Phytomedicine 21 (2014) 745–752

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Withania somnifera root extract prolongs analgesia and suppresses hyperalgesia in mice treated with morphine

Alessandro Orrù[a,*], Giorgio Marchese[a], Gianluca Casu[a], Maria Antonietta Casu[a], Sanjay Kasture[b], Filippo Cottiglia[c], Elio Acquas[d,e,f], Maria Paola Mascia[g], Nicola Anzani[c], Stefania Ruiu[a,*]

[a] *CNR-Institute of Translational Pharmacology, U.O.S. of Cagliari, Science and Technology Park of Sardinia Polaris, Pula, Italy*
[b] *Sanjivani College of Pharmaceutical Education & Research, Kopargaon, India*
[c] *Department of Life and Environmental Sciences – Drug Sciences Section, University of Cagliari, Italy*
[d] *Department of Life and Environmental Sciences – Pharmaceutical, Pharmacological and Nutraceutical Sciences Section, University of Cagliari, Italy*
[e] *Centre of Excellence on Neurobiology of Addiction, University of Cagliari, Italy*
[f] *National Institute of Neuroscience – INN, University of Cagliari, Italy*
[g] *CNR-Institute of Neuroscience, Cagliari, Cittadella Universitaria, 09042 Monserrato, Italy*

ARTICLE INFO

Article history:
Received 23 August 2013
Received in revised form
16 September 2013
Accepted 17 October 2013

Keywords:
Withania somnifera
Morphine
Antinociception
Hyperalgesia
Binding assay

ABSTRACT

Previous studies demonstrated that *Withania somnifera* Dunal (WS), a safe medicinal plant, prevents the development of tolerance to the analgesic effect of morphine.

In the present study, we investigated whether WS extract (WSE) (100 mg/kg, i.p.) may also modulate the analgesic effect induced by acute morphine administration (2.5, 5, 10 mg/kg, s.c.) in the tail-flick and in the hot plate tests, and if it may prevent the development of 2.5 mg/kg morphine-induced rebound hyperalgesia in the low intensity tail-flick test. Further, to characterize the receptor(s) involved in these effects, we studied, by receptor-binding assay, the affinity of WSE for opioid (μ, δ, k), cannabinoid (CB_1, CB_2), glutamatergic (NMDA), GABAergic ($GABA_A$, $GABA_B$), serotoninergic ($5HT_{2A}$) and adrenergic (α_2) receptors.

The results demonstrated that (i) WSE alone failed to alter basal nociceptive threshold in both tests, (ii) WSE pre-treatment significantly protracted the antinociceptive effect induced by 5 and 10 mg/kg of morphine only in tail-flick test, (iii) WSE pre-treatment prevented morphine-induced hyperalgesia in the low intensity tail-flick test, and (iv) WSE exhibited a high affinity for the $GABA_A$ and moderate affinity for $GABA_B$, NMDA and δ opioid receptors.

WSE prolongs morphine-induced analgesia and suppresses the development of morphine-induced rebound hyperalgesia probably through involvement of $GABA_A$, $GABA_B$, NMDA and δ opioid receptors. This study suggests the therapeutic potential of WSE as a valuable adjuvant agent in opioid-sparing therapies.

Introduction

Pain has negative consequences on health status and life quality, and its relief is considered one of the major and complex medical concerns. To date, opioid analgesics remain the most efficacious pharmacological agents for the treatment of moderate to severe pain, but their therapeutic benefit is often hampered by the development of analgesic tolerance and hyperalgesia (Benyamin et al. 2008). Opioid-induced hyperalgesia (OIH), defined as a paradoxical increase in sensitivity to noxious stimuli (Pasero and McCaffery 2012), is a side effect difficult to recognize because its clinical manifestations are similar to those observed in opioid-induced analgesic tolerance (DuPen et al. 2007; Raffa and Pergolizzi 2011). Despite such difficulty, OIH incidence is growing as opioid prescriptions are increasing (Raffa and Pergolizzi 2011). A considerable effort has been then expended in the development of novel therapeutic strategies that may maintain adequate opioid-induced analgesia and, at the same time, mitigate the appearance of tolerance and OIH. Adjuvant pharmacological therapies are among the therapeutic approaches used to reach this purpose, and consist in the association of opioid analgesics with a second non-opioid agent (DuPen et al. 2007; Khan et al. 2011). This approach has been found to improve opioid-induced analgesia at different levels: enhancing pain relief (with a concurrent reduction of opioid dosage) and preventing, at the same time, the development of

