International Scholars Journals

International Journal of Pharmacy and Pharmacology ISSN: 2326-7267 Vol. 7 (12), pp. 001-004, December, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

L-Arginine abolishes the anxiolytic-like effect of withaferin A in the elevated plus-maze test in rats

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Accepted 25 October, 2018

The involvement of nitrergic mechanisms in the behavioural effects of withaferin A in rats was studied in the elevated plus-maze, open-field and rotarod tests. Administration of the nitric oxide (NO) precursor L-arginine (100 mg/kg, i.p.), assumed to increase the synthesis of NO, abolished the anxiolytic-like effect of withaferin A (40 mg/kg, i.p.) in the elevated plus-maze, whereas the inactive enantiomer D-arginine (100 mg/kg) did not. Neither withaferin A alone nor in combination with L- or D-arginine affected the exploratory activity of animals in the open field. Pretreatment with L-arginine (100 and 200 mg/kg) did not modify the motor impairment of rats after withaferin A (40 mg/kg) in the rotarod test. Withaferin A (40 mg/kg i.p.) did inhibit the brain NO synthase activity measured *EX VIVO* by NOx assay. We conclude that a suppression of NO synthase activity may be important in the anxiolytic-like effect of withaferin A.

Key words: Withaferin A, nitric oxide (NO) synthase, L-Arginine, anxiety, elevated plus maze test.

INTRODUCTION

Nitric oxide (NO) has been recognized as an intercellular messenger in the central nervous system (Bredt and Snyder, 1989). Interestingly, withaferin A share have an anxiolytic-like effect, decrease motor activity, possess an anticonvulsant effect and decrease cyclic GMP content (Khan and Ghosh, 2010; Kulkarni and Dhir, 2008; Bhattacharya, 2002; Bredt and Snyder, 1989). Therefore, we hypothesized that the brain nitrergic system may be involved in some of the effects of withaferin A. The aim of the present study was to elucidate whether the behavioural effects of an anxiolytic, withaferin A, could be modulated by the NO precursor L-arginine. Moreover, the effect of withaferin A on the brain NO synthase activity was measured in *ex vivo*.

MATERIALS AND METHODS

Animals

Male Wistar rats (150 to 200 g) were used in the study. Rats were housed at a constant temperature of 20±2°C under a 12 h light; 12 h dark cycle. The animals (n = 6 to 10 per group) had free access to food and water throughout the experiments. Animal care was as per Indian National Science Academy (INSA) Guidelines for the Care and Use of Animals in Scientific Research, and the study had the approval of Institutional Animal Ethical Committee (IAEC).

Stress procedure

The animals were subjected to restraint stress for 1 h at room temperature by immobilizing them in adjustable Plexiglas restrainers (INCO, Ambala). Immediately after the restraint stress procedure, the rats were exposed to the behavioral tests.

Elevated plus-maze test

The apparatus and procedure have been previously described (Volke et al., 1997). During a 5 min observation period, the following parameters were measured: number of open arm entries, time spent on open arms and number of closed arm entries. Subsequently, the percentage of the number of entries into the open arms of the total number of entries into all arms and percentage of time spent on open arms were calculated.

Open field test

Immediately after the plus-maze test, the rats were placed singly into an open field (1 m×1 m, divided by lines into 16 equal squares)

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and observed in 4 min to measure locomotor activity. The number of line crossings and rearing were counted.

Rotarod

The rotarod performance was measured as described by Suzdak et al. (1992). The rats were trained on the day before the test to stay on the rotating wooden bar (diameter 8 cm, speed 9 rpm) for at least 3 min. On the test day, the animals were put on the rotarod and the number of falls during a 3 min session was registered.

NO synthase assay

Brain NO*x* contents were determined as described by Tracey et al. (1995). Brain samples were homogenized in 5 ml distilled water and centrifuged at 10 000×*g* for 15 min at 4°C. 50 µl of supernatant was mixed with 20 µl of 0.11 mM FAD and 20 mU of nitrate reductase. Samples were allowed to incubate for 1 h at room temperature in the dark. Then 5 µl of 1 M $ZnSO_4$ was added to the samples in order to precipitate the proteins. Samples were centrifuged at 6000 ×*g*, for 5 min at 4°C and the supernatants were removed. 100 µl of Griess reagent (1:1 mixture of 1% sulphanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)-ethylenediamine) was added to 50 µl of supernatant and the mixture was incubated for 10 min at room temperature. Absorbance was measured at 540 nm in a microassay plate by microscan MS 5605A (Electronics Corporation of India) and converted to NO*x* content, using a nitrate standard curve. Brain supernatant protein was estimated by Lowry's method (Lowry et al., 1951). The data were expressed as nmol NO*x*/mg protein.

