Reversal of NO-induced nociceptive hypersensitivity by St. John's wort and hypericin: NF-κB, CREB and STAT1 as molecular targets

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ORIGINAL INVESTIGATION

Reversal of NO-induced nociceptive hypersensitivity by St. John's wort and hypericin: NF-κB, CREB and STAT1 as molecular targets

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Abstract
Rationale Hypericum perforatum, popularly called St. John's wort (SJW), is a medicinal plant mainly used as antidepressant with a favorable safety profile than standard antidepressants. Some studies have also documented other SJW bioactivities, including pain modulation.
Objectives The aim of this study was to demonstrate the capability of SJW to relieve nitric oxide (NO)-induced nociceptive hypersensitivity and identify the effective component.
Methods Nociceptive hypersensitivity induced by administration of the NO donors nitroglycerin (GTN) and sodium nitroprusside (SNP) was assessed by cold and hot plate tests. The cellular pathways and molecular targets involved were investigated by Western blotting.
Results GTN and SNP produced a prolonged allodynia and hyperalgesia in mice. A single oral administration of low doses of an SJW dried extract or purified hypericin reversed the NO donor-induced nociceptive behavior whereas hyperforin and flavoinoids were ineffective. Investigating into the cellular pathways involved, an increased CREB and STAT1 phosphorylation, and activation of NF-κB were detected within PAG and thalamus following NO donors' administration. These cellular events were prevented by SJW or hypericin. Since hypericin showed PKC blocking properties, a role of PKC as an upstream modulator of these transcription factors was hypothesized. NO donors increased expression and phosphorylation of protein kinase C (PKC) γ and ε isoforms, molecular events prevented by SJW or hypericin.
Conclusions SJW reversed NO-induced nociceptive hypersensitivity through the blockade of a supraspinal signaling pathway involving a PKC-dependent CREB, STAT1 and NF-κB activation due to presence of hypericin. These data indicate SJW/hypericin as a therapeutic perspective for pain treatment.

Keywords Pain · St. John's wort · Protein kinase C · CREB · STAT1 · NF-κB

Abbreviations

CREB	Cyclic AMP response element binding protein
GTN	Nitroglycerin
i.c.v.	Intracerebroventricularly
i.p.	Intraperitoneally
iNOS	Inducible NO synthase
LPS	Lipopolysaccharide
NO	Nitric oxide
PAG	Periaqueductal grey matter
PKC	Protein kinase C
SJW	St. Jonh's wort
SNP	Sodium nitroprusside
STAT	Signal transducer and activator of transcription

Introduction

Nitric oxide (NO) is a free radical that is synthesized by three isoforms of NOS: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Bredt et al. 1991; Lamas et al. 1992; Nishida et al. 1992). NO produces a variety of biological actions under physiological and pathological conditions (Harrison 1997; Brown and Bal-Price 2003; Tieu et al. 2003). Among them, the involvement of NO in acute and chronic pain has been shown. Many animal studies have shown that NO contributes to the sensitization during inflammatory and neuropathic pain. The administration of a NO precursor or a NO donor decreases pain threshold causing hyperalgesia (Machelska et al. 1998;

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Tassorelli et al. 2003) and potentiates thermal hyperalgesia and tactile allodynia in neuropathic rats (Naik et al. 2006). Inhibition of NO synthesis can considerably reduce both inflammatory and neuropathic pain. NOS inhibitors such as L-NAME and L-NMMA, which inhibit all three NOS isoforms in a nonspecific manner, led to a reduction of the nociceptive behavior in several animal models of inflammatory and neuropathic pain (Luo and Cizkova 2000). More recent experiments with selective NOS inhibitors and in NOS-deficient mice confirmed the importance of NO-producing enzymes in the spinal cord during the development and maintenance of inflammatory and neuropathic pain (Boettger et al. 2007; Guan et al. 2007; Chu et al. 2005; Kuboyama et al. 2011). Furthermore, different studies have demonstrated the implications of the NO in the analgesic effect of several drugs that are indicated for the treatment of neuropathic pain, such as tramadol (Dal et al. 2006) and gabapentin (Mixcoatl-Zecuatl et al. 2006), and also in the antinociceptive effect of anti-inflammatory drugs such as indomethacin (Ventura-Martinez et al. 2004). It has been suggested that the analgesic effect of one of the most popular drugs in the pain treatment, paracetamol (acetaminophen), is partially mediated through the inhibition of NO generation (Björkman et al. 1994). Therefore, blocking the strong pain-sensitizing effects of NO-dependent signaling pathways could be potentially useful for the management of inflammatory and neuropathic pain.

Hypericum perforatum L., commonly called St. John's wort (SJW), is one of the oldest and best investigated medicinal herbs. Depressive disorders are the best-known indication for SJW and preclinical and clinical investigations have showed its efficacy against mild to moderate depression with a favorable incidence of side effects than standard antidepressants (Kasper et al. 2010). In addition to the antidepressant activity, some studies have documented other bioactivities produced by SJW as antibacterial (Saddiqe et al. 2010) and antiviral (Birt et al. 2009) activities. SJW is also involved in pain processing. It is endowed with anti-inflammatory activity following topic (Sosa et al. 2007) and systemic administration (Mattace Raso et al. 2002). Recently, the analgesic activity against acute pain (Galeotti et al. 2010a) and the capability to relieve from neuropathic pain (Galeotti et al. 2010b) were observed after oral administration of SJW.

A large body of evidence indicates that NO essentially contributes to the processing of nociceptive signals in the spinal cord. NO might signal by various mechanisms including cGMP production upon activation of NO-sensitive guanylyl cyclase (NO-GC), *S*-nitrosylation, tyrosine nitration and the interaction with superoxide to form peroxynitrite (Bian et al. 2006), even if there is considerable evidence that NO-GC is the most important NO target during spinal nociceptive processing. Conversely, the role of NO in supraspinal pain modulation and the molecular events involved in the NO-mediated signaling pathway are poorly elucidated. In the present study, we investigated the supraspinal cellular and molecular components involved in the NO-induced pronociceptive activity in an animal model of NO-induced nociceptive behavior obtained by a peripheral administration of NO donors. We also investigated the efficacy of an SJW dried extract to relieve the NO-induced hypernociception. The capability of SJW to counteract the molecular events modulated by NO donors was studied.

Materials and methods

Animals

Male Swiss albino mice (20–22 g) from the Morini (San Polo d'Enza, Italy) breeding farm were used. Ten mice were housed per cage (26×41 cm). The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed a standard laboratory diet and tap water ad libitum and kept at 23±1 °C with a 12-h light/dark cycle, lights on at 7 A.M. The principles of laboratory animal care were followed. All experiments were carried out in accordance with the "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council 2003). All efforts were made to minimize animal suffering, and to reduce the number of animals used.

Drug administration

Animals were randomly assigned to treatment groups. The NO donors nitroglycerin (GNT) (10 mg/kg; Bioindustria L.I.M., Italy), dissolved in 10 % ethylene glycol in saline (0.9 % NaCl), and sodium nitroprusside (SNP) (1 mg/kg; Sigma, Milan Italy), dissolved in saline, were administered intraperitoneally (i.p.), and the behavioral tests or sample collection for Western blotting experiments were performed 1–6 h after i.p. administration.

Hypericum perforatum (SJW) dried extract containing 0.32 % of total hypericins (Indena Research Laboratories, Settala, Milan Italy) hypericin, hyperforin, hyperoside, quercetin and amentoflavone (Sigma) were dissolved in 1 % carboxymethylcellulose (CMC) solution immediately before use and administered by oral gavage. The doses of hypericin (0.01 mg/kg), hyperforin (0.21 mg/kg), quercetin (0.0415 mg/kg), amentoflavone (0.0029 mg/kg), hyperoside (0.3175 mg/kg) correspond to the amount of each component present in a 5-mg/kg preparation of SJW dried, the minimal SJW dose able to counteract NO donors' nociceptive hypersensitivity.

To investigate the SJW supraspinal mechanism of action, hypericin (0.1 μg per mouse) and the PKC blocker calphostin

C (0.2 μg per mouse) (Calbiochem, Milan, Italy), dissolved in 0.5 % DMSO, were administered intracerebroventricularly (i.c.v.), as previously described (Galeotti et al. 2003).

Behavioral studies

SJW and components were administered 150 min after GNT or SNP treatment.

Western blotting experiments

Experiments were conducted on periaqueductal grey matter (PAG) and thalamus of naïve and GTN- and SNP-treated mice. Brain areas were removed 1, 2, 4 and 6 h after NO donors' administration.

