academicJournals

Vol. 8(30), pp. 781-785, 15 August, 2014
DOI: 10.5897/AJPP2013.3989
Article Number: 17DB60946929
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Study of visceral antinociceptive potential of bee *Apis mellifera* venom

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Received 30 December, 2013; Accepted 16 July, 2014

Pain is one of the most common reasons for patients to seek medical care. Bee *Apis mellifera* venom (AMV) has traditionally been used to treat inflammatory diseases and the alleviation of pain. Herein, we aimed to investigate the visceral antinociceptive potential of *A. mellifera* bee venom and its possible mechanism of action. Acetic acid-induced writhing assay was used in mice to determine the degree of visceral antinociception. Visceral antinociceptive activity was expressed as the reduction in the number of abdominal constrictions. Mice received an intraperitoneal injection of acetic acid after administration of AMV (0.08 or 0.8 mg/kg; intraperitoneally (i.p.)). In mechanistic studies, separate experiments were realized to examine the role of α2-receptors, nitric oxide, calcium channels, K^+_{ATP} channel activation, TRPV1 and opioid receptors on the visceral antinociceptive effect of AMV (0.8 mg/kg), using appropriate antagonists, yohimbine (2 mg/kg), L-NG-Nitroarginine methyl ester (L-NAME, 10 mg/kg), verapamil (5 mg/kg), glibenclamide (5 mg/kg), ruthenium red (3 mg/kg) or naloxone (2 mg/kg). AMV presented visceral antinociceptive activity in both doses tested (0.08 and 0.8 mg/Kg). Visceral antinociceptive effect of AMV was resistant to all the antagonists used. Mice showed no significant alterations in locomotion frequency, indicating that the observed antinociception is not a consequence of motor abnormality. Although AMV efficient diminished the acetic acid-evoked pain-related behavior, its mechanism is unclear from this study and future studies are needed to verify how the venom exerts its antinociceptive action.

Key words: *Appis mellifera venom*, antinociceptive, visceral pain.

INTRODUCTION

Pain is one of the most common reasons for patients to seek medical care. Current analgesics fall into two major classes: non-steroidal anti-inflammatory drugs (NSAIDs) and opioids, both of which have critical liabilities and limitations. Opioids are tightly controlled because of their addictive effects and other serious side effects (McQuay,

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1999). Gastrointestinal side effects and effectiveness only in cases of mild to moderate pain limit NSAIDs use (Frölich,1997; Kingery, 1997). Pain control is an important medical problem. Much research has gone towards the identification of agents that can relieve chronic pain with out unwanted side effects.

Bee *Apis mellifera* venom (AMV) has traditionally been used to treat inflammatory diseases and the alleviation of pain (Lee et al., 2005; Son et al., 2007). Various components of AMV have been identified, but there is not a consensus about their concentration. The predominant component of the dried AMV is melittin (40 to 50%), a peptide of 26 amino acid residues. Moreover, many components with much lower concentration have been identified including hyaluronidase, acid phosphatase, apamin, mast cell degranulating peptide, adolapin, secapin, minimine, phospholipase A2 (PLA2) histamine, glycosidase, tertiapin, dopamine and carbohydrates (Gauldie et al., 1976; Habermann, 1972; Nelson and O'Connor, 1968; Vetter and Visscher, 1998; Vetter et al., 1999). These AMV components were reported to have a wide variety of pharmacological properties (Lariviere and Melzack, 1996).

An animal study suggested that melittin, the main component of whole AMV, is a likely candidate for the anti-inflammatory and antinociceptive effects observed in AMV treatment (Lee et al., 2004). According to Li et al. (2010), the mechanism of the antinociceptive effect of melittin is unknown, but several studies revealed that this effect may be partially explained by the following findings: the repeated application of capsaicin is followed by a prolonged period of hypoalgesia (Nolano et al., 1999); the initial nociceptive effect of melittin may function similarly to capsaicin by increasing the pain thresholds and desensitizing the nociceptor; the α2-adrenoceptor is involved in the anti-nociceptive effect of whole bacterial vaginosis (BV) (Kwon et al., 2001a,b,c); and the antinociceptive effect is dependent on the site-specific acupoint (Oliver et al., 2006).

