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Further antinociceptive effects of myricitrin in chemical models of overt nociception in mice

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ABSTRACT

The present work explored the antinociceptive effects of the flavonoid myricitrin in models of overt nociception triggered by intraplantar injection of chemical algogens into the hind paw of mice. The nociception induced by bradykinin (3 nmol/paw i.pl.) was abolished by prior treatment with myricitrin (10–100 mg/kg, i.p.) with ID_{50} of 12.4 (8.5–18.1) mg/kg. In sharp contrast, myricitrin failed to affect the nociception elicited by prostaglandin E_2 (3 nmol/paw i.pl.). Cinnamaldehyde (10 nmol/paw i.pl.)-induced nociception was reduced by myricitrin (100 mg/kg, i.p.) and camphor (7.6 mg/kg, s.c.) in 43 ± 10% and 57 ± 8%, respectively. Myricitrin (30–100 mg/kg, i.p.) and amiloride (100 mg/kg, i.p.) inhibited nociceptive responses induced by acidified saline (pH 5/paw i.pl.), with ID_{50} of 22.0 (16.1–30.0) mg/kg and inhibition of 71 ± 6% and 64 ± 5%, respectively. Moreover, myricitrin (10–30 mg/kg, i.p.) and ruthenium red (3 mg/kg, i.p.) significantly reduced the nociception induced by menthol (1.2 μmol/paw i.pl.) with the mean ID_{50} of 2.4 (1.5–3.7) mg/kg and inhibition of 95 ± 3% and 51 ± 7%, respectively. In addition, myricitrin administration (30 and 100 mg/kg, i.p.) markedly reduced menthol-induced mechanical allodynia. However, myricitrin (100 mg/kg, i.p.) prevented (only in time of 60 min) cold allodynia induced by menthol. Collectively, the present results extend prior data and show that myricitrin promotes potent antinociception, an action that is likely mediated by an inhibition of the activation of nociceptors by bradykinin and TRPs agonist (i.e. cinnamaldehyde, acidified saline and menthol), probably via inhibition of PKC pathways. Thus, myricitrin could constitute an attractive molecule of interest for the development of new analgesic drugs.

Acute nociceptive pain is the consequence of primary afferent nociceptive C and Aδ fibers activation by intense mechanical, chemical, or thermal stimuli [18]. Thus, such noxious stimuli excite peripheral nociceptors by activation of distinct types of ionotropic channels and metabotropic receptors [18]. In fact, transient receptor potential (TRP) and acid-sensing ion channels (ASIC) play important roles in generating nociceptive signals in response to various specific noxious stimuli [10]. In addition, following tissue injury or during certain inflammatory processes occurs the release of pro-inflammatory mediators, namely bradykinin, prostaglandins and protons that contribute to elicit nociceptive signals as well as to promote hyperalgesia and allodynia [7,18]. Finally, the activity of some of these receptors can be facilitated (or upregulated) by protein kinases A and C (PKA and PKC), which sensitize them or amplify their responses by phosphorylating key residues in their structures, as well as of other proteins implicated in nociceptive signaling pathways in sensory neurons [32,33].

Although a considerable number of antinociceptive drugs are available, novel substances could contribute to our current understanding of the nociceptive signaling pathways to improve the treatment of painful conditions. Many plant-derived substances are attractive sources for developing new analgesic agents, among these, myricitrin, a naturally occurring flavonoid obtained from genus *Eugenia* displays marked anti-inflammatory and antinociceptive effects in rodents [24–27]. Our group has shown that myricitrin inhibited nociception caused by acetic acid and by intraplantar injection of glutamate, capsaicin and phorbol myristate acetate (PMA) in mice [25]. In addition, myricitrin reduced mechanical hyperalgesia induced by intraplantar injection of bradykinin in rats as well as the inflammatory and neuropathic allodynia caused by intraplantar injection of complete Freund's adjuvant and

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by partial sciatic nerve ligation in mice [25,26]. Interestingly, the mechanisms involved in the antinociceptive action of myricitrin include inhibition of PKC and of p38 MAPK phosphorylation [25,27].

