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Involvement of p38^{MAPK} on the antinociceptive action of myricitrin in mice

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ARTICLE INFO

Article history:
Received 9 April 2007
Accepted 18 June 2007

Keywords:
Myricitrin
Nociception
Interleukin-1β
Tumor necrosis factor-α
Extracellular-signal regulated kinase
c-Jun amino-terminal kinases
p38^{MAPK}

ABSTRACT

Previous studies from our group investigated the analgesic and anti-inflammatory properties of the flavonoid myricitrin. Here, we demonstrated the role of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and mitogen-activated protein kinases (MAPKs) on the antinociceptive action of myricitrin. The nociceptive response was evaluated by monitoring biting behaviour following intratecal (i.t.) administration of IL-1β and TNF-α in mice. Western blot analyses of total and phosphorylated MAPKs: p38^{MAPK}, extracellular-signal regulated kinase (ERK1/2) and c-Jun amino-terminal kinases (JNK1/2) from the spinal cord of mice injected with cytokines were measured. Myricitrin (0.03–30 mg/kg) or vehicle (control) was administered 30 min beforehand by intraperitoneal (i.p.) injection. Myricitrin pretreatment prevented cytokine-induced biting behaviour. The calculated ID_{50} of myricitrin were 6.8 (4.6–9.0) and 2.6 (0.3–4.9) mg/kg and maximal inhibition of 83 ± 9 and 100 ± 0% for IL-1β and TNF-α, respectively. Intrathecal injection of IL-1β and TNF-α significantly increased p38^{MAPK} phosphorylation and this was inhibited by myricitrin treatment. Cytokines administration did not alter ERK1/2 and JNK1/2 phosphorylation. Myricitrin prevented cytokine-induced biting behaviour and inhibited p38^{MAPK} phosphorylation in response to cytokines stimulation. Taken together, it suggests that the mechanism for antinociceptive action of myricitrin in response to cytokines may involve a blockage on p38^{MAPK} pathway. This finding could explain, at least in part, the antinociceptive action of this flavonoid in process like neuropathic and inflammatory chronic pain.

1. Introduction

Under pathological conditions like neuropathies, rheumatoid arthritis and process related to the chronic pain, cytokines are released from leucocytes, fibroblasts, Schwann cells and endothelial cells [1–3]. These peptides, together with other mediators, are responsible for initializing and maintaining pain [1,4,5]. The cytokines IL-1β and TNF-α are over expressed in the spinal cord on peripheral inflammatory and neuropathic experimental models of pain [1,4,6,7]. In addition, IL-1β and TNF-α cause sensitization of peripheral nociceptors (hypernociception) and cause nociception when administrated intrathecally [1,5,8]. The intracellular mechanisms by which IL-1β and TNF-α cause nociception involve activation of tyrosine kinases, protein kinase C (PKC) and MAPKs [1,4,6].

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0006-2952/$ – see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.bcp.2007.06.024

MAPKs are a family of highly conserved enzymes which comprise ubiquitous groups of signalling proteins that regulate transduction, amplification and integration of cell signalling from the cell surface to the nucleus [9–11]. These proteins are regulated by extra-cellular stimuli like growth factors, cytokines and environmental stress, controlling complex programs, such as cell differentiation and proliferation, embryogenesis and cell death [9–11]. The MAPK family includes ERK1/2 and stress activated protein kinases (SAPKs), JNK1/2/3 and $p38^{MAPK}$ α, β, γ, δ [9,10,12]. The ERK1/2 activation has been mainly associated with cell survival and proliferation [13]. JNK and $p38^{MAPK}$ cascades are preferentially activated by environmental stresses and pro-inflammatory cytokines, and are largely associated with the promotion of inflammation, pain and programmed cell death [4,12,14–16].

Inflammatory mediators and aberrant neuronal activity activate MAPKs pathways in primary sensory and dorsal horn neurons of the spinal cord. This event mediates the induction and maintenance of neuropathic pain through both post-translational and transcriptional mechanisms [4,17–20]. Inhibitors of $p38^{MAPK}$ decrease neuropathic pain and inflammation because they reduce synthesis of cytokines [4,16,21]. Therefore, compounds that exhibit an inhibitory activity on MAPKs pathways could be successfully employed as therapeutic agents on the pain states, including chronic pain [4,16]. These substances may also be useful pharmacological tools for the investigation of pain transmission mechanisms.

It is known that myricitrin, a flavonoid presents in plants of genus *Eugenia,* comprises antinociceptive effects in experimental models of acute and chronic pain [22,23]. The antinociceptive mechanisms of myricitrin include inhibition of PKC, decrease in nitric oxide (NO) production, activation of $G_{i/o}$ protein pathway and alteration of Ca^{2+} and K^+ movement [22,24]. However, the relationship between myricitrin, cytokine-induced MAPKs phosphorylation and nociception is still unknown. The aim of present study was to investigate the myricitrin effects on the ERK1/2, JNK and $p38^{MAPK}$ phosphorylation and nociception caused by intrathecal administration of IL-1β and TNF-α.

