Analysis of the Antinociceptive Effect of the Flavonoid Myricitrin: Evidence for a Role of the L-Arginine-Nitric Oxide and Protein Kinase C Pathways

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ABSTRACT
The present study investigated the antinociceptive effects of the flavonoid myricitrin in chemical behavioral models of pain in mice and rats. Myricitrin given by i.p. or p.o. routes produced dose-related antinociception when assessed on acetic acid-induced visceral pain in mice. In addition, the i.p. administration of myricitrin exhibited significant inhibition of the neurogenic pain induced by intraplantar (i.pl.) injection of capsaicin. Likewise, myricitrin given by i.p. route reduced the nociception produced by i.pl. injection of glutamate and phorbol myristate acetate (PMA). Western blot analysis revealed that myricitrin treatment fully prevented the protein kinase C (PKC) and PKCe activation by PMA in mice hind paws. Myricitrin given i.p. also inhibited the mechanical hyperalgesia induced by bradykinin, without affecting similar responses caused by epinephrine and prostaglandin E₂. The antinociception caused by myricitrin in the acetic acid test was significantly attenuated by i.p. treatment of mice with the nitric oxide precursor, L-arginine. In contrast, myricitrin antinociception was not affected by naloxone (opioid receptor antagonist) or neonatal pretreatment of mice with capsaicin and myricitrin antinociceptive effects is not related to muscle relaxant or sedative action. Together, these results indicate that myricitrin produces pronounced antinociception against chemical and mechanical models of pain in rodents. The mechanisms involved in their actions are not completely understood but seem to involve an interaction with nitric oxide-L-arginine and protein kinase C pathways.

Flavonoids are a γ-benzopyrone family that occur naturally and are widely spread in higher plants (Ramelet, 2000). They are plant secondary metabolites and are chemically defined by their common structure, which is composed of diphenylpropanes (C₆-C₃-C₆) and consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (Harborne, 1989). In mammals, flavonoids occur only through dietary intake. The average daily human intake in the United Kingdom and United States has been present to be 20 mg to 1 g. These compounds are present in fruits, vegetables, grains, nuts, tea, and wine. However, little is reported about quantify flavonoid in food and thus, only a few studies have attempted to assess the relationship between consumption of foods rich in flavonoids and the prevention of certain diseases (Birt et al., 2001).

A variety of biological effects have been ascribed to flavonoids (Birt et al., 2001; Havsteen, 2002; Calixto et al., 2003, 2004). Much attention has been given to their antioxidant (Edenharder and Gruhnage, 2003) and anti-inflammatory properties, in vitro and in vivo (Calixto et al., 2003, 2004). In addition, some studies report antitumoral (Clifford et al., 1996) and hepatoprotective (Ferrándiz et al., 1994) action. Flavonoids inhibit cytokine release from RAW264.7 cells (Xagorari et al., 2002) and may modulate the increasing number of cellular processes involving redox reaction, including the regulation of tyrosine phosphatase activity (Ganet-Payrastre et al., 1999). In contrast, little is known about the effects of flavonoids on the modulation of pain transmission.

Myricitrin (Fig. 1) is a flavonoid that belongs to the flavonol subgroup. This flavonoid is found in fruits of genus

ABBREVIATIONS: NO, nitric oxide; i.pl., intraplantar(ly); PMA, phorbol 12-myristate 13-acetate; L-NOARG, N^-nitro-L-arginine; PKC, protein kinase C; PBS, phosphate-buffered saline; ANOVA, analysis of variance; PGE₂, prostaglandin E₂; PKA, protein kinase A; TRPV1, transient receptor potential vanilloid type 1.
of myricitrin. Attempts have been made to further investigate some of the mechanical models of nociception in mice and rats. At this date. Here, we have therefore attempted to examine the possible antinociceptive effects of this flavonoid up to this date. Here, we have therefore attempted to examine the possible antinociceptive effects of this flavonoid up to this date.

Previous reports demonstrate that myricitrin is able to inhibit nitric oxide (NO) production. In addition, it reduces the overexpression of nitric-oxide synthase and nuclear factor-κB activation induced by lipopolysaccharide on RAW264.7 cells (Chen et al., 2000).