Here, we evaluated the effect of myricitrin on nociception triggered by intraplantar injection of bradykinin, prostaglandin E2, protons (activator of ASIC/TRPV1 channels), cinnamaldehyde (agonist of TRPA1 channel), menthol (agonist of TRPM8 channel). Finally, we evaluated the effect of myricitrin on cold and mechanical allodynia induced by intraplantar injection of menthol in mice.

Male Swiss mice (25–35 g) were housed (22 ± 2 °C, 12 h light–dark cycle) with food and water *ad libitum*. Mice were acclimatized to the laboratory for at least 1 h before testing. All experiments were previously approved by the UFSC's Committee on the Ethical Use of Animals, and conducted under the ethical guidelines of the International Association for the Study of Pain [42].

First, we investigated whether myricitrin would be able to antagonize bradykinin- and prostaglandin-induced nociception in the mouse paw. Animals used were individually adapted into a glass funnel. Myricitrin (1–100 mg/kg, i.p.) or vehicle (control, 10 ml/kg, i.p.) was administered 30 min before intraplantar (i.pl.) injection of the chemical algogens [12,19]. Following myricitrin or vehicle treatment, mice received a 20 μl i.pl. injection of either bradykinin (3 nmol/paw) or prostaglandin E2 (3 nmol/paw). Immediately after the injection, each animal was placed into individual glass cylinder of 20 cm and paw licking or biting was measured for 15 min, and considered as indicative of nociception.

In another set of experiments, to test if TRPA1, TRPM8 and ASIC receptors constitute potential specific targets for the antinociceptive actions of myricitrin, we tested the effects of this flavonoid against nociceptive responses elicited by activators of each channel. To this effect, following treatment with myricitrin, camphor [7.6 mg/kg, subcutaneous (s.c.) used as positive control], amiloride (100 mg/kg, i.p. used as positive control), ruthenium red (3 mg/kg, i.p. used as positive control) or vehicle, mice received a 20 μl intraplantar (i.pl.) injection of either cinnamaldehyde (10 nmol/paw), acidified saline (2% acetic acid in 0.9% saline, pH 5/paw) or menthol (1.2 μmol/paw). Paw licking or biting were recorded for 5 min (for cinnamaldehyde), 15 min (for acidified saline) or 20 min (for menthol).

Myricitrin (30 mg/kg) was administered by i.p. route at different times (15–240 min) before i.pl. injection of menthol to determine the duration of its antinociceptive effect. Next, we examined the influence of myricitrin (30 and 100 mg/kg, i.p.) in menthol-induced mechanical and cold allodynia. For assessment, mice were placed in clear plexiglas observation chambers (9 cm × 7 cm × 11 cm) on elevated wire mesh platforms. Animals were habituated to the chamber for 30 min. Hind paw responsiveness to mechanical or cold stimuli were assessed before menthol (1.2 μmol/paw) injection and then again, repeatedly, at several time points thereafter, but animals were exposed only to a single modality of sensory stimulus.

Mechanical allodynia was evaluated as the withdrawal response frequency to 10 applications of 0.4 g of von Frey hairs (Stoelting, Chicago, USA) [6]. Cold allodynia was assessed through spraying 50 μl of acetone on the plantar surface of the hind paw. Behavioral response was analyzed during 20 s and then recorded in scores: 0 – no response; 1 – quick withdrawal, flick or stamp of the paw; 2 – prolonged withdrawal or repeated flicking of the paw; 3 – repeated flicking of the paw with licking directed at the ventral side of the paw. Acetone application was repeated three times for each hind paw, in 5 min intervals, and the sum of three scores was used for data analysis [13].

