Oxysophocarpine induces anti-nociception and increases the expression of GABA$_{\alpha1}$ receptors in mice

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Received November 26, 2012; Accepted March 27, 2013

DOI: 10.3892/mmr.2013.1414

Abstract. Oxysophocarpine (OSC) is an alkaloid extracted from Siphocampylus verticillatus. The aim of this study was to investigate the anti-nociceptive effects of OSC through systemic and intracerebroventricular administration in mice. Moreover, to evaluate its effectiveness and mechanism of action, this study investigated whether OSC altered the expression of $\gamma$-aminobutyric acid type A $\alpha_1$ (GABA$_{\alpha1}$) receptors in the central nervous system. Thermal and chemical behavioral models of nociception were used to assess the anti-nociceptive action of OSC. The warm water tail-flick test, the hot-plate test, acetic acid-induced abdominal constriction and formalin-induced pain were used in mice. OSC was administered intraperitoneally (i.p.) or intracerebroventricularly (i.c.v.). Results showed that OSC (80 mg/kg, i.p.) significantly increased the tail withdrawal threshold with a peak of 25.46% maximal possible effect (MPE) at 60 min (P<0.01). Additionally, OSC (80 mg/kg) increased the positive staining of GABA$_{\alpha1}$ receptors in cells. In conclusion, OSC administration is suggested to have anti-nociceptive effects on the central and peripheral nervous systems. The involvement of GABA$_{\alpha}$ receptors in the anti-nociceptive activity of OSC is currently being investigated.

Introduction

Opioids are known to be potent drugs used for acute and chronic pain, although some effects limit their use, including respiratory depression and possibly dependence. Non-steroidal anti-inflammatory drugs (NSAIDs) are also not viable options due to their weak response and more adverse effects such as gastrointestinal disturbances and renal damage. Therefore, the identification of analgesic agents that cause no side-effects and retain opioid-like potency is required. There are several ways to identify new targets for this purpose. Previous studies have shown the involvement of the GABAergic system in the modulation of pain at the supraspinal and spinal level (1,2). Oxysophocarpine (OSC) is an alkaloid extracted from Siphocampylus verticillatus (Campanulaceae) (3). Findings of previous studies showed that the contents extracted from Siphocampylus verticillatus have certain pharmacological effects. Rodrigues et al (4) demonstrated that OSC has a significant antidepressant-like effect following assessment using the tail suspension and forced swimming tests in mice. Trentin et al (5) demonstrated that the hydroalcoholic extract of Siphocampylus verticillatus causes long-lasting anti-nociception when assessed against neurogenic and inflammatory models of nociception in mice. Findings of a pharmacological and clinical study demonstrated that sophocarpine has pain-relieving effects (6). Oxysophocarpine and sophocarpine have a similar molecular structure (7). We hypothesized that OSC is an anti-nociceptive drug with greater efficacy, lower toxicity and fewer side-effects. To test this hypothesis, thermal- and chemical-based behavioral models of nociception were used and different routes of administration were assessed to investigate the analgesic effects of OSC and to determine the primary analgesic sites of OSC in mice. Additionally, immunohistochemistry was used to investigate whether the anti-nociceptive effects of OSC are associated

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Key words: oxysophocarpine, analgesia, $\gamma$-aminobutyric acid, $\gamma$-aminobutyric acid type A
with the expression of GABA$_A$ receptors in the cerebral cortex and the hippocampus in ICR mice.

Materials and methods

Animals. Institute of Cancer Research (ICR) mice (18-22 g) were provided by the Experimental Animal Center of Ningxia Medical University (Yinchuan, China) (certificate no. SYXK Ningxia 2005-0001). Mice were housed at 22±2°C and 50±5% relative humidity under a 12-h light/dark cycle, and they had access to food and water ad libitum. The experiments were performed during the light phase. Mice were acclimatized to the laboratory for ≥1 h prior to testing. For the test each mouse was used only once. The mice were randomly divided into the negative control (saline group), positive control (morphine, aspirin) and different OSC dose groups (n=10/group).

The drugs were injected intraperitoneally (i.p.) or intracerebroventricularly (i.c.v.), with the exception of aspirin, which was administered intragastrically (i.g.). The experiments were performed in accordance with the Guidelines for the Care of Laboratory Animals of Ningxia Medical University.

