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Hesperidin, a flavonoid glycoside with sedative effect, decreases brain pERK1/2 levels in mice

M. Cecilia Martínez [a,1], Sebastian P. Fernandez [b,2], Leonardo M. Loscalzo [b,2], Cristina Wasowski [b,2], Alejandro C. Paladini [b,2], Mariel Marder [b,2], Jorge H. Medina [a,1], Haydée Viola [a,c,3,*]

[a] Instituto de Biología Celular y Neurociencias, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 3 °Piso, C1121ABG, Argentina
[b] Instituto de Química y Fisicoquímica Biológicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 6 °Piso, C1113AAD, Argentina
[c] Departamento de Fisiología, Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Intendente Güiraldes 2160, 2 °piso, Pabellón II, Ciudad Universitaria, C1428EGA, Argentina

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ABSTRACT

The aim of this work was to evaluate if the intraperitoneal administration of the natural compound hesperidin, in a sedative dose, and neo-hesperidin, a hesperidin structural analog that exerts minor sedative effect, were able to induce changes in intracellular signaling cascades in different areas of the brain.
The systemic administration of hesperidin produced a marked reduction in the phosphorylation state of extracellular signal-regulated kinases 1/2 (ERK 1/2), but not of Ca^{+2}/calmodulin-dependent protein kinase II α subunit (αCaMKII), in the cerebral cortex, cerebellum and hippocampus. In contrast, neo-hesperidin did not markedly affect the activity of ERK 1/2 in both the cortex and the cerebellum.
Taken together, these results demonstrated that intracellular signalling involving a selective decrease in ERK1/2 activation accompanied the depressant action of hesperidin. Even more, the low sedative action of neo-hesperidin correlates with a negligible decrease in phosphorylation state of ERK 1/2 (pERK 1/2), suggesting that low levels of pERK 1/2 in CNS could be a marker of sedative efficacy of flavonoids.

1. Introduction

Many sedative drugs which are massively consumed in Europe contain extracts from *Valeriana officinalis* (Reynolds, 1996). Despite its wide acceptance in theraupetics, the compounds responsible of *V. officinalis* actions have remained unknown for ages. In the last 25 years, the combination of in vitro and in vivo assays, allowed a better comprehension of the active principles and their effects. However, in many cases there was a lack of correlation between activity in vitro and the observed clinical effect. The presence of volatile oil components and valepotriates has been reported in several Valeriana species, among other constituents such as lignans, alkaloids and amino acids (Houghton, 1999). We have already shown that two compounds of flavonoid nature isolated from *V. officinalis* and *Valeriana wallichii* had central actions. These compounds were identified as hesperidin and 6-methylapigenin and exhibited sedative actions when administered intraperitoneally i.p. in mice (Marder et al., 2003; Wasowski et al., 2002). The apigenin derivative exhibits affinity for the benzodiazepine binding site of the gamma-aminobutyric acid type-A ($GABA_A$) receptor and has anxiolytic effects at doses of 1 mg/kg (Viola et al., 1995). This pharmacological profile is compatible with other natural aglycone flavonoids isolated from plants used in folkloric medicine (Medina et al., 1998; Viola et al., 1994; Viola et al., 1995; Viola et al., 1997; Wolfman et al., 1994). In contrast, hesperidin has sedative and sleep-enhancing properties in a dose-dependent manner from doses starting at 2 mg/kg, but it is not a ligand of the benzodiazepine binding site (Fernandez et al., 2005). Despite hesperidin has a pharmacological profile of a sedative and sleep-inducing compound, its effect is not dependent on binding to receptors typically associated to depressant actions as glutamatergic (AMPA), serotonin ($5\text{-}HT_{1A}$ y $5\text{-}HT_2$) or adenosine (type I) receptors (Marder et al., 2003). However, the participation of the opioid receptors in the depressant activities of hesperidin was recently demonstrated (Loscalzo et al., 2008).

