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Hesperidin induces antinociceptive effect in mice and its aglicone, hesperetin, binds to μ-opioid receptor and inhibits GIRK1/2 currents

Leonardo M. Loscalzo [a,b], Tin T. Yow [b], Cristina Wasowski [a], Mary Chebib [b], Mariel Marder [a,*]

[a] Instituto de Química y Fisicoquímica Biológicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 (C1113AAD), Buenos Aires, Argentina
[b] Faculty of Pharmacy, The University of Sydney, NSW 2006, Australia

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ABSTRACT

This paper extended the evaluation of the depressant and antinociceptive activities of hesperidin in order to determine its effectiveness by the intraperitoneal and oral routes, its pharmacological interaction with diverse pathways of neurotransmission and the role of its aglycone, hesperetin. The capacity of hesperidin and hesperetin to bind to μ-opioid receptor and their actions on μ-opioid receptor co-expressed with GIRK1/GIRK2 channels (G protein-activated inwardly rectifying K^+ channels) in *Xenopus laevis* oocytes were also determined.

Hesperidin exhibited a depressant activity in the hole board and locomotor activity tests, antinociceptive activities in the abdominal writhing and hot plate tests and no motor incoordination in the inverted screen and rotarod assays, only by the intraperitoneal route. Hesperetin did not show any effects *in vivo* in mice in these models, but *in vitro* it displaced the [^{3}H]DAMGO binding with low-affinity and inhibited inward currents through the expressed GIRK1/2 channels. Although hesperidin actions *in vivo* demonstrated to be mediated by an opioid mechanism of action, it failed to directly bind to and activate the μ-opioid receptor or produce any change on inward GIRK1/2 currents *in vitro*. However, it should be considered that hesperidin may be metabolized, possibly resulting in crucial changes in its biological activity.

1. Introduction

Hesperidin (hesperetin-7-rhamnoglucoside) is an abundant and inexpensive by-product of citrus cultivation and is the major flavonoid in sweet oranges and lemons. A deficiency of this substance in the diet has been linked with abnormal capillary leakiness as well as pain in the extremities causing aches, weakness and night leg cramps. Therapeutically useful properties of hesperidin have also been described, among them hypolipidemic, anti hypertensive, anti oxidative, anticarcinogenic effects and the like, whereas its aglycone, hesperetin, has many beneficial effects including antioxidant, neuroprotective, antivirus and cholesterol-lowering effects (Cho 2006; Garg et al., 2001). Also, hesperidin possesses significant anti-inflammatory and analgesic effects in rats and mice (Emim et al., 1994; Galati et al., 1994).

Absorption and metabolism of flavonoids are complex processes that determine their bioavailability and there is much controversy as to whether natural flavonoid glycosides can be absorbed by the gastrointestinal tract, or whether they are hydrolyzed in the small intestine prior to absorption (Kobayashi et al., 2008). Several studies have reported that dietary hesperidin is absorbed as its aglycone, hesperetin, after the removal of the disaccharide by intestinal bacteria and that it is immediately metabolized to glucuronides in the intestinal epithelium and liver (Nielsen et al., 2006). Glucuronides and sulfoglucuronides of hesperetin and homoeriodictyol are the major conjugates derived from hesperidin in rat plasma, and they reached a peak between 4 h and 6 h (Matsumoto et al., 2004).

Previous studies reported the presence of hesperidin in the roots and rhizomes of *Valeriana officinalis* and *V. wallichii* and described its sedative and sleep enhancing activities in mice (Marder et al., 2003) after its intraperitoneally (i.p.) administration. In contrast, its aglycone hesperetin did not show any activity at the doses tested (Fernández et al., 2006).

We have demonstrated that hesperidin's depressant action in mice could be partially blocked by naltrexone, a nonselective opioid receptor antagonist and by nor-binaltorphimine, a potent and highly selective κ-opioid receptor antagonist. These results indicate that the depressant effect of hesperidin is mediated, at least partially, by an opioid mechanism of action (Loscalzo et al., 2008). In this paper we have extended the evaluation of the depressant and antinociceptive activities of hesperidin in order to determine its effectiveness by the i.p. and oral routes of administration, the pharmacological interaction with diverse pathways of neurotransmission and neuromodulation on its antinociceptive effects and the role of hesperetin, its aglycone.

μ-opioid receptors dominate in rat and mouse brain and exist alongside smaller populations of δ and κ opioid receptors (Robson et al.,

* Corresponding author at: Instituto de Química y Fisicoquímica Biológicas, Facultad de Farmacia y Bioquímica, Junín 956 (C1113AAD), Buenos Aires, Argentina. Tel.: +54 11 4964 8289; fax: +54 11 4962 5457.
E-mail address: mmarder@qb.ffyb.uba.ar (M. Marder).

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1985). Opioid receptors are members of the G protein-coupled receptor (GPCR) family. These receptors activate several effectors including G protein-activated inwardly rectifying K^+ (GIRK) channels through direct action of G protein βγ-subunits released from pertussis toxin-sensitive G proteins (North et al., 1987; Reuveny et al., 1994; Wimpey and Chavkin, 1991). GIRK channel opening induces membrane hyperpolarization of the neurons via efflux of K^+ and reduces neuronal excitability (Ikeda et al., 2002). Neuronal GIRK channels are predominantly heteromultimers composed of GIRK1 and GIRK2 subunits in many brain regions in the rat and even more so in the mouse (Karschin et al., 1994; Kobayashi et al., 1995; Liao et al., 1996). The functional coupling of μ-opioid receptor with GIRK1/2 channels in *Xenopus* oocytes was previously reported by Ikeda et al. (1996, 2003).

The capacities of hesperidin and its aglycone hesperetin to bind to μ-opioid receptor in rat brain membranes and their actions on μ-opioid receptor co-expressed with GIRK1/2 channels in *X. laevis* oocytes were also evaluated.

2. Materials and methods

2.1. Drugs

Hesperidin, hesperetin, ketanserin tartrate, prazosin hydrochloride, nor-binaltorphimine dihydrochloride, naltrexone hydrochloride, caffeine and yohimbine hydrochloride were purchased from Sigma-Aldrich Chemical Company (USA). Flumazenil was obtained from Richet (S.A., Argentina). Naltrindole hydrochloride was obtained from Tocris Bioscience (UK), naloxone hydrochloride and [D-Ala^2, N-Me-Phe^4, Gly-ol^5] enkephalin (DAMGO) acetate salt from Tocris Bioscience (USA). [^{3}H]DAMGO was purchased from PerkinElmer (Boston, USA). Diazepam was obtained from Roche Diagnostics (Argentina). Morphine hydrochloride was purchased from Gramon, Argentina.