Taking into account the biological activities of myricitrin, it is surprising that no pharmacological study has been carried out on the possible antinociceptive effects of this flavonoid up to this date. Here, we have therefore attempted to examine the possible antinociceptive action of myricitrin in chemical and mechanical models of nociception in mice and rats. Attempts have been made to further investigate some of the possible mechanisms that underlie the antinociceptive action of myricitrin.

Materials and Methods

Animals
Experiments were conducted using Wistar rats (200–300 g) or Swiss mice (25–35 g) of both sexes, housed at 22 ± 2°C under a 12-h light/dark cycle (lights on at 6:00 AM) and with access to food and water ad libitum. Animals (male and female rat or mice were homogeneously distributed among the groups) 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 protocol approval by the Institutional Ethics Committee and were carried out in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals as specified (Zimmermann, 1983). The number of animals and intensity of the noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

Abdominal Constriction Induced by Acetic Acid
The abdominal constrictions were induced according to procedures described previously (De Campos et al., 1997) and resulted in contraction of the abdominal muscle together with a stretching of the hind limbs in response to an i.p. injection of acetic acid (0.6%, 0.45 ml/mouse) at the time of the test. Mice were pretreated with myricitrin by i.p. (0.01–10 mg/kg) or p.o. (1–100 mg/kg) routes, 30 or 60 min before the irritant injection. Control animals received a similar volume of vehicle (10 ml/kg). After the challenge, the mice were individually placed into glass cylinders of 20-cm diameter, and the abdominal constrictions were counted cumulatively over a period of 20 min.

Algogen-Induced Overt Nociception in Mice
Glutamate-Induced Nociception. In an attempt to provide more direct evidence concerning the possible interaction of myricitrin with the glutamatergic system, we separately investigated whether myricitrin would be able to antagonize glutamate-induced licking in the mouse paw. The procedure used was similar to describe previously (Beirith et al., 2002). A volume of 20 μl of glutamate (10 μmol/paw prepared in saline) was injected intraplantarly (i.pl.) in the ventral surface to the right hind paw. The mice were observed individually for 15 min following glutamate injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of nociception. Mice were treated with myricitrin (1–100 mg/kg i.p.) or vehicle (10 ml/kg i.p.) 30 min before glutamate injection.

Phorbol Myristate Acetate (PMA)-Induced Nociception. The procedure used was similar to that described previously (Siebel et al., 2004). A volume of 20 μl of PMA (50 pmol/paw prepared in saline) was injected i.pl. in the ventral surface of the right hind paw. After the challenge, the mice were individually placed into glass cylinders of 20-cm diameter, serving as observation chambers. The mice were observed individually from 15 to 45 min after PMA injection, and the amount of time spent licking the injected paw timed with a chronometer was considered indicative of nociception. The mice were treated with myricitrin (0.01–10 mg/kg i.p.) or vehicle (10 ml/kg i.p.) 30 min before PMA injection.

Capsaicin-Induced Nociception. The procedure used was similar to that described previously (De Campos et al., 1997). After an adaptation period, 20 μl of capsaicin (1.6 μg/paw) was injected i.pl. in the ventral surface of the right hind paw. The mice were observed individually for 5 min following capsaicin injection. The amount of time spent licking the injected paw was recorded with a chronometer and was considered as indicative of nociception. The mice were treated with myricitrin (1, 10, and 100 mg/kg i.p.) 30 min before capsaicin injection. Control animals received vehicle (10 ml/kg).

Bradykinin-, Epinephrine-, or Prostaglandin E2-Induced Hyperalgesia. The procedures used were similar to those described previously (De Campos et al., 1997). The rats were pretreated i.p. with myricitrin (30 mg/kg) or vehicle (10 ml/kg) 30 min before injection of 100 μl of bradykinin (3 nmol/paw), epinephrine (450 nmol/paw), prostaglandin E2 (10 nmol/paw), or only saline solution alone (control group) in the ventral surface of the right hind paw. The nociception threshold (of squeak response or paw withdrawal) was assessed by applying increasing pressure to the dorsal site of inflamed or control paws, using a Basile algometry meter (Ugo Basile, Comerio, Italy) according to the method of Randall and Selitto (1957). The weight on the algometry meter ranged from 0 to 750 g, and the threshold was expressed as load (grams) tolerated. When bradykinin was used, animals were pretreated with the angiotensin-converting enzyme inhibitor captopril (5 mg/kg s.c.) 60 min before experiments to prevent its degradation (De Campos et al., 1997).