It was demonstrated that subcutaneous bee venom injection produces a robust antinociceptive effect in several different rodent models of both somatic and visceral pain (Known et al., 2001a,b,c). These preliminary data imply that bee venom is useful for the management of both somatic and visceral pain, but it is not clear which constituent is responsible for its antinociceptive effect.

The effects induced by AMV and its components in experimental models of nociceptive and inflammatory pains have been reported (Merlo et al., 2011). These studies demonstrated that AMV antinociception involves the action of different components and does not result from non-specific activation of endogenous antinociceptive mechanisms activated by exposure to noxious stimuli. In the present study, we aimed to investigate the visceral antinociceptive potential of *A. mellifera* bee venom and its possible mechanism of action.

MATERIALS AND METHODS

Animals

Swiss mice (20 to 25 g) were used. Experimental groups consisted of 6 animals per group. They were housed at 22 ± 2°C under a 12 h light/12 h dark cycle and had free access to standard pellet diet (Purina chow) and tap water. Each animal was used only once for experimentation. The experimental protocols were in accordance with the ethical guidelines of the Brazilian Council for the Control of Animal Experiments (CONCEA) and were approved by the Animal Research Ethics Committee of the Federal University of Ceará, under entry #90/2011.

Venom

The bee AMV was donated by Professor Marcos Hiraki Toyama from Univerdidade Estadual do Litoral Paulista (UNESP). For the tests, it was prepared a stock solution (1 mg/ml) of the venom in phosphate buffered saline (PBS), pH 7.4, sterile.

Acetic acid-induced visceral nociception

Abdominal constrictions were induced by intraperitoneal injection of acetic acid (0.6%). The animals were pretreated with AMV (0.08 or 0.8 mg/kg, intraperitoneally (i.p.)), indomethacin (10 mg/kg, i.p.) or vehicle (PBS 10 ml/kg, i.p.) 30-min prior to acetic acid injection. After the challenge, each mouse was placed in a separate glass funnel and the number of contractions of the abdominal muscles, together with stretching, was cumulatively counted over a period of 20 min. Antinociceptive activity was expressed as the reduction in the number of abdominal contractions, comparing the control animals with the mice pretreated with AMV.

In order to verify the possible involvement of noradrenergic, nitrergic, calcium, K^+_{ATP}, $TRPV_1$ and opioid mechanisms in the effects of AMV, the animals were treated with yohimbine (2 mg/kg, i.p), L-NAME (10 mg/kg, i.p), verapamil (5 mg/kg i.p.), rutehnium red (3 mg/kg subcutaneously (s.c.)), glibenclamide (5 mg/kg i.p.) or/and naloxone (2 mg/kg i.p.) 30 min before the administration of the AMV (0.8 mg/kg).

Evaluation of the motor activity

The motor coordination and performance of each mouse was evaluated in a rota-rod apparatus, 30 min after the intraperitoneal treatment with AMV (0.08 or 0.8 mg/kg), vehicle (PBS, 10 ml/kg) or Diazepam (1 mg/kg). This apparatus has a 2.5 cm diameter bar, divided into six parts, and it is placed at a height of 25 cm, rotating at 7 rpm. Latency to fall from the rotating bar during a 1 min period was registered.

Statistical analysis

The results are presented as the mean ± standard error of mean (SEM) of 8 animals per group. Statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Tukey *post hoc* test for multiple comparisons. *P*-values less than 0.05 ($p < 0.05$) were considered as indicative of statistical significance.

