Effect of Modification of Renin-Angiotensin System (RAS) on Diazepam Withdrawal-Induced Anxiogenesis in Albino Mice

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Abstract:
The present study evaluated the correlation between renin-angiotensin system (RAS) and anxiety in adult male albino mice using elevated plus-maze (EPM).
Angiotensin-converting enzyme (ACE) inhibitor captopril (CPL), angiotensin II (ANG II) receptor antagonist losartan (LRN), and anxiolytic drug diazepam (DZP) were used to examine the role of RAS in the central nervous system (CNS).
The acute administration of captopril, losartan and diazepam all caused the reduction of anxiety-like behaviour. They also antagonised the anxiety-like behavior caused by withdrawal of chronic administration of diazepam. Losartan showed better effect than captopril in reversing the anxiety-like behaviour, indicating a possible role of angiotensin receptor in the mediation of anxiety. Therefore it may be concluded that renin-angiotensin system may have a role to play in the genesis of anxiety.

Key words: EPM, RAS, Withdrawal, Captopril, Diazepam, Losartan.

Introduction:
The anxiety disorders constitute one of the most frequent classes of psychiatric illnesses. It has been found that 30.5% of women and 19.2% of men in the United States are affected by an anxiety disorder at some time in their lives.[1]
Benzodiazepines (BZDs), are widely prescribed and clinically effective anxiolytics and produce their pharmacological actions via specific high affinity binding sites on a supra-molecular complex composed of α-amino-butyric acid (GABA) and a BZD receptor coupled with a chloride ion channel.[2,3] Although several other neurotransmitter systems have been implicated in anxiety, the precise neurochemical mechanisms are not yet clear.
Initial findings suggested that central ANG II is likely to play a role in behavioural effects as its injection in rodents before or after a conditioned avoidance test facilitated learning and retention.[4] Since the selective AT_1 antagonist losartan was found to abolish the ANG II-induced improvement in object recognition, the cognition-improving effects of ANG II were suggested to be transmitted by AT_1 receptor.[5] Recently, it has been reported that the ANG II attenuates GABA release and excites hypothalamus paraventricular nucleus (PVN).[6,7] Therefore, administration of AT_1 receptor antagonist, losartan may be responsible for an anxiolytic effect.[8]
Investigations using ACE inhibitors, supported the hypothesis that ANG II suppression may have anxiolytic effects,[9] and may have cognitive enhancing effects.[10] Central ANG II administration initially causes a decrease in exploratory behaviour in rats reflecting increased anxiety followed by increased exploratory behavior.[10-12]
The aim of present study therefore was to investigate possible anxiolytic effects of captopril and losartan in the EPM, a behavioural test for anxiolytic drugs. Furthermore, the effects of these drugs and diazepam were compared to determine whether the behavioural profile of captopril and losartan differs from an established anxiolytic drug, diazepam. Standard anxiolytic drugs, such as diazepam, increase both the percentage of entries, rats make into the open arms of the maze and the percentage of time they spend in these open arms.[13,14] The effects of the drugs were studied in albino mice on two parameters – time spent and number of entries in open arms in the present investigation.

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Equipments, materials and methods:

Animals:

Male albino mice weighing (27 ± 3 g), were obtained from animal house of Faculty of Pharmacy, Al-Fateh University of Medical Sciences, Tripoli - Libya. The animals were housed in colony cages, at constant room temperature (24 ± 2ºC), on a 12/12h light - dark cycle. Food and water were given ad libitum, food was obtained from ALCO, Sfax - Tunisia.

Drugs and chemicals:

Losartan potassium, obtained from Merk, USA. Captopril malate and diazepam were obtained from Sigma Aldrich, Germany. Tween 80 was obtained from Riedel-De Haen AG Seelzf-Hannover.

Drugs administration and doses:

All drugs were dissolved in normal saline containing tween 80 (1%) (vehicle) and were administered intraperitoneally (i.p.), at a volume of 5ml/kg body weight. All drugs were freshly prepared. The ACE inhibitor captopril (10 mg/kg),[15,16] angiotensin AT_1 receptor antagonist losartan (10 mg/kg),[17,18] and anxiolytic drug diazepam (1.5 mg/kg)[19,20] were used.