Measurement of Motor Performance, Locomotor Activity, and Corporal Temperature. To evaluate the possible nonspecific muscle-relaxant or sedative effects of myricitrin, mice were submitted to the Rotarod task (Vaz et al., 1996) and open-field test (Rodrigues et al., 2002). Rotarod apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into four compartments by disks 25 cm in diameter. The bar rotated at a constant speed of 17 revolutions/min. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 120 s. Animals were treated with myricitrin (30 or 100 mg/kg i.p.) or vehicle (10 ml/kg i.p.) 30 min before being tested. The results are
expressed as the time(s) for which animals remained on the Rotarod. The cut-off time used was 120 s.

The ambulatory behavior was assessed in an open-field test as described previously (Rodrigues et al., 2002). The 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 with all paws crossing was counted in a 6-min session. Mice were treated with myricitrin (30 and 100 mg/kg i.p.) or vehicle (10 ml/kg i.p.) 30 min beforehand.

In addition, some compounds cause antinociception by decreasing basal cerebral temperature (hypothermia). To exclude this possibility, we assessed the skin temperature of mice 30 min after they received vehicle (saline) or myricitrin (30 and 100 mg/kg i.p.). A thermosensor (Mallory Ltda., Ceará, Brazil) was placed on the skin in the sacral region, and the procedure was carried out in accordance with the manufacturer’s instructions.

Analysis of the Possible Mechanism of Action of Myricitrin

Involvement of the Opioid System. To investigate the possible participation of the opioid system in the antinociceptive effect of myricitrin, mice were pretreated with naltaloxone (a nonselective opioid receptor antagonist, 5 mg/kg i.p.), and after 20 min, the animals received an injection of myricitrin (1 mg/kg i.p., morphine (5 mg/kg s.c.), or vehicle (10 ml/kg i.p.). Other groups were pretreated with vehicle and after 20 min received myricitrin, morphine, or vehicle, 30 min before acetic acid injection.

Involvement of the L-Arginine-Nitric Oxide Pathway. To investigate the role played by the nitric oxide-L-arginine pathway in the antinociception caused by myricitrin, mice were pretreated with L-arginine (40 mg/kg i.p., a nitric oxide precursor) or D-arginine (40 mg/kg i.p., an inactive isomer of L-arginine), and after 20 min, they received myricitrin (1 mg/kg i.p.), Nω-nitro-L-arginine (L-NOARG; 25 mg/kg i.p., an inhibitor of nitric oxide synthetase), or vehicle (10 ml/kg i.p.). The nociceptive responses to acetic acid were recorded 30 min after the administration of myricitrin, L-NOARG, or vehicle. Other groups were pretreated with vehicle (10 ml/kg i.p.) and after 20 min received myricitrin, L-NOARG, or vehicle 30 min before acetic acid injection (Abacioglu et al., 2001).

Involvement of Capsaicin-Sensitive Fibers. To explore the role of capsaicin-sensitive fibers in the antinociceptive effect of myricitrin, newborn mice were anesthetized with ether and treated s.c. with 50 mg/kg myricitrin on the 2nd day of life with the purpose of inducing the irreversible degeneration of unmyelinated afferent neurons, especially C fibers (Holzer, 1991). Control animals received, by the same route, the same volume of vehicle used to dissolve capsaicin with 50 mg/kg capsaicin on the 2nd day of life with the purpose of inducing the irreversible degeneration of unmyelinated afferent neurons, especially C fibers (Holzer, 1991). Control animals received, by the same route, the same volume of vehicle used to dissolve capsaicin with 50 mg/kg capsaicin on the 2nd day of life with the purpose of inducing the irreversible degeneration of unmyelinated afferent neurons.

Effect of Myricitrin on the PKCα and PKCe Activation by PMA

Preparation of Tissue for Western Blot Studies. The mice received myricitrin (1 mg/kg i.p.) or vehicle (10 ml/kg i.p.), 30 min before PMA (50 pmol/paw) injection. The injected paw was isolated 15 min after PMA or phosphate-buffered saline (PBS) injection (Ferreira et al., 2005). The skin and connective tissues of the plantar portion of the hind paws were removed and disrupted using a glass Potter homogenizer in an ice-cold buffer containing protease and phosphatase inhibitors (100 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 μg of aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 200 mM NaF, and 2 mM sodium orthovanadate). The homogenate was centrifuged at 1000g for 10 min at 4°C; the pellet was discarded, and the supernatant was further centrifuged at 35,000g for 30 min at 4°C. The supernatant was collected as a cytoplasm-rich fraction. The resulting pellet was resuspended and considered as a membrane-rich fraction. The protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA). The samples were aliquoted and stored at −80°C until their Western blot analysis.