Extraction of withaferin A and drugs

The roots of *Withania somnifera* grown in natural habitat and purchased from an authorized dealer were air-dried in shade and finely powdered. The chief botanist at Indian Agricultural Research Institute (IARI), New Delhi, India, identified the roots and a voucher specimen (accession number NISCAIR/RHM/F-3/2008/Consult/473) has been deposited at the herbarium of IARI. The root powder was exhaustively extracted with methanol:water (4:1, v/v) under reflux (WS). This extract was partitioned with chloroform and water to give WS-chloroform and WS-water, respectively. WS-chloroform was subjected to 12 successive elutions of water and acetonitrile (ACN) and these elutions were labeled from A1 to A12.

HPLC (Waters, Milford, U.S.A.) of *W. somnifera* extracts was performed using Kromasil C8 column (4.6 mm × 25 cm, 5 µm), and the mobile phase consisted of ACN and water (1:1, v/v) at a flow rate of 1 ml/min for a run time of 30 min. The HPLC of fractions was conducted using Novapak C18 column (3.9 mm × 15 cm, 4 µm) and the mobile phase consisted of potassium-dihydrogen orthophosphate (0.05 M) and ACN (3:7, v/v) at the flow rate of 1.5 ml/min for a run time of 30 min. The photodiode array (PDA) detector was set to detect at 229 nm and scan spectral data from 190 to 400 nm. Using the standard Withaferin A (Natural Remedies Private Ltd., Bangalore, India), the bioactive constituents of the extracts were quantified by external calibration method. The following drugs were used: L-Arginine and D-Arginine (Sigma Aldrich). All drugs were freshly prepared and given intraperitoneally (i.p.) in a volume of 0.1 ml/100 g body weight of rats at different time intervals before the testing.

Statistics

The data were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test for post hoc comparisons. A P-value of at least 0.05 was considered as the level of significance in all statistical tests.

RESULTS

Elevated-plus-maze and open field

The results are shown in Figures 1 and 2, one-way ANOVA indicated significant drug effects on percentage of time spent on, percentage of entries into open arms and total number of arm entries ($F = 3.75$, $P<0.05$; $F = 8.12$, $P<0.001$ and $F = 3.81$, $P<0.05$, respectively). Withaferin A (40 mg/kg i.p.) caused a significant increase in percen-tage of open arm visits ($P<0.05$), in total number of arm entries ($P<0.05$) and in percentage of time spent on open arms ($P<0.05$). Pretreatment with L-arginine (100 mg/kg i.p. 60 min before testing), but not with D-arginine, abo-lished the anxiolytic-like effect of withaferin A ($P<0.05$, Figure 1A). ANOVA did not show any drug effect on the ambulation in Figure 1B ($F = 1.25$, $P>0.1$). L-Arginine seems to cause a true change in the anxiolytic-like action of withaferin A.

Rotarod performance

Treatment had significant effect on the motor coordination of rats on rotarod ($F = 6.0$, $P<0.01$). Withaferin A (40 mg/kg) increased the number of falls during the 3 min test session from 0.4±0.2 to 9.4±2.4 ($P<0.01$). Pretreatment with L-arginine (100) did not modify the motor in coordination caused by withaferin A (number of falls 8.4±1.9 and 9.6±1.9, respectively). There is no change in motor activity with treatment of both the drugs.

NO synthase activity

ANOVA revealed a significant drug effect on the brain NO synthase measured *ex vivo* ($F = 60$, $P<0.0001$ and $F = 46$, $P<0.0001$, respectively). The withaferin A (40 mg/kg i.p. 30 min prior sampling) decreased NO synthase activity by more than 65% ($P<0.001$) in cortex and 70% ($P<0.001$) in brain, as shown in Figure 3. There is significant change in NO level with withaferin A and L-arginine but not with D-arginine.