2. Materials and methods

2.1. Reagents

Myricitrin was isolated from plants of the genus *Eugenia* at the Department of Chemistry, Universidade Federal de Santa Catarina, Brazil. It was identified by spectral analyzes (RMN-1H) and (RMN-13C) and by comparison with the published spectra [32]. It showed a degree of purity greater than 98%. The myricitrin was dissolved in Tween 80 plus saline. The pro-inflammatory cytokines (IL-1β and TNF-α), protease inhibitor cocktail and the primary antibodies anti-ERK1/2, anti-JNK1/2 and anti-$p38^{MAPK}$ were obtained from Sigma (St. Louis, USA). Primary antibodies anti-phospho-ERK1/2, anti-phospho-JNK1/2, anti-phospho-$p38^{MAPK}$ and secondary antibody anti-rabbit IgG horseradish peroxidase conjugated (IgG-HRP) were obtained from Cell Signalling Technology (Danvers, MA, USA). All other chemicals were of analytical grade and obtained from standard commercial suppliers. The cytokines were dissolved in phosphate-buffered saline pH 7.4.

2.2. Animals

Experiments were conducted using 80 adult Swiss female mice (25–35 g) housed at 22 ± 2 °C under a 12-h light:12-h dark cycle (lights on at 6:00) and with access to food and water *ad libitum*. All animals were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. The experiments were performed after approval of protocol by the Institutional Ethics Committee from Universidade Federal de Santa Catarina (UFSC) (protocol number 23080.0011700/2005-03/UFSC). All experiments were carried out in accordance with current guidelines for care of laboratory animals and investigation of experimental pain in conscious animals, as specified by Ref. [25]. The number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

2.3. Pro-inflammatory cytokines-induced overt nociception in mice

The antinociceptive effects of myricitrin were evaluated against nociception induced by intrathecal injection of IL-1β and TNF-α, as previously described in Ref. [8] with minor modifications [26]. Mice received an i.p. injection of myricitrin (0.03–30 mg/kg) or vehicle (3% Tween 97% saline, 10 ml/kg). Myricitrin stock solutions of 10 mg/ml were prepared in 10% Tween 90% saline. This stock solution was further diluted in saline to give the solution of use. Thus, a solution of 3 mg/ml was injected in a volume of 10 ml/kg as the highest dose of myricitrin (30 mg/kg, 3% Tween 97% saline). Further dilutions were done to achieve determined concentrations of myricitrin. Thirty minutes after myiricitrin administration, an i.t. injection of 5 μl of IL-1β (1 pg/site), TNF-α (0.1 pg/site) or vehicle (buffered saline) were given to fully conscious mice awake, using the method described by Ref. [27]. The concentrations of cytokines were chosen as determined previously in our laboratory [26]. It did not cause any overt sign of inflammation in the local of injection up to 15 min. Briefly, the animals were restrained manually and a 30-gauge needle, attached to a 25 μl microsyringe, was inserted through the skin and between the vertebrae into the subdural space of the L5-L6 spinal segments. Injections were given over a period of 5 s. The amount of time that the animal subsequently spent biting was evaluated for 15 min. A bite was defined as a single head movement directed at the flanks or hind limbs, resulting in contact of the animal's snout with the target organ. Groups were divided in: (S) saline i.p. and saline i.t.; (C) saline i.p. and cytokines i.t.; (M) myricitrin i.p. and cytokines i.t.

2.4. Assessment of MAPKs phosphorylation through Western blot assay

In this experiment, the doses of 3 and 10 mg/kg of myricitrin were chosen to evaluate its effects against cytokine-induced MAPKs phosphorylation. The groups were divided: (1) mice treated with vehicle (10 ml/kg, i.p.) 30 min before an i.t.

injection of buffered saline (5 μl); (2) mice treated with vehicle (10 ml/kg, i.p.) 30 min before an i.t. injection of IL-1β (1 pg/site) or TNF-α (0.1 pg/site); (3) mice treated with myricitrin (3 or 10 mg/kg) before an i.t. injection of IL-1β (1 pg/site) or TNF-α (0.1 pg/site). The i.t. injection was carried out as described in Section 2.2. After 15 min the mice were killed by cervical dislocation and the lumbar region of spinal cord was removed for Western blot analysis. The tissue was homogenized at 4 °C in a buffer (pH 7.0) containing 50 mM Tris, 10 mM β-mercaptoethanol, 1 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride, 20 μM Na_3VO_4, 100 μM sodium fluoride and Sigma cocktail protease inhibitor [28]. The homogenates were centrifuged at 1000 × *g* for 10 min at 4 °C and the supernatants (S1) collected. Proteins were quantified as described before in Ref. [29]. Samples (100 μg of protein) were separated by SDS-PAGE using 10% gels and they were transferred to nitrocellulose membrane using 400 mA current (3 h at 4 °C) [30,31]. The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS; Tris 10 mM, NaCl 150 mM, pH 7.4) for 1 h, followed by a second blockage (1 h) with 2.5% gelatin in TBS [29,30]. ERK1/2, $p38^{MAPK}$ and JNK1/2, total and the activated (bi-phosphorylated) forms, were detected by Western blot [30,31]. Briefly, the primary antibodies (Cell Signalling) to anti-phospho ERK1/2, anti-phospho $p38^{MAPK}$ and anti-phospho JNK1/2 were diluted 1:1000 and they were incubated for 24 h at 4 °C. The primary antibodies (Sigma) to anti-ERK1/2 (1:40,000), anti-$p38^{MAPK}$ and anti-JNK1/2 (1:10,000) were incubated for 2 h at room temperature. Secondary antibody anti-rabbit IgG horseradish peroxidase conjugated (IgG-HRP; Cell Signalling) were diluted at 1:2000 and incubated for 1 h. All steps of blocking and incubation were followed by three times of 5 min washes with TBS-T (Tris 10 mM, NaCl 150 mM, Tween 20 0.05%, pH 7.5).