For Western blotting experiments, animals were divided in two groups: (1) SJW (5 mg/kg, p.o.), hypericin (0.01 mg/kg, p.o.; 0.1 μg per mouse, i.c.v.) or calphostin C administered 10 min before NO donors injection and protein expression detected 1 or 2 h after GNT/SNP administration; (2) SJW (5 mg/kg, p.o.), hypericin (0.01 mg/kg, p.o.; 0.1 μg per mouse, i.c.v.) or calphostin C administered 3 h after NO donors and protein expression detected 4 or 6 h after GNT/SNP. Doses and administration schedules were chosen on the basis of time-course and dose–response curves performed in our laboratory (Galeotti et al. 2003).

Behavioral testing

Animals were habituated to the experimental room and were investigated by observers blinded for treatment of the animals. Ten animals per group were used.

Cold plate

For assessment of cold allodynia, mice were placed on a cold plate (Ugo Basile, Varese, Italy) maintained at a temperature of 4±0.1 °C. Reaction times (s) were measured with a stopwatch before and 1, 2, 4 and 6 h after administration of the NO donors. The time between placements of a mouse on the plate and licking or lifting of a hind paw was measured with a digital timer. An arbitrary cut-off time of 60 s was adopted.

Hot plate

Mice were placed inside a stainless steel container, which was set thermostatically at 50.0±0.1 °C in a precision waterbath from KW Mechanical Workshop (Siena, Italy). Reaction times (s) were measured with a stopwatch before and 1, 2, 4 and 6 h after administration of the NO donors. The endpoint used was the licking of the fore or hind paws. An arbitrary cut-off time of 60 s was adopted.

Western blotting

Preparation of total cell lysates, membrane and cytosol fractions

Mice were perfused transcardially with 0.9 % NaCl. Brain areas to conduct Western blotting experiments were collected 1, 2, 4 and 6 h after the GTN (10 mg/kgi.p.) or SNP (1 mg/kg i.p.) treatment. Mouse brains were dissected to separate specific areas. Dura mater, PAG and thalamus were homogenized in an homogenization buffer containing 25 mM Tris–HCl pH=7.5, 25 mM NaCl, 5 mM EGTA, 2.5 mM EDTA, 2 mM NaPP, 4 mM PNFF, 1 mM Na_3VO_4, 1 mM PMSF, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 0.1 % SDS. The homogenate was centrifuged at 9,000×*g* for 15 min at 4 °C, the low speed pellet was discarded. The supernatant (whole cell lysate) was centrifugated at 100,000×*g* for 60 min at 4 °C. The resulting supernatant was the cytosol fraction, and the pellet was resuspended in the homogenizing buffer containing 0.2 % (wt/vol) Triton X-100. The homogenate was kept at 4 °C for 60 min with occasional stirring and then centrifugated at 100,000×*g* for 60 min at 4 °C. The resultant supernatant was used as membrane fraction. Protein concentration was quantified using Bradford's method (protein assay kit; Bio-Rad Laboratories, Milan, Italy).

Western blot analysis

Membrane homogenates (10–50 μg) made from PAG and thalamus regions of GTN-, SNP-, LPS-treated and naïve mice were separated on 10 % SDS-PAGE and transferred onto nitrocellulose membranes (90 min at 120 V) using standard procedures. Membrane were blocked in PBST (PBS containing 0.1 % Tween) containing 5 % nonfat dry milk for 120 min. Following washings, blots were incubated overnight at 4 °C with specific antibodies against PKCγ phosphorylated on Thr514 (pPKCγ, 1:1,000 dilution) (Biosource, Camarillo, CA, USA); PKCγ (1:1,000); PKCε (1:800); PKCε phosphorylated on Ser729 (pPKCε, 1:750); IκBα (1:1,000); STAT1 phosphorylated on Tyr701 (pSTAT1, 1:500); β-actin (1:1,000 dilution) (Santa Cruz Biothechnology Inc., Santa Cruz, CA, USA); CREB (1:500) or CREB phosphorylated on Ser133 (pCREB, 1:500) (cell Signalling Technology). After being washed with PBS containing 0.1 % Tween, the nitrocellulose membrane was incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antisera (1:10,000) and left for 1 h at room temperature. Blots were then extensively washed according to the manufacturer's instruction and developed using enhanced chemiluminescence detection system (Pierce, Milan, Italy). Exposition and developing time used was standardized for all the blots. Optical density measurements were performed by dividing the intensity of the

bands by the intensity of the housekeeping protein β-actin, used as loading control, at each time point. Measurements in control samples were assigned a relative value of 100 %.

Statistical analysis

All experimental results are given as the mean ± SEM. Analysis of variance (ANOVA) followed by Tukey's post-hoc test was used for statistical analysis.

Results

SJW prevented nociceptive hypersensitivity induced by NO donors' administration

Peripheral administration of GTN (10 mg/kg, i.p.; Fig. 1a) and SNP (1 mg/kg, i.p.; Fig. 1b) produced a prolonged heat hyperalgesia as revealed by the hot plate test. The licking latency values were reduced 2 and 4 h after NO donors' administration and returned to control values 6 h after GNT. The effect of SJW was, then, evaluated 4 h after NO donors' treatment. Oral administration of a 1 mg/kg SJW dried extract was ineffective whereas at 5 mg/kg completely reversed the heat hyperalgesia induced by GTN (Fig. 1c) and SNP (Fig. 1d). Investigating into the main components responsible for the SJW effect, we observed that the administration of hypericin (hyp; 0.01 mg/kg, p.o.), when administered in a concentration corresponding to the content of hypericin present in a 5 mg/kg preparation of SJW, produced similar results (Fig. 1c,d). Conversely, the administration of hyperforin (hyf; 0.21 mg/kg) and flavonoids (flav) hyperoside, amentoflavone and quercetin, was ineffective (Fig. 1e,f). Flavonoids modified NO donor-induced painful behaviors neither administered alone (data not shown) nor in association (Fig. 1e,f).

Following NO donors' treatment, a cold allodynia was also observed in the cold plate test that peaked 2 and 4 h after GTN (Fig. 2a) and SNP (Fig. 2b) administration. Similar to heat hyperalgesia, cold allodynia induced by GTN (Fig. 1c) and SNP (Fig. 1d) was completely prevented by oral administration of SJW and hypericin. Hyperforin and flavonoids were devoid of any effect (Fig. 2e,f).

Prevention by SJW of activation of the NF-κB pathway

The activation of NF-κB following NO donors' administration was examined by immunoblotting in homogenates of PAG and thalamus after administration of GTN (10 mg/kg, i.p.) and SNP (1 mg/kg, i.p.). NF-κB is constitutively inhibited by IκBα. Upon IκBα phosphorylation and degradation, the p65–p50 dimer translocates to the nucleus, promoting gene transcription. After SNP (Fig. 3a) and GTN (Fig. 3b) administration, we observed a degradation of IκBα in homogenates of PAG, as indicated by the significant decrease of protein levels, that peaked after 2 h. At later time points, the levels of IκBα progressively increased. SJW administration prevented the activation of the NF-κB pathway as indicated by the increased levels of IκBα at any time point. Single oral administration of hypericin (hyp) or i.c.v. injection of the PKC blocker calphostin C (calph) significantly reverted the activation of NF-κB pathway produced by SNP (Fig. 3a) and GTN (data not shown).

A similar pattern of NF-κB activation was observed in the thalamus following SNP (Fig. 3c) and GTN (Fig. 3d) administration with a peak of IκBα down-regulation 2 h after NO donors' treatment. Oral administration of SJW and hypericin, or i.c.v. injection of calphostin C prevented the decrease of the of IκBα content.

SJW prevented the NO donor-induced pSTAT1 up-regulation

A dramatic cerebral increase in the phosphorylated form of STAT1 was detected following SNP treatment. In the thalamus (Fig. 4a) and in the PAG (Fig. 4b), the increase of the pSTAT1 expression was detected from 1 to 4 h after NO donor administration with a peak at 2 h. The pSTAT1 contents returned to control levels 6 h after treatment. The oral administration of an SJW dried extract completely prevented pSTAT1 up-regulation. A further confirmation of the modulation of the STAT1 pathway by SJW arises from experiments conducted in the membrane fraction (Fig. 4c). A similar time-course and intensity of SNP-induced STAT1 phosphorylation, in comparison with the total lysates, was obtained in the membrane fraction, effect that was completely prevented by SJW administration (Fig. 4c). The absence of any variation in the pSTAT1 contents was detected in the cytosolic fraction (Fig. 4d). No difference between the effects produced by GTN and SNP was observed (data not shown).