The following substances were used: myricitrin was isolated from the plant of genus *Eugenia* in the Department of Chemistry, Federal University of Santa Catarina, Brazil. Analysis of the 1H NMR and 13C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure [1]. The chemical purity of myricitrin (more than 98%) was determined by GC/HPLC. Myricitrin was dissolved in Tween 80 plus saline. The final concentration of Tween did not exceed 10% and did not cause any effect per se. Bradykinin, prostaglandin E2, cinnamaldehyde, menthol amiloride, camphor and ruthenium red were from Sigma–Aldrich (St. Louis, MO). All other chemicals were of analytical grade and obtained from standard commercial suppliers. Drugs were dissolved in 0.9% NaCl solution, with the exception of menthol, which was dissolved in 1.6% ethanol/0.01% Tween 80 in saline. In both of these conditions, the final solutions containing ethanol failed to cause any nociceptive effects per se when administered alone. All procedures, doses and administration routes of the various drugs were chosen on the basis of previous studies [3,4,12,19,23,36] or in preliminary experiments carried out in our laboratory (data not shown).

Results are presented as mean ± S.E.M., except the ID_{50} values (i.e. the dose of myricitrin that reduce the nociceptive response by 50% relative to the control values), which are reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} values were determined by nonlinear regression from individual experiments using GraphPad software (GraphPad software, San Diego, CA, USA). The percentages of inhibition were calculated for the most effective dose used. Data concerning nociception to chemical stimuli were analyzed statistically using one-way ANOVA followed by Newman–Keuls' post hoc test. Statistical comparisons of mechanical and cold allodynia data were performed by two-way ANOVA, followed by Bonferroni's multi-comparison post hoc test. In all cases, differences were considered to be significant when $P < 0.05$.

Myricitrin (3–100 mg/kg, i.p.) produced dose-dependent inhibition (83 ± 2%) of bradykinin-induced nociception with mean ID_{50} of 12.4 (8.5–18.1) mg/kg (Fig. 1A). Post hoc comparisons with the Newman–Keuls test detected a significant difference with myricitrin treatment at doses of 10 mg/kg ($P < 0.01$), 30 mg/kg ($P < 0.001$) and 100 mg/kg ($P < 0.001$). Like bradykinin, i.pl. injection of prostaglandin E2 also induced nociceptive behaviors in mice. However, myricitrin had no effect on nociceptive response triggered by prostaglandin E2 ($P > 0.05$) (Fig. 1B).

As shown in Fig. 1C, only the highest dose of myricitrin (100 mg/kg, i.p.) inhibited the nociceptive behavior induced by cinnamaldehyde, with inhibition of 43 ± 10% ($P < 0.001$). Pretreatment of camphor (7.6 mg/kg, s.c.), a nonspecific TRP blocker, inhibited the cinnamaldehyde-induced nociception in 57 ± 8% ($P < 0.01$). Fig. 1D shows that myricitrin (1–100 mg/kg, i.p. 30 min before) also inhibited (71 ± 6%) the nociceptive response induced by acidified saline with the mean ID_{50} value of 22.0 (16.1–30.0) mg/kg. Post hoc comparisons with the Newman–Keuls test detected a significant difference with myricitrin at doses of 30 mg/kg ($P < 0.001$) and 100 mg/kg ($P < 0.01$). Moreover, the blocking of the acid-sensitive ion channels (ASICs) by amiloride (100 mg/kg, i.p.) also decreased the nociception mediated by acidified saline in 64 ± 5% ($P < 0.001$).

Intraplantar injection of menthol produced a marked nociception in mice. Previous treatment with the general TRP blocker ruthenium red (3 mg/kg, i.p.) reduced nociception evoked by menthol in 51 ± 7% ($P < 0.05$). Furthermore, myricitrin (1–30 mg/kg, i.p.) inhibited (95 ± 3%) menthol-induced nociceptive behavior with the mean ID_{50} value of 2.4 (1.5–3.7) mg/kg (Fig. 2A). Post hoc comparisons with the Newman–Keuls test detected significant differences with myricitrin treatment at doses of 10 mg/kg ($P < 0.001$) and 30 mg/kg ($P < 0.001$). A time-course analysis of the antinociceptive effect of myricitrin is shown in Fig. 2B. Myricitrin produced marked antinociception as early as 15 min after administration, an action that remained significant up to 60 min (Fig. 2B). Furthermore, menthol promoted mechanical and cold allodynia, lasting 2 and 4 h, respectively. Myricitrin (30 and 100 mg/kg, i.p.) reduced