In order to detect phosphorylated and total forms of each MAPK in the same membrane, the immune complexes were stripped. Briefly, membranes were washed once with double deionized water (5 min), followed by incubation with NaOH 0.2 N (5 min), washing with double deionized water (5 min) and with TBS-T (10 min). The membranes stripped of immune complexes were blocked as required for the next round of Western blotting [30,31].

The optical density (O.D.) of the bands was quantified using Scion Image software®. The phosphorylation level of each MAPK was determined as a ratio of O.D. of phosphorylated band/O.D. of total band [28]. The data are expressed as percentage of the saline/saline group (considered as 100%) and the values are presented as mean ± S.E.M.

2.5. *Statistical analysis*

Results are presented as mean ± S.E.M., except the ID_{50} values (i.e., the dose of myricitrin that decreased the nociceptive response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} value was determined using three doses of myricitrin by linear regression from individual experiments using linear regression software (GraphPad software, San Diego, CA). Percentage of maximal inhibition was calculated at the most effective dose used (30 mg/kg). The results were analyzed by one-way ANOVA and post hoc analysis was performed by Duncan's test when appropriate. A value of $P < 0.05$ was considered to be significant.

3. Results

As shown in Fig. 1, the i.t. administration of IL-1β or TNF-α caused marked nociceptive biting behaviour in mice ($P < 0.001$ from saline i.t. group). The myricitrin i.p. administration significantly reduced the biting behaviour induced by both cytokines in a dose-dependent manner (Fig. 1A and B). The myricitrin ID_{50} values were 6.8 (4.6–9.0) and 2.6 (0.3–4.9) mg/kg for IL-1β and TNF-α, respectively. The percentages of maximal inhibition (MI) of 30 mg/kg myricitrin were 83 ± 9 and 100% for IL-1β and TNF-α, respectively. No biting behaviour was observed in the group that received vehicle i.p plus buffered saline (5 μl, i.t.) (Fig. 1).

In another set of experiments, we investigated the myricitrin effect on MAPKs phosphorylation induced by i.t. administration of IL-1β and TNF-α. Western blot analysis from spinal cord homogenates showed $p38^{MAPK}$, ERK1/2 and JNK1/2

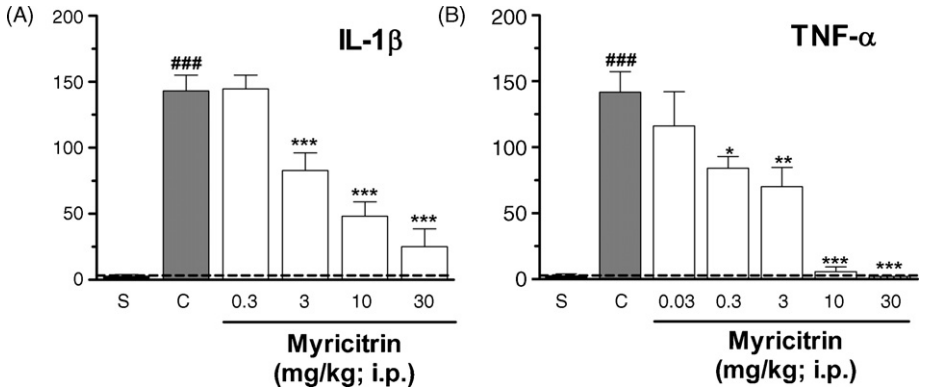

Fig. 1 – Effect of myricitrin on nociceptive biting behaviour induced by i.t. injection of cytokines (A) IL-1β (1 pg/site) and (B) TNF-α (0.1 pg/site). Each bar represents the average ± S.E.M. of six animals treated with saline i.p. and buffered saline i.t. (S), saline i.p. and IL-1β or TNF-α i.t. (C), myricitrin i.p. and IL-1β or TNF-α i.t. (white bars). Statistical analysis were performed by analysis of variance (ANOVA/one-way), followed by Duncan's test. The symbols represent a significant difference $^{###}P < 0.001$ from saline/saline group (S) and $^{*}P < 0.05$; $^{**}P < 0.01$ or $^{***}P < 0.001$ from saline/cytokines group (C).