Many flavonoids interact in vitro with protein kinases involved in different signaling pathways such as protein kinase C, tyrosine kinases (Abou-Shoer et al., 1993; Agullo et al., 1997), serine/threonine kinases (Hagiwara et al., 1988) and mitogen-activated protein kinase (MAPK) (Khan et al., 2006; Schroeter et al., 2002) among others. Particularly, much recent interest has been concentrated on their interactions with ERK1/2 (Chung et al., 2001; Neuhaus et al., 2004; Sah et al., 2004; Spencer et al., 2003).

* Corresponding author. Instituto de Biología Celular y Neurociencias, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 3 °Piso, CP 1121, Argentina. Tel.: +54 11 5950 9500x2210; fax: +54 11 5950 9626.
E-mail address: hviola@fmed.uba.ar (H. Viola).
[1] Tel.: +54 11 5950 9500x2210; fax: +54 11 5950 9626.
[2] Tel.: +54 11 4964 8200.
[3] Tel.: +54 11 4576 3300.

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Therefore, the objective of this work is to evaluate if the i.p. administration of the natural compound hesperidin in a sedative dose is able to induce changes in intracellular signaling cascades in different areas of the brain. With the aim to study the correlation between sedation and kinases activity in the CNS, we also compare the actions of hesperidin with its structural analog neo-hesperidin which possesses minor sedative effects.

2. Materials and methods

2.1. Subjects

Adult male Swiss mice (weight 25–30 g) were used for behavioral studies and biochemical experiments. Animals were housed in a controlled environment with free access to food and water, maintained in a 12:12 h day/night cycle.

Housing, handling and experimental procedures complied with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publications No. 80-23, revised 1996). All the experiments were performed between 9 a.m. and 2 p.m.

2.2. Drug and injection procedures

Drugs used in all the experiments were: hesperidin (purity >98% by HPLC), isolated by us from *V. wallichii* (Marder et al., 2003) and neohesperidin (purity >98% by HPLC), isolated from small oranges aborted from the tree *Citrus hamlin*, kindly provided by Dr. Guillermo Ellenrieder of the Universidad Nacional de Salta, Argentina. They were dissolved by the sequential addition of dimethylsulfoxide, up to a final concentration of 10%; ethanol, up to a final concentration of 10%, and saline solution to complete the volume.

Drugs were injected i.p. (10 mg/kg) in a volume of 0.15 ml/30 g of body weight, 30 min before performing the behavioral tests in mice. In each session, a control group receiving vehicle only was tested in parallel with those animals receiving drug treatment.

2.3. Behavioral studies

2.3.1. Locomotor activity assay

Locomotor activity was measured in a box by optical means (Opto-Varimex apparatus) for a 5-min interval according to Viola et al. (1995). A reduction in the number of transitions through the light beams in relation to the control group reflects a decrease in locomotor activity, which is considered an index of sedative behavior.

2.3.2. Holeboard test

Exploratory activity was measured according to Fernandez et al. (2005). Briefly, the number of head dips and the time spent head-dipping as well as the number of rearings were registered for 5 min. A decrease in these parameters reveals a sedative behavior (File and Pellow, 1985).

2.3.3. Sodium thiopental-induced sleeping time assay

A subhypnotic dose of sodium thiopental (35 mg/kg) was i.p. injected to mice 20 min after vehicle or drug injections. Sleeping time was determined as the interval between the loss and the recovery of righting reflex (Viola et al., 1997). There was a ceiling time limit of 30 min.

2.4. Biochemical assays

To perform these assays mice received an i.p. injection of vehicle or drugs (10 mg/kg dose). Thirty minutes after drug or vehicle administration mice were sacrificed by decapitation and, immediately after, their cerebral cortex, cerebellum and hippocampus were dissected out and stored at −70 °C until further processing.

Brain structures were mechanically homogenized in cold buffer solution (20 mM Tris–HCl, pH 7.4, 0.32 M saccharose, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, 15 μg/ml leupeptin, 50 mM sodium fluoride, 1 mM sodium orthovanadate) and samples were stored at −70 °C.

