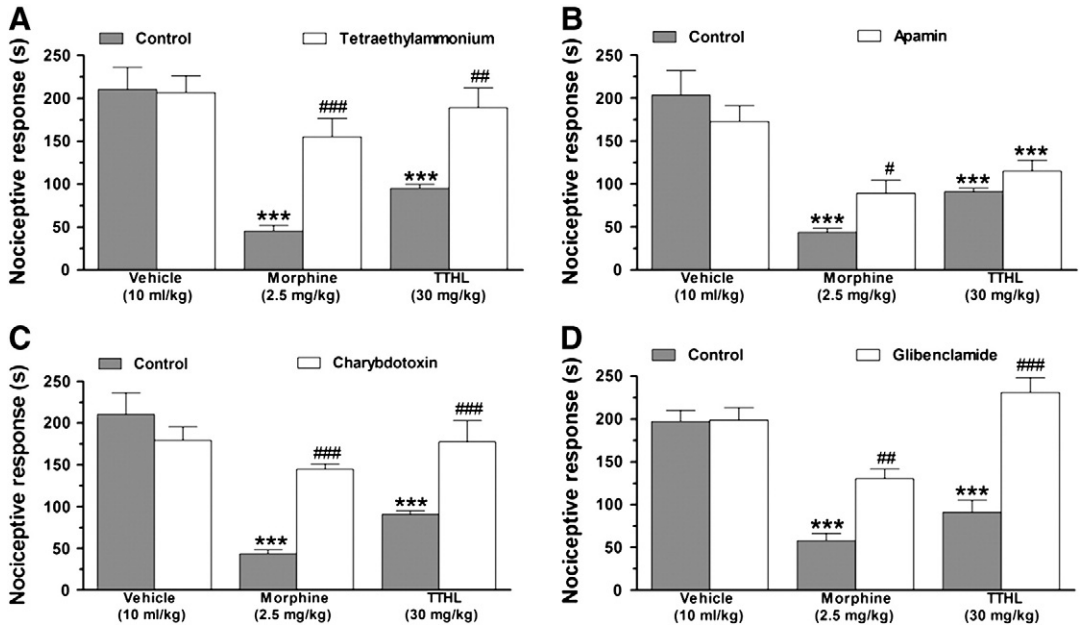

Fig. 7. Effect of i.t. pre-treatment with tetraethylammonium (1 μg/site, panel A), apamin (50 ng/site, panel B), charybdotoxin (250 pg/site, panel C) and glibenclamide (80 μg/site, panel D) on the antinociceptive effect of TTHL (30 mg/kg, i.g.) against glutamate-induced nociception in mice. Each column represents the mean of 9 animals and the error lines indicate the S.E.M. in the absence (grey bars) or presence (open bars) of K^+ channel blockers. The symbols denote significance levels: ***$P<0.001$ compared to the control group (animals injected with vehicle alone); $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared to the TTHL group. Two-way ANOVA followed by the Tukey HSD test.

(late phase) pain responses induced by formalin injection in mice. Furthermore, it has been reported that several drugs that antagonize ionotropic and metabotropic glutamate receptors (Bhave et al., 2001; Coderre et al., 1990; Fisher and Coderre, 1996; Yoon et al., 2006) produce significant antinociceptive action on formalin-induced nociceptive behavior. However, these drugs produced significant disturbances in locomotor activity (Parsons, 2001; Pietraszek et al., 2007; Takahashi and Afford, 2002). Of interest, the previous results (Longhi-Balbinot et al., 2009) and data of the present study showed that the antinociception caused by TTHL administrated either intrathecally or orally, besides promoting significant antinociception, did not produce locomotor impairment. It is important to mention that the nociceptive response induced by formalin and glutamate results from a combination of peripheral input and spinal cord sensitization (Sakurada et al., 2003; Santos and Calixto, 1997; Tjølsen et al., 1992). The i.pl. injection of formalin and glutamate releases excitatory amino acids, PGE_2, NO, neuropeptides and kinins in the spinal cord (Beirith et al., 2002; Sakurada et al., 2003; Tjølsen et al., 1992). Thus, the antinociceptive effect evoked by TTHL could be dependent on either peripheral or central sites of action. In this regard, we demonstrated that the intrathecal (central) injection of TTHL significantly inhibited nociception caused by either the peripheral or spinal administration of glutamate. Based on these data, we suggest that this effect could be by a direct action on glutamatergic neurons located in the spinal cord (Longhi-Balbinot et al., 2009).

Here, in order to investigate some of the mechanisms underlying the antinociceptive response produced by systemically administered TTHL, we employed the model of nociception by glutamate because this triterpene causes consistent antinociception with good reproducibility.

Despite of the relation between glutamate and NO cascades (Garthwaite, 1991; Meller and Gebhart, 1993; Meller et al., 1996), the L-arginine–NO pathway is unlikely to be involved in the antinociceptive effect of TTHL. This conclusion is drawn from the fact that pre-treatment with the substrate for NOS, L-arginine, at a dose that produces no significant effect on glutamate-induced nociception, significantly reversed the antinociception caused by L-NAME (a nitric oxide synthase inhibitor), but did not alter the antinociceptive effect of TTHL.