SJW prevention of CREB activation

A robust increase of the phosphorylated form of CREB (pCREB) was detected in the whole cell lysate from PAG after SNP (Fig. 5a) and GTN (Fig. 5b) administration. The NO donor-induced increase of pCREB was significant 1 h after administration, peaked between 2 and 4 h and then the pCREB levels returned comparable to control values. The oral administration of SJW and hypericin prevented the increase of pCREB expression at any time point. Similar results were obtained from experiments conducted on the total cell lysates from the thalamus following SNP (Fig. 5c) and GTN (Fig. 5d) administration. The levels of pCREB on the membrane fraction from PAG showed the same time-course observed in the whole cell lysate (Fig. 5e). The

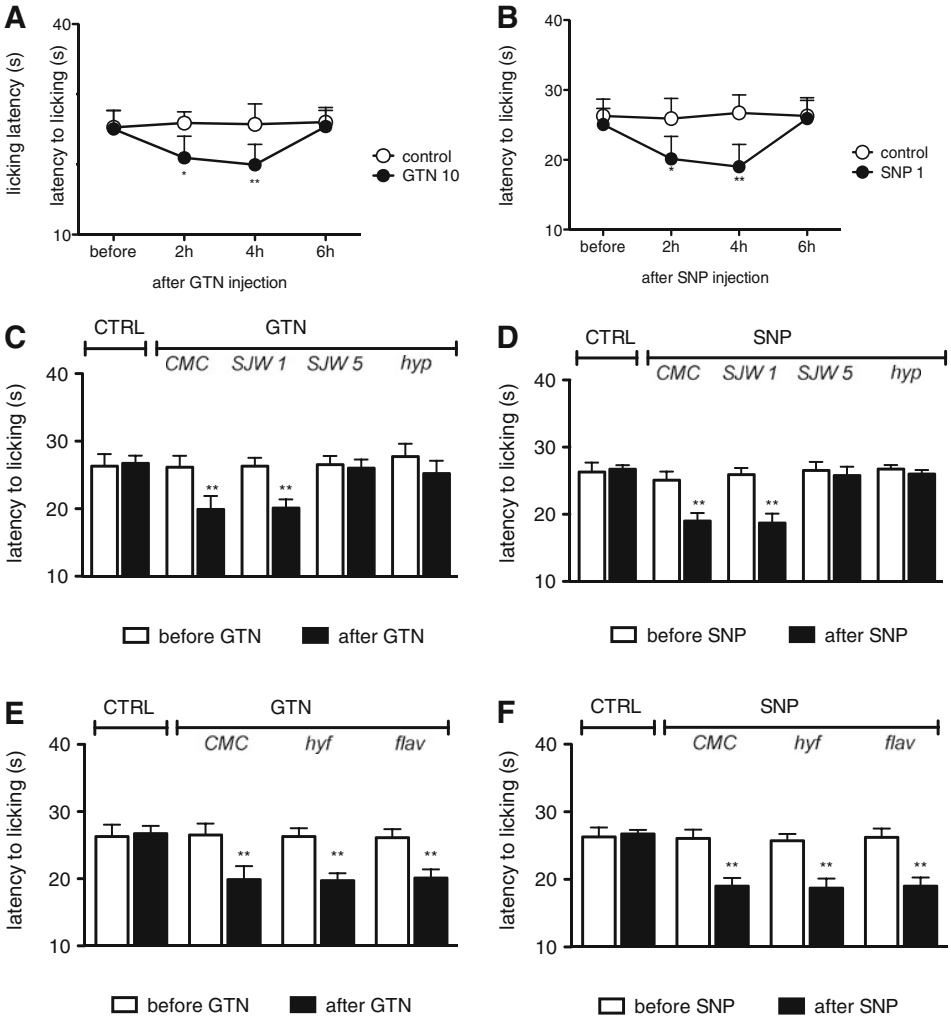


Fig. 1 St. John's wort (*SJW*) prevents the NO donor-induced nociceptive behavior. Systemic administration of the NO donors nitroglycerin (*GTN*) (**a**) or sodium nitroprusside (*SNP*) (**b**) induced heat hyperalgesia in the hot plate test. A single oral administration of SJW and hypericin (*hyp*), a main component of SJW, prevented the pronociceptive activity of GTN (**c**) and SNP (**d**). Oral administration of hyperforin (*hyf*) and flavonoids (*flav*) did not prevent the GTN (**e**) and SNP (**f**) heat hyperalgesia. *$P<0.05$, **$P<0.01$ compared with control group

modulation by SJW of the phosphorylation of CREB was demonstrated by the prevention of the increase of pCREB contents after SJW and hypericin administration (Fig. 5a–e).

SNP produced a drastic decrease of CREB levels in the whole cell lysates from PAG (Fig. 5f). The reduction of CREB expression was evident 1 and 2 h after NO donor administration, then the levels increased and returned to control values. The reduction of CREB expression was modulated by SJW since the oral administration of SJW and its main component hypericin completely reversed the SNP-induced decrease of CREB protein levels. A reduction of CREB expression was also observed in the thalamus, and no difference between the effects produced by GTN and SNP was observed (data not shown).

SJW counteracts the increased expression and phosphorylation of PKCγ and PKCε

PAG and thalamus of mice treated with GTN and SNP were examined for the protein expression and phosphorylation of the main PKC isoforms involved in pain modulation. The protein levels were detected in the whole cell lysates, membrane and cytosolic fractions. SNP (Fig. 6a) and GTN (Fig. 6b) treatment produced a robust and progressive increase of pPKCγ within PAG that peaked 4 h after treatment, returning to control values at 6 h. Oral administration of SJW dried extract and hypericin (hyp) completely prevented the increase of PKCγ phosphorylation. Within the thalamus, a PKCγ activation of similar intensity was detected after SNP (Fig. 6c) and GTN (Fig. 6d) administration. The time-course of the pPKCγ up-regulation was comparable to that observed in the PAG. A complete prevention of pPKCγ expression was obtained after SJW and hypericin administration. To further confirm the supraspinal activation of the PKCγ mediated pathway, a dramatic increase of the phosphorylation of PKCγ was observed in the membrane fraction between 1 and 4 h after NO donor administration, whereas at 6 h the pPKCγ levels returned to control values. Hypericin prevented the PKCγ hyperphosphorylation at any time point