DISCUSSION

The main finding of the present study is that pretreatment with L-arginine, abolished the anxiolytic-like effect of withaferin A in the elevated plus-maze. L-Arginine seems to cause a true change in the anxiolytic-like action of withaferin A, since Faria et al. (1997) as well as our group (Volke et al., 1997) have shown that L-arginine, (60 and 100 mg/kg i.p., respectively) administered alone, does

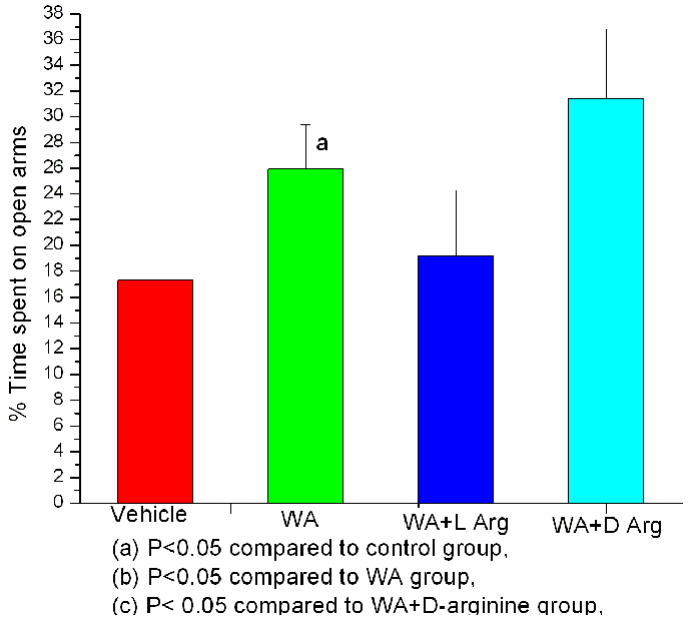

Figure 1A. Effect of pretreatment with L- or D-arginine (100 mg/kg i.p. 60 min prior to test) on the behaviour action of WA (40 mg/kg i.p. 30 min prior to test) in EPM open arm entries.

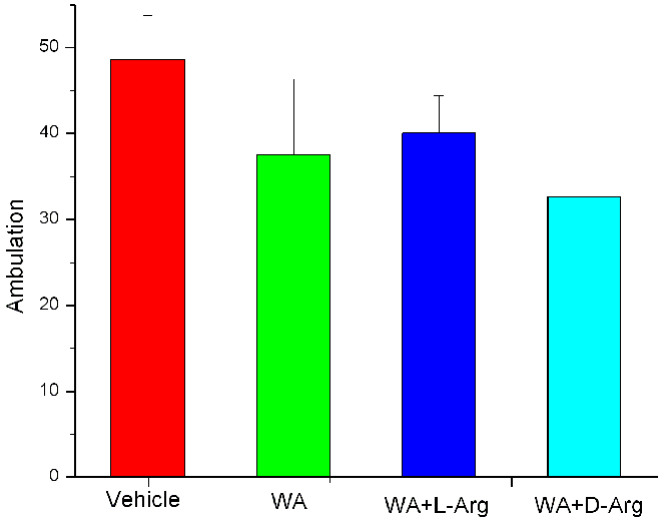

Figure 1B. Effect of pretreatment with L- or D-arginine (100 mg/kg i.p. 60 min prior to test) on the behaviour action of WA (40 mg/kg i.p. 30 min prior to test) in OFT ambulation.

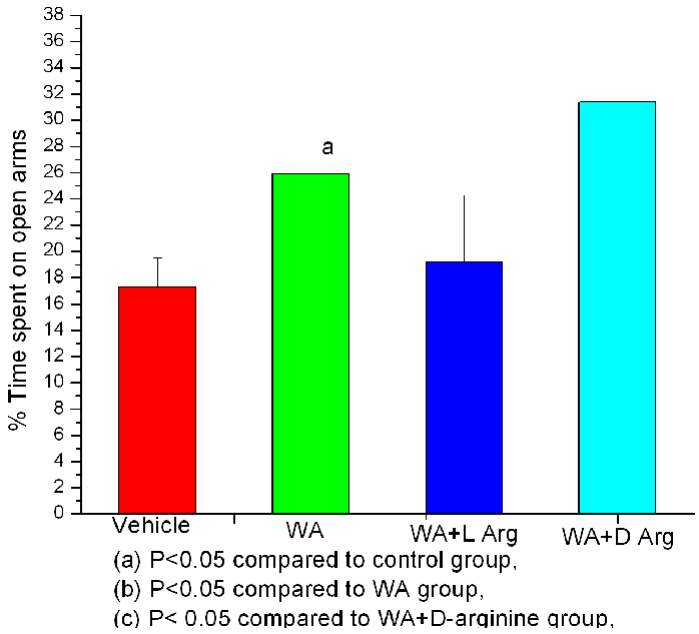

Figure 2. Effect of pretreatment with L- or D-arginine (100 mg/kg i.p. 60 min prior to test) on the behaviour action of WA (40 mg/kg i.p. 30 min prior to test) in EPM open arm time spent.

