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Antinociceptive effect of astragalosides and its mechanism of action

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KEY WORDS astragalosides; *Astragalus membranaceus*; morphine; naloxone; *L*-arginine; nitric oxide

ABSTRACT

AIM: To study the effect and mechanism of astragalosides (AST) related to the antinociceptive activity. **METHODS**: The standardized formalin test was performed to induce the direct stimulation of nociceptors followed by inflammatory process in the Kunming strain mice. The involvement of opioid and nitric oxide was studied by subcutaneous injection of morphine with/without naloxone 30 min before formalin test, or peritoneal injection of *L*-arginine with/without *L*-NAME 20 min before formalin. **RESULTS**: AST 20, 40, and 80 mg/kg significantly lowered pain score of the second phase of formalin response as compared with control group ($P < 0.01$). The maximum analgesic effect of AST 40 mg/kg was found at 4 h after the administration of AST (34.4 % inhibition at the second phase). Injection of morphine 5 mg/kg significantly inhibited pain response of both phases ($P < 0.01$) and this was reversed by naloxone 2 mg/kg ($P < 0.01$). However, naloxone did not alter the effect of AST on the second phase. Antinociceptive effect of AST 40 mg/kg was partially blocked by *L*-arginine 400 or 800 mg/kg ($P < 0.01$). **CONCLUSION**: AST has an antinociceptive effect on formalin test in mice that is not mediated by the endogenous opioid system but related to its inhibitory effect on the production of NO.

INTRODUCTION

Astragalus is one of the valuable Chinese tonic herbs. Astragalosides, extracted from the root of *Astragalus membranaceus*, is the active compound, in addition to *astragalus* polysaccharides. Previous studies from our laboratory showed that astragalosides possesses anti-inflammatory properties[1]. However, it is unknown whether astragalosides has an antinociceptive effect by either direct stimulation of the nociceptors or the inflammatory process and if it does, what the mechanism could be.

The pain produced during the inflammatory process may be related to prostanoids (PGE_2)[2], nitric oxide (NO)[3], and other substances. A pilot study from our laboratory demonstrated that astragalosides had analgesic effect in mice (data not published) and we hypothesized that such analgesic effect may also exist in the inflammatory process.

The present study was therefore designed to investigate the possible antinociceptive effect of astragalosides during formalin test that produced a two-phase response, ie, the direct stimulation of nociceptors followed by the production of inflammatory mediators. Furthermore, we studied the mechanism of such effect with regard to the opioid system and the nitric oxide release.

MATERIALS AND METHODS

Mice Kunming strain mice (aged 6 – 8 weeks, ♂, weighing 22 g ± 2 g) were supplied by Experimental Animal Department, Anhui Institute of Medicine (Grade Ⅱ, Certificate № 96001).

Materials Astragalosides (AST) was provided by Jiangsu Institute of Materia Medica (Nangjing, China), and dissolved in 1 % sodium carboxymethylcellulose (CMC-Na). The content of AST was 100 %. Formalin was purchased from Shanghai Pacific Chemical Manufacturer. *L*-NAME was obtained from Sigma. *L*-arginine, morphine, and naloxone were bought from Shanghai Lizhu Dongfeng Biological Technique Co, Dongbei № 6 Pharmaceutical Factory, and Shanghai № 12 Pharmaceutical Factory, respectively.

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Formalin test The formalin test was performed according to the method of Hunskaars *et al*[4]. Formalin (1 %, 20 μL) was injected intraplantarly into the right hind paw of mice. Behavioral responses were recorded, the first phase was 0 – 5 min after the injection of formalin, and the second phase was 20 – 30 min after the injection. A modified Dubuission method[4,5] was used to evaluate the pain response as follows. Score 0: walking as usual; Score 1: limping, not moving with the injected paw on the floor; Score 2: raising the injected paw; Score 3: paw licking or gnawing. The duration of different behavior described above was recorded separately. The pain score was calculated by the following formula: $(0t_1 + 1t_2 + 2t_3 + 3t_4)/(t_1 + t_2 + t_3 + t_4)$, t_1, t_2, t_3, and t_4 are the duration time for score 0, 1, 2, and 3, respectively.

Experimental protocols Mice were randomly divided into 5 – 7 groups ($n = 10$ in each group). The following protocols were used for the experiments.

(1) Time course of antinociceptive effect of AST on formalin test There were 6 groups. In 5 groups, AST 40 mg/kg was orally given 1, 2, 3, 4, and 5 h before the formalin injection, respectively. In the 6th group which served as control, 1 % CMC-Na solution was given.

(2) Effect of AST on formalin test There were 5 groups. In three groups, AST 20, 40, and 80 mg/kg was orally given respectively 4 h before the formalin injection whereas in the control group 1 % CMC-Na solution was given. In the 5th group, as the positive control, morphine (5 mg/kg, sc) was injected 30 min before the formalin injection.