2.4.1. Western blot analysis

Samples were subjected to SDS–PAGE (10% polyacrilamide in the presence of SDS 10%, 20 μg per lane) and western blot analysis was carried out as described by Cammarota et al. (2000).

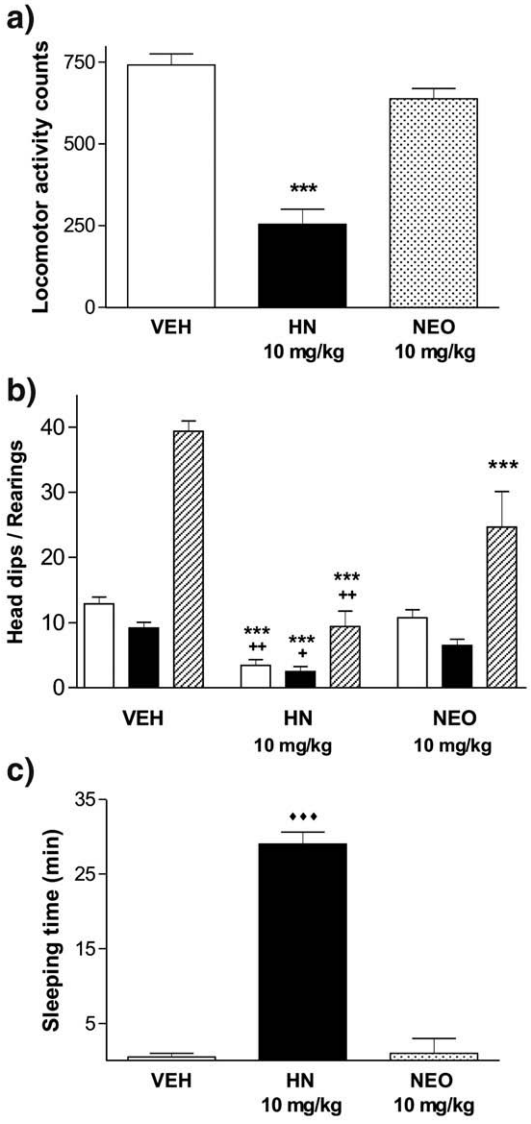

Fig. 1. Effects of hesperidin and neo-hesperidin on sedative and hypnotic behavior. Vehicle (VEH), hesperidin (HN) or neo-hesperidin (NEO) were injected i.p. 30 min before test (n=8–12). a) Mean±S.E.M. of the locomotor activity counts registered during a 5-min test session in an Opto-Varimex apparatus. ***$p<0.001$ vs. VEH and NEO, Newman–Keuls test after one-way ANOVA ($P<0.0001$; $F(2,23)=44.42$). b) Mean±S.E.M. of the parameters registered during a 5-min session in the holeboard test. One way ANOVA detected significant differences between groups for the number of head dips (open bars: $P<0.0001$; $F(2,31)=14.74$), time spent head-dipping (closed bars: $P=0{,}0003$; $F(2,31)=10.42$) and the number of rearings (hatched bars: $P<0.0001$; $F(2,33)=27.09$). ***$p<0.001$ vs. VEH; $^{++}p<0.01$, $^{+}p<0.05$ vs. NEO, Newman–Keuls test after one-way ANOVA. c) Median (interquartile range) of sleeping time in mice. Sleeping time was measured as the time spent between disappearance and reappearance of the righting reflex. Drugs were administered 20 min before a sub threshold dose of sodium thiopental. ♦♦♦$p<0.001$ vs. VEH and NEO; Dunns' multiple comparison test after Kruskal–Wallis test ($P<0.0001$; Kruskal–Wallis statistic ($2,\alpha=0.05$)=27.71).