Drugs. OSC was supplied by the Zi Jin Hua Pharmaceutical Co., (Ningxia University, Ningxia, China) (lot no. 071218, purity >98%), which was dissolved in 0.9% (w/v) NaCl solution. Morphine was obtained from the Shenyang Pharmaceutical Co. (Shenyang, China), and aspirin from the Yongning Pharmaceutical Factory (Yinchuan, China); both were dissolved in normal saline. The drugs were administered i.p. in a volume of 0.1 ml/10 g, with the exception of aspirin, which was administered i.g. in a volume of 0.2 ml/10 g. Intracerebroventricular administration was performed in a volume of 10 µl for each mouse. The drug solutions were prepared immediately prior to initiation of the experiment. The doses of OSC (10, 40 and 80 mg/kg) were selected based on the results of the preliminary experiments. A single dose of aspirin (400 mg/kg) was chosen according to a previous study conducted in our laboratory (400 mg/kg) in the formalin model (8), and various doses of morphine (5, 20, 40 and 50 mg/kg) were chosen according to previous studies (7,9).

Warm water tail-flick test. The warm water tail-flick test is a common method used to immobilize the animal in a restrainer with its tail extending through the hole. The lower 2/3 of the tail was immersed in hot water maintained at a constant temperature of 50±0.5°C. Changes in nociception were determined by the changes in the latency between the tail immersion and withdrawal from the hot water bath (10). The animals were immobilized in the tube briefly (25-30 sec) during the tail-flick measurements. To minimize tissue injury caused by exposure to heat stimulus, a cut-off time of 15 sec was applied. Pretreatment latencies were determined twice with an interval of 10 min prior to drug administration to obtain a stable pre-drug response (baseline withdrawal latency). Mice with a basal tail-flick latency of 2-5 sec were used. Animals with a significantly different baseline value from that of the control mice were not included in the study. The reaction time was recorded for OSC (10, 40 and 80 mg/kg) or morphine (40 mg/kg). Control animals were administered a similar volume of 0.9% NaCl solution (10 ml/kg). Following drug administration, tail immersion latency was measured at 15, 30, 60, 90 and 120 min post-injection. The tail immersion response was expressed as a percentage of basal latency. Percentage analgesia was calculated using the formula: analgesia (%) = [(post-drug latency - pre-drug tail withdrawal latency)/(15 sec - pre-drug tail withdrawal latency)] x100.

Acetic acid-induced abdominal constriction. Abdominal constriction was induced by i.p. injection of acetic acid (0.6%), which consisted of abdominal muscle constriction, together with stretching of hind limbs, and was performed according to previously described methods (13). The animals were pretreated with OSC (10, 40 and 80 mg/kg) or morphine (20 mg/kg). Control mice were administered a similar volume of normal saline (10 ml/kg). A lethal intravenous dose of OSC (LD$_{50}$) was 250.37 mg/kg in mice (12). The time of latency was determined at 15, 30, 60, 90 and 120 min post-injection. A latency period (cut-off) of 60 sec was evaluated as complete analgesia.

Percentage analgesia was calculated using the formula: analgesia (%) = [(post-drug latency - pre-drug latency)/ (60 sec - pre-drug latency)] x100.

Formalin-induced pain test. This procedure was performed according to previously described methods (14-16). Twenty microliters of 0.2% formalin solution (0.92% formaldehyde) dissolved in saline solution were injected under the paw surface of the right hind paw. The amount of time spent licking the injected paw was considered to be indicative of pain. The initial nociceptive time usually peaked 0-5 min (first phase) and 10-60 min (second phase) after the formalin injection. These peaks represented the tonic and inflammatory pain response, respectively. The animals were treated with OSC (10, 40 and 80 mg/kg), morphine (5 mg/kg), or aspirin (400 mg/kg). Control animals were administered a similar volume of 0.9% NaCl solution (10 ml/kg). The drugs were administered 60 min prior to formalin injection. Following intraplantar injection of formalin, the animals were immediately placed in a glass
cylinder with a diameter of 20 cm. The time spent licking the injected paw was considered to be indicative of pain and was timed using a chronometer.

**Intracerebroventricular injections.** Animals were administered a unilateral intracerebroventricular injection (2.0 mm caudal and 2.0 mm lateral with respect to bregma and -2.5 mm ventral from the skull surface) (17). OSC (0.25-4 mg/kg) and morphine (2 mg/kg) were dissolved in saline (0.9% NaCl), in a volume of 10 µl/mouse (for ~10 sec). Control mice were administered the same volume of vehicle. Following the intracerebroventricular injection, the animals were tested using the tail immersion test described above.