(3) Relationship between antinociceptive effect of AST and endogenous opioid system There were 5 groups. In two groups, AST 40 mg/kg was given, and in other two groups, morphine 5 mg/kg was given as in the second protocol. In addition, in one of each two groups, naloxone (2 mg/kg, sc) was also injected 45 min before the formalin injection. In the control group, 1 % CMC-Na solution was orally given.

(4) Relationship between antinociceptive effect of AST and NO system There were 7 groups. In three groups, AST 40 mg/kg was given as in the second protocol and in two of these three groups, *L*-arginine (*L*-Arg) 400 or 800 mg/kg was also injected intraperitoneally (ip) 20 min before the formalin injection. In other three groups, *L*-NAME 37.5 mg/kg was injected ip 15 min before the formalin injection and in two of these three groups, *L*-Arg was given as above. In the control group, 1 % CMC-Na solution was orally given.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared by the student's *t*-test.

RESULTS

Time course of antinociceptive effect of AST AST 40 mg/kg had no effect on the first phase of the formalin test 1 – 5 h after administration, while it inhibited the second phase of the response. The inhibition of the pain score at the second phase was 17.0 % at 1 h, 21.6 % at 2 h, 28.9 % at 3 h, 34.4 % at 4 h, and 26.7 % at 5 h, respectively. As shown above, the maximum analgesic effect (34.4 % inhibition at the second phase) was demonstrated in the mice in which AST was given 4 h before the formalin injection (scored 1.26 ± 0.13 *vs* 1.92 ± 0.10 in control, $P < 0.01$).

Effect of AST on formalin test All applied doses of AST lowered the pain score of the second phase of the formalin response. However, it had no effect on the first phase. In contrast, injection of morphine markedly inhibited the pain response of both phases (Tab 1).

Tab 1. Antinociceptive effect of AST evaluated by the formalin test in mice. $n = 10$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* **control.**

Group	Dose /mg·kg^{-1}	Pain score		Inhibition/%	
		1st phase	2nd phase	1st phase	2nd phase
Control	–	1.99 ± 0.26	1.93 ± 0.11	–	–
AST	20	1.97 ± 0.12	1.63 ± 0.12^c	1.0	15.5
	40	1.87 ± 0.14	1.27 ± 0.11^c	6.0	34.2
	80	1.89 ± 0.14	1.25 ± 0.11^c	5.0	35.2
Morphine	5	1.43 ± 0.17^c	0.74 ± 0.08^c	28.1	61.7

Relationship between antinociceptive effect of AST and endogenous opioid system As aforementioned, AST attenuated the pain response of the second phase but had no effect on the first phase and morphine markedly inhibited the pain response of both phases. With regard to the effect of opioid antagonists, naloxone reversed the effect of morphine on both phases of the formalin response ($P < 0.01$) but did not alter the effect of AST on the second phase (Tab 2).

Relationship between antinociceptive effect of AST and NO production Both AST and

L-NAME (a NO synthase inhibitor) inhibited the second phase of the formalin response. Furthermore, *L*-Arg (a substrate of the NO synthesis) at 400 mg/kg partially and 800 mg/kg completely blocked the antinociceptive effect of *L*-NAME while both doses of *L*-Arg only partially blocked the effect of AST (Tab 3).

DISCUSSION

The main findings from the present study are 1) AST posseses an antinociceptive effect in mice and the effect reaches the maximum when AST is given 4 h before the formalin test and 2) the antinociceptive effect of AST is related to the decrease of the production of NO. Therefore, AST may be used to reduce the pain related to the inflammatory process.

The formalin test in mice is a useful test for evaluating mild analgesics[4]. Compared with other frequently used test for analgesic, in this model, the pain stimulus is continuous and may thus bear strong similarity with some kinds of pain encountered in the clinical setting. The test has two different phases which reflect different types of pain: the first phase seems to be due to the direct stimulation of nociceptors while the second phase may be due to the production and release of inflammatory mediators[4-6].

In this study, we have found that AST at 20, 40, and 80 mg/kg markedly lowered the pain score of the second phase in the formalin test although it had no effect on the first phase. With regard to the effect of the individual doses, the effect of AST at 40 and 80 mg/kg was significantly different from that of AST at 20 mg/kg ($P < 0.05$). However, the effect of AST 40 and 80 mg/kg was similar ($P > 0.05$). We also investigated the time course of the administration of AST. When given 4 h before the formalin test, the effect of AST reached the maximum.