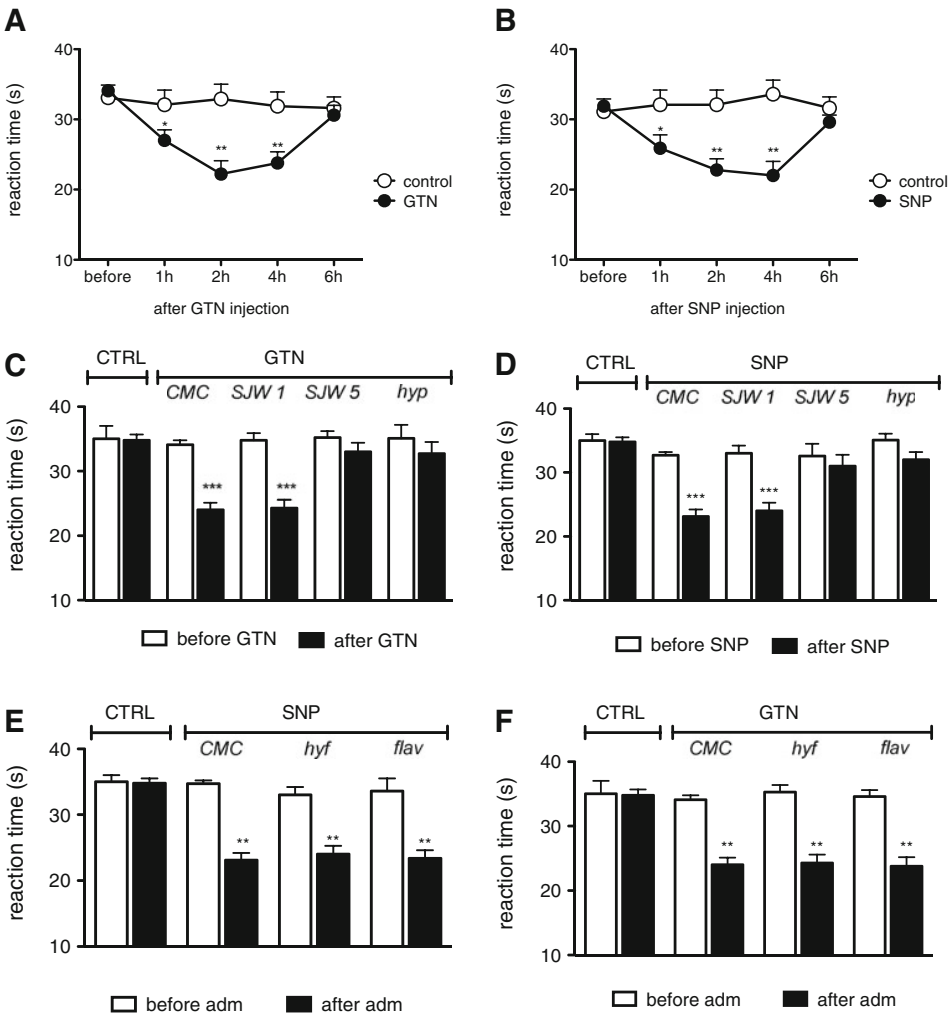


Fig. 2 St. John's wort (*SJW*) prevents the NO donor-induced allodynia. GTN (**a**) and SNP (**b**) induced cold allodynia in the cold plate test. Oral administration of SJW and hypericin prevented the cold allodynia induced by GTN (**c**) and SNP (**d**), whereas hyperforin (hyf and flavonoids (*flav*) were ineffective against GTN (**e**) and SNP (**f**) allodynic effect. **P*< 0.05, ***P*<0.01, ****P*<0.001 compared with the control group

(Fig. 6e). In the cytosolic fraction, the levels of pPKCγ remained unmodified (Fig. 6f). PKCγ protein expression was also increased 2 and 4 h after SNP treatment within PAG (Fig. 6g) and thalamus (Fig. 6h). This increase was prevented by oral administration of SJW and hypericin.

A robust increase of the phosphorylation of PKCε was produced by both SNP (Fig. 7a) and GTN (Fig. 7b) within PAG. Similar to pPKCγ, the pPKCε levels increased between 2 and 4 h and disappeared 6 h after NO donors' administration. A similar PKCε activation was observed within thalamus following SNP (Fig. 7c) and GTN (Fig. 7d) treatment. SJW and hypericin, orally administered, were able to prevent the pPKCε up-regulation at any time point with a similar efficacy in both cerebral areas (Fig. 7a–d). Total PKCε protein contents were also increased 2 and 4 h after NO donor treatment in the PAG (Fig. 7e) and 2 h after treatment in the thalamus (Fig. 7f) with a similar degree of activation. Treatment of animals with SJW and hypericin prevented the PKCε up-regulation.

Hypericin prevented NO-induced nociceptive hypersensitivity through a supraspinal mechanism

The intracerebroventricular administration of hypericin completely reversed the heat hyperalgesia (Fig. 8a) and cold allodynia (Fig. 8b) induced by GTN and SNP. Investigating into the supraspinal cellular events modulated by hypericin, we observed that the i.c.v. administration of hypericin prevented the decrease of IκBα content and counteracted the overexpression of pSTAT1, pCREB, pPKCγ and pPKCε induced by the administration of SNP in the PAG (Fig. 8c) and in the thalamus. The results obtained were of similar intensity to those detected following p.o. administration of SJW and hypericin.

Discussion

We report here the first description of an SJW dried extract to prevent NO-induced nociceptive hypersensitivity after a

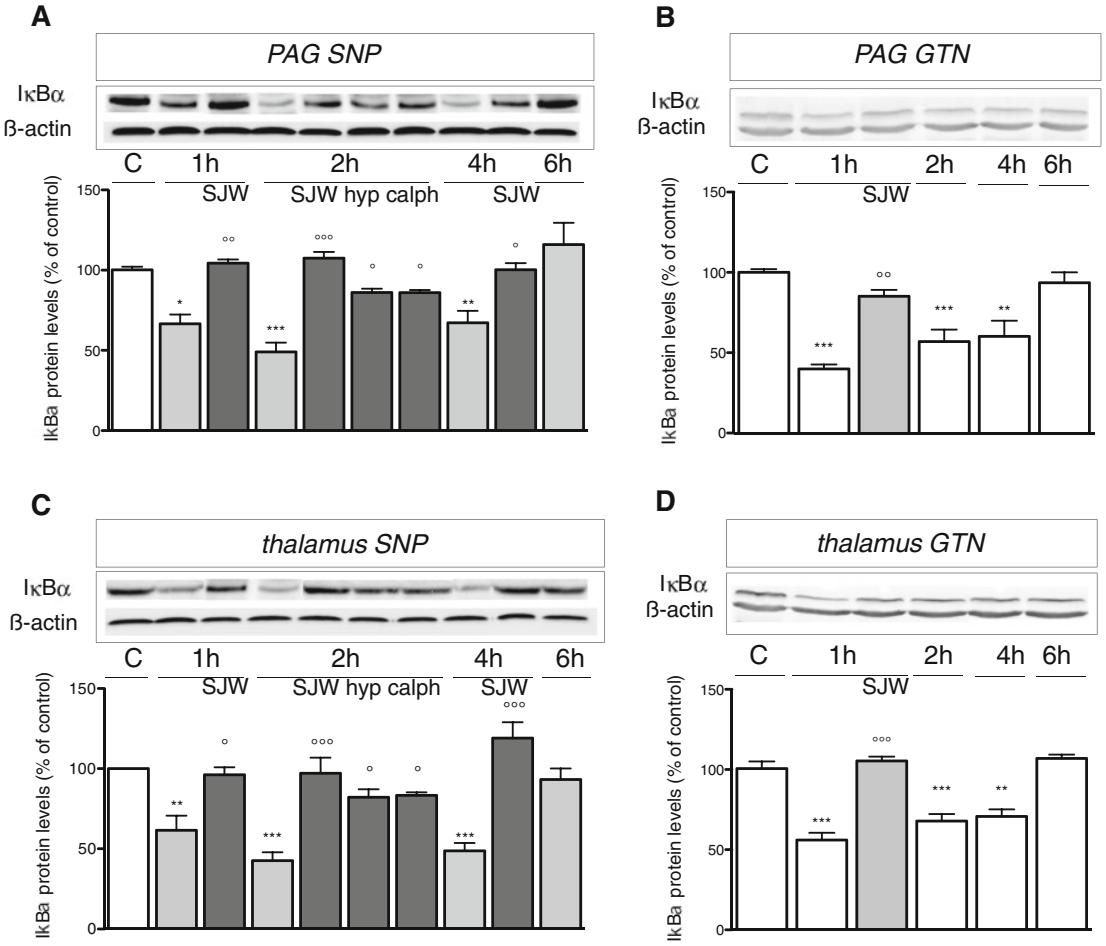


Fig. 3 Effect of SJW on IκBα expression. **a** SNP activated supraspinal NF-κB pathway within PAG as indicated by the reduced levels of IκBα. The activation of the NF-κB pathway was prevented by SJW, hypericin (*hyp*) and the PKC blocker calphostin C (*calph*). **b** Within PAG SJW also prevented the IκBα down-regulation produced by GTN administration. A cerebral activation of the NF-κB pathway is indicated by the reduction of IκBα levels in the thalamus following SNP (**c**) and GTN (**d**). A single oral administration of SJW, hypericin and calphostin C prevented the NO donor-induced activation of the NF-κB pathway. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with the control group (C). °$P<0.05$, °°$P<0.01$, °°°$P<0.001$ compared with corresponding NO donor-treated group

single oral administration. The systemic administration of the NO donors GTN and SNP produced a prolonged thermal allodynia and hyperalgesia in mice, further confirming the essential contribution of NO for nociceptive processing. A single oral administration of a low dose of an SJW dried extract reversed the pronociceptive effect induced by NO donors. The dose of SJW used (0.016 mg of total hypericins) was considerably lower than those required to induce antidepressant (1.8–2.7 $mg\,day^{-1}$ of total hypericins) (Kasper et al. 2010) and analgesic activities (0.96 mg of total hypericins) (Galeotti et al. 2010a,b). We can suppose that SJW relief from NO-induced nociceptive hypersensitivity in this animal model is not secondary to its antidepressant or analgesic property.