Elevated plus-maze (EPM):

The apparatus was made of Plexiglass and consisted of two opposite open arms (5 x 30 cm) crossed with two opposite enclosed arms of the dimension with 15 cm height. The arms were connected with a central square (5x 5 cm) to give the apparatus a plus sign appearance. The maze was kept elevated 30 cm above the floor in a dimly lit room.[21,22]

The plus-maze test was conducted in a closed room with low level of illumination,[15] the mice were individually placed on the central square of the plus maze facing an enclosed arm. The time spent and number of entries made by the mice, during the next 4 min on open and closed arms were recorded. An arm entry was defined when all the four limbs were on the arm .The apparatus was cleaned after each use. An increase in open arm enteries and increase in time spent in open arms is indicative of potential anxiolytic activity, as mice naturally prefer the closed arms.

Mice were randomly assigned to four groups (n=8, each group); group1, diazepam (10 mg/kg) + 2doses of captopril (10 mg/kg); group2, diazepam (10 mg/kg) + 2doses of losartan (10 mg/kg); group3, diazepam (10 mg/kg) + 2doses of diazepam (1.5 mg/kg) (anxiolytic dose); group4, diazepam (10 mg/kg) + 2doses of vehicle (5 ml/kg) "control". All groups of mice received diazepam (10mg/kg) (i.p) twice daily (9-10am, 18-19pm), for fourteen days then withdrawn for 24hr. Anxiogenic response induced by diazepam withdrawal was investigated by losartan, captopril and standard anxiolytic drug diazepam during the period of withdrawal. Losartan (10 mg/kg) and diazepam (1.5 mg/kg) were administered 12h and 30 min before testing , and captopril (10 mg/kg) was given 12h and 45 min before test. All drugs were freshly prepared, and administered in a constant volume of injection 5 ml/kg body weight. All treated groups were compared with a diazepam + vehicle treated group which received vehicle 12h and 30 min before test.

Statistical analysis:

Descriptive statistical analysis was performed, on the parameters of samples within each experiment, to find out whether the observed samples were normally distributed, using the non-parametric Kolmogorove-Smirnov maximum deviation test for goodness of fit. If the parameters were normally distributed, the treatments were compared by applying one-way ANOVA. For multiple compression Post hoc tests, additional LSD tests were performed, when appropriate, to detect any significant differences between the treated groups and the control group, and between the combined drugs and drug itself. The differences were considered to be significant at ($p<0.05$). All analyses were conducted using the SPSS (software packing version 13) for IBM compatible computer.

Results:

Acute effects of drugs on time spent on open arms:

The mean duration of time spent in open arms by the group that was treated with vehicle "control group" was 53 ± 7.093 seconds which was highly significantly lower than diazepam (108 ± 16.673 sec) and losartan (98 ± 5.8 sec) and significantly different from captopril (90.375 ± 7.889 sec) treated groups respectively. In contrast, there was no significant difference between diazepam, losartan and captopril treated groups ($p>0.05$) (see figure 1).