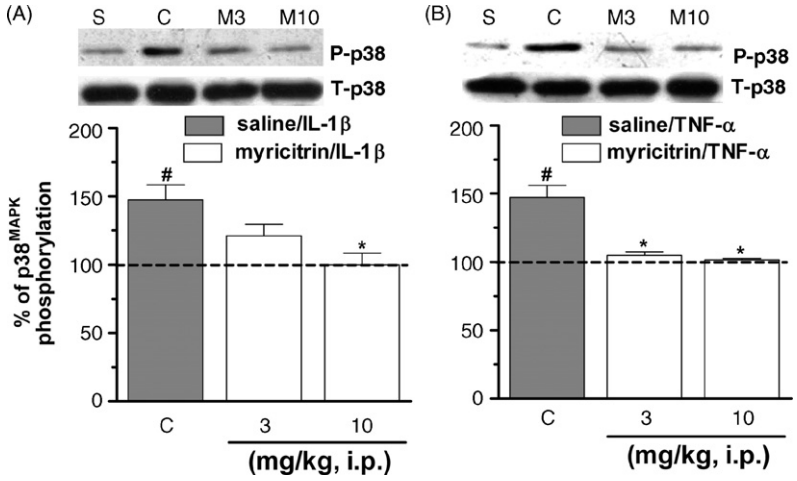

Fig. 2 – Effect of myricitrin on p38^{MAPK} phosphorylation induced by i.t. injection of cytokines (A) IL-1β (1 pg/site) and (B) TNF-α (0.1 pg/site) in spinal cord of mice. Each bar represents the average ± S.E.M. of four animals. The data are expressed as ratio between phosporylated and total form of p38^{MAPK}. The percentage of phosphorylation was calculated considering control group (saline/saline) as 100% (horizontal dotted line). The mice received saline i.p. plus IL-1β or TNF-α i.t. (C, grey bars); myricitrin i.p. plus IL-1β or TNF-α i.t. (white bars). Statistical analysis were performed by analysis of variance (ANOVA/one-way), followed by Duncan's test. The symbols denote a significant difference $^{\#}P < 0.001$ from saline/saline group (horizontal dotted line) and $^{*}P < 0.05$ from saline/cytokines group (C). The bands on the top show the p38^{MAPK} expression on phosphorylated form (upper bands) and total form (lower bands) referent to saline/saline (S), saline/cytokines (C), myricitrin 3 mg kg^{-1}/cytokines (M3) and myricitrin 10 mg kg^{-1}/cytokines (M10) groups.

were constitutively expressed and phosphorylated in tissue of mice. Cytokines administration did not alter significantly the total amount of p38^{MAPK}, ERK1/2 and JNK 1/2 in 15 min ($P > 0.05$ from saline/saline group, Figs. 2–4). However, the treatment with IL-1β and TNF-α caused a significant increase in p38^{MAPK} phosphorylation (Fig. 2A and B, $P < 0.05$ from saline/saline group). The pre-treatment with myricitrin (3 or 10 mg/kg; i.p.) totally inhibited p38^{MAPK} phosphorylation induced by TNF-α. Mice treated with myricitrin had similar level of p38^{MAPK} phosphorylated than untreated mice (saline/saline group) (Fig. 2B). The myricitrin significantly inhibited p38^{MAPK} phosphorylation induced by i.t. injection of IL-1β. However, myricitrin displayed this effect only at the highest dose employed (10 mg/kg) (Fig. 2A).

Administration of IL-1β or TNF-α did not alter ERK 1/2 and JNK1/2 phosphorylation in spinal cord of mice 15 min after

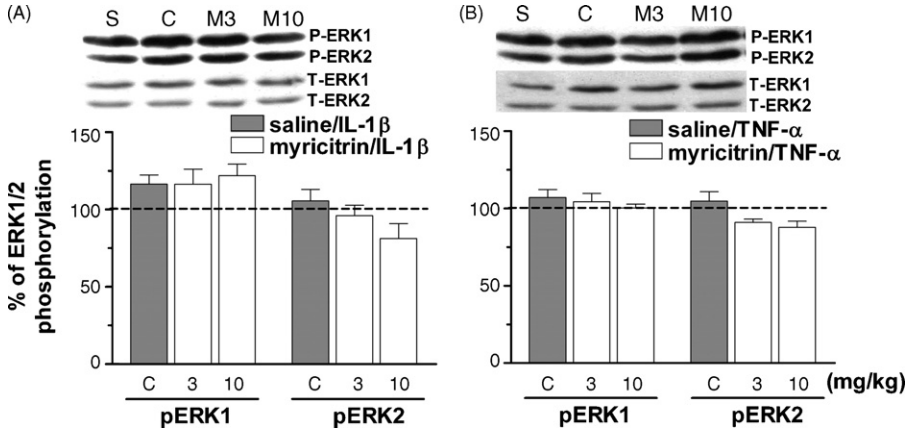

Fig. 3 – Effect of myricitrin on ERK1/2 phosphorylation induced by i.t. injection of cytokines (A) IL-1β (1 pg/site) and (B) TNF-α (0.1 pg/site) in spinal cord of mice. Each bar represents the average ± S.E.M. of four animals. The data are expressed as ratio between phosporylated and total form of ERK1/2. The percentage of phosphorylation was calculated considering control group (saline/saline) as 100% (horizontal dotted line). The mice received saline i.p. plus IL-1β or TNF-α i.t. (C, grey bars); myricitrin i.p. plus IL-1β or TNF-α i.t. (white bars). Statistical analysis were performed by analysis of variance (ANOVA/one-way), followed by Duncan's. The bands on the top show the ERK1/2 expression on phosphorylated form (upper bands) and total form (lower bands) referent to saline/saline (S), saline/cytokines (C), myricitrin 3 mg kg^{-1}/cytokines (M3) and myricitrin 10 mg kg^{-1}/cytokines (M10) groups.