Membranes were incubated with the following antibodies: anti pERK 1/2 (1:4000, pThr202/pTyr204, Cell Signaling Technologies, USA) and anti pαCaMKII (1:2000, pThr286, Santa Cruz Biotechnology, USA). For total protein levels of ERK 1/2 s and αCaMKII, membranes were incubated for 120 min in stripping buffer (8 mM Tris–HCl, 7 mM SDS, 0.5 M NaCl, 100 mM urea, pH 4.5), washed and incubated with anti ERK 1/2 (1:3000; Cell Signaling Technologies, USA) or anti αCaMKII (1:1000, Santa Cruz Biotechnology, USA). Densitometric analysis of the films was performed by using Gel-pro Analyzer (Media Cybernetics, USA). Western blots were developed for linearity within the range used for densitometry. pERK/ERK and pαCaMKII/αCaMKII ratios was calculated as follows: first it was obtained a value of pERK or pαCaMKII for each experimental animal relative to the media of control group. After stripping the membranes, ERK or αCaMKII levels were obtained and a relative value with respect to the media of control group, was calculated for each animal. Finally, using these values the ratio was calculated as pERK/ERK or pαCaMKII/αCaMKII.

2.5. Data analysis

Newman–Keuls multiple comparison test after repeated measures one-way analysis of variance (ANOVA) was applied for the statistical analysis of behavioral and biochemical assays data. Non-paired Student's *t* test was used when two independent groups were compared. Dunns' multiple comparison test after Kruskal–Wallis test was applied for the statistical analysis of behavioral non-parametrical data.