* Corresponding authors. Tel.: +39 070 9241057; fax: +39 070 9241057.
E-mail addresses: alessandro.orru@ift.cnr.it (A. Orrù), stefania.ruiu@cnr.it (S. Ruiu).

0944-7113/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.phymed.2013.10.021

side effects, specifically tolerance and OIH (DuPen et al. 2007). The main adjuvant agents that are currently associated to opioids in the management of pain are NMDA receptor antagonists, α_2 receptor agonists and cyclooxigenase-2 inhibitors (Low et al. 2012; Pasero and McCaffery 2012).

Medicinal plants are among the potential adjuvant agents that could be also evaluated in this therapeutic approach. Phytotherapy offers, in fact, a valid support to conventional medicine in a number of diseases (Patwardhan 2005) and this also applies to the management of pain (Low Dog 2008). *Withania somnifera* Dunal (WS, family: Solanaceae) also known as Ashwagandha or Winter Cherry is a plant commonly used in Ayurvedic medicine to treat several diseases (Alam et al. 2012). A growing number of pharmacological studies demonstrated that WS extract (WSE) has anticancer, anti-inflammatory, immunomodulatory, adaptogenic and neuroprotective properties (Alam et al. 2012). Interestingly, Kulkarni and Ninan (1997) demonstrated that, in mice chronically treated with morphine, WSE prevented the development of tolerance to the antinociceptive effect of morphine.

The molecular underpinnings responsible for the development of analgesic tolerance show similarities with those involved in OIH (DuPen et al. 2007; Raffa and Pergolizzi 2011), and are the consequence of plastic changes in neurotransmitter systems that contribute to mediate pain transmission and opioid-induced analgesia. Accordingly, pre-clinical studies demonstrated that several pharmacological agents prevent the development both of antinociceptive tolerance and OIH (Bryant et al. 2006; Gupta et al. 2011), such as NMDA antagonists, among others. Moreover, the same compounds have been found to prolong or increase the acute analgesic effect of opioids (Fischer et al. 2005).

Starting from these studies, since the administration of WSE blocked the development of tolerance to the analgesic effect of morphine (Kulkarni and Ninan 1997), we hypothesize that WSE could modulate additional aspects of morphine-induced analgesia. Therefore, we aimed to determine whether the combined administration of WSE with morphine could enhance or prolong the antinociceptive effect of morphine in the tail-flick and hot plate tests. Moreover, we investigated the potential ability of WSE to block the development of morphine-induced hyperalgesia using the low intensity tail-flick test, a validated model of OIH (Gupta et al. 2011). Finally, since little information exists regarding the sites of action of WSE in the brain and about the receptor(s) involved in its pharmacological and behavioural properties, performing competition studies we determined WSE affinity for opioid (μ, δ, k), cannabinoid (CB_1, CB_2), glutamatergic (NMDA), GABAergic ($GABA_A$, $GABA_B$), serotoninergic ($5HT_{2A}$) and adrenergic (α_2) receptors.

Materials and methods

Subjects

Male CD1 mice (Charles River, Calco, Italy) 20–25 g were used for both behavioural and receptor binding studies. Animals were housed in an animal facility on a 12 h light/dark cycle (lights on from 07:00 AM), at a constant room temperature of $21 \pm 1\,^{\circ}C$ (relative humidity approximately 60%). Standard rodent chow and water were available ad libitum. Animals were allowed to adapt to the animal facility conditions for at least two weeks after arrival.