**Immunohistochemistry.** Mice were treated i.p. with 80 mg/kg of OSC, and the control mice were administered a similar volume of 0.9% NaCl solution (10 ml/kg). The drugs were administered 45 min prior to perfusion. Animals were deeply anesthetized i.p. in a volume of 0.1 ml/10 g pentobarbital sodium (1%) and were perfused with 40 ml of saline for 2 min, followed by 100 ml of 4% paraformaldehyde (Sigma, St. Louis, MO, USA) at 4°C for 20 min [0.01 mol/l phosphate-buffered saline (PBS), pH 7.4]. Following fixation, the brain was removed and post-fixed in the same fixation solution for 48 h. For immunohistochemical analysis, brain sections were mounted onto slides, air-dried, dehydrated in alcohol (differentiation time was evaluated under the microscope), cleared in xylenes and cover slipped.

Brain sections (5 µm) were treated with 3% H2O2 to quench endogenous peroxidase activity, then washed in PBS 3x5 min, and transferred to 0.1 M citrate buffer in 5% normal bovine serum albumin (BSA) for 20 min at room temperature. The sections were then incubated with GABAARα1 receptor primary antibody at 4°C for 24 h, which was an anti-rabbit polyclonal antiserum (1:100 in PBS; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). As a negative control, additional sections were treated in a similar manner although the primary antibody was omitted. Subsequent to incubation with primary antibody, sections were washed in PBS 3x5 min. The sections were incubated at 37°C for 2 h with the secondary antibody (rabbit anti-goat IgG) and avidin-biotin complex (ABC). The specimens were then washed as described above and visualized using 3,3′-diaminobenzidine (DAB; Wuhan Boster Biological Technology, Ltd., Wuhan, China).

**Image analysis and counting.** Following immunostaining, brain specimens were thoroughly rinsed in water and then examined under a light microscope. Digital images of GABAARα1 receptor neurons were captured using an Olympus BH-2 microscope (Olympus Optical Co., Ltd., Tokyo, Japan), with an attached digital microscope camera and a personal computer. Cells stained positive for GABAARα1 receptors were marked on a computer screen (magnification, ×400) and counted in every two random visual fields/well, in the cerebral cortex and hippocampus of the brain specimens. Up to four stained sections/mouse were obtained from the segment and analyzed using the Motic Images Advanced 3.2 software developed by Motic China Group Co., Ltd. (Xiamen, China). The number of stained neurons/section were calculated [means ± standard deviation (SD); one-way ANOVA]. *P*<0.05 was considered to indicate statistically significant intergroup differences.

**Statistical analysis.** The data are presented as the means ± SD, except the mean ED50 values, which were reported as geometric means accompanied by their respective 95% confidence limits. Data were analyzed using one-way analysis of variance (ANOVA) or t-test, with between-group comparisons for drug treatment and within-group comparisons for time. *P*<0.05 was considered to indicate a statistically significant difference.

**Results**

**Warm water tail-flick test.** An injection of 80 mg/kg OSC (i.p.) increased the tail-curving latency in the warm water tail-flick test and a maximal inhibition of 25.46% was observed. This effect persisted for up to 120 min. Under similar conditions, morphine 40 mg/kg (i.p.) caused a significant increase (the maximal inhibition was 85.05% and persisted for ≥120 min) in the latency in the tail immersion assay (Fig. 1).

**Hot-plate test.** Compared with the control group, i.p. injection of OSC at doses of 40 and 80 mg/kg or morphine at a dose of 20 mg/kg caused a significant increase in the response latency in the hot-plate test (14.57±4.81 sec in the control group, 24.20±12.67 sec in the OSC-treated groups, and 60.00 sec in the morphine-treated group). The analgesic effect persisted for ≥120 min post-injection (Fig. 2).

**Acetic acid-induced abdominal constriction.** OSC administered i.p. at 10, 40 and 80 mg/kg 15 min prior to the test, causing a significant dose-dependent inhibition of acetic acid-induced abdominal constriction, with a maximal inhibition of 47.02% at 80 mg/kg. Morphine (50 mg/kg) and aspirin (400 mg/kg) induced an anti-nociceptive response with a maximal inhibition of 99.56 and 93.38%, respectively (Fig. 3).

**Formalin-induced pain test.** In the formalin test, pretreatment (60 min) with i.p. injection of different doses of OSC (10, 40

Figure 1. Latency changes of OSC in the warm water tail-flick test in mice. Animals were treated i.p. with saline, morphine or OSC (10, 40 and 80 mg/kg). Animals/group, n=10. *P*<0.05, **P*<0.01 vs. the control group. Data are presented as the means ± SD.
XU et al: OSC INDUCES ANTI-NOCICEPTION AND INCREASES THE EXPRESSION OF GABA\(_{\alpha1}\) RECEPTORS