2.2. Animals

Adult male Swiss mice weighing 25–30 g were used in the pharmacological assays and adult male rats (200–300 g) Wistar strain for biochemical studies, both were obtained from the Central Animal House of the School of Pharmacy and Biochemistry, University of Buenos Aires. For behavioral assays mice were housed in groups of five in a controlled environment (20–23 °C), with free access to food and water and maintained on a 12 h/12 h day/night cycle, light on at 06:00 AM. Housing, handling, and experimental procedures complied with the recommendations set forth by the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and CICUAL (Institutional Committee for the Care and Use of Laboratory Animals, University of Buenos Aires, Argentina). All efforts were taken in order to minimize animal suffering. The number of animals used was the minimum number consistent with obtaining significant data. The animals were randomly assigned to any treatment groups and were used only once. The behavioral experiments were performed between 10:00 AM and 2:00 PM.

2.3. Apparatus and pharmacological tests

2.3.1. Drugs solutions and administration procedures

Hesperidin, hesperetin and diazepam were dissolved by the sequential addition of dimethylsulfoxide up to a final concentration of 5%, a water solution of 0.25% Tween 80 up to a final concentration of 20% and saline to complete 100% volume, for the intraperitoneally (i.p.) injections. For oral administrations (p.o.) drugs were suspended in an aqueous solution of carboxymethylcellulose 0.5%.

Ketanserin tartrate, prazosin hydrochloride, nor-binaltorphimine dihydrochloride, naltrexone hydrochloride, naltrindole hydrochloride, yohimbine hydrochloride, flumazenil, caffeine and morphine hydrochloride were dissolved in saline.

The choice of the doses of hesperidin and hesperetin was based on our published data (Fernández et al., 2005; Loscalzo et al., 2008) and on preliminary experiments. Higher doses of hesperetin were not attempted due to solubility issues.

Drugs and vehicle administered i.p., p.o. and sub cutaneously (s.c.) were given in volumes of 5 μl/g, 7 μl/g and 3 μl/g of body weight, respectively. Control animals received vehicle only. The animals were fasted for 12 h–14 h when p.o. administrations were performed.

2.3.2. Hole-board assay

This assay was conducted in a walled black Plexiglass arena with a floor of 60 cm × 60 cm and 30 cm high walls, with four centered and equally spaced holes in the floor, 2 cm in diameter each as previously described (Fernández et al., 2006) and illuminated by an indirect and dimly light. Each animal was placed in the center of the hole-board and allowed to freely explore the apparatus for 5 min and the number of holes explored, the time spent head dipping and the number of rearings were measured.

2.3.3. Locomotor activity test

The spontaneous locomotor activity was measured in a box made of Plexiglass, with a floor of 30 cm by 15 cm and 15 cm high walls as previously described (Fernández et al., 2006) and was expressed as total light beam counts per 5 min.

The locomotor activity and the hole board tests were performed 20 min after the i.p. injection, and 1 h or 4 h after oral gavage administrations of vehicle, hesperidin, hesperetin or diazepam.

2.3.4. The abdominal writhing test

The writhing test was carried out according to the method described by Koster et al. (1959); 0.75% acetic acid aqueous solution was injected i.p. (0.2 ml/30 g body weight) and the animals were placed in a glass observation chamber (15 cm × 15 cm × 30 cm). The number of writhing responses (abdominal cramps) was counted for 20 min after the injection of the acetic acid solution.

The writhing test was performed 30 min after the i.p. injections of hesperidin or hesperetin and 1 h or 4 h after oral administration of hesperidin.

2.3.5. Hot plate test

The sensitivity of animals to painful stimuli was tested in the hot plate test heated to 50 °C ± 0.1 °C. Each mouse was placed into a transparent beaker made of Plexiglas with a height of 18 cm and a diameter of 10 cm to avoid the animals escaping from the plate. The latency to licking and shaking the hind paws or jumping was recorded as the reaction time. A 90 s cut-off time was used to prevent tissue damage to the paws. This test was performed 30 min after the i.p. injection of vehicle, hesperidin or morphine.

2.3.6. Rotarod test

The motor effect of hesperidin was assessed in the rotarod test using a Mouse Rota Rod apparatus (Dunham and Miya, 1957). Each mouse was trained to run on the rotarod at a constant speed (6 rpm) until it could remain there for 120 s without falling. Mice that were unable to remain on the rod for three consecutive trials were discarded. Latency to fall (s) was determined 30 min after the i.p. injection of vehicle or hesperidin. Cut off time was equal to 120 s.

2.3.7. Inverted screen test

The inverted screen test was used to assess the motor toxicity of hesperidin (Coughenour et al., 1977). Mice were placed on a 13 cm × 13 cm wire mesh screen elevated 40 cm above the ground. After slowly inverting the screen through an angle of 180° the mice were tested for their ability to climb to the top. Mice were trained for two consecutive trials and were i.p. injected with vehicle or hesperidin 30 min

before the assay. Mice not climbing to the top (all four paws on upper surface) were counted as failures.

2.3.8. Blockade experiments

The involvement of various brain receptors on hesperidin antinociceptive effects in mice were investigated by measuring its blockade with different specific antagonists.

Several doses of each antagonist were tested to find the maximal dose devoid of intrinsic action to be used in the blockade experiments.

To assess the participation of the opioid system, mice were pretreated with vehicle, naltrexone (5 mg/kg, i.p., a nonselective opioid receptor antagonist), naltrindole (3 mg/kg, i.p., a potent and highly selective δ opioid receptor antagonist) or nor-binaltorphimine (10 mg/kg, s.c., a potent and highly selective κ opioid receptor antagonist), and after 20 min or 24 h (for nor-binaltorphimine), the animals received an injection of hesperidin (1 mg/kg, i.p.) or vehicle. The writhing test and the hot plate assay were performed 30 min later.

To examine the possible participation of the benzodiazepine binding sites of the gamma amino butyric acid type A receptors ($GABA_A$), serotonin receptors, the adrenoceptors and adenosine receptors on the antinociceptive effect of hesperidin, mice were pretreated with flumazenil (10 mg/kg, i.p., a selective benzodiazepine binding site antagonist), ketanserin (0.5 mg/kg, i.p., a selective antagonist for the 5-$HT_{2A/C}$ receptor), prazosin (0.1 mg/kg, i.p., an α_1-adrenoceptor antagonist), yohimbine (3 mg/kg, i.p., an α_2-adrenoceptor antagonist) or caffeine (30 mg/kg, i.p., a non selective adenosine antagonist). After 5 min, the animals received an injection of hesperidin (1 mg/kg) or vehicle and mice were evaluated on the writhing test 30 min later.

2.4. [^{3}H]DAMGO binding assay

A crude membrane fraction was prepared from male Wistar rat forebrains according to the method of Ioja et al. (2007) with small modifications. Rats (from the Central Animal House of the School of Pharmacy and Biochemistry, University of Buenos Aires) were humanely killed by decapitation and the brains without cerebellum were rapidly removed and washed several times in ice-cold 50 mM Tris–HCl buffer pH 7.4. The forebrains were homogenized in 10 volumes of 0.32 M sucrose at 0 °C and the homogenate was centrifuged at 900×*g* for 10 min at 4 °C. Supernatant was decanted and centrifuged at 100,000×*g* for 30 min at 4 °C, and the resulting pellet was resuspended in 50 mM Tris–HCl buffer pH 7.4 and incubated for 30 min at 37 °C to remove any endogenous opioids. Centrifugation was repeated and the final pellet was resuspended in 50 mM Tris–HCl buffer pH 7.4. The membranes were stored at −20 °C until used.