Western Blot Analysis. To confirm the activation of PKCsα and PKCe after PMA injection into the mouse paw, Western blot analysis was carried out as previously described (Ferreira et al., 2005). Equivalent amounts of protein (10 and 50 μg for membrane- and cytoplasm-rich fractions, respectively) were mixed in buffer (200 mM Tris, 10% glycerol, 2% SDS, 2.75 mM β-mercaptoethanol, and 0.04% bromphenol blue) and boiled for 5 min. Proteins were resolved in 10% SDS-polyacrylamide gel electrophoresis and transferred on to polyvinylidene difluoride membranes, according to the manufacturer’s instructions (Millipore Corporation, Billerica, MA). The membranes were saturated by incubation overnight with 10% nonfat dry milk solution and then incubated with anti-PKCα and anti-PKCe (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing, the membranes were incubated with adjusted peroxidase-coupled secondary antibodies. The immunocomplexes were visualized using the enhanced chemiluminescence detection system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Drugs. The following substances were used: acetic acid, morphine hydrochloride (Merck, Darmstadt, Germany), bradykinin, epinephrine, prostaglandin E2, capsaicin, naltaloxone hydrochloride, phenol 12-myristate 13-acetate, glutamic acid, L-arginine, D-arginine, L-NOARG, and PBS tablets (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 myricitrin, which was dissolved in Tween 80 plus saline and capsaicin, dissolved in ethanol plus Tween 80 plus saline. The final concentration of Tween and ethanol did not exceed 5% and did not cause any “per se” effect. The myricitrin, isolated from the plant of genus Eugenia by the Department of Chemistry (Federal University of Santa Catarina, Brazil), was characterized by spectral analyses (RMN-H) and (RMN-13C) and by comparison with the spectrum literature data (Agrawal, 1989) and showed a degree of purity greater than 98%.

Statistical Analysis. The results are presented as mean ± S.E.M., except the ID50 values (i.e., the dose of myricitrin reducing the nociceptive response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. When possible, the ID50 value was determined using at least three dosages of myricitrin by linear regression from individual experiments, using linear regression software (GraphPad Software Inc., San Diego, CA). Maximal inhibition values were calculated at the most effective dose used. The statistical significance of differences between groups was performed by ANOVA followed by Newman-Keuls test. When p < 0.05 was considered as indicative of significance.

Results

Abdominal Constriction Induced by Acetic Acid. The results in Fig. 2A show that myricitrin, given i.p. 30 min prior to testing, produced dose-related inhibition of the acetic acid-induced abdominal constrictions in mice, with a mean ID50 value (and their respective 95% confidence limits) of 0.33 (0.20–0.54) mg/kg, and the inhibition observed was 84 ± 5% for the dose of 10 mg/kg. Furthermore, given by p.o. route 60 min beforehand, myricitrin (100 mg/kg) caused a partial but significant inhibition (39 ± 4%) of the acetic acid-induced pain (Fig. 2B). Hence, myricitrin was less efficacious and potent in preventing the nociception caused by acetic acid when it was given orally in comparison with when it was
given i.p. Thus, the administration of myricitrin by the i.p. route (time point 30 min beforehand) was chosen for all further studies with independent groups of animals.

Glutamate-Induced Nociception. The results presented in Fig. 3A show that myricitrin caused a dose-dependent and significant inhibition of the glutamate-induced nociception, with a mean ID$_{50}$ value of 16.8 (10.3–27.5) mg/kg, and the inhibition observed was 81 ± 10% for the dose of 100 mg/kg.

Capsaicin-Induced Nociception. The i.p. administration of myricitrin produced partial, but significant, inhibition of the capsaicin-induced neurogenic nociception (Fig. 3B). The inhibition observed was 13 ± 8, 42 ± 8, and 37 ± 6% for the doses of 1, 10, and 100 mg/kg, respectively (Fig. 3B).