To clarify the supraspinal cellular pathways involved in the NO-induced nociceptive behavior, we detected the expression of transcription factors involved in the modulation of inflammation and pain processing, such as nuclear factor-κB (NF-κB), Signal Transducer and Activator of Transcription (STAT)-1, cyclic AMP response element binding protein (CREB), within PAG and thalamus. The activation of the NF-κB pathway after GTN and SNP was demonstrated by the consistent down-regulation of IκBα protein expression. NF-κB consists of dimers that under basal condition are sequestered within the cytoplasm by the IκB family of inhibitory proteins. Phosphorylation of IκBα triggers its ubiquination and rapid degradation, thereby releasing NF-κB to initiate expression of iNOS, cytokines (IL-1β, IL6) and molecules involved in inflammation and pain perception (Oeckinghaus et al. 2011). The decrease of IκBα protein expression was prevented by oral administration of an SJW dried extract showing the capability of this herbal medicine to counteract supraspinal cellular events involved in inflammation and nociceptive hypersensitivity

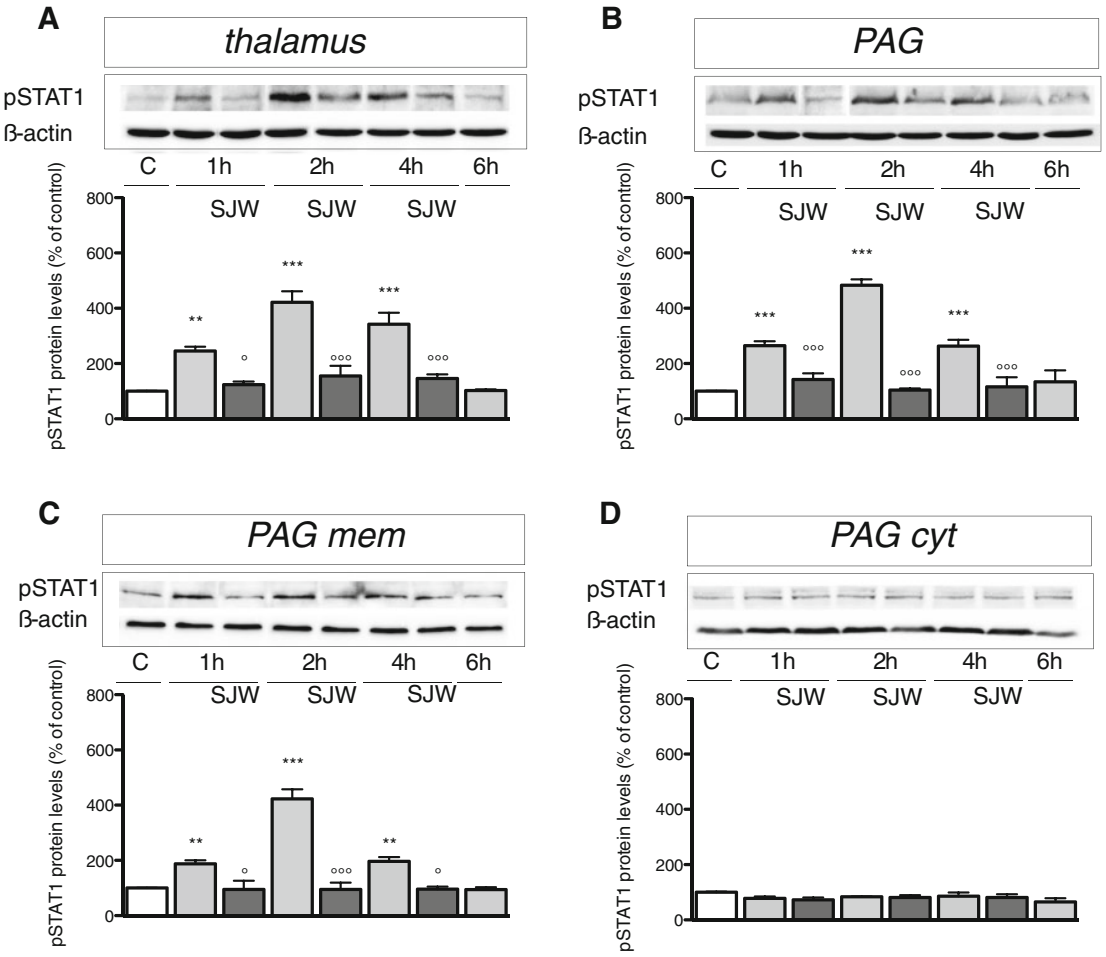


Fig. 4 Prevention by SJW of STAT1 hyperphosphorylation. Phosphorylated STAT1 (*pSTAT1*) protein levels were increased 1, 2 and 4 h after SNP treatment within the thalamus (**a**) and the PAG (**b**). Oral administration of SJW (5 mg/kg) prevented the NO donor–induced increase of pSTAT1 expression. **c** pSTAT1 protein levels were increased in the PAG membrane fraction, effect prevented by SJW administration. **d** The absence of any variation of pSTAT1 levels was observed in the cytosolic fraction. $^{**}P<0.01$, $^{***}P<0.001$ compared with the control group (*C*). $^{\circ}P<0.05$, $^{\circ\circ}P<0.01$, $^{\circ\circ\circ}P<0.001$ compared with corresponding SNP-treated group

produced by NO. These results are in agreement with previous reports attributing the anti-inflammatory property of SJW to the inhibition of the NF-κB activation (Bork et al. 1999).

STAT proteins possess the ability to transduce signals from the cell membrane to the nucleus to activate gene transcription, thus bypassing the involvement of secondary messengers. STAT proteins are activated by a plethora of cytokines, including interferons and interleukins, as well as growth factors and hormones (Lim and Cao 2006) and the relevance of the STAT pathways in the response to cytokines and inflammation is widely known. In the mouse model of NO-induced nociceptive behavior we observed a dramatic increase in the expression of the phosphorylated form of STAT1 (pSTAT1) following NO donors' injection within PAG and thalamus with a time-course consistent with the nociceptive behavior. We, then, might suggest that the NO donor-induced supraspinal pSTAT1 up-regulation might participate to the pronociceptive activity. The administration of SJW completely prevented the pSTAT1 up-regulation at any time point detected. The capability of SJW to inhibit iNOS expression by down-regulating STAT1α activation was observed in human epithelial cell lines at concentrations consistent with those used in the present study (Tedeschi et al. 2003), confirming the involvement of this transcription factor in the mechanism of anti-inflammatory action of the investigated herbal drug.

CREB plays an important role in the inflammatory processes and in the modulation of pain perception. In the early stages of inflammation (Ji and Rupp 1997) and sciatic nerve injury (Ma and Quirion 2001), the phosphorylation of CREB is increased. Furthermore, this transcription factor regulates long-lasting effects of NO in neurons (Contestabile 2008). Following GTN and SNP treatment, we detected a robust