For the binding assay, membranes were thawed and suspended in 50 mM Tris–HCl pH 7.4 to a final protein concentration of 0.25–0.35 mg/ml. The incubation was carried out at 25 °C for 1 h in a final volume of 1 ml of membrane suspension (in duplicate) in the presence of the sample assayed and with 1 nM of [^{3}H]DAMGO (56.8 Ci/mmol). Non specific binding was determined in parallel incubations in the presence of 10 μM naltrexone. The assays were terminated by filtration under vacuum through Whatman GF/A glass-fiber filters and three washes with 3 ml each of incubation medium. Filters were counted after addition of Optiphase "Hisafe" 3 (Wallac, Turku, Finland) liquid scintillation cocktail.

2.5. Electrophysiological studies

cDNA for rat μ-opioid receptor subcloned into pCDNA1 was kindly provided by Dr. Charles E. Spivak (National Institute on Drug Abuse, Baltimore, Maryland, USA). Human GIRK1 and GIRK2 DNA were purchased from ORIGENE (MD, USA). Rat μ-opioid receptor cDNA was linearized using HpaI, human GIRK1 plasmid with XbaI and human GIRK2 plasmid was linearized with SacI and SmaI, and the DNA extracted via QIAquick gel extraction kit (QIAGEN Pty Ltd, Australia). mRNAs were transcribed *in vitro* using T7 mMessage mMachine™ transcription kit (Ambion Inc., Austin, TX, USA).

Female *Xenopus laevis* frogs were anesthetized with tricaine (850 mg/500 ml) and several ovarian lobes were surgically removed by a small incision on the abdomen. The lobes were cut into small pieces, rinsed thoroughly with oocyte releasing buffer 2 (OR2: 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES (hemi-Na)) and digested with collagenase A (2 mg/ml in OR2; Boehringer Manheim, Germany) at room temperature for 2 h. The oocytes were further washed with OR2 and stored in ND96 wash solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES (hemi-Na)) supplemented with 2.5 mM sodium pyruvate and 0.5 mM theopylline until ready for injection. Stage V–VI oocytes were selected and injected (Nanoliter 2000, World Precision Instrument, Inc.) with 50.6 nl of mRNA in a 1:1:10 ratio of GIRK1:GIRK2: μ-opioid receptor. The injected oocytes were maintained at 18 °C in ND96 wash solution supplemented with 2.5 mM sodium pyruvate, 0.5 mM theopylline and 50 μg/ml gentamycin, for 3–5 days before use in electrophysiological studies.

Whole-cell currents were recorded using two-electrode voltage clamp setup incorporating a Digidata 1200, Geneclamp 500B amplifier and pClamp 8 for Windows (Axon instruments Inc., Foster City, CA), together with a Powerlab/200 (AD Instruments, Sydney, Australia) and Chart version 3.5 program for a PC. The recording microelectrodes were filled with 3 M KCl and had resistance between 0.2 and 1 mΩ. The oocytes were placed in a 100 μl chamber, impaled and the membrane potential was clamped at −60 mV. While recording, oocytes were initially superfused with ND96 wash solution until it achieved a stable base current and then was switched to a high-potassium solution (90 mM) containing 90 mM KCl, 8 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES (hemi-Na). Six minutes of washing intervals were performed between doses with high K^+ solution.

Naloxone and DAMGO solutions were prepared in distilled water. Stock solutions of hesperidin and hesperetin were prepared in DMSO and stored at −20 °C. These compounds were further diluted with ND96 or 90 mM K^+ solution prior to recording, and the final concentrations of DMSO were less than 0.8%. This concentration of DMSO produces no effect on μ-opioid receptor or GIRK1/2 channels.

2.6. Statistical analyses

The effects of the compounds in mice were analyzed by one-way analysis of variance (ANOVA) and post-hoc comparisons between treatments and vehicle were made using Dunnett multiple comparison test. The blockade experiments were analyzed by one-way analysis of variance (ANOVA) and post-hoc comparisons between individual treatments were made using Newman–Keuls multiple comparison test. Data from the rotarod and the inverted screen tests were analyzed with the Chi-square test. Significance levels were set at $P<0.05$.

For the competition binding, data were analyzed by nonlinear regression fit to one site of specific bound vs radioligand concentration. Ki values were calculated using the Cheng–Prusoff/Chou equation: $Ki = IC_{50}/[1+(L/Kd)]$, where Ki refers to the inhibition constant of the unlabeled ligand, IC_{50} is the concentration of unlabeled ligand required to reach half-maximal binding, Kd refers to the equilibrium dissociation constant of the radioactive ligand and L refers to the concentration of radioactive ligand.

For the electrophysiological analysis, the dose response curves (variable slope model) were obtained using the equation below:

$$Percentage\,response = 100 / (1 + 10\text{^}((\log IC50 - \log[drug]) * HillSlope))$$

where IC_{50} represents the concentration of drug that inhibits the half-maximal response. When two groups were compared in the same

oocyte, paired *t*-test was used and when the groups were from different oocytes, unpaired *t*-test was applied. A P value <0.05 was considered statistically significant. All data were expressed as mean ± S.E.M. and analyzed with GraphPad Prism 5.00 software.

3. Results

3.1. Behavioral effects of hesperidin and hesperetin in the holeboard and the locomotor activity tests

The effects of hesperidin and hesperetin in the hole board and locomotor activity tests are shown in Table 1.

For hesperidin i.p. injected at 2 mg/kg, 4 mg/kg and 30 mg/kg, ANOVA indicated a significant effect on the number of mouse rearings [$F(3,28)=13.28$, $P<0.0001$], the number of holes explored [$F(3,28)=8.092$, $P=0.0005$], the time spent head dipping [$F(3,28)=4.191$, $P=0.0143$] and the locomotor activity counts [$F(3,30)=11.78$, $P<0.0001$]. Comparisons between the vehicle control group and experimental groups (Dunnett's procedure) indicated that hesperidin significantly decreased the number of head dips, the time spent head dipping, the number of rearings and the locomotion, as expected (Marder et al., 2003). Considering that hesperidin exhibited a depressant action by the i.p. route, its effects were then evaluated when it was orally administered. Hesperidin (p.o.) did not produce a significant effect in mice, in these assays and at the doses tested, 1 h or 4 h after its administration, proving to be ineffective by this route.

Hesperetin (i.p.) failed to show any depressant effect, at the doses tested. Diazepam, used as a reference compound, evidenced its sedative effects, as indicated by a decreased in the number of holes explored, the time spent head dipping and the locomotor activity counts ($P<0.0001$, $P=0.0012$, $P=0.0008$; unpaired *t*-test, respectively).