The present study showed that i.p. treatment with OSC induces a dose-dependent and significant anti-nociception in models of chemical nociception, i.e., acetic acid-induced abdominal constriction and formalin-induced licking response. Moreover, it was found that OSC induces significant anti-nociceptive effects in mice when assessed using the warm water tail-flick and hot-plate tests.
The warm water tail-flick test is a model of nociceptive pain produced via thermal stimuli of short duration (i.e., phasic pain), which is commonly used to measure responses to somatic stimulation and observe whether the drug has a central anti-nociceptive effect, since the tail-flick is mainly a reflex to the spinal cord. This test measures changes in the pain threshold that produces a tail-flick response. The response is mainly a spinal reflex, which is modulated by supraspinal mechanisms (18). The results of the effect on the warm water tail-flick test increased the response latency time by 25.46%. The result was less active compared to that caused by morphine in the same test, while the effectiveness of OSC in the warm water tail-flick test showed that OSC acts on the central nervous system, particularly on the spinal cord, which was modulated by supraspinal mechanisms. However, further investigation is needed to fully elucidate the mechanism of action.

The hot-plate test is a common sensorimotor task that identifies drugs with a supraspinal analgesic effect, such as opioid-derived analgesics (19). In other words, analgesic agents play a primary role in the spinal medulla and/or higher central nervous system levels or by an indirect mechanism (20). Therefore, analgesic drugs, which were known to be selectively effective against painful thermal stimuli, act on the central nervous system rather than the peripheral system (21).

The present study demonstrated that OSC (80 mg/kg, i.p.) was effective in the hot-plate test, where the response latency time was increased by 26.60%. The effectiveness of OSC in the hot-plate test showed that OSC acts on the central nervous system, which was in agreement with the results obtained by the warm water tail-flick test. The anti-nociceptive effect of OSC is associated with supraspinal components as shown by the warm water tail-flick and the hot-plate tests, respectively. The results of these tests indicated that OSC has a central anti-nociceptive effect as demonstrated by the increased response time of the mice in the hot-plate test, and also by the prolonged delay in reaction when mice were subjected to a nociceptive stimulus during the warm water tail-flick test.

Intracerebroventricular administration is a common method to determine whether a drug has a central analgesic effect. The mechanism by which OSC produces systemic, spinal or supraspinal anti-nociception in mice has yet to be

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<th>Group</th>
<th>Cerebral cortex</th>
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<td>169±49&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup>P<0.01 vs. the control group. Animals were treated i.p. with 80 mg/kg of saline or OSC. Animals/group, n=10. Data are presented as the mean ± SD.

Figure 6. Neurons in the brain specimens of mice stained positive for GABA<sub>A,α1</sub> receptors following immunostaining. Photomicrographs of GABA<sub>A,α1</sub> receptors in (A and B) the cerebral cortesxes and hippocampal regions of control mice (magnification, x400) and (C and D) the cerebral cortesxes and hippocampal regions of the experimental mice (magnification, x400). Arrows point to typical deposited GABA<sub>A,α1</sub> receptor immunoreactive neurons with plasma membrane labeling.
fully elucidated. Nevertheless, OSC (1 and 4 mg/kg, i.c.v.; 1/10 of the dose used in i.p. administration) produced anti-nociceptive effects in mice when assessed using the warm water tail-flick test. These results suggest that the anti-nociceptive effects of OSC occur through a central mechanism.

The formalin-induced pain test is a valid and reliable model of nociception and is sensitive to various classes of analgesic drugs. It assesses the way an animal responds to moderate, continuous, long-lasting pain generated by injured tissue (20). The formalin-induced pain test produced a distinct biphasic response, these two different phases have different mechanisms of action in the test. The first phase reflected direct chemical stimulation, which appeared to be predominantly caused by the nociceptive afferent fibers, mainly C-fibers, and the release of substance P (22). This phase may be inhibited by centrally acting analgesic drugs. The second phase may be associated with the release of inflammatory mediators locally, such as prostaglandins, serotonin, histamine and bradykinin, and with enhanced synaptic transmission in the spinal cord neurons (23). Therefore, this test could be used to clarify the potential mechanism of a proposed analgesic. Drugs, such as morphine, act primarily on the central nervous system and inhibit the first and second phases equally, while peripherally acting drugs such as aspirin inhibit only the second phase (24). The i.p. pre-administration of OSC (40 and 80 mg/kg) exerted dose-dependent and significant anti-nociceptive effects when assessed against neurogenic pain (first phase) and inflammatory pain (late phase) caused by intraplantar injection of formalin in mice. Furthermore, the results of this test were in agreement with those obtained in thermal behavioral models of nociception, whereas OSC was also demonstrated to have central anti-nociceptive effects.