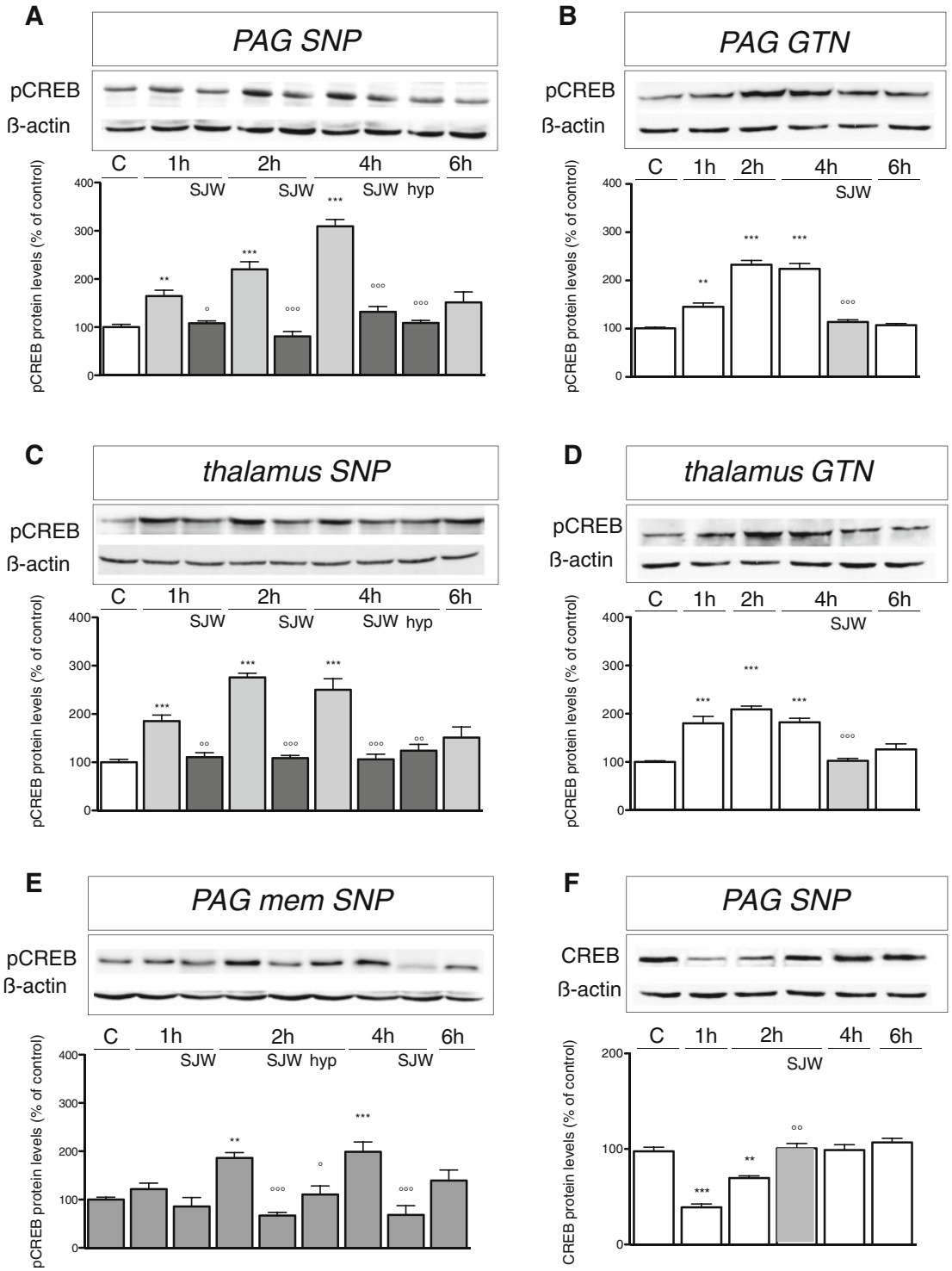
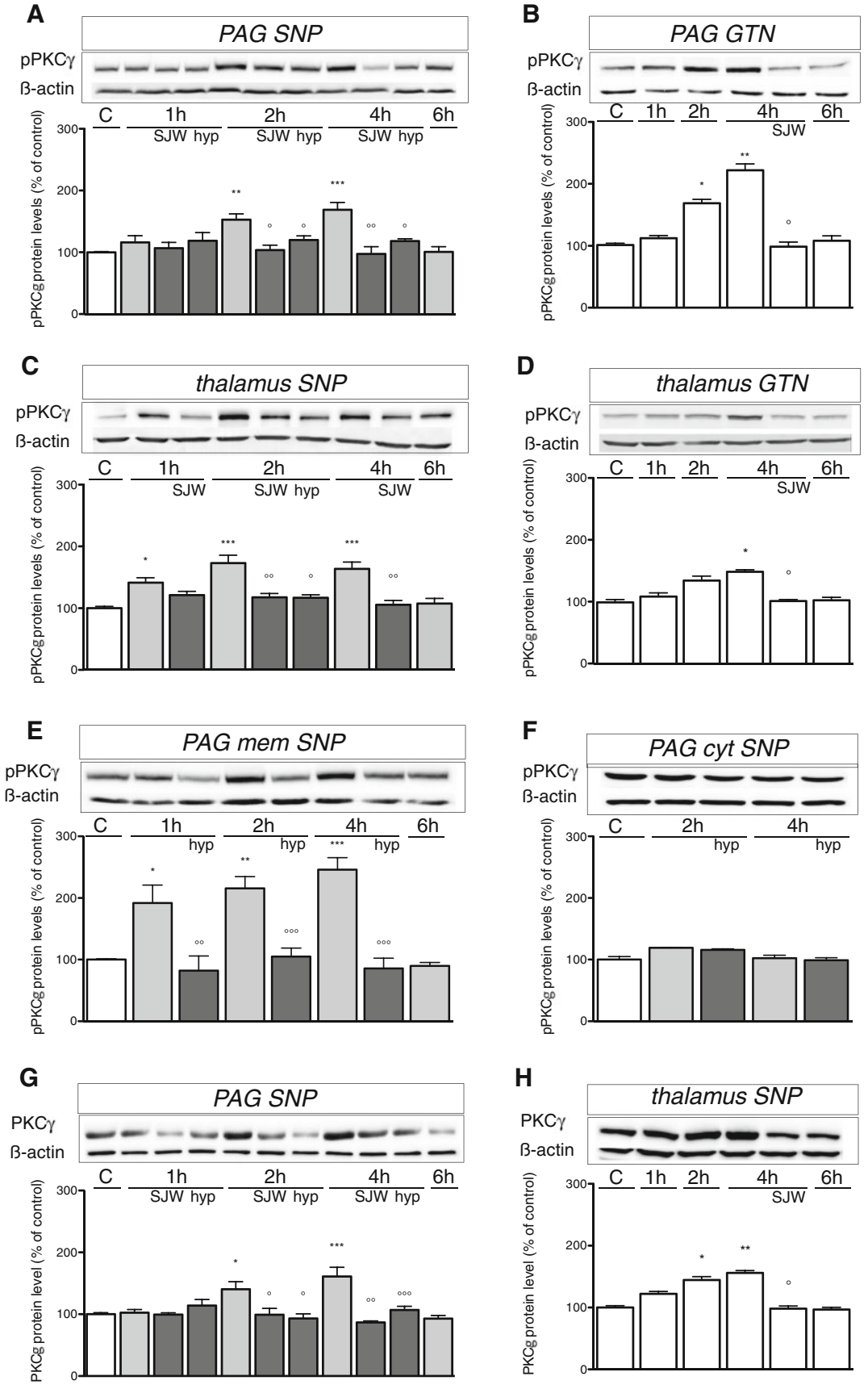


Fig. 5 SJW and hypericin prevention of the increased supraspinal expression of phosphorylated CREB (*pCREB*) induced by NO donors. SNP (**a**) and GNT (**b**) increased the pCREB protein levels within PAG 1, 2 and 4 h after administration. Pretreatment with SJW (5 mg/kg, p.o.) and hypericin (*hyp*) prevented the NO donors increase pCREB expression. Within thalamus SNP (**c**) and GTN (**d**) increased pCREB content with a time-course similar to that observed in the PAG. Orally administered SJW and hypericin prevented the pCREB up-regulation. **e** SNP increased pCREB expression in the PAG membrane fraction, effect prevented by SJW and hypericin administration. **f** CREB protein levels were down-regulated 1 and 2 h after SNP treatment within PAG. SJW oral administration reversed the SNP-induced effect. $^{**}P<0.01$, $^{***}P<0.001$ compared with the control group (*C*). $^{\circ}P<0.05$, $^{\circ\circ}P<0.01$; $^{\circ\circ\circ}P<0.001$ compared with corresponding NO donor-treated group


A
PAG SNP
pPKCγ
ß-actin
C
1h
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4h
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SJW hyp
SJW hyp
SJW hyp
pPKCg protein levels (% of control)
300
200
100
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B
PAG GTN
pPKCγ
ß-actin
C
1h
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4h
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SJW
pPKCg protein levels (% of control)
C
thalamus SNP
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pPKCγ
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SJW
PKCg protein level (% of control)

◀ **Fig. 6** SJW prevented the up-regulation and increased phosphorylation of PKCγ induced by NO donors' administration. Systemic administration of GNT (**a**) and SNP (**b**) increased the expression of pPKCγ within PAG 2 and 4 h after administration that was completely prevented by oral administration of SJW and hypericin. SNP (**c**) and GTN (**d**) up-regulated pPKCγ within the thalamus. SJW and hypericin counteracted the PKCγ hyperphosphorylation. **e** SNP produced a cerebral activation of the PKCγ pathways as confirmed by the increased expression of pPKCγ within the PAG membrane fraction that was prevented by hypericin. **f** Lack of effect on pPKCγ protein levels in the cytosolic fraction following NO donor and hypericin administration. SJW and hypericin prevented the PKCγ up-regulation induced by SNP treatment within the PAG (**g**) and thalamus (**h**). *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with the control group (*C*). °$P<0.05$, °°$P<0.01$, °°°$P<0.001$ compared with the corresponding NO donor-treated group

increase in the phosphorylation of CREB within PAG and thalamus, in coincidence with the presence of allodynia and hyperalgesia. Oral administration of SJW drastically reduced pCREB levels. These results illustrate a relevant influence of SJW on CREB expression and phosphorylation and support the possibility that CREB signaling pathway may play an important role in the modulation of pain processing produced by NO.