3.2. Effects of hesperidin and hesperetin in the acetic acid-induced nociception and hot plate test

The results of the abdominal writhing test are shown in Fig. 1. Hesperidin exhibited an antinociceptive action against the mouse abdominal constrictions induced by 0.75% acetic acid by the i.p. route (Fig. 1A) [$F(4,27)=34.52$, $P<0.0001$]. The comparison between the vehicle control group and experimental groups by the Dunnett's test indicated that hesperidin at 0.6 mg/kg and 1 mg/kg significantly decreased the number of writhes ($P<0.001$) with antinociceptive efficacies (%) of 53.9 ± 10.2 and 90.2 ± 5.4, respectively. Hesperetin (i.p.) did not induce antinociceptive effects (Fig. 1B), and was no further evaluated *in vivo*.

Hesperidin did not cause antinociception 1 h or 4 h after its oral administration (Fig. 1C). Thus, i.p. administrations of this compound, 30 min before the experiments, were selected for all further *in vivo* studies.

ANOVA indicated a significant effect in the reaction time measured in the hot plate test after the i.p. injection of hesperidin (Fig. 2) [$F(3,27)=4.981$, $P=0.0070$]. Mice receiving hesperidin 10 mg/kg and 30 mg/kg had significantly longer hot plate latencies than control mice ($P<0.01$, $P<0.05$, respectively, Dunnett's procedure).

3.3. Effect of hesperidin in motor coordination tests in mice

Hesperidin (1 mg/kg, 10 mg/kg and 30 mg/kg) did not alter the motor coordination performance of mice in the inverted screen (Chi-Square = 0.556; $P=0.7573$) and rotarod tests (no mice fell the rod), doses at which it is active in the abdominal constrictions and the hot plate tests.

3.4. Analysis of the possible mechanism of the antinociceptive action of hesperidin

The results are summarized in Table 2 and Fig. 3. As shown in the Table 2, pretreatment of the animals with flumazenil (10 mg/kg, i.p., a selective benzodiazepine binding site antagonist), ketanserin (0.5 mg/kg, i.p., a selective antagonist for the 5-$HT_{2A/C}$ receptor), prazosin (0.1 mg/kg, i.p., an α_1-adrenoceptor antagonist), yohimbine (3 mg/kg, i.p., an α_2-adrenoceptor antagonist) or caffeine (30 mg/kg, i.p., a non selective adenosine antagonist) given 5 min before the injection of hesperidin (1 mg/kg, i.p.) failed in to revert the analgesic effect of the flavonoid when analyzed in the mouse abdominal constrictions induced by 0.75% acetic acid.

In order to confirm the participation of the opioid system, mice were pretreated with naltrexone (a nonselective opioid receptor antagonist),

Table 1
Effect of hesperidin and hesperetin on the locomotor activity and in the hole-board test in mice.

Treatment	Hole-board (mean ± S.E.M.)			Locomotor activity (mean ± S.E.M.)
	Number of rearings	Number of head dips	Time head dipping (s)	Counts
VEH (i.p.)	41.6 ± 5.2	13.6 ± 1.1	9.1 ± 0.7	720.1 ± 40.8
HN (2 mg/kg, i.p.)	20.0 ± 5.3[b]	8.1 ± 1.5[b]	6.3 ± 1.4[a]	528.9 ± 43.9[a]
HN (4 mg/kg, i.p.)	22.4 ± 4.3[b]	7.6 ± 1.1[b]	5.6 ± 0.4[a]	453.4 ± 71.7[b]
HN (30 mg/kg, i.p.)	7.2 ± 1.7[c]	6.4 ± 0.9[c]	5.4 ± 0.8[a]	238.8 ± 29.0[c]
VEH (p.o., 1 h)	31.0 ± 4.1	10.4 ± 1.2	8.1 ± 1.6	614.5 ± 29.1
HN (60 mg/kg, p.o., 1 h)	33.4 ± 6.2	10.3 ± 1.4	6.6 ± 1.2	669.0 ± 30.2
HN (300 mg/kg, p.o., 1 h)	31.1 ± 4.1	8.1 ± 1.5	6.4 ± 1.7	562.6 ± 31.7
VEH (p.o., 4 h)	23.4 ± 4.9	7.9 ± 1.2	5.6 ± 0.8	551.5 ± 19.2
HN (60 mg/kg, p.o., 4 h)	29.0 ± 5.8	7.7 ± 1.2	6.5 ± 1.3	575.4 ± 18.5
HN (300 mg/kg, p.o., 4 h)	15.8 ± 4.3	6.2 ± 1.1	6.4 ± 1.5	605.7 ± 37.2
VEH (i.p.)	31.0 ± 3.8	14.6 ± 1.9	10.1 ± 1.3	704.5 ± 30.8
HT (15 mg/kg, i.p.)	28.6 ± 4.3	19.4 ± 2.6	14.5 ± 2.7	731.0 ± 41.9
HT (30 mg/kg, i.p.)	35.4 ± 3.5	16.1 ± 2.0	12.2 ± 3.1	668.3 ± 30.6
VEH (i.p.)	34.5 ± 6.4	13.2 ± 1.4	8.1 ± 1.2	648.4 ± 37.3
DZ (10 mg/kg, i.p.)	42.3 ± 4.9	4.4 ± 1.1[d]	2.7 ± 0.8[e]	423.4 ± 44.2[f]

Performance of mice on the locomotor activity and in the hole board test 20 min after the i.p. injection, and 1 h or 4 h after oral gavage administrations of vehicle (VEH), hesperidin (HN), hesperetin (HT) or diazepam (DZ).

[a] $P<0.05$.
[b] $P<0.01$.
[c] $P<0.001$ significantly different from the corresponding vehicle; Dunnett multiple comparison test after ANOVA.
[d] $P<0.0001$.
[e] $P=0.0012$.
[f] $P=0.0008$; significantly different from the corresponding vehicle, unpaired *t*-test.