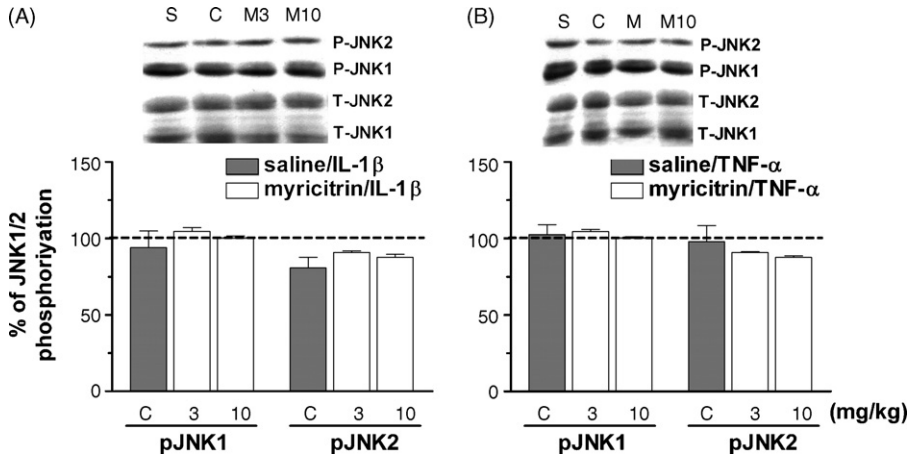

Fig. 4 – Effect of myricitrin on JNK1/2 phosphorylation induced by i.t. injection of cytokines (A) IL-1β (1 pg/site) and (B) TNF-α (0.1 pg/site) in spinal cord of mice. Each bar represents the average ± S.E.M. of four animals. The data are expressed as ratio between phosporylated and total form of JNK1/2. The percentage of phosphorylation was calculated considering control group (saline/saline) as 100% (horizontal dotted line). The mice received saline i.p. plus IL-1β or TNF-α i.t. (C, grey bars); myricitrin i.p. plus IL-1β or TNF-α i.t. (white bars). Statistical analysis were performed by analysis of variance (ANOVA/one-way), followed by Duncan's test. The bands on the top show the JNK1/2 expression on phosphorylated form (upper bands) and total form (lower bands) referent to saline/saline (S), saline/cytokines (C), myricitrin 3 mg kg^{-1}/cytokines (M3) and myricitrin 10 mg kg^{-1}/cytokines (M10) groups.

administration. As shown in Figs. 3 and 4 the phosphorylated forms of these kinases stayed at basal levels ($P > 0.05$ from saline/saline group). Furthermore, myricitrin pre-treatment did not change ERK 1/2 and JNK1/2 phosphorylation.

4. Discussion

It is known that flavonoids interact with enzymes and proteins within cells and therefore, they regulate processes including cell growth, apoptosis, immunological response and energetic metabolism [33–36]. Previous studies from our group showed that the flavonoid myricitrin has antinociceptive effects in acute model of nociception in rodents, mainly by its ability to inhibit PKC activation [22]. Myricitrin antinociceptive effects were also attributed to inhibition of NO production and nuclear factor-κB (NF-κB) activation [22,37]; activation of protein $G_{i/o}$ pathway, increase in K^+ efflux, decrease in Ca^{2+} movement and anti-inflammatory activity [23,24].

The antinociception afforded by myricitrin was widened to chronic models of nociception like sciatic nerve injury and chronic inflammation induced by intraplantar injection of complete Freund's adjuvant (CFA) [22]. In both situations, an augment of cytokines, IL-1β and TNF-α, in the dorsal horn of medulla spinal contributes to neuronal sensitization through direct opening of ionic channels, activation of intracellular mediators and induction of expression of molecules involved with pain transmission and maintenance [1,4,7,38–40]. In this study, we observed that systemic administration of myricitrin induced potent antinociception against biting behaviour induced by IL-1β and TNF-α.

The biting behaviour is a common method employed to evaluate nociceptive transmission in rodents. The intrathecal injection of glutamatergic receptor agonists, kinins or cytokines directly sensitises neuron in the spinal medulla. As a consequence, the pain behaviour (caudal direct biting, licking and scratching) starts immediately after the injection of the mediators [8,24,27,41]. The molecular mechanisms involved in the sensitization of neurons in the spinal medulla including increases in nitric oxide production, protein kinase C activity and calcium influx [4,41]. Here, we demonstrated for the first time, that the biting behaviour evoked by intrathecal injection of IL-1β and TNF-α is also associated to phosphorylation of $p38^{MAPK}$.

During nociceptive and inflammatory process IL-1β and TNF-α trigger $p38^{MAPK}$. The phosphorylation of $p38^{MAPK}$ generates a positive feedback in pain and inflammation because it activates transcriptional factors that regulate the gene transcription of pro-inflammatory cytokines [4,16,39,42]. Hence, inhibitors of $p38^{MAPK}$ decrease neuropathic pain and inflammation because they reduce synthesis of cytokines [4,16,21]. $p38^{MAPK}$ phosphorylation increases inflammatory and nociceptive response also at post-translational levels, via phosphorylation of receptors, ion channels, protein kinases and increase of phospholipases activity [4,39]. Consequently, $p38^{MAPK}$ phosphorylation can modify membrane excitability and contribute directly to pain transmission [4,39].