SJW contains a number of bioactive compounds (Barnes et al. 2001). Among them, hypericin appears of particular relevance since behavioral experiments showed that hypericin was able to reverse cold allodynia and thermal hyperalgesia induced by NO donors with the same pharmacological profile of SJW whereas hyperforin and flavonoids were ineffective. To clarify the cellular mechanism of SJW, we focused our attention on the molecular pathways modulated by hypericin. Enzyme assays performed on rat brain demonstrated that hypericin is a potent and selective inhibitor of the protein kinase C (PKC) (Takahashi et al. 1989), enzyme highly involved in pain modulation (Velazquez et al. 2007). PKC is a family of serine/threonine kinases that are divided into three groups based on calcium and diacylglicerol dependence: conventional (α, βI, βII, γ), novel (δ, ε, η, θ), atypical (ζ, λ/ι) (Way et al. 2000). We examined the involvement of PKCε and PKCγ since they appear to be the isoforms with a prominent role in the modulation of pain perception (Velazquez et al. 2007). We detected a specific up-regulation and increased phosphorylation of PKCε and PKCγ isoforms in PAG and thalamus of GTN and SNP treated mice. The oral administration of SJW and hypericin prevented the NO donor-induced hyperphosphorylation of both PKC isoforms. The blockade of PKC activity appears a fundamental step in the mechanism of action through which SJW modulates pain processing since also its analgesic and antineuropathic activity is related to the prevention of cerebral PKC hyperphosphorylation (Galeotti et al. 2010a,b). The translocation of these enzymes from cytosol to the synaptic membrane is thought to be necessary for their activation (Nishizuka 1992). We observed a robust increase of the PKCε and PKCγ isoform phosphorylation in the membrane fraction, whereas a lack of increase in any of the phosphorylation of PKCε and PKCγ in the cytosol was detected, confirming the specific PKC activation in this murine model. Treatment with SJW, hypericin or the PKC blocker calphostin C prevented the increased phosphorylation of PKCε and PKCγ in the membrane fraction, further demonstrating that the inhibition of PKC hyperphosphorylation induced by SJW is consequent to the PKC blocking property of hypericin. Furthermore, similarly to SJW, purified hypericin was also able to counteract the activation of the NF-κB, STAT1 and CREB signaling pathways, further confirming hypericin as a major SJW component involved in the reduction of NO-induced nociceptive behavior. Following the i.c.v. administration of hypericin, the reversal of the nociceptive behavior induced by NO donors' administration was obtained. The same treatment also prevented the NO-induced IκBα decrease and the pSTAT1, pCREB, pPKCγ and pPKCε overexpression in the PAG and thalamus with intensity comparable to that observed after oral administration. These results further confirm a supraspinal mechanism of action of hypercin.

These results have highlighted the presence of a PKC-mediated pathway in the supraspinal nociceptive processing following NO donor administration that might represent a molecular target of SJW. In particular, we can suppose that PKC might act as an upstream regulator of NF-κB, STAT1 and CREB. The hypothesis of the activation of the PKC/NF-κB pathway following NO donors' administration, that contributes to the induction of allodynia/hyperalgesia, was confirmed by studies conducted on murine microglia showing that LPS- and peptidoglycan-induced iNOS production appears to be mediated by a signaling pathway involving the sequential of PKC and NF-κB activation (Bhatt et al. 2010; Wen et al. 2011). Similarly, we can suppose that PKC might be involved in the NO-induced supraspinal activation of STAT1. This hypothesis is supported by the observation that blockade of conventional PKC isoenzymes down-regulate STAT1 activation in LPS-treated murine macrophages (Salonen et al. 2006). We, then, might hypothesize that CREB, STAT1 and NF-κB, acting as downstream effectors of PKC, can cooperatively modulate the sensation of pain to produce a condition of hypersensitivity to noxious stimuli. It appears plausible to suppose that SJW prevents the nociceptive behavior in response to NO donor administration by inhibiting a PKC-dependent intracellular pathway involving CREB, STAT1 and NF-κB through the activity of hypericin (see Fig. 9).

A number of antidepressants are routinely used in the treatment of chronic pain conditions and the involvement of the monoaminergic pathways in the modulation of pain perception has long been known. Receptor binding and enzyme inhibition assays carried out using hypericum extract demonstrated significant affinity for MAO_A, MAO_B and inhibitory activity towards synaptosomal uptake of serotonin, dopamine and noradrenaline. The MAO-inhibitory properties of SJW

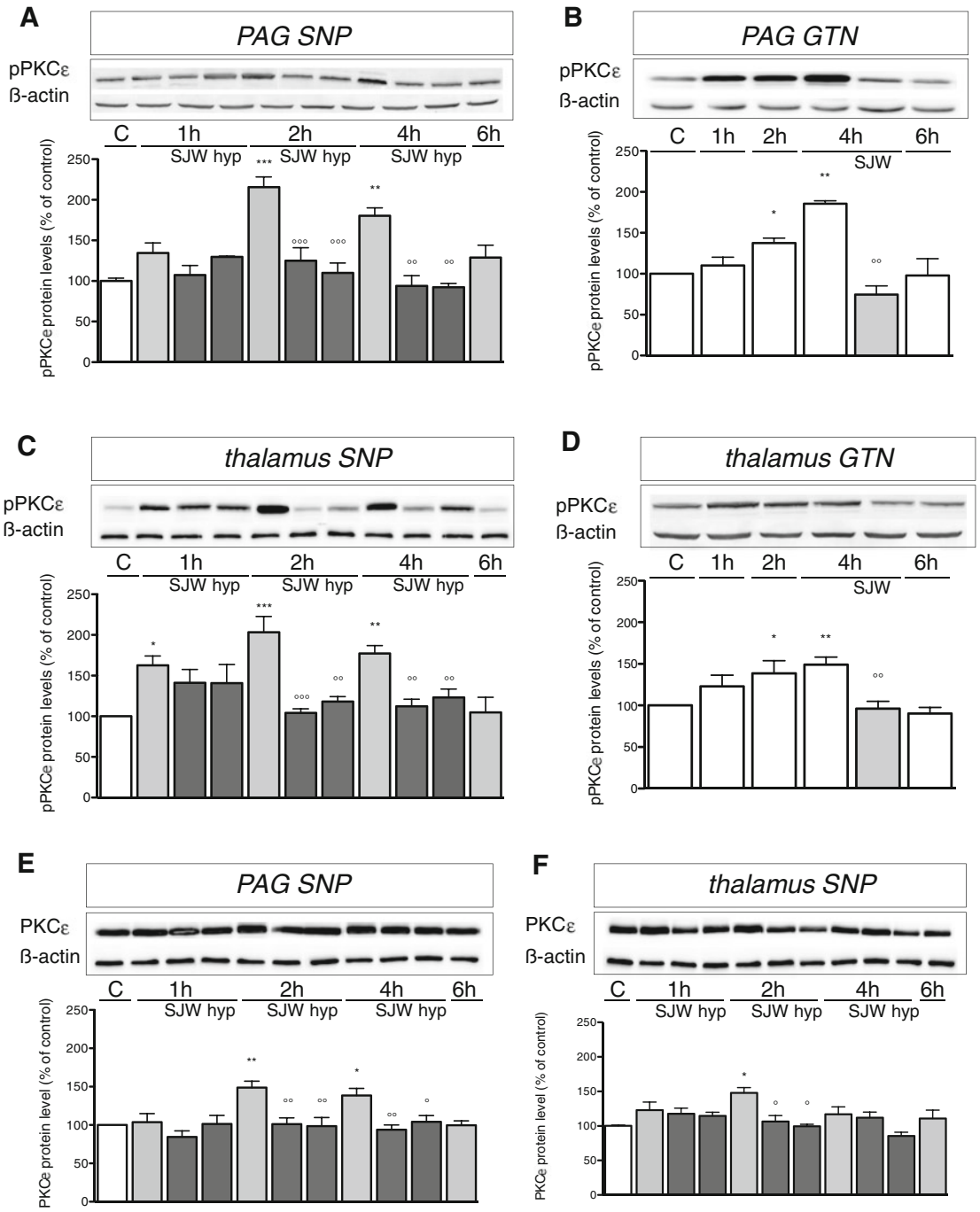

Fig. 7 Oral administration of SJW and hypericin prevented NO donor-induced supraspinal PKCε activation. SNP (**a**) and GTN (**b**) activated the PKCε pathway as indicate by the increased expression of pPKCε within PAG that was prevented by administration of SJW and hypericin. Within the thalamus, SNP (**d**) and GTN (**e**) systemic treatment up-regulated pPKCε. Oral administration of SJW and hypericin prevented the No donor-induced increase of pPKCε expression. The same treatment also prevented the total PKCε protein increase within PAG (**e**) and thalamus (**f**). *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with control group (*C*); °$P<0.05$, °°$P<0.01$, °°°$P<0.001$ compared with corresponding NO donor-treated group

were mainly due to hypericin, but the MAO inhibition was obtained in vitro with concentrations of extracts too high (>100 μg/ml) to be achieved in vivo (Bladt and Wagner 1994; Yu 2000). The inhibition of monoamine synaptosomal uptake of SJW is related to the presence of hyperforin. The amount of hyperforin present in the SJW concentration used in the present study was ineffective on modulating the pain threshold and, therefore, we can exclude the inhibition of the