Procedures involving animals and their care were conducted in accordance with the institutional guidelines that are in compliance with national (D.L. 116/1992) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Every effort was made to minimize animal pain and discomfort and to reduce the number of experimental subjects.

Drugs and chemicals

Morphine hydrochloride (Salars, Como, Italy) was dissolved in saline (NaCl 0.9%) and administered subcutaneously (s.c.) in a volume of 5 ml/kg. The standardized root methanolic extract of WS (*Withania somnifera* Dunal, Natural Remedies Pvt. Ltd., Bangalore, India) was dissolved in saline for analgesia experiments [administered intraperitoneally (i.p.) in a volume of 5 ml/kg] and in dimethyl sulfoxide (DMSO, Sigma–Aldrich, Milan, Italy) for binding assay. The dose of WSE for analgesia experiments was selected on the basis of previous studies (Kasture et al. 2009; Ruiu et al. 2013). [^{3}H]-DAMGO ([d-Ala2, N-Me-Phe4,Gly-ol5]-enkephalin), [^{3}H]-DPDPE ([D-Pen^2, D-Pen^5]-enkephalin), [^{3}H]-U-69,593, [^{3}H]-CP55,950, [^{3}H]-MK801, [^{3}H]-Muscimol, [^{3}H]-Baclofen, [^{3}H]-Clonidine and [^{3}H]-Ketanserine were purchased from Perkin Elmer, Monza (MB), Italy. CP-55,940, Naloxone, U-69,593, muscimol, MK801, yohimbine and methysergide were obtained from Tocris Cookson Ltd. (Bristol, UK).

Plant materials

The standardized WSE was kindly provided by Natural Remedies Pvt. Ltd., Bangalore, India.

High-performance liquid chromatographic (HPLC) analysis

The WSE has been characterized by an HPLC-fingerprint analysis, as certified by Natural Remedies Pvt. Ltd., with identification of the main withanolides. This analysis with the necessary description of the technique has been published in Neurotoxicity Research by Kasture et al. (2009). An HPLC system (Shimadzu, LC 2010 A, Japan) equipped with UV detector, auto-injector, and column oven with class VP software was used. The stationary phase was an octadecylsilane column (Phenomenex-Luna, C18, 5 μm, 250 mm × 4.6 mm). The mobile phase was a mixture of phosphate buffer (Solvent A) [prepared by dissolving 0.136 g of KH_2PO_4 in 900 ml of HPLC grade water and by adding 10% dilute aqueous H_3PO_4, adjusting the pH to 2.8 ± 0.05 and making the volume of 1000 ml with water] and acetonitrile (Solvent B). The following withanolides were identified: withanoside-IV, 0.49%w/w; physagulin D, 0.11%w/w; 27-hydroxywithanone, 0.01%w/w; withanoside-V, 0.33%w/w; withaferin-A, 0.11%w/w; 12-deoxy withastramonolide, 0.16%w/w; withanolide-A, 0.19%w/w; withanone 0.004%w/w, and withanolide-B, 0.03%w/w.

Analgesia experiments

The antinociceptive effects were quantified using the tail-flick test and the hot plate test (Ruiu et al. 2003). An automated device (model 7360, Ugo Basile, Italy) was used to determine the tail-flick latency, defined by the time (s) at which the animals withdraw the tail from a radiant heat source. Mice were held and gently restrained above the apparatus; the light beam was focused 1.5 cm from the tip of the ventral surface of the tail. The stimulus intensity was adjusted to result in a mean pre-drug control latency of 2–3 s, and a cut-off time of 12 s was applied to avoid tissue damage.

A semi-automated device (model 7280, Ugo Basile, Italy) was used to determine the reaction of mice placed on the hot plate, defined by the time (s) at which mice exhibited a nociceptive response or discomfort (licking or fanning the paws, jumping). A 50 cm high Plexiglas cylinder was suspended over the hot plate and the temperature was maintained at $55 \pm 0.2\,^{\circ}C$; to avoid skin damage, after 15 s mice were removed from the hot plate. Mice were