PMA-Induced Overt Nociception. The i.p. administration of myricitrin also produced a marked and dose-dependent inhibition of PMA-induced licking (Fig. 3C). The mean ID$_{50}$ value from this result was 0.56 (0.33–0.95) mg/kg, and the inhibition was 100% when administrated at 10 mg/kg.

Bradykinin-, Epinephrine-, or Prostaglandin E$_2$-Induced Hyperalgesia. The results of Fig. 4 show that intraplantar administration of bradykinin (3 nmol/paw), epinephrine (450 nmol/paw), and prostaglandin E$_2$ (PGE$_2$; 10 nmol/paw) significantly increased ($p < 0.01$) the sensibility to mechanical stimuli (hyperalgesia) in rats when assessed in the Randall-Selitto model. In addition, bradykinin, epinephrine, and PGE$_2$ caused a decrease of 40 ± 7, 58 ± 2, and 75 ± 3% on pressure supported in grams compared with control group value. Furthermore, the treatment of rats with myricitrin (30 mg/kg i.p.) completely reversed the hyperalgesic effect caused by bradykinin ($p < 0.01$) but did not reduce epinephrine- or PGE$_2$-induced hyperalgesia (Fig. 4).

Measurement of Motor Performance, Locomotor Activity, and Basal Temperature. The myricitrin treatment (30 and 100 mg/kg i.p.) did not alter response of mice in both tests, motor performance on the Rotarod task and locomotor activity in the open-field test compared with animals that received saline (control group) (Table 1). In addition, the basal temperature of mice was not altered by myricitrin (30 and 100 mg/kg) treatment (Table 1).

Involvement of the Opioid System. The results presented in Fig. 5 show that the pretreatment of mice with
Involvement of the L-Arginine-Nitric Oxide Pathway. The results presented in Fig. 6 show that mice pretreatment with nitric oxide precursor L-arginine (40 mg/kg i.p.), given 20 min prior to testing, but not with D-arginine (40 mg/kg i.p.) significantly prevented (p < 0.05) the antinociception caused by L-NOARG (25 mg/kg i.p.) and by myricitrin (1 mg/kg i.p.) when analyzed against acetic acid-induced abdominal contractions.

Involvement of Capsaicin-Sensitive Fibers. Finally, the neonatal capsaicin (50 mg/kg s.c.) treatment of mice produced partial, but significant, inhibition (27 ± 7%) of the acetic acid-induced nociception (Fig. 7). In contrast, the same treatment of mice with capsaicin did not significantly change the antinociceptive effect of myricitrin (1 and 10 mg/kg i.p.) compared with the neonatal vehicle treatment group (Fig. 7). Furthermore, successful capsaicin treatment of newborn mice was confirmed by a significant reduction (p < 0.001) in the response to topical application of capsaicin to the cornea in capsaicin-treated mice. The mean number of writhing motions was 4.6 ± 1.6 and 18.5 ± 1.7 in capsaicin- and vehicle-treated mice, respectively.

Western Blot Analysis of Protein Kinase C. The possible participation of the PKC pathway on the antinociceptive effect of myricitrin was confirmed through Western blot analysis. Injection of PMA (50 pmol/site) into the mouse paw activated PKCα and, to a lesser extent, PKCe isoforms, as indicated by their translocation from cytosol- to membrane-rich homogenates achieved in administered tissues (Fig. 8, A–D). In addition, myricitrin (1 mg/kg) pretreatment significantly prevented the activation of both PKCα and PKCe isoforms caused by PMA injection (Fig. 8, A–D).