In this study, the increase in $p38^{MAPK}$ phosphorylation, as a consequence of the injection of IL-1β and TNF-α, was believed to contribute to nociceptive behaviour in mice. In agreement, it was previously reported that the treatment with $p38^{MAPK}$ inhibitor, SB203580, inhibited hyperalgesia induced by an intrathecal injection of IL-1β [6]. Considering that $p38^{MAPK}$ phosphorylation mediated nociceptive response within 15 min after cytokines injection, the nociceptive effects of $p38^{MAPK}$ phosphorylation were probably associated to a direct activation of receptors, ion channels and enzymes, but not associated to the synthesis of new mediators [4,39].

Interestingly, the injection of IL-1β or TNF-α did not stimulate ERK1/2 and JNK1/2. Hence, ERK1/2 and JNK1/2 are activated during neuropathic pain and referred hyperalgesia [4,17,19,20]. Although it was an unexpected finding, our data corroborate with those found by Sung et al. [6], which demonstrated that intrathecal administration of IL-1β induced phosphorylation of $p38^{MAPK}$ but not of ERK1/2 and JNK1/2 in the spinal cord of rats. In fact, $p38^{MAPK}$ is more susceptible to activation by cytokines than ERK1/2 and JNK1/2 in the spinal cord and the activation of ERK1/2 and JNK1/2 by IL-1β or TNF-α is only achieved with few hours of exposure or by chronic treatments [39,42–44].

The myricitrin administration fully prevented $p38^{MAPK}$ phosphorylation induced by IL-1β and TNF-α. Through these results, we suggest an involvement of $p38^{MAPK}$ phosphorylation in the antinociceptive effects of myricitrin. Myricitrin inhibited more potently the nociception and $p38^{MAPK}$ phosphorylation induced by TNF-α than that induced by IL-1β. The reason why these cytokines had different sensitivities to myricitrin is unknown, but a rational explanation could be because upstream IL-1β and TNF-α have different pathways leading to $p38^{MAPK}$ phosphorylation [39,45]. These results suggest that myricitrin could affect upstream kinases responsible by $p38^{MAPK}$ phosphorylation. However, further studies are necessary to test this hypothesis.

Considering that $p38^{MAPK}$, PKC and protein $G_{i/o}$ are integrated signalling cascade in some cells [46,47], we could not exclude an indirect effect of myricitrin on the PKC and protein $G_{i/o}$ pathways driving the inhibition of $p38^{MAPK}$ phosphorylation. However, this indirect way would explain myricitrin effects only on $p38^{MAPK}$ phosphorylation induced by IL-1β but not that induced by TNF-α. In fact, the binding of IL-1β to its receptor activates tyrosine kinases and calcium-independent PKC [48]. This event is likely associated with $p38^{MAPK}$ activation [47]. On the other hand, the binding of TNF-α to its receptor triggers $p38^{MAPK}$ phosphorylation independently of PKC or protein $G_{i/o}$ pathways [43,49–51].

As reported previously, myricitrin antinociceptive effects are not associated with non-specific effects as impairment motor, ataxia, depression of central nervous system or hypothermia. Hence, a higher dose than utilized in this study did not alter time of permanency on the rota-rod, locomotion on the open-field, body temperature or body posture [22].

Given that flavonoids inhibit protein kinases [22,33,35] and reduce ERK1/2 and JNK1/2 phosphorylation in carcinoma cells [36,52], we may not rule out the capacity of myricitrin to act as an inhibitor of ERK1/2 or JNK1/2 activation in different conditions unlike those used in this study. In our model, IL-1β and TNF-α treatment were devoid of ERK1/2 and JNK1/2 activation.

Inhibitors of $p38^{MAPK}$ are promising therapeutic agents for the treatment of inflammatory diseases. These inhibitors have efficacy against arthritic and inflammatory disease and some compounds have reached clinical studies in humans [53]. Considering the key role of $p38^{MAPK}$ phosphorylation during chronic painful and inflammatory process, the effects of myricitrin in counteracting $p38^{MAPK}$ phosphorylation could explain, at least in part, the beneficial action of myricitrin against neuropathic and inflammatory nociception [23].

In summary, the findings of the present work demonstrated a role of $p38^{MAPK}$ phosphorylation in acute nociception induced by cytokines. The flavonoid myricitrin was able to counter both nociception and $p38^{MAPK}$ phosphorylation induced by i.t. administration of IL-1β and TNF-α. From the results obtained here, we may infer a role of $p38^{MAPK}$ phosphorylation on the antinociceptive action of myricitrin.

Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Programa de Apoio aos Núcleos de Excelência (PRONEX) and Fundação de Apoio a Pesquisa Científica e Tecnológica do Estado de Santa Catarina (FAPESC). F.C. Meotti and T. Posser are Ph.D. student in Biochemistry, they thank CAPES for fellowship support. The authors thank Mark Hampton and Rufus Turner for critical review of the manuscript.