The acetic acid-abdominal constriction test is generally used for evaluating peripheral analgesic activity (25,26). Previous studies have shown that acetic acid acts indirectly by inducing the release of endogenous mediators that stimulate the nociceptive nerves sensitive to opioids and non-steroidal anti-inflammatory drugs (NSAIDs) (27). Moreover, abdominal constriction induced by acetic acid is usually used for screening synthetic and natural compounds (11). In this behavioral model, an i.p. injection of OSC (10, 40 and 80 mg/kg) caused a dose-dependent inhibition of acetic acid-induced abdominal constriction. The maximum inhibition observed was 47.02%. Based on these results, this test indicated that OSC treatment has anti-nociceptive effects on central mechanisms.

γ-aminobutyric acid (GABA) is the major inhibitory transmitter in the adult mammalian central nervous system and has been reported as the principle neurotransmitter of the circadian system. It is also found in >50% of neurons in the central nervous system (28). GABA neurons and synapses are widely distributed throughout the peripheral and central nervous systems, which are primarily located on the outer layer of the cerebral cortex and the hippocampus (29). Moreover, accumulating evidence suggests that the GABAergic system is important in the pre-synaptic inhibition of primary afferents (primary afferent depolarization), thus sensory transmission, motor activity and modulating nociception. Motor activity on pre- and post-synaptic levels (30).

The present study indicated that the anti-nociceptive effect of OSC resulted from the activation of the GABA<sub>A</sub> receptor. Most of the synaptic inhibitory action of GABA is mediated by GABA<sub>A</sub> receptors, which constitute hetero-oligomeric chloride channels encoded by a family of ≥16 known different subunit genes including 6α, 3β, 3γ, δ, ε, θ and π subunits (31), among which GABA affinity is mainly regulated by a subunit and plays an important pharmacological role (32,33). Putative ligands and drugs are known to interact at one of the major sites associated with the GABA<sub>A</sub> receptors and to modulate GABA-gated chloride ion conductance positively or negatively. The increased chloride conductance regulates the membrane potential towards the reversal potential of the chloride ion which inhibits the firing of new action potentials and initiates inhibitory postsynaptic potential (IPSPs) in order to produce an anti-nociceptive effect (34,35). Molecular studies have identified the GABA<sub>A</sub> receptor as a macromolecular complex consisting of ≥5 membrane-spanning subunits. Five subunit types have been described, α, β, γ, δ and ϵ (33). A limited number of subunits is present in the mammalian central nervous system, mainly α1βγ2. Different subunit isoforms (α1-6, β1-4, γ1-4, δ, ϵ, θ) give rise to a considerable diversity of GABA<sub>A</sub> receptors (36,37) that are differentially expressed in the brain and localized in different cell types and subcellular areas (38). Subtypes of GABA<sub>A</sub> receptors are important in the development of drugs which selectively influence this transmitter system (39). The differential expression of GABA<sub>A</sub> receptor subtypes between the superficial layers of the dorsal horn and projection neurons may be of particular relevance within the framework of the ‘gate control’ theory (40,41), according to which pain perception has been suggested to be modulated in the substantial gelatinosa (lamina II), which functions as a gate controlling impulse transmission from primary afferents to projecting neurons. Moreover, the α1 subunit acts particularly on the function of GABA<sub>A</sub>α1 receptors (42), which are compared with controls in the cerebral cortex and the hippocampal regions in mice.

In conclusion, OSC induces systemic anti-nociception in mice when assessed using some classical behavioral tests, such as thermal stimuli (hot-plate and warm water tail-flick tests) and chemical stimuli (acetic acid and formalin-induced pain). The results obtained in the present study indicate that OSC has significant analgesic effects that may be crucial in the central and peripheral nervous systems. This study also showed that OSC produced possible alterations in the expression of GABA<sub>A</sub>α1 receptors in the central nervous system. However, the mechanism by which OSC induces anti-nociception in the models of nociception has yet to be fully elucidated. Investigations on the potential involvement of the GABA receptors in the analgesic action of OSC are currently in progress.

Acknowledgements

The study was supported by the National Natural Science Foundation of China (grant nos. 30960506 and 81160524), the Key Scientific Research Projects of Ningxia Health Department (2012059), the Scientific Research Starting Foundation for Special Talent (XT2012015) and Ningxia Education Department (NGY2012055). We are indebted to the staff in the Animal Center and the Science and Technology Centre for providing assistance during this study. The authors would like to thank Dr Dingfeng Su, Professor Wannian...
Zhang, Miss Jie Wang, Miss Shujing Wang and Yan Zhang who provided technical support during the implementation of this study.

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