Discussion

The present study demonstrates, for the first time, that systemic (i.p. or p.o.) administration of the flavonoid myricitrin, at doses that did not produce any important motor dysfunction, alterations in basal temperature, or any other obvious side effects induced a dose-dependent inhibition of acetic acid-induced visceral nociceptive response in mice. The most relevant additional findings of the present work were that i.p. administration of myricitrin caused significant inhibition of glutamate- and capsaicin-induced nociception and dose-dependent inhibition of the nociceptive response caused by intraplantar injection of PMA; the myricitrin antinociceptive effect in PMA-induced nociception is closely related to inhibition of both PKCα and PKCe; myricitrin comprised an antihyperalgesic effect upon the intraplantar injection of bradykinin, but not with epinephrine or PGE2, in rats; its antinociceptive effect against the acetic acid test was significantly reversed by i.p. treatment of mice with L-arginine, but not by naloxone; and the neonatal treatment of mice with capsaicin completely failed to affect myricitrin-induced antinociception in mice.
Flavonoids exert important pharmacological actions, such as antioxidant, anti-inflammatory, antiallergic, and anti-ischemic properties, suggesting their potential protective function against cardiovascular and coronary heart diseases and against certain forms of cancer (Gamet-Payrastre et al., 1999; Birt et al., 2001; Havsteen, 2002; Calixto et al., 2003, 2004; Edenharder and Grünhage, 2003). The flavonoid myricitrin produces important antioxidant and antimutagenic effects, which are attributed or not to its free radical scavenger action (Yasukawa et al., 1990; Edenharder and Grünhage, 2003). Although myricitrin is known to possess a strong antioxidant effect, the putative antinociceptive activities of myricitrin, as well as the mechanisms of its action in vivo, have never been reported.

The results reported here indicate that i.p. administration of myricitrin produced consistent and dose-related antinociception when assessed in acetic acid-induced visceral nociception in mice. Compared with standard analgesic drugs (results obtained by our group), myricitrin was about 36.6- to 72.7-fold more potent than acetaminophen, aspirin, and diclofenac in attenuating acetic acid-induced pain (Vaz et al., 1996; Santos et al., 1998). As expected, oral administration of myricitrin was less potent and efficacious than its i.p. administration in preventing the nociception. In fact, it is generally recognized that intact flavonoid glycosides, like myricitrin, are poorly absorbed when given by oral route (Murota and Terao, 2003). Thus, the present result is in agreement with data from the literature demonstrating that the bioavailability (antinociceptive activity) of myricitrin is notably decreased when given orally in comparison with when given i.p.

The acetic acid-induced writhing reaction in mice, described as a typical model for inflammatory pain, has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new agents (Collier et al., 1968). This method presents a good sensitivity; however, it shows poor specificity, leaving scope for the misinterpretation of results. This can be avoided by complementing the test with other models of nociception and by a performance motor
test. For this reason, myricitrin was examined for its possible inhibitory action in the Rotarod and open-field tests. In both tests, we could observe that there was no statistically significant interference in performance motor patterns at higher doses than those producing marked suppression of the writhing response.

Another interesting finding of the present study is the demonstration that myricitrin, given i.p., produced a dose-dependent inhibition of the nociceptive response caused by injection of glutamate into the mouse hind paw. Glutamate noceception seems to involve peripheral, spinal, and supraspinal sites of action, and it is greatly mediated by both N-methyl-D-aspartate and non-N-methyl-D-aspartate receptors, as well as by the release of nitric oxide and nitric oxide-related substances (Beirith et al., 2002). Thus, these previous findings and data of the present results may indicate, at least in part, that the antinociceptive effect of myricitrin could be associated with its ability to inhibit NO production or through interaction with the glutamatergic system.

Results of the present study also strongly suggest the involvement of protein kinase C, but not protein kinase A, in the antinociception caused by myricitrin. This notion derived from the data showing that i.p. administration of myricitrin dose-dependently inhibited the overt nociception by intraplantar PMA injection (a protein kinase C activator). Another piece of evidence that supports this view was the results demonstrating that myricitrin suppressed the mechanical hyperalgesia induced by bradykinin but not that induced by prostaglandin E2 in rats. Some studies propose that, in nociceptor, bradykinin binds to the B2 receptor, causing a direct activation of protein kinase C and the indirect activation of the protein kinase A (Ferreira et al., 2004). Furthermore, the results of the current study show that myricitrin, at a dose that abolished bradykinin-induced mechanical hyperalgesia, was not able to inhibit epinephrine-induced mechanical hyperalgesia. It has been suggested that mechanical hyperalgesia produced by epinephrine depends on the activation of both PKC and PKA (Khasar et al., 1999). Thus, we can speculate that epinephrine produced a powerful hyperalgesic effect by acting in the β-adrenergic receptor via cAMP/PKA independent of PKC second messenger pathways.