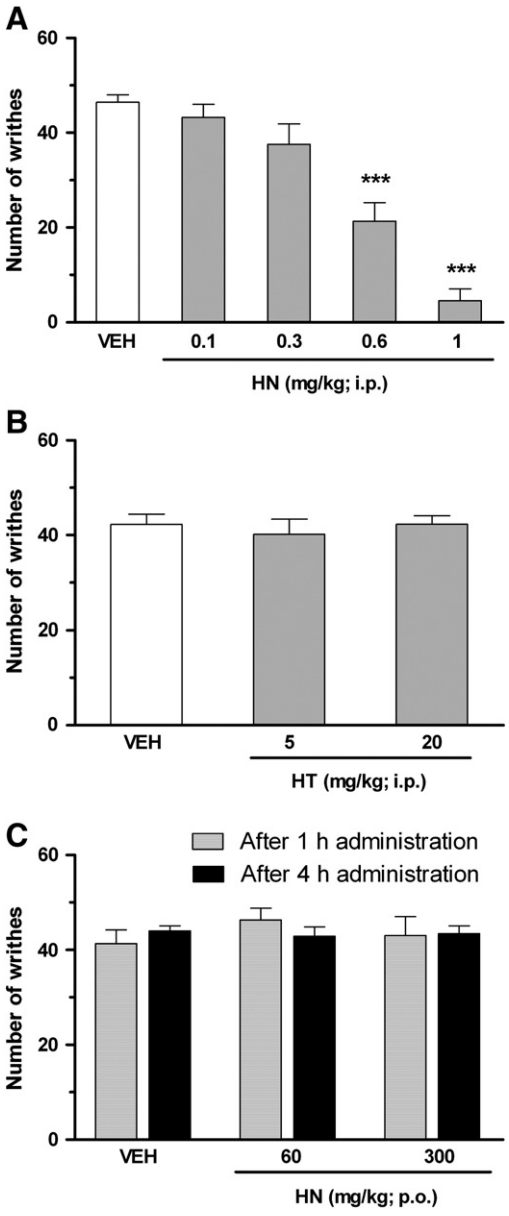

Fig. 1. Effect of hesperidin and hesperetin on the acetic acid induced writhing in mice. Results are expressed as mean ± S.E.M of the number of writhes. The writhing test was performed A) 30 min after the i.p. injection of hesperidin (HN), B) 30 min after the i.p. injection of hesperetin (HT) and C) 1 h or 4 h after oral administration of hesperidin. The individual doses of the compounds tested are indicated in the abscissa. ***$P<0.001$ compared with control group, Dunnett multiple comparison test after ANOVA. Number of mice per group ≥6.

nor-binaltorphimine (a potent and highly selective κ-opioid receptor antagonist) or naltrindole (a potent and highly selective δ opioid receptor antagonist).The pretreament with naltrexone (5 mg/kg, i.p.) given 20 min before the injection of hesperidin (1 mg/kg, i.p.) caused marked inhibition of the analgesic effect induced by the flavonoid in the abdominal writhing test (Table 2) (Loscalzo et al., 2008). In turn, naltrexone completely abolished the antinociceptive action caused by injection of morphine (2 mg/kg, i.p.).

Nor-binaltorphimine (10 mg/kg, s.c.) or naltrindole (3 mg/kg, i.p.), given 24 h and 20 min, respectively, before the administration of hesperidin (1 mg/kg, i.p.) did not antagonize its antinociceptive action in the abdominal constriction test.

The pretreatment with naltrexone (5 mg/kg, i.p.) 20 min beforehand completely reversed the analgesic response of hesperidin (10 mg/kg, i.p.) and morphine (6 mg/kg, i.p.), respectively, measured in the hot plate test (Fig. 3).

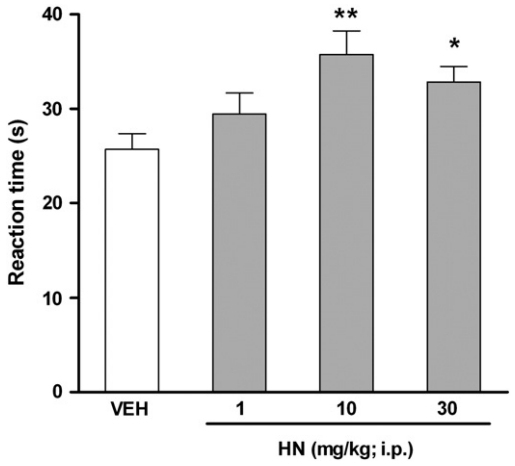

Fig. 2. Dose–response effect of hesperidin in the hot plate test in mice. Mean ± S.E.M. of the reaction time of mice in the hot plate test, 30 min after an i.p. injection of vehicle (VEH) or hesperidin (HN, 1,10, 30 mg/kg). *$P<0.05$, **$P<0.01$ significantly different from vehicle; Dunnett multiple comparison test after ANOVA. Number of mice per group varied between 6 and 9.

3.5. [^{3}H]DAMGO binding assay

The glycosylated flavonoid hesperidin did not displace [^{3}H]DAMGO binding even at concentrations as high as 300 μM (data not shown). On the other hand, its aglycone hesperetin inhibited the specific binding of

Table 2
Effect of different antagonists pretreatments on the antinociceptive effect of HN in mice measured on the acetic acid induced writhing assay.

Pretreatment	Treatment	Time before measure (min)	Number of writhes (mean ± S.E.M)
VEH	VEH	35	40.0 ± 3.1
VEH	HN (1 mg/kg)	35	5.8 ± 2.7[a]
FMZ (10 mg/kg)	VEH	35	35.2 ± 3.2
YOH (3 mg/kg)	VEH	35	41.4 ± 4.4
KET (0.5 mg/kg)	VEH	35	39.2 ± 2.9
PRZ (0.1 mg/kg)	VEH	35	42.5 ± 2.6
CAF (30 mg/kg)	VEH	35	45.6 ± 2.4
FMZ (10 mg/kg)	HN (1 mg/kg)	35	4.7 ± 2.0[a]
YOH (3 mg/kg)	HN (1 mg/kg)	35	11.1 ± 3.5[a]
KET (0.5 mg/kg)	HN (1 mg/kg)	35	2.3 ± 0.9[a]
PRZ (0.1 mg/kg)	HN (1 mg/kg)	35	3.8 ± 1.7[a]
CAF (30 mg/kg)	HN (1 mg/kg)	35	12.0 ± 2.6[a]
VEH	VEH	50	40.8 ± 2.8
VEH	HN (1 mg/kg)	50	5.7 ± 2.8[a]
NLD (3 mg/kg)	VEH	50	42.8 ± 2.3
NLD (3 mg/kg)	HN (1 mg/kg)	50	3.4 ± 2.2[a]
VEH (s.c.)	VEH (i.p.)	1470	46.5 ± 1.9
VEH (s.c.)	HN (i.p.)	1470	4.6 ± 2.5[a]
NBT (10 mg/kg, s.c.)	VEH (i.p.)	1470	46.6 ± 1.3
NBT (10 mg/kg, s.c.)	HN (i.p.)	1470	4.2 ± 2.2[a]
VEH	VEH	50	49.6 ± 1.5
VEH	HN (1 mg/kg)	50	3.4 ± 1.3[a]
VEH	MOR (2 mg/kg)	50	16.0 ± 1.9[a]
NTX (5 mg/kg)	VEH	50	51.6 ± 2.9
NTX (5 mg/kg)	HN (1 mg/kg)	50	25.6 ± 2.8[a,b]
NTX (5 mg/kg)	MOR (2 mg/kg)	50	51.8 ± 4.0[c]

Results are expressed as mean ± S.E.M. of the number of writhes. Mice were pretreated with vehicle (VEH), flumazenil (FMZ), yohimbine (YOH), ketanserin (KET), prazosin (PRZ), caffeine (CAF), naltrindole (NLD), nor-binaltorphimine (NBT) or naltrexone (NTX). The writhing test was performed 30 min after the i.p. injection of hesperidin (HN), morphine (MOR) or vehicle (VEH). Number of animals per group ranged between 6 and 10. The symbols denote significance levels.

[a] $P<0.001$, significantly different from VEH–VEH.