RESULTS

In acetic acid-induced writhing test, AMV suppressed the

mean number of writhes, when compared with vehicle-treated control group (Table 1). These were in the order of 52.17 ± 5.05, 24.67 ± 4.59 and 1.17 ± 0.83, respectively, for the control and AMV at the tested doses of 0.08 and 0.8 mg/kg. The positive control group treated with indomethacin (10 mg/kg, i.p.) also manifested significantly diminished number of stretches (26.00 ± 3.90).

The acetic acid-induced visceral nociception was not significantly blocked by yohimbine, L-NG-Nitroarginine methyl ester (L-NAME), verapamil, rutehnium red, glibenclamide or naloxone. Their combinations with AMV failed to modify AMV antinociception (Table 2).

At the doses tested (0.08 and 0.8 mg/kg), AMV failed to produce any significant effect on motor coordination on rota-rod in mice. In contrast, diazepam (1 mg/kg, i.p.) significantly lowered the motor coordination (data not shown).

DISCUSSION

In this present study, it wad observed that the acetic acid-evoked visceral nociceptive behavior was significantly attenuated in mice pretreated with AMV. The acetic acid-induced writhing is a standard test for visceral pain, sensitive to opiates as well non-opiates analgesics (Steranka et al., 1987).

The α_2-adrenoceptor agonist has been shown to induce antinociceptive effect in the experimental model of formalin-induced colitis in rats and reduce visceral hypersensitivity in clinical settings (Lima-Júnior et al., 2006; Miampamba et al., 1992). Therefore, a possible involvement of α_2-adrenoceptors in the antinociceptive effect of AMV, using the antagonist yohimbine was investigated. Yohimbine could not reverse the antinociception produced by AMV.

Know et al. (2005) reported that a water soluble fraction (BVAF3, <10 kDa) from bee venom selectively activates the descending adrenergic system through α_2-adrenergic receptors and that activation is associated with the antinociceptive effect observed in the abdominal visceral pain model. BVAF3 produced a significant antinociceptive effect similar to that observed following injection of AMV. This discrepancy may be due to differences in experimental conditions and the route of AMV delivery.

This study also examined the possible participation of NO/cGMP/K^+_{ATP} pathway. Pretreatments with a K^+_{ATP} blocker, glibenclamide or the non-specific NOS inhibitor, L-NAME, could not reverse the antinociceptive effect of AMV, suggesting that AMV antinociception do not result from the modulation of K^+_{ATP} currents. Besides this, verapamil, blocker of Ca^{2+} channels, could not reverse the antinociception produced by AMV.

It is currently accepted that an endogenous opioid analgesic system is present at peripheral level (Smith, 2008; Alves et al., 2012), and most of opioid antinociceptive effects are mediated via activation of opioid receptors (Stein and Lang, 2009) and opioid receptors have been identified on peripheral terminals of afferent nerves, which can be the sites of the intrinsic modulation of nociception (Vadivelu et al., 2009). Attempts to mimic or augment such peripheral analgesia may potentially lead to analgesic effects in the absence of the central adverse effects caused by opioids.

The antinociceptive effect of AMV was not modified by a non-selective $TRPV_1$ antagonist ruthenium red. Roh et al. (2010) found that the destruction of capsaicin-sensitive primary afferents by resiniferatoxin (RTX) pre-treatment selectively decreased AMV-induced spinal Fos expression, but did not affect AMV-induced antinociception in the formalin test. They suggested that subcutaneous AMV stimulation of the Zusanli point activates central catecholaminergic neurons via capsaicin-insensitive afferent fibers without induction of nociceptive behaviour or by naloxone, a non-selective μ-opioid receptor antagonist, suggesting that there is no participation of an opioid mechanism. Kwon et al. (2001a,b,c) also showed that AMV acupoint stimulation can produce visceral antinociception that is not associated with naloxone-sensitive opioid receptors

The demonstration of the antinociceptive activity of AMV is in line with the demonstrations that AMV inhibits the nociceptive response induced by acetic acid in mice (Kim et al., 2007). In this study, AMV was injected into specific points of acupuncture. As the doses (0.06 to 6 mg/kg) used by these authors are in the range of those used in the present study, it is suggested that the antinociceptive effect induced by AMV is not related to injection into a specific point of acupuncture, but results from a systemic action.