Of note, mice treated with myricitrin did not show PKC activation, as indicated by Western blot analyses. PKC activity requires an intracellular translocation from cytosol to cytoskeletal and membrane sites of action. Thus, translocation of PKC from a cytosolic to a membrane-associated location within the cell is a sensitive indicator of activation (Ferreira et al., 2005). In accordance with previous results (Ferreira et al., 2005), we found that i.pl. injection of PMA induced the translocation of a classic (PKCα) and, to a lesser extent, a novel protein kinase C (PKCe) isoforms from cytosolic to membrane. The present results show that myricitrin given systemically was able to prevent the activation of both PKC isoforms. Taken together, these results strongly suggest that the antinociceptive effect of myricitrin in PMA-induced nociception is closely related to inhibition of PKC. In fact, experimental evidence now indicates that flavonoids, such as myricitrin, can inhibit phosphoinositide 3-kinases, and, consequently, they inhibit protein kinase C isoenzyme activation (Gamet-Payrastre et al., 1999). However, flavonoids can also inhibit PKC directly (Agullo et al., 1997). The major structural characteristics in potent phosphoinositide 3-kinases and PKC inhibition by flavonoids are the presence of the 3′,4′-OH group on the B ring (Gamet-Payrastre et al., 1999), and myricitrin shares this condition.

Another interesting result of the present study was the demonstration that the L-arginine-nitric oxide pathway is likely to be involved in the antinociception caused by myricitrin. This conclusion derives from the fact that the pretreatment of mice with the substrate of nitric-oxide synthase, L-arginine, at a dose that produced no significant effect on acetic acid-induced pain, significantly reversed the antinociception caused by both myricitrin and L-NOARG (a known nitric oxide inhibitor). In marked contrast, the pretreatment of animals with the inactive isomer of L-arginine, D-arginine, had no significant effect against both myricitrin- and L-NOARG-induced antinociception. In agreement with these findings, it has been reported that myricitrin inhibits NO production and reduces the overexpression of inducible nitric-oxide synthase in RAW264.7 cells (Chen et al., 2000). Our data also demonstrate that the activation of the opioid naloxone-sensitive pathway is probably not involved in the antinociception produced by myricitrin because naloxone significantly reversed morphine, but not myricitrin, antinociception in the acetic acid test.

Previous studies have demonstrated the involvement of vanilloid receptor (TRPV1) in acetic acid-induced writhing (Ikeda et al., 2001). Hence, TRPV1 is stimulated by capsaicin, heat and pH alterations (Julius and Basbaum, 2001). Capsaicin activates TRPV1, which, in turn, induces membrane depolarization and increases cation influx, leading to noxious stimulus (Julius and Basbaum, 2001). Capsaicin administration in newborn mice (48 h old) causes persistent desensitization due to a nonselective loss of small sensory fibers, mostly C fibers (Jancsó et al., 1977). Our results confirm these observations by demonstrating that neonatal treatment of mice with capsaicin significantly blocked the writhing responses induced by acetic acid. However, the capsaicin newborn treatment did not significantly modify myricitrin-induced antinociception. Furthermore, the i.p. administration of myricitrin produces partial, but significant, inhibition of capsaicin-induced neurogenic nociceptive behavior on mouse hind paw. Capsaicin is known to evoke the release of neuropeptides, excitatory amino acids, nitric oxide, and proinflammatory mediators from the peripheral C fibers, and transmits nociceptive information to the spinal cord or causes spinal sensitization through protein kinase C and A activation (Calixto et al., 2005). Thus, the ability of myricitrin to interact with PKC might explain, at least partially, its antinociceptive effect on acetic acid-, glutamate-, capsaicin-, and PMA-induced nociceptive responses. In addition, it has been demonstrated that inhibitors of PKC prevent the phosphorylation of TRPV1, reducing the sensitization of this capsaicin sensitive receptor (Phekumar and Ahern, 2000; Ferreira et al., 2004; Calixto et al., 2005), thus, making it less responsive to agonist action.

In conclusion, the present results provide convincing evidence indicating that myricitrin, a flavonoid occurring naturally and widespread in higher plants, produces systemic antinociception when assessed in chemical models of nociception in mice, as well as producing antihyperalgesic effects in models of painful mechanical hypersensitivity in rats, at
doses that reveal no interference with locomotor activity. The precise mechanisms through which myricitrin exerts its action are currently under investigation, but inhibition of the L-arginine-nitric oxide and blockade of the PKC pathways seems largely to account for myricitrin antinociceptive effect. However, the opioid system, unmyelinated C fibers sensitive to capsaicin and PKA pathway, seem unlikely to participate in the antinociception caused by myricitrin.

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References


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