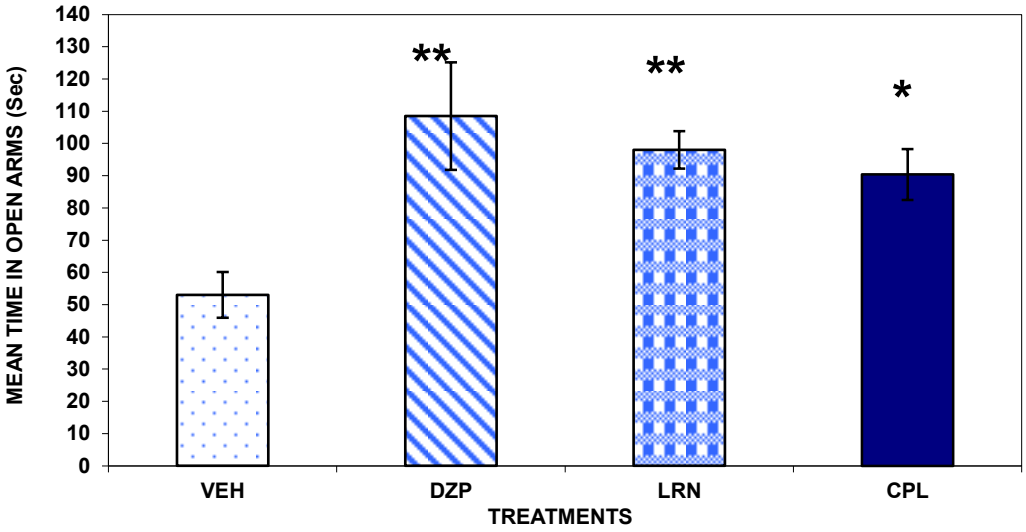

Fig. 1: Effects of diazepam (DZP), losartan (LRN) captopril (CPL) and vehicle (VEH) on the time spent in open arms of the elevated plus-maze (n = 8 in each group).
**p<0.01 = Highly significant difference. *p<0.05 = Significantly different.
All data are mean values with (±S.E.M) and compared with control.

Acute effects of drugs on number of entries on open arms:
The number of entries into open arms by vehicle treated group "control group" was (4.5 ± 0.534) which was highly significantly different from diazepam (anxiolytic dose) (9.75 ± 1.319) and losartan (8.375 ± 0.924) treated groups. However, it was only significantly different from captopril (7.75 ± 0.958) treated group. In contrast, there was no significant difference between diazepam, losartan and captopril (p>0.05) treated groups (see figure 2).

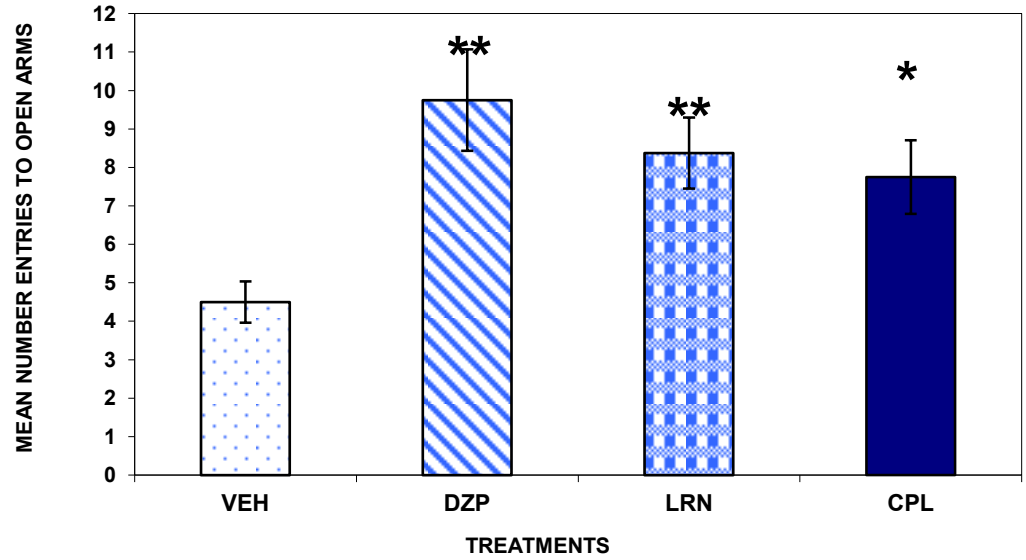

Fig. 2: Effects of diazepam (DZP), losartan (LRN), captopril (CPL) and vehicle (VEH) on the number of open arms entries in elevated plus-maze (n = 8 in each group).
**p<0.01 = Highly significantly difference. *p<0.05 = Significantly different.
All data are mean values with (±S.E.M) and compared with control.

Acute effects of drugs on time spent on open arms after chronic diazepam withdrawal:
The mean duration of time spent in open arms by the group that was treated