[b] $P<0.001$ significantly different from VEH–HN.

[c] $P<0.001$ significantly different from VEH–MOR (one-way ANOVA followed by Newman–Keuls test).

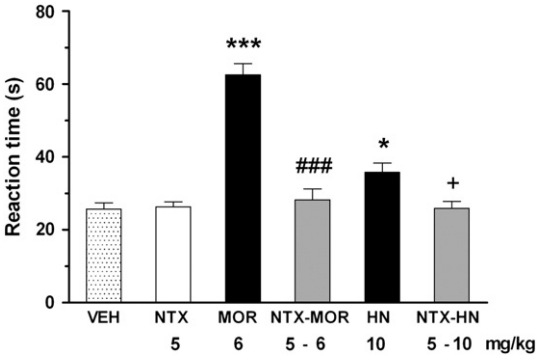

Fig. 3. Effect of naltrexone pretreatment on the antinociceptive effect of hesperidin in mice on the hot plate test. Results are expressed as mean ± S.E.M. of the reaction time (s). Mice were pretreated with vehicle (VEH) or naltrexone (NTX) 20 min before the i.p. injection of hesperidin (HN), morphine (MOR) or vehicle (VEH). The hot plate test was performed at 50 ± 0.1 °C, 30 min after the last injection. The doses of the compounds are indicated in the abscissa. Number of animals per group ranged between 6 and 9. The symbols denote significance levels: $^{***}P<0.001$, $^{*}P<0.05$, significantly different from VEH–VEH; $^{+}P<0.001$ significantly different from VEH–HN; $^{\#\#\#}P<0.001$ significantly different from VEH–MOR (one-way ANOVA followed by Newman–Keuls test).

[^{3}H]DAMGO with a Ki value of 39.04 ± 1.32 μM (n = 4) (Fig. 4). Naltrexone, used as a control, gave a Ki value of 0.21 ± 0.01 nM (n = 3).

3.6. Electrophysiological studies

3.6.1. Effects of hesperidin and hesperetin in oocytes expressing μ-opioid receptor and GIRK1/2 channels

The application of 90 mM K^+ solution produce a small inward-rectifying K^+ current (83.2 ± 6.4 nA, n = 11) in uninjected oocytes, indicating the presence of intrinsic channels sensitive to K^+. Hesperetin, at concentrations ranging from 1 to 1000 μM, reduced this K^+ current in a concentration-dependent manner showing an inhibitory effect on intrinsic oocytes channels sensitive to K^+, with a maximum percentage of inhibition of 58 ± 3 at 1000 μM. The magnitude of hesperetin current responses in oocytes expressing GIRK1/2 channels were corrected by subtracting the hesperetin response on intrinsic channels from the currents measured for each concentration. In uninjected oocytes superfused with ND96 solution, hesperetin produced no significant responses.

In oocytes co-injected with GIRK1, GIRK2 and μ-opioid receptor mRNAs, large basal GIRK currents (1419 ± 110 nA; n = 25) were observed upon application of 90 mM K^+. These basal currents were abolished in the presence of 3 mM Ba^{2+}, which blocks the inward-rectifier K^+ (Kir) channel family and not the endogenous K^+ currents.

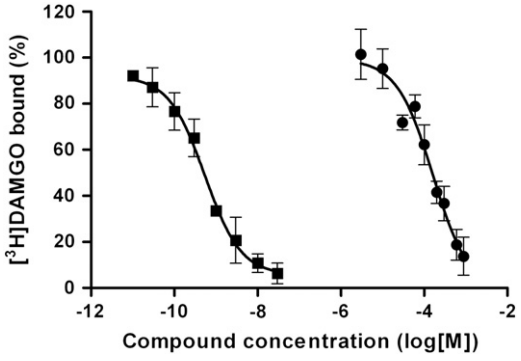

Fig. 4. Representative competition curves of naltrexone (■) and hesperetin (●) for [^{3}H]DAMGO binding to Wistar rat forebrain membranes. Each point represents the mean ± S.E.M., expressed as a percentage of [^{3}H]DAMGO bound, of three independent experiments performed in duplicate.

Thus the 3 mM Ba^{2+}-sensitive current observed in oocytes expressing GIRK1/2 channels were considered to correspond to the magnitudes of GIRK currents (Kobayashi et al., 2004). The functional expression of the opioid receptor was confirmed by the increased K^+ conductance upon application of 1 μM DAMGO, a selective μ-opioid agonist (939 ± 119 nA; n = 23). This response was completely blocked by the opioid antagonist naloxone (10 μM) (Fig. 5A). In oocytes expressing GIRK1/2 channels without the μ-opioid receptor, 1 μM DAMGO produced no significant response.

Extracellular application of the glycosylated flavonoid hesperidin, up to a concentration of 600 μM, induced no significant responses in oocytes co-injected with GIRK1, GIRK2 and μ-opioid receptor mRNAs (data not shown). In contrast, in the presence of high K^+ solution, hesperetin, immediately and reversibly, caused a reduction of the basal currents that occurs through the GIRK1/2 channels. These responses were observed only at high micromolar concentrations of the aglycone and showed a concentration-dependent effect (Fig. 6A and B). To investigate whether this effect was opioid receptor mediated, hesperetin responses were measured in oocytes expressing GIRK1/2 channels with and without the μ-opioid receptor, and there was no significant difference in the magnitude of the responses produced by hesperetin (Fig. 6C).

Moreover, naloxone (10 μM) was not able to block 200 μM hesperetin current responses, confirming that the flavonoid responses were not mediated by the μ-opioid receptor (Fig. 5B). In addition, the current responses of 300 μM hesperetin were partially blocked during the application of 100 μM Ba^{2+} ($P<0.05$; Fig. 7).

4. Discussion

The present study extended our previous data and corroborated that hesperidin, administered by the i.p. route in mice, exhibited depressant

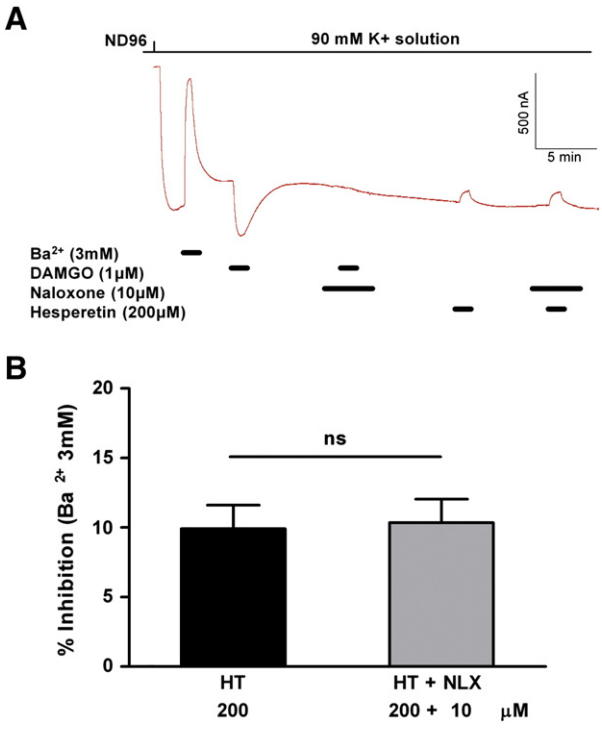

Fig. 5. Effect of naloxone on hesperetin responses in *Xenopus* oocytes expressing μ-opioid receptor and GIRK1/2 channels. (A) Representative current traces (nA vs min) of 1 μM DAMGO (reference compound), 10 μM naloxone and 200 μM hesperetin. Bars show the duration of application. (B) Percentage inhibition (mean ± S.E.M., n = 5 oocytes) of 3 mM Ba^{2+}-sensitive current components. Current responses were measured at a membrane potential of −60 mV in a high-potassium solution containing 90 mM K^+. HT = hesperetin, NLX = naloxone; ns = not significantly different; paired *t*-test.