Motor deficits may create confounds in studies in which antinociception is measured. To clarify if the analgesic effect is not a result of motor deficits, the effects of AMV on rota-rod test was assessed, that is, a classical model for screening central nervous system actions providing information on myorelaxant activity. AMV did not present myorelaxant activity as demonstrated in the rota-rod test that measures grip strength, suggesting that the AMV antinociception observed in this investigation is not exerted through induction of sedation. According to Heneine et al. (2007), AMV lacks analgesic action in test of hot-plate suggesting that its analgesic effect is only peripheral but not central.

It seems that antiinflammatory property of AMV may contribute to its antinociceptive effect. Recent studies have shown that bee venom treatment can induce a significant antiinflammatory response mediated by inhibition of inflammatory mediators, similar to what is achieved with the administration of non-steroidal anti-inflammatory drugs (Miampamba et al., 1992). In experimental rheumatoid arthritis, bee venom treatment significantly decreased the expression of inflammation-related

Table 1. Visceral antinociceptive activity of *Apis mellifera* venom.

Group	Dose (mg/kg)	Number of animal constrictions/20
Control	-	52.16 ± 5.05
Indomethacin	10	26.00 ± 3.90*
AMV	0.08	24.67 ± 4.59*
-	0.8	1.16 ± 0.83***

*p < 0.05 and ***p < 0.001 compared to the vehicle-administered control group (Control).

Table 2. Effect of yohimbine and L-NAME on visceral antinociception induced by of *Apis mellifera* venom.

Group	Dose	Number of animal constrictions/20
Control	-	40.166 ± 3.19
AMV	0.8	1.54 ± 0.63***
Yohimbine	2	44.00 ± 4.58
L-NAME	10	46.66 ± 3.74
Verapamil	5	46.16 ± 3.10
Glibenclamide	5	45.66 ± 3.57
Ruthenium red	3	28.83 ± 8.60
Naloxone	2	19.40 ± 2.13
Yohimbine+AMV	2 + 0.8	8.00 ± 3.15***
L-NAME+AMV	10 + 0.8	4.16 ± 2.22***
Verapamil+AMV	5 + 0.8	17.56 ± 7.17**
Glibenclamide+AMV	5 + 0.8	11.33 ± 9.94*
Ruthenium red+AMV	3 + 0.8	1.83 ± 0.65***
Naloxone+AMV	2 + 0.8	8.00 ± 3.74**

*p < 0.05 compared to the vehicle-administered control group (Control).

cytokines such as cyclooxygenase-2 (COX-2), phospholipase A2 (PLA2), tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, nitric oxide (NO) and reactive oxygen species (ROS) (Son et al., 2007) via NF-κB (Kim et al., 2013). It is known that after intraperitoneal administration of acetic acid, inflammatory reactions develop in the peritoneum (Clementi et al., 1999).

According to Know et al. (2005), the soluble fraction (BVF3) contains several small peptides including melittin, apamin, mast cell degranulating (MCD) peptide and minimine. The authors believed that BVAF3-induced antinociception may be produced by the interaction of several constituents of BVAF3 rather than by one specific antinociceptive component.

Conclusion

In conclusion, although AMV efficient diminished the acetic acid-evoke pain-related behaviour, its mechanism is unclear from this study and future studies are needed to verify how the venom exerts its antinociceptive action. Since AMV contains a wide number of constituents, attempts to investigate them individually are slightly challenging, because synergistic interaction among the AMV components may occur.

However, in order to gain better insight into the mechanisms of action of AMV, further efforts, including molecular biology methods, are necessary in the future.

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