It is well known that the sensation of pain is modified by endogenous pain inhibitory systems, such as endogenous opioids (β-endorphin and dynorphin) and descending serotonin (5-HT). Opioid receptors are widely distributed in several peripheral tissues (Wittert et al., 1996) and cutaneous nerves (Ständer et al., 2002; Stein et al., 1990), as well as the central nervous system, especially in the pain transmission pathways like the dorsal horn of the spinal cord (Lao et al., 2008; Ninkovic et al., 1982) and small cells in dorsal root ganglia (Botticelli et al., 1981). There are three classical opioid receptors: μ, κ and δ. All are G protein-coupled receptors. They also signal in similar ways, coupling to guanine nucleotide binding regulatory proteins ($G_{i/o}$) to inhibit adenylyl cyclase, open inner-rectifying K^+ channels and inactivate L-type and N-type Ca^{2+} channels. All three of these receptors mediate analgesia in the spinal cord (Budai and Fields, 1998; Chen and Pan, 2006; Chen et al., 2007; Takemori and Portoghese, 1993).

In the present study, we showed that the opioid system is involved in the antinociceptive effect induced by TTHL. This observation is based on the fact that naloxone, a non-selective opioid receptor antagonist, as well as CTOP (a selective μ-opioid receptor antagonist), nor-binaltorphimine (a selective κ-opioid receptor antagonist) and naltrindole (a selective δ-opioid receptor antagonist) not only reversed the antinociceptive effect of morphine (a non-selective opioid agonist, positive control), but also reversed TTHL-induced antinociception in mice. In view of these results, we can speculate that TTHL may elicit an antinociceptive effect either by releasing endogenous opioid that can bind to their specific opioid receptors, or by interacting with opioid receptors. However, other studies are necessary to further substantiate this assertion.

Data in the literature has demonstrated a possible interaction between 5-HT and the opioid systems. In the CNS, serotonergic neurons are involved in the pain inhibition induced by opioid agonists. Furthermore, an enhanced rate of release of serotonin into perfusates of rat spinal cord occurred when morphine was injected into periaqueductal grey matter (Hamon et al., 1991; Fürst, 1999). Serotonin was also observed to modulate the pain transmission in the spinal cord and in the brain (Yaksh and Wilson, 1979). Autoradiographic studies showed that $5\text{-}HT_{1A}$, $5\text{-}HT_{1B}$, $5\text{-}HT_{1D}$ and $5\text{-}HT_3$ binding sites are abundant in the spinal cord. However, their roles in the control of

nociception are sometimes controversial. In this regard, there are studies showing that 5-HT antagonists, i.e. 5-HT_3 antagonists, can produce both pronociceptive (Ali et al., 1996; Green et al., 2000; Oatway et al., 2004; Suzuki et al., 2002) and antinociceptive effects (Alhaider et al., 1991; Glaum et al., 1990; Paul et al., 2001). On the other hand, others demonstrated that the activation of spinal cord serotonin subtype receptors 5-HT_{1A} and 5-HT_2 produce antinociception (Bardin et al., 2000; Millan, 2002). The data presented here suggest that the serotonergic system could be involved in the antinociceptive effect of TTHL in mice. This supposition is based on the fact that the depletion of endogenous serotonin with tryptophan hydroxylase inhibitor PCPA, aside from significantly reversing morphine antinociception, largely antagonized the antinociceptive action of TTHL. Furthermore, pre-treatment of mice with selective antagonists of 5-HT_{1A}, WAY100635, 5-HT_{2A}, ketanserin, but not with 5-HT_3 antagonist, ondansetron, significantly reversed the antinociception caused by systemic administration of TTHL.

Another interesting finding demonstrated in the present study is that inactivation of the $G_{i/o}$ protein through i.t. pertussis toxin treatment reversed the antinociceptive effect induced by TTHL and morphine. Pertussis toxin (PTX) inactivates the $G_{i/o}$ by ADP-ribosylation of the α-subunit of this protein (Wen et al., 2003), disrupting inhibitory $G_{i/o}$ protein-coupled signal transduction, and consequently, leading to a predominance of the spinal excitatory receptor system. Additionally, inhibitory neurotransmitter receptors (i.e., opioid and 5-HT) are linked to PTX-sensitive proteins (Aghajanian and Wang, 1986; Jeong and Ikeda, 2001), and inactivation of these G-proteins may contribute to the activation of excitatory receptors, such as the NMDA receptor (Jeong and Ikeda, 2001). Furthermore, Ito et al. (2000) showed that activation of 5-HT_{1A} promotes the opening of K^+ channels and closing of Ca^{2+} channels through coupling negatively to adenylyl cyclase, therefore inhibiting sensory transmission. In addition, activation of $G_{i/o}$ protein is considered an inhibitory signaling event, since it inhibits adenylyl cyclase and opens two different types of K^+ inward rectifier (Kir) channels: K^+-ATP dependent and G-protein-regulated inwardly rectifying K^+ (GIRK) channels (Childers and Deadwyler, 1996; Ocaña et al., 2004).

Corroborating these data, we demonstrated that pre-treatment with glibenclamide, a blocker of the K^+-ATP dependent channel, charybdotoxin, a blocker of large (or fast)-conductance calcium-gated K^+ channels and tetraethylammonium, a blocker of voltage-gated K^+ channels, reverse the TTHL antinociceptive effect. However, pre-treatment with apamin, a blocker of small (or low)-conductance calcium-gated K^+ channels, did not prevent the TTHL action.

In conclusion, the present results are in agreement with previous data and demonstrate that TTHL produces antinociception when systemically administered. Furthermore, these evidences showed that the TTHL-induced antinociception involves the opioidergic (i.e., through μ, κ and δ receptors) and serotonergic systems (i.e., through 5-HT_{1A} and 5-HT_{2A} receptors), $G_{i/o}$ protein activation and opening of specific K^+ channels (ATP dependent, voltage- and large-conductance Ca^{2+}-gated). Together, the present results indicate that TTHL might be of potential interest in the development of new clinically relevant drugs for the management of pain. Although, additional studies are necessary to confirm this hypothesis.

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