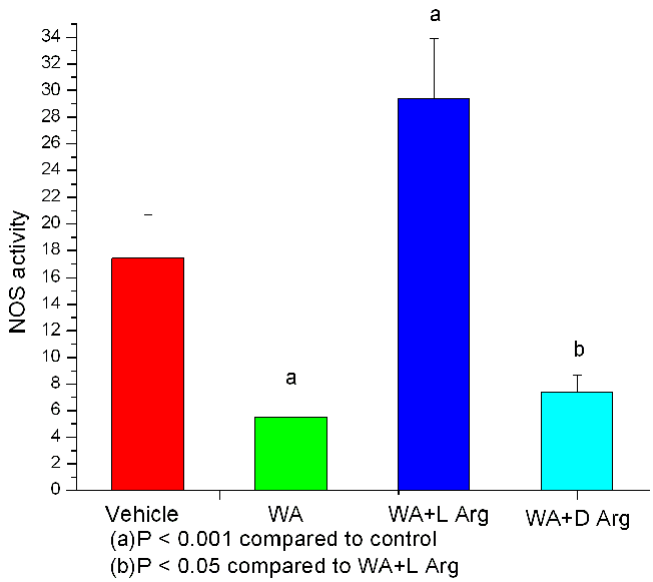

Figure 3. Effect of pretreatment with L- or D-arginine (100 mg/kg i.p. 60 min prior to test) on the behaviour action of WA (40 mg/kg i.p. 30 min prior to test) NO synthase activity.

not modify the behaviour of rats in the elevated plus-maze test. Moreover, the effect of the L-arginine treatment to decrease entries into open arms of the maze could not be explained by nonspecific changes in motor activity, since L-arginine combined with withaferin A did not affect locomotion of rats in the open field test. Since only the NO precursor L-arginine, but not the biologically inactive enantiomer D-arginine, modified the behaviour of the rats, the increased synthesis of NO seems likely to

to account for the mentioned action. L-arginine (150 and 300 mg/kg i.p.) has been shown to increase brain NO synthesis by about 50% (Salter et al., 1996). The present results are in line with the previous findings showing that, specific herbal NO synthase inhibitors induce an anxiolytic-like effect in the elevated plus-maze (khan and Ghosh,2010; Volke et al., 1997; Faria et al., 1997) and

indicating that NO may be an anxiogenic stimulus. However, not all the evidence is unanimous. Quock and Nguyen (1992) have described that the NO synthase inhibitor, L-NG-nitro arginine (L-NOARG) antagonizes the anxiolytic-like effect of chlordiazepoxide. Moreover, De Oliveira et al. (1997) showed that L-NOARG has an anxiogenic-like effect in the elevated plus-maze. We do not have any explanation for these discrepancies, but non-specific effects of the drug on locomotion cannot be ruled out. We are unable to assess whether an increase in NO synthesis affects the benzodiazepine induced hypolocomotion, because the dose of withaferin A used (10 mg/kg) did not significantly suppress the motor activity. L- Arginine did not modify the motor impairment caused by withaferin A (40 mg/kg), even though the dose of L-arginine was increased to 200 mg/kg, demonstrating that not all the effects of withaferin A are sensitive to an increase of the NO synthesis.

In another set of experiments, we tested the hypothesis that withaferin A may directly affect the NO synthase activity, which in turn would explain the behavioural effect of L-arginine. However, withaferin A did modify NO synthase activity as measured *ex vivo*, decreased activity by more than 60%. In conclusion, the present study indicates that the anxiolytic-like effect of withaferin A depends on the level of NO synthesis. However, withaferin A is an inhibitor of NO synthase, but may affect the NO synthase balance indirectly via some unknown mechanism which can be further investigated.

ACKNOWLEDGEMENT

Funding for the research was provided by the School of Bio Sciences and Technology, VIT University, Vellore.

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