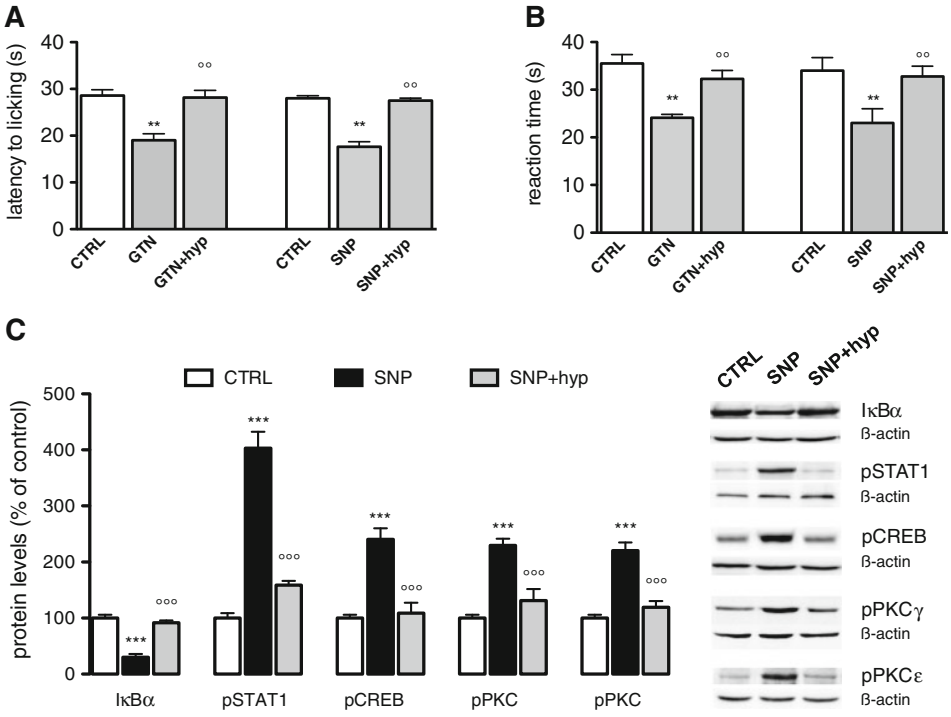

Fig. 8 Intracerebroventricular (i.c.v.) administration of hyperforin prevents the NO donor-induced nociceptive behavior and cellular modulation. **a** Nitroglycerin (*GTN*) or sodium nitroprusside (*SNP*) induced heat hyperalgesia in the hot plate test prevented by a single i.c.v. injection of hypericin (*hyp*). **b** Intracerebroventricular hypericin also prevented NO-induced cold allodynia. **c** Following i.c.v. administration, hypericin (*hyp*) counteracted the SNP-induced activation of the NF-κB pathways, as demonstrated by the reduction of the IκBα levels, and hyperphosphorylation of STAT1, CREB, PKCγ and PKCε within PAG. $^{**}P<0.01$, $^{***}P<0.001$ compared with the control group (*CTRL*); $^{\circ\circ}P<0.01$, $^{\circ\circ\circ}P<0.001$ compared to the corresponding NO donor-treated group

monoamine reuptake as a mechanism of the SJW antihyperalgesic activity in this model. The involvement of the adrenergic system can be further ruled out since the α_2-adrenoceptor antagonist yohimbine did not prevent SWJ, hyperforin and hypericin induced antinociception, as recently demonstrated (Galeotti et al. 2010a).

With regard to the development of novel analgesics that might act through NO, it is important to consider that this

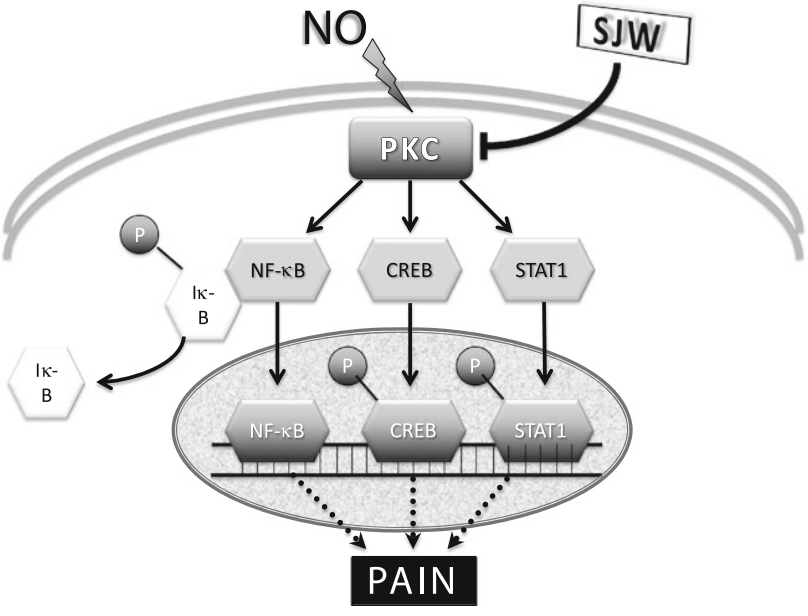

Fig. 9 A proposed model for NO-induced nociceptive behavior in the mouse brain. Under resting conditions, the transcription factors NF-κB, CREB and STAT1 reside inactive in the cytoplasm. NF-κB resides inactivated by inhibitory proteins κB (*IκB*). CREB and STAT1 are non-phosphorylated. NO increases phosphorylation of PKCγ isoform that phosphorylates CREB, STAT1 and IκB proteins. Once phosphorylated, IκB family members dissociate from NF-κB. NF-κB, pCREB and pSTAT1 act in the nucleus, binding specific recognition elements in the promoter regions of genes, thereby activating their transcription and leading to nociceptive hypersensitivity. SJW, through inhibition of the PKC phosphorylation, inhibits the NF-κB-CREB- and STAT1-dependent transcriptional activation to prevent nociceptive behavior

molecule is involved in many physiological and pathophysiological processes throughout the body. Thus, if unspecific inhibitors of NO are administered systemically, a variety of potential side effects, especially in the vascular system, have to be considered. Conversely, SJW is endowed with a favorable tolerability and safety profile (Rahimi et al. 2009) and might represent a valid alternative to NO inhibitors to produce pain relief. Recently, interactions of SJW with prescription drugs have been reported. SJW, at the dose recommended for the treatment of mild to moderate depression, is a potent inducer of cytochrome P450 enzymes resulting in decrease plasma concentration of a number of drugs used in co-medication (Whitten et al. 2006). Recent studies show that the degree of enzyme induction by SJW correlates strongly with the amount of hyperforin found in the product. High-dose hyperforin extracts (>10 mg day^{-1}) had outcomes consistent with CYP3A induction whereas low-dose hyperforin extracts (<4 mg day^{-1}) demonstrated no significant effect on CYP3A (Whitten et al. 2006; Madabushi et al. 2006). We observed that SJW reduced NO induced nociceptive behavior at very low doses containing an amount of hyperforin (0.21 mg) unable to produce clinical significant interactions.

In conclusion, present results demonstrated the activation of a supraspinal signaling pathway involving a PKC-dependent NF-κB, STAT1 and CREB activation following systemic NO donors' administration. A single oral administration of SJW or hypericin reverted the nociceptive behavior and the hyperphosphorylation of the transcription factors. Due to the efficacy and favorable safety profile, we can suppose that this herbal plant represents an important therapeutic perspective for the treatment of nociceptive hypersensitivity.

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Conflict of interest The authors indicate no potential conflicts of interest

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