REFERENCES

[1] Sommer C, Kress M. Recent findings on how pro-inflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. Neurosci Lett 2004;361:184–7.
[2] Sommer C, Petrausch S, Lindenlaub T, Toyka KV. Neutralizing antibodies to interleukin 1-receptor reduce pain associated behaviour in mice with experimental neuropathy. Neurosci Lett 1999;270:258.
[3] Watkins LR, Maier SF. Implications of immune-to-brain communication for sickness and pain. Proc Natl Acad Sci USA 1999;96:7710–3.
[4] Ji RR, Stricharstz G. Cell signaling and the genesis of neuropathic pain. Science 2004;252:1–19.
[5] Cunha TM, Verri WA, Silva JS, Poole S, Cunha FQ, Ferreira SH. A cascade of cytokines mediates mechanical inflammatory hypernociception in mice. Proc Natl Acad Sci USA 2005;102:1755–60.
[6] Sung C, Wen Z, Chang W, Chan K, Ho S, Tsai S, et al. Inhibition of p38 mitogen-activated protein kinase attenuates interleukin-1β-induced thermal hyperalgesia and inducible nitric oxide synthase expression in the spinal cord. J Neurochem 2005;94:742–52.
[7] Li M, Shi J, Tang J, Chen D, Ai B, Chen J, et al. Effects of complete Freund's adjuvant on immunohistochemical distribution of IL-1β and IL-1R I in neurons and glia cells of dorsal root ganglion. Acta Pharmacol Sin 2005;26:192–8.
[8] Choi SS, Han KJ, Lee JK, Lee HK, Han EJ, Kim DH, et al. Antinociceptive mechanisms of orally administered decursinol in the mouse. Life Sci 2003;73:471–85.
[9] Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature 2001;410:37–40.
[10] Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, et al. MAP kinases. Chem Rev 2001;101:2449–76.
[11] Thomas GM, Huganir RL. MAPK cascade signalling and synaptic plasticity. Nat Rev Neurosci 2004;5:173–83.
[12] Mielke K, Herdegen T. JNK and p38 stresskinases-degenerative effectors of signal-transduction-cascades in the nervous system. Prog Neurobiol 2000;61:45–60.
[13] Hetman M, Gozdz A. Role of extracellular signal regulated kinases 1 and 2 in neuronal survival. Eur J Biochem 2004;271:2050–5.