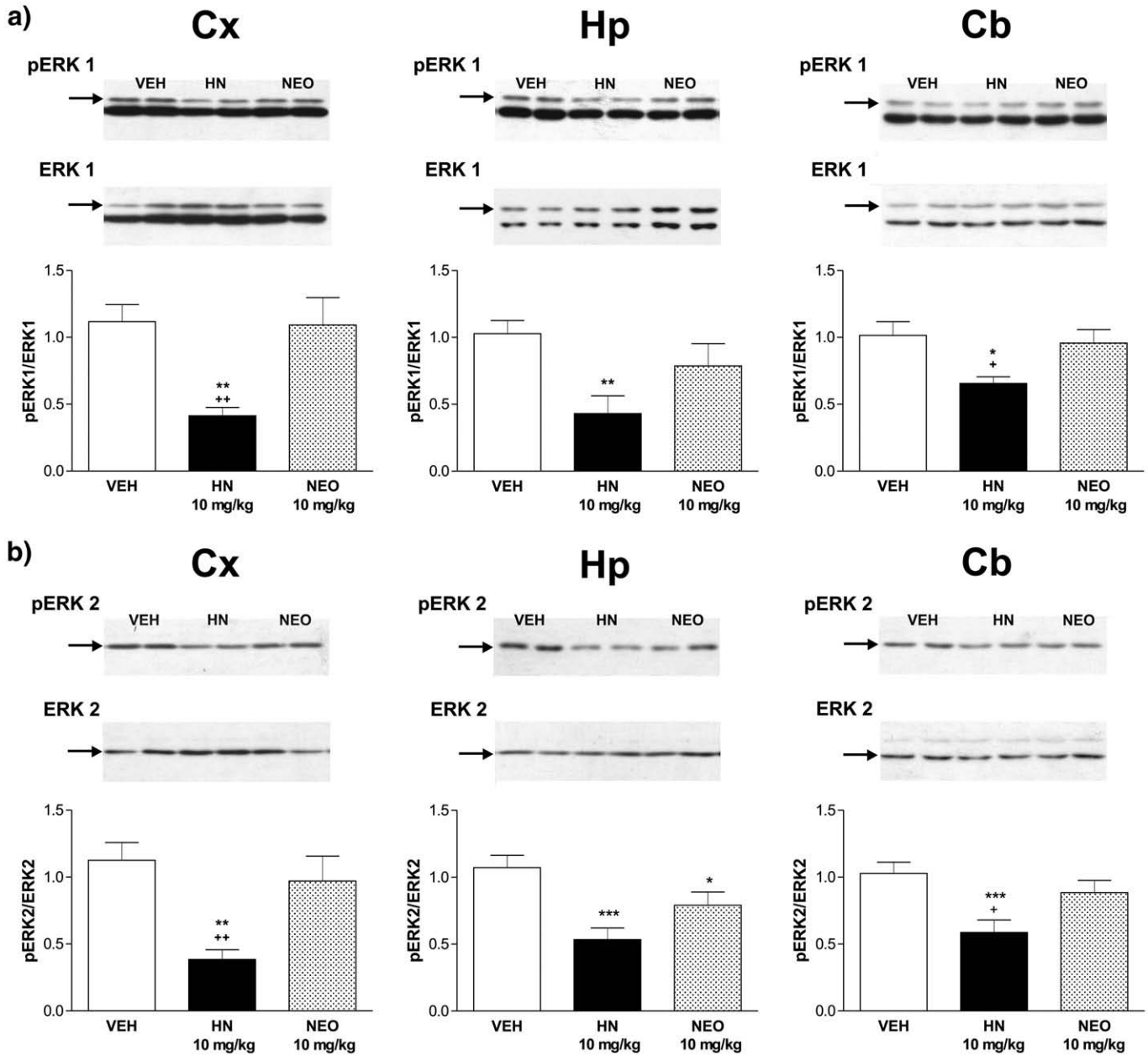

Fig. 2. Effect of hesperidin and neo-hesperidin on ERK 1/2 phosphorylation state. a) Representative western blots and densitometric quantification of pERK1/ERK1. One-way ANOVA detected significant differences between groups in cerebral cortex (Cx: $P=0.0028$; $F(2,35)=7.004$), hippocampus (Hp: $P=0.0093$; $F(2,33)=5.409$) and cerebellum (Cb: $P=0.0120$; $F(2,38)=4.977$). b) Representative western blots and densitometric quantification of pERK2/ERK2. One-way ANOVA detected significant differences between groups in cerebral cortex (Cx: $P=0.0021$; $F(2,35)=7.363$), hippocampus (Hp: $P=0.0012$; $F(2,32)=8.390$) and cerebellum (Cb: $P=0.0024$; $F(2,40)=7.029$). Animals were treated with vehicle (VEH), hesperidin (HN) or neo-hesperidin (NEO) (10 mg/kg, i.p). Values are expressed as mean ± SEM ($n=10$–15). $^{***}p<0,001$, $^{**}p<0,01$, $^{*}p<0,05$ vs. VEH; $^{++}p<0,01$, $^{+}p<0,05$ vs. NEO, Newman–Keuls test after one-way ANOVA.

3. Results

3.1. Sedative and sleep-enhancing effects of hesperidin compared to those of neo-hesperidin

A clear-cut depressant action of hesperidin was found in three different behavioral tests. As shown in Fig. 1a, an i.p. administration of hesperidin (10 mg/kg), induced a significant reduction in the ambulatory locomotor activity ($p<0.001$) whereas neo-hesperidin at the same dose was ineffective ($p>0.05$).

The effect of hesperidin on exploratory activity was studied in the holeboard test, which constitutes a sensitive method to evaluate sedative behavior in rodents. Hesperidin significantly reduced the number of rearings and head-dips, as well as the time spent head-dipping (Fig. 1b; $p<0.001$ vs. VEH, $p<0.01$–0.05 vs. NEO). By contrast, neo-hesperidin had mild effects on exploratory activity detected only in the number of rearings ($p<0.001$ vs. VEH).

Hesperidin also had marked effects on the loss of righting reflex induced by a sub threshold dose of sodium thiopental. Fig. 1c shows that the systemic administration of hesperidin, but not of neo-hesperidin, promoted the loss of righting reflex for a time period of approximately 30 min ($p<0.001$).

3.2. Effect of hesperidin on ERK 1/2 phosphorylation state

Considering that many flavonoids exert inhibitory effects on ERKs activity in in vitro assays, we next determined the phosphorylation state of ERK 1/2 in homogenates from three different brain regions, obtained 30 min after an i.p. administration of 10 mg/kg of hesperidin or neo-hesperidin.