It has been demonstrated that NO is involved in and is a modulator of the pain response at different levels[3]. Studies have shown that in formalin test, ip injection of *L*-NAME has no effect on the first phase but inhibits the second phase response[5]. Considering that AST also has similar inhibitory effect on the second phase response, we have hypothesized that there may be similarities between the mechanism of the effect of AST and that of *L*-NAME. The present study showed that AST and *L*-NAME presented 34.9 % and 39.9 % inhibition at the second phase of the formalin response respectively.

Tab 2. Influence of naloxone on the antinociceptive effect of AST 40 mg/kg evaluated by the formalin test in mice. $n = 10$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* morphine.

Group	Dose/mg·kg^{-1}	Pain score		Inhibition/%	
		1st phase	2nd phase	1st phase	2nd phase
Control	–	1.99 ± 0.14	1.91 ± 0.08	–	–
AST	40	1.95 ± 0.11	1.26 ± 0.12^c	2.0	34.0
AST + Naloxone	40 + 2	1.84 ± 0.26	1.32 ± 0.12^c	7.5	30.9
Morphine	5	1.38 ± 0.13^c	0.72 ± 0.07^c	30.7	62.3
Morphine + Naloxone	5 + 2	1.92 ± 0.20^f	1.84 ± 0.11^f	3.5	3.7

Tab 3. Influence of *L*-arginine on the antinociceptive effect of AST 40 mg/kg evaluated by the formalin test in mice. $n = 10$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* AST. $^iP < 0.01$ *vs* *L*-NAME.

Group	Dose/mg·kg^{-1}	Pain score		Inhibition/%	
		1st phase	2nd phase	1st phase	2nd phase
Control	–	1.96 ± 0.13	1.98 ± 0.08	–	–
AST	40	1.94 ± 0.16	1.29 ± 0.09^c	1.0	34.9
AST + *L*-Arg	40 + 400	1.83 ± 0.17	1.50 ± 0.15^{cf}	6.6	24.2
	40 + 800	1.92 ± 0.15	1.74 ± 0.10^{cf}	2.0	12.1
L-NAME	37.5	1.84 ± 0.15	1.19 ± 0.14^c	8.7	39.9
L-NAME + *L*-Arg	37.5 + 400	1.88 ± 0.18	1.62 ± 0.11^{ci}	4.1	18.2
	37.5 + 800	1.90 ± 0.19	2.04 ± 0.09^i	3.1	0

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Furthermore, *L*-Arg completely reversed the antinociceptive effect of *L*-NAME whereas only partially blocked the effect of AST. These results suggest that the effect of AST might be partially related to the inhibition of NO synthesis/release. We recently demonstrated that AST might inhibit the production of NO[1]. In the present study, we showed that the mode of the antinociceptive activity of AST was at least partially related to its decreasing effect on the production of NO. However, the mechanism of AST could not be simply explained by the decrease of NO production, because unlike the effect of *L*-NAME, the effect of AST was only partially reversed by *L*-Arg, suggesting that inhibition of the production of NO might be one of the multiple mechanisms of AST.

We also explored the possible mechanism of AST with regard to the endogenous opioid system. In contrast to the effect of AST, morphine markedly inhibited the pain response of both phases. The opioid antagonist naloxone did not reverse the effect of AST, suggesting that the antinociceptive effect of AST is opioid-independent.

In conclusion, AST has an antinociceptive effect on formalin test in mice that is not mediated by the endogenous opioid system but related to its inhibitory effect on the production of NO.

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黄芪总甙的镇痛作用及其作用机制

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关键词 黄芪总甙; 膜荚黄芪; 吗啡; 纳络酮; *L*-精氨酸; 一氧化氮

目的: 研究黄芪总甙(astragalosides, AST)的镇痛作用及其作用机制. **方法**: 采用小鼠福尔马林致痛模型, 对痛反应进行评分. 通过福尔马林试验前 30 min 皮下注射吗啡和纳络酮或福尔马林试验前 20 min 腹腔内注射 *L*-精氨酸和 *L*-NAME 以研究阿片肽和一氧化氮在此疼痛模型中的作用并对 AST 的作用与吗啡和 *L*-NAME 进行比较. **结果**: AST 20, 40 和 80 mg/kg 可显著降低小鼠福尔马林致痛后第二时相的疼痛反应($P<0.01$). AST 40 mg/kg 最大镇痛作用见于给药后 4 h (第二时相疼痛反应的抑制率为 34.4 %). 吗啡 5 mg/kg 对两个时相的疼痛反应均有明显抑制作用($P<0.01$), 此抑制作用能被纳络酮 2 mg/kg 拮抗($P<0.01$), 而 AST 的镇痛作用不受纳络酮影响. *L*-精氨酸(400 或 800 mg/kg)可部分抑制 AST 的作用($P<0.01$). **结论**: AST 所具有的镇痛作用不是通过内源性阿片肽系统介导, 而可能与抑制 NO 等参与疼痛反应的炎症介质的生成有关.

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