[14] Tibbles LA, Woodgett JR. The stress-activated protein kinase pathways. Cell Mol Life Sci 1999;55:1230–54.
[15] Takeda K, Ichijo H. Neuronal p38 MAPK signalling: an emerging regulator of cell fate and function in the nervous system. Genes Cells 2002;7:1099–111.
[16] Kaminska B. MAPK signaling pathways as molecular targets for anti-inflamatory therapy-from molecular mechanisms to therapeutic benefits. Biochim Biophys Acta 2005;1754:253–62.
[17] Galan A, Cervero F, Laird JMA. Extracellular signalling-regulated kinase-1 and -2 (ERK1/2) mediate referred hyperalgesia in murine model of visceral pain. Brain Res Mol Brain Res 2003;116:126–34.
[18] Ji R, Baba H, Brenner GJ, Woolf CJ. Nociceptive-specific activation of ERK in spinal neurons contributes to pain hyper-sensitivity. Nat Neurosci 1999;2:1114–9.
[19] Zhuang ZY, Woolf CJ, Ji RR. Differential activation of ERK and p38 MAPK in the dorsal root ganglion and spinal cord by spinal nerve ligation and its implication for neuropathic pain. Soc Neurosci Abstr 2003;29: 586.16.
[20] Zhuang ZY, Zhang DR, Ji RR. JNK MAP kinase is activated in spinal cord by spinal nerve ligation and contributes to neuropathic pain. Soc Neurosci Abstr 2004;30:980.14.
[21] Inoue K, Tsuda M, Koizumi S. ATP- and adenosine-mediated signalling in the central nervous system: chronic pain and microglia: involvement of the ATP receptor P2X4. J Pharmacol Sci 2004;94:112–4.
[22] Meotti FC, Luiz AP, Pizzolatti MG, Kassuya CAL, Calixto JB, Santos ARS. Analysis of the antinociceptive effect of the flavonoid myricitrin. Evidence for a role of the L-arginine-nitric oxide and protein kinase C pathways. J Pharmacol Exp Ther 2006;316:789–96.
[23] Meotti FC, Missau FC, Ferreira J, Pizzolatti MG, Mizuzaky C, Nogueira CW, et al. Anti-allodynic property of flavonoid myricitrin in models of persistent inflammatory and neuropathic pain in mice. Biochem Pharmacol 2006;72:1707–13.
[24] Meotti FC, Fachinetto R, Maffi L, Missau FC, Pizzolatti MG, Rocha JBT, et al. Antinociceptive action of Myricitrin: involvement of the K^+ and Ca^{2+} channels. Eur J Pharmacol 2007;567:198–205.
[25] Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16:109–10.
[26] Paszcuk AF, Gadotti VM, Tibola D, Quintão NLM, Rodrigues ALS, Calixto JB, et al. Anti-hypernociceptive properties of agmatine in persistent inflammatory and neuropathic models of pain in mice. Brain Res 2007;1159:123–33.
[27] Hylden JL, Wilcox GL. Intrathecal substance P elicits a caudally-directed biting and scratching behavior in mice. Brain Res 1981;217:212–5.
[28] Calloni GW, Penno CA, Cordova FM, Trentin AG, Neto VM, Leal RB. Congenital hypothyroidism alters the phosphorylation of ERK1/2 and p38MAPK in the hippocampus of neonatal rats. Brain Res Dev Brain Res 2005;154:141–5.
[29] Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1977;72:248–54.
[30] Cordova FM, Rodrigues AL, Giacomelli MB, Oliveira CS, Posser T, Dunkley PR, et al. Lead stimulates ERK1/2 and p38MAPK phosphorylation in the hippocampus of immature rats. Brain Res 2004;998:65–72.
[31] Leal RB, Ribeiro SJ, Posser T, Cordova FM, Rigon AP, Zaniboni Filho E, et al. Modulation of ERK1/2 and p38(MAPK) by lead in the cerebellum of Brazilian catfish Rhamdia quelen. Aquat Toxicol 2006;77:98–104.
[32] Agrawal PK. Studies in Organic Chemistry. New York: Elsevier Science Publishers; 1989.
[33] Gamet-Payrastre L, Manenti S, Gratacap MP, Tulliez J, Chap H, Payrastre B. Flavonoids and the inhibition of PKC and PI 3-kinase. Gen Pharmacol 1999;32:279–86.
[34] Middleton Jr E, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 2000;52:673–751.
[35] Havsteen BH. The biochemistry and medical significance of the flavonoids. Pharmacol Ther 2002;96:67–202.
[36] Ko CH, Shen SC, Hsu CS, Chen YC. Mitochondrial-dependent, reactive oxygen species-independent apoptosis by myricetin: roles of protein kinase C, cytochrome c, and caspase cascade. Biochem Pharmacol 2005;69: 913–27.
[37] Chen YC, Yang LL, Lee TJF. Oroxylin A inhibition of lipopolysaccharide-induced iNOS and COX-2 gene expression via suppression of nuclear factor-κB activation. Biochem Pharmacol 2000;59:1445–57.
[38] DeLeo JA, Colburn RW, Ricknian AJ. Cytokine and growth factor immunohistochemical spinal profiles in two animals models of neuropathy. Brain Res 1997;759:50–7.
[39] Ji RR, Woolf CJ. Neuronal plasticity and signal transduction in nociceptive neurons: implications for the initiation and maintenance of pathological pain. Neurobiol Dis 2001;8:1–10.
[40] McMahon SB, Cafferty WBJ, Marchand F. Immune and glial cell factors as pain mediators and modulators. Exp Neurol 2005;192:444–62.
[41] Liu H, Mantayh PW, Basbaum AI. NMDA-receptor regulation of substance P release from primary afferent nociceptors. Nature 1997;386:721–4.
[42] Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 1999;79: 143–80.
[43] Gao B, Calhoun K, Fang D. The proinflammatory cytokines IL-1β and TNF-α induce the expression of Synoviolin, an E3 ubiquitin ligase, in mouse synovial fibroblasts via the Erk1/2-ETS1 pathway. Arthritis Res Ther 2006;8:R172.
[44] Bhattacharjee R, Li T, Koshy S, Beard LL, Sharma K, Carter EP, et al. Stimulation of MAP kinase pathways after maternal IL-1β exposure induces fetal lung fluid absorption in guinea pigs. Respir Res 2007;8:27.
[45] Kyriakis JM, Avruch J. Mammalian Mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 2001;81:807–69.
[46] Chang LC, Wang JP. The upstream regulation of p38 mitogen-activated protein kinase phosphorylation by arachidonic acid in rat neutrophils. J Pharm Pharmacol 2000;52:539–46.
[47] Kandere-Grzybowska K, Kempuraj D, Cao J, Cetrulo CL, Theoharides TC. Regulation of IL-1-induced selective IL-6 release from human mast cells and inhibition by quercetin. Br J Pharmacol 2006;148:208–15.
[48] Obreja O, Rathee PK, Lips KS, Distler C, Kress M. 1IL-1β potentiates heat-activated currents in rat sensory neurons: involvement of IL-1RI, tyrosine kinase and protein kinase C. FASEB J 2002;16:1497–503.
[49] Schafers M, Svensson CI, Sommer C, Sorkin LS. Tumor necrosis factor-α induces mechanical allodynia after spinal nerve ligation by activation of p38 MAPK in primary sensory neurons. J Neurosci 2003;23:2517–21.
[50] Nguyen J, Gogusev J, Knapnougel P, Bauvois B. Protein tyrosine kinase and p38MAP kinase pathways are involved in stimulation of matrix metalloproteinase-9 by TNF-α in human monocytes. Immunol Lett 2006;106:34–41.

[51] Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol 2003;3:745–56.
[52] Gopalakrishnan A, Xu CJ, Nair SS, Chen C, Hebbar V, Kong AN. Modulation of activator protein-1 (AP-1) and MAPK pathway by flavonoids in human prostate cancer PC3 cells. Arch Pharm Res 2006;29:633–44.
[53] Kumar S, Boehm J, Lee JC. p38MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. Nature 2003;2:717–26.