Western blot assays revealed a decrease in pERK1/ERK1 ratio in cerebral cortex, hippocampal and cerebellar homogenates from mice injected with hesperidin (Fig. 2a, $p<0.05$–0.01 vs. VEH). In contrast, in mice receiving a dose of 10 mg/kg of neo-hesperidin there was no significant change in pERK1/ERK1 ratio compared to control group (Fig. 2a $p>0.05$). Similarly, the ratio pERK2/ERK2 decreased in the three brain regions in mice injected with hesperidin (Fig. 2b, $p<0.01$–0.001 vs. VEH) while there was only a significant decrease in pERK2/ERK2 ratio the hippocampus of mice treated with neo-hesperidin (Fig. 2b, $p<0.05$).

To examine whether these effects were a result of a global reduction in the phosphorylated state of several kinases by the central actions of flavonoids, we compared the effects of hesperidin and neo-hesperidin on the phosphorylation state of αCaMKII in cerebral cortex and hippocampus homogenates. In contrast to the observed reduction in pERKs/ERKs ratios, neither hesperidin nor neo-hesperidin altered pαCaMKII/αCaMKII ratio in these structures (Fig. 3, $p>0.05$).

4. Discussion

The general aim of this work was to study the behavioral and molecular effects of the systemic administration of hesperidin and to compare them with its structural analog neo-hesperidin.

We found that hesperidin reduced the exploratory activity as well as the exploratory parameters in the holeboard test, evidencing the sedative effects as previously described (Fernandez et al., 2005; Fernandez et al., 2006; Marder et al., 2003). In contrast, animals treated with neo-hesperidin showed the same exploratory activity as the control group and a small reduction of the number of rearings in the holeboard test. In addition, animals that received hesperidin prior to a sub threshold dose of sodium thiopental lost the righting reflex for approximately 30 min, while neo-hesperidin did not show significant effects compared to the control group. These results confirm and extend previous findings on the sedative and sleep-inducing actions of hesperidin. We can conclude that hesperidin has a higher biological efficacy than its structural analog neo-hesperidin.

We have previously studied the pharmacological properties of a series of flavonoid glycosides but the mechanisms involved in their CNS depressant action are not fully understood. All the reported data strongly suggest that the behavioral effects induced by these compounds do not involve classical $GABA_A$ receptors, at least not directly (Fernandez et al., 2005; Fernandez et al., 2006). We have also explored the participation of other neurotransmitter receptors including opioid, serotonin (5-hydroxytryptamine type 2, 5-HT_2) and α_1-adrenoceptors, on hesperidin sedative and antinociceptive actions and we provided the first pharmacological evidence about the involvement of opioid receptors in the sedative and antinociceptive effects of hesperidin (Loscalzo et al., 2008).

Therefore, we decided to search for other molecular substrates which could be related to the sedative–hypnotic actions of hesperidin, studying proteins involved in intracellular signaling. Results obtained in western blot assays of ERK 1/2 show a marked reduction of the phosphorylation state levels in animals treated with hesperidin in three brain regions analyzed (cerebral cortex, hippocampus and cerebellum). In contrast, we observed a mild reduction in phosphorylation state of ERK2 only in hippocampus of mice treated with neo-hesperidin, associated with its weak sedative effect. Thus, our results suggest a correlation between sedative action of flavonoids and its