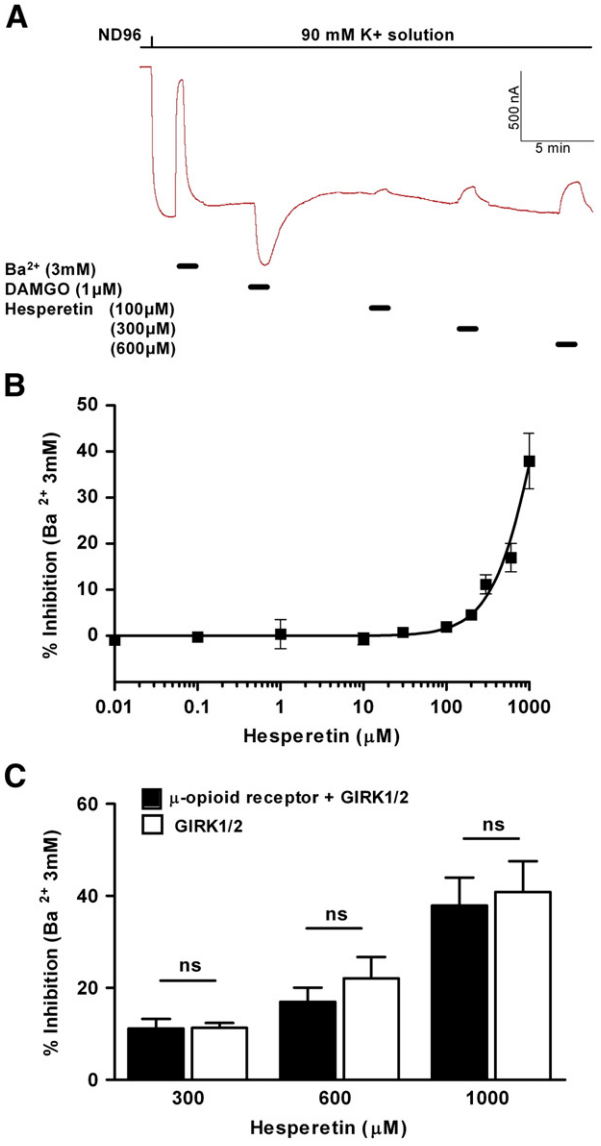

Fig. 6. Effect of hesperetin on GIRK1/2 currents in *Xenopus* oocytes expressing GIRK1/2 channels with μ-opioid receptor. (A) Representative current traces (nA vs min) of 1 μM DAMGO (reference compound) and 100, 300 and 600 μM hesperetin. Bars show the duration of application. (B) Concentration–response curve of the inhibition of GIRK1/2 currents by hesperetin. (C) Comparative responses of 300, 600 and 1000 μM hesperetin in *Xenopus* oocytes expressing GIRK1/2 channels with or without μ-opioid receptor. The magnitude of hesperetin currents were expressed as the percentage inhibition of the 3 mM Ba^{2+}-sensitive current components. Current responses were measured at a membrane potential of −60 mV in a high-potassium solution containing 90 mM K^+. Data points represent mean ± S.E.M. (n = 3–7 oocytes). ns = not significantly different; unpaired *t*-test.

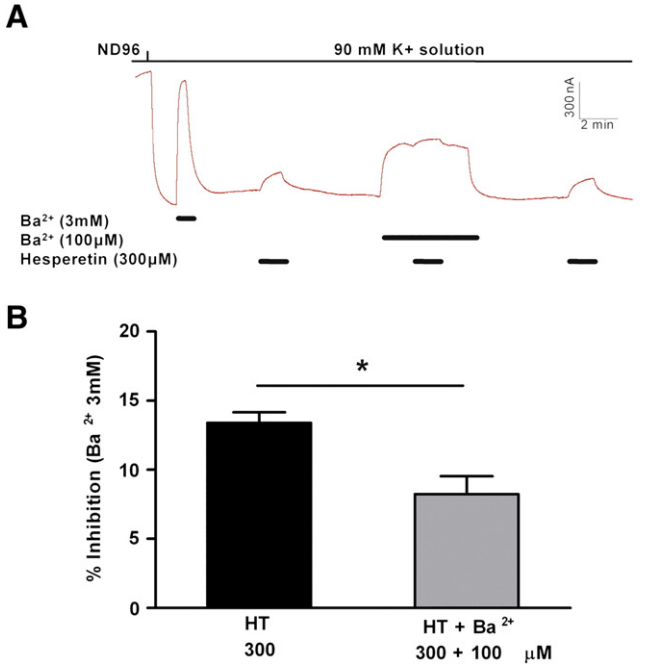

Fig. 7. Blocking effect of Ba^{2+} on hesperetin current responses in *Xenopus* oocytes expressing GIRK1/2 channels. (A) Representative current traces (nA vs min) of 300 μM hesperetin and 300 μM hesperetin during the application of 100 μM Ba^{2+}. Bars show the duration of application. (B) Percentage inhibition (mean ± S.E.M., n = 5 oocytes) of 3 mM Ba^{2+}-sensitive current components. Current responses were measured at a membrane potential of −60 mV in a high-potassium solution containing 90 mM K^+. HT = hesperetin; $^*P<0.05$, significantly different; paired *t*-test.

action on the locomotor and exploratory activities, and exerted antinociceptive effects. Here we have found that hesperidin tested 1 h and 4 h after its administration by the oral route produced no effects in the hole board, locomotor activity and writhing tests. Matsumoto and co-workers (2004) thoroughly studied the conjugated metabolites derived from the oral administration of hesperidin in rats. They demonstrated that hesperidin metabolized to hesperetin–glucuronides, primarily comprised of hesperetin-7-*O*-β-D-glucuronide and hesperetin-3′-*O*-β-D-glucuronide. Furthermore, not only hesperetin conjugates but also homoeriodictyol conjugates were observed in rat plasma. To understand the precise mechanism showing the biological activities of the i.p. injection of hesperidin, it might be important to identify the really active chemical entity. One plausible explanation is that the rutinose moiety is important for hesperidin's activity. In addition, the i.p. administration of the aglicone hesperetin failed to show any effects in mice.