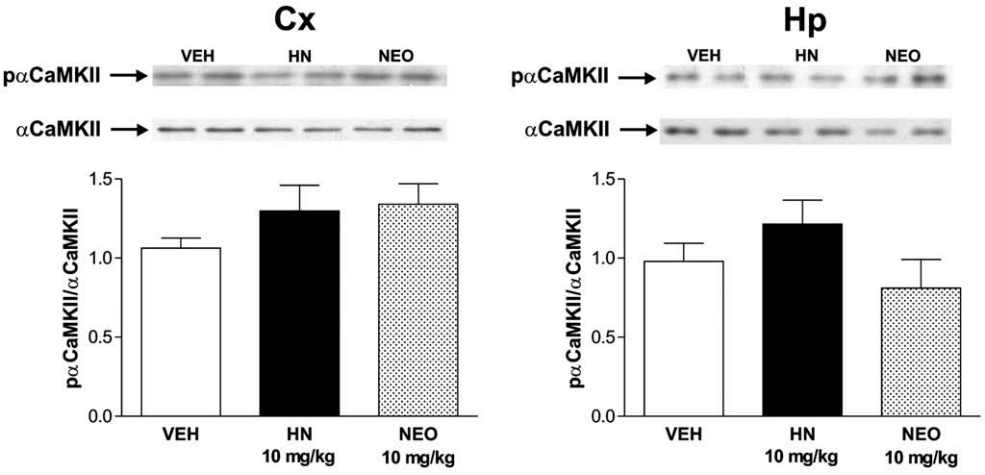

Fig. 3. Neither hesperidin or neo-hesperidin alter αCaMKII phosphorylation state. Representative western blots and densitometric quantification of pαCaMKII/αCaMKII. One-way ANOVA did not detect significant differences between groups in cerebral cortex (Cx: $P=0.1686$; $F(2,32)=1.883$) nor in hippocampus (Hp: $P=0.2014$; $F(2,33)=1.683$) Animals were treated with vehicle (VEH), hesperidin (HN) or neo-hesperidin (NEO) (10 mg/kg, i.p). Values are expressed as mean ± SEM ($n=10$–12).

capacity to decrease ERK activity in CNS. In that sense, the increase in the level of ERK1/2 phosphorylation induced by hesperetin (the aglycone of hesperidin and neo-hesperidin) in cortical neuron cultures (Rainey-Smith et al., 2008; Vauzour et al., 2007), is in accordance with the absence of sedative–hypnotic action when it was injected in mice (Fernandez et al., 2006).

It is important to point out that ERK 1/2 is a target of mitogen-activated protein kinase/ERK kinase (MEK), and is active when phosphorylated on tyrosine 202 and threonine 204 which are specifically detected by our immunoblot assay (Sweatt, 2001; Thomas and Huganir, 2004). The effect of hesperidin was specific on ERK 1/2 kinases and did not affect the phosphorylation state of threonine 286 of αCaMKII, which is also related with its activity (Chang et al., 2001).

Our findings on the decrease of ERK phosphorylation state are in agreement with previous experimental evidences related to the reduction in the activity of several kinases induced by other flavonoids (Schroeter et al., 2002). Remarkably, many results from different groups suggest that several flavonoids interact selectively with MAPK signaling pathway. For example, quercetin can downregulate the expression of intercellular cell adhesion molecule-1 (ICAM-1) by inhibition of c-jun N-terminal kinase (JNK) activity (Kobuchi et al., 1999). In addition, it has been shown that incubation with quercetin and its metabolites can also induce a reduction of the phosphorylation of AKT/PKB and ERK in cortical neurons (Spencer et al., 2003). Besides, the green tea polyphenols epigallocatechin-3-gallate (EGCG) and theaflavin-3,3'-digallate (TFdiG) decreased the phosphorylation state of ERK 1/2 and MEK in two different cell lines, and it was proposed that EGCG interacts directly with a region rich in proline residues in MEK, disrupting its association with Raf-1 (Chung et al., 2001). Moreover, EGCG inhibited epidermal growth factor receptor (EGFR) in vitro, resulting in a very selective decrease in ERK 1/2 phosphorylation state and activity of these kinases which are downstream in the signaling pathway (Sah et al., 2004).

In accordance to our results, some evidences connect CNS actions of drugs with the activity of ERKs. Recent reports indicate that the levels of pERK increased significantly during anxiety. Thus, ERK signal transduction pathway might play an important role in anxiety and its inhibition could produce anxiolytic effects (Ailing et al., 2008). This fact is in agreement with the observed anxiolytic activity after acute administration of EGCG in mice (Vignes et al., 2006); which is related with its ability to selective decrease ERK 1/2 phosphorylation state (Chung et al., 2001; Sah et al., 2004). Furthermore, intraperitoneal injection of sedative doses of ethanol or flurazepam, decreased pERKs levels in mouse cerebral cortex in vivo (Kalluri and Ticku, 2002a). Potentiation of GABA receptor activity following acute ethanol administration may have a negative regulatory role on the MAP kinase pathway (Kalluri and Ticku, 2002b). Taken together with our results, the possibility exists that inactivation of ERKs may be considered a final common cellular step involved in sedative actions of different drugs.

In sum, our results show a sedative action of hesperidin isolated from *V. wallichii*, associated with a decrease in pERK 1/2 kinases in different brain regions. It has been reported that extracts of Valerian with sedative actions had affinity for serotonin receptor 5-HT_{5A} (Dietz et al., 2005). 5-HT_{5A} receptor is associated to G_i protein, which inhibits adenylate cyclase. Therefore, this signalling pathway could be coupled to ERK 1/2 by means of Raf and cAMP as described by Morozov et al. (2003). Further research is needed to evaluate whether hesperidin interacts with 5-HT_{5A}, leading to a possible decrease in pERK1/2 levels.

Regarding hesperidin interactions with neurotransmitter synaptic receptors, it has been recently found that the sedative actions of hesperidin can be partially blocked by the administration of opioid receptor antagonists (Loscalzo et al., 2008). These results cannot be related straightforward to our present findings, as the opioid receptors are linked to the activation of MAPKs in presence of their agonists (Gutstein et al., 1997). However, it was observed a decrease in ERK activation in limbic areas after acute systemic morphine administration. One possible explanation is that μ opioids receptors induce the activation of Akt pathway that inhibits ERK signaling (Eitan et al., 2003). Moreover, chronic morphine also diminished pERK staining in some brain regions including the cerebral cortex (Schulz and Hollt, 1998). It is plausible that higher doses of this drug have direct or indirect inhibitory effects on the ERK pathway that correlate with their sedative behavioral properties (Valjent et al., 2004). In conclusion, depending on the dose and duration of the treatment and the brain region studied, opioid agonists exert activating or inactivating actions on ERK 1/2.

Our results indicate that the effect of systemic administration of hesperidin in mice is specific for ERK 1/2, provided that no changes were found in phosphorylation levels of αCaMKII. Regarding the behavioral and biochemical effects of hesperidin in relation to neo-hesperidin, there was a clear difference in the efficacy of both flavonoids. Hesperidin exerted inhibitory actions in behavioral and biochemical parameters as well, while neo-hesperidin had mild effects noticeable only in some of the parameters measured. In agreement, a recent study showed the differential effects of these flavanones on the reduction of the gluthation peroxidase activity, which was suppressed in cells pretreated with hesperidin but not with neo-hesperidin (Hwang and Yen, 2008). Hesperidin and neo-hesperidin share the same flavan ring system but they differ in the bond between rhamnose and glucose. Thus, linkage to sugar confers to hesperidin and neo-hesperidin different biological properties probably due to a differential permeability, metabolism and/or absorption of the compounds (Serra et al., 2008; Youdim et al., 2003).

In sum, our results demonstrate that the sedative and sleep-inducing effects of the systemic administration of hesperidin are associated with a marked reduction in the phosphorylation state of ERK 1/2 kinases, but not of αCaMKII, in the cerebral cortex, cerebellum and hippocampus. In addition, an analog with less sedative actions did not markedly affect the activity of ERK 1/2 in both the cortex and the cerebellum. Thus, a selective pERKs decrease correlates with the depressant efficacy of these flavonoids, suggesting that a mechanism involving the inactivation of ERKs may account for the sedative and sleep-inducing actions of hesperidin.

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