The use of different models is significant in the detection of antinociceptive properties in a substance, considering that the use of a variety of stimuli recognizes different types of pain and reveals the actual nature of the antinociceptive test drug (Bergerot et al., 2006).

Acetic acid induced writhing reaction in mice, a model for inflammatory pain, has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new agents. The acetic acid acts indirectly by inducing the release of endogenous mediators, stimulating the nociceptive neurons which are sensitive to nonsteroidal anti inflammatory drugs and opioids. The hot plate test is used to assess acute pain sensitivity to a thermal stimulus. Hesperidin, administered i.p., evidenced antinociceptive effects in both tests.

Motor coordination is a complex behavioral domain, and can reflect balance, muscle strength, and patterned gait, as well as sensory competence. Difficulties in motor performance can confound behavioral assays of learning and memory, exploration, motivation and nociception (Rustay et al., 2003). Hesperidin, i.p. administered, did not produced motor incoordination at the doses it reduced the exploration and the locomotor activity, and produced antinociceptive effects in mice, suggesting that its activities were not due simply to sedation and may have a motivational component (Rustay et al., 2003; Stone et al., 1999).

Recently, Martínez and co workers (2011) gave evidence that systemic administration of hesperidin produces antinociceptive effect in the pain-induced functional impairment model (PIFIR model) in the rat, a well known test validated to search for new or alternative treatments for arthritic pain, that uses diluted uric acid injected into the knee joint of the rat right hind limb to render a good representation of clinical gout arthritis. Although the active doses of hesperidin i.p. administered were as high as 1666.72 mg/kg, no motor dysfunction was observed. It was also found that the transient receptor potential vanilloid 1 (TRPV1) receptor was partially involved in this particular action and that hesperidin produced a significant antinociceptive response at 30 mg/kg

and 100 mg/kg i.p. in mice on the capsaicin-induced nociception assay. Unfortunately, no studies on the mechanism of action were performed on the capsaicin-induced nociception test in mice.

Concerning the mechanism of the sedative action of hesperidin, all the reported data up to now strongly suggest that the behavioral effect induced by this compound does not involve classical $GABA_A$ receptors, at least not directly (Fernández et al., 2005, 2006). Also, the 5-HT_2 receptor and the α_1-adrenoceptor seem unlikely to be involved in the behavioral effect of hesperidin, since ketanserin and prazosin were ineffective to antagonize hesperidin sedative action at the doses tested (Loscalzo et al., 2008).

The sensitivity of hesperidin to specific serotonin, adenosine, adrenergic and benzodiazepine binding site of the $GABA_A$ receptor antagonists was examined using the acetic acid induced abdominal constrictions. These pathways of neurotransmissions might not mediate hesperidin's antinociceptive effects as any of the selective antagonists used failed to blockade the action of hesperidin.

The selective κ and δ opioid receptor antagonists, nor-binaltorphimine and naltrindole, were ineffective in blocking hesperidin antinociception in the abdominal constriction test. Only naltrexone, a nonselective opioid receptor antagonist, was able to partially reverse the antinociceptive effect of hesperidin, confirming our previous reported data (Loscalzo et al., 2008). In addition, pretreatment of mice with naltrexone was also effective in totally reversing the reaction time elicited by hesperidin in the hot plate test, further reinforcing the notion that its antinociceptive effect is mediated by an opioid mechanism of action, most probably by the μ opioid receptor.

In the rat cerebral cortex the majority of the opioid binding sites are μ (56%) and δ opioid receptors (40%), with a very small population of κ receptors (4%) (Thomasy et al., 2007). In the present study, the capacities of hesperidin and its aglycone hesperetin to inhibit the specific binding of [^{3}H]DAMGO to μ-opioid receptor were evaluated. Hesperidin was unable to modify [^{3}H]DAMGO binding to rat forebrain membranes, showing that this flavonoid has no affinity for the μ-opioid receptor. In contrast, the aglycone hesperetin displaced the [^{3}H]DAMGO binding with low-affinity, suggesting that the sugar moiety could impede the binding capacity of hesperidin.

Several flavonoids have already been evaluated for opioid receptor activity using the [^{35}S]GTP-γ-S binding assay and CHO cell membrane homogenates expressing the human opioid receptors (Katavic et al., 2007). These authors reported that some flavonoids exerted opioid antagonist activity *in vitro*, most of them working as κ-antagonists.

In order to determine if hesperidin and hesperetin produce any effect on μ-opioid receptor, *X. laevis* oocytes co-expressing heteromultimeric GIRK1/2 channels with rat μ-opioid receptor were used. Hesperidin induced no significant responses, showing that this compound cannot directly activate μ-opioid receptor or produce any change on inward GIRK1/2 currents. In contrast, application of hesperetin, since 100 μM, immediately and reversibly inhibited the basal inward rectifying GIRK1/2 currents in a concentration-dependent manner. This effect was independent from the co-expressed μ-opioid receptor since naloxone, a non-selective opioid antagonist, was an ineffective blocker. Moreover, when hesperetin was applied to oocytes expressing GIRK1/2 channels without μ-opioid receptor, GIRK currents were inhibited in a similar way. Furthermore, the hesperetin responses were reduced during the application of Ba^{2+}, a Kir channel pore blocker. All these results indicate that hesperetin, at high micromolar concentrations, inhibits inward currents through the expressed GIRK1/2 channels in *Xenopus* oocytes. However, this effect seems not to be specific for GIRK1/2 channels because hesperetin was able to also reduce the endogenous inward-rectifying K^+ currents. These endogenous inward currents have been described in native oocytes of some *X. laevis* donors (Bauer et al., 1996) and could comprise an assembly of GIRK5, an endogenous isoform cloned from *Xenopus* oocytes (Salvador et al., 2001). Interestingly, hesperetin also inhibits cardiac hERG (human ether-á-go-go-related gene) K^+ channels heterologously expressed in *Xenopus* oocytes (Scholz et al., 2007). Thus hesperetin affects various K^+ channels but additional studies are needed to clarify the inhibitory mechanism of hesperetin on GIRK channels.

Taken together, the results confirm and extend our previous studies, indicating that hesperidin presents antinociceptive effects in two pharmacological pain models and it was effective when administered by the i.p. pathway. Although hesperidin's sedative and antinociceptive activities in mice were demonstrated to be blocked by the non-selective opioid antagonist naltrexone, this compound failed to directly bind to and activate μ-opioid receptor *in vitro*. Moreover, as nor-binaltorphimine, a potent and highly selective κ opioid receptor antagonist; and naltrindole, a selective δ opioid receptor antagonist; were ineffective in reversing hesperidin antinociceptive effects, the participation of these opioid receptors could be discarded.

The results indicate that hesperetin binds to μ-opioid receptor and inhibits inward currents through the expressed GIRK1/2 channels in *Xenopus* oocytes. Due to the high effective concentrations of hesperetin, the influence of this compound on μ-opioid receptor or GIRK1/2 channels functions seems unlikely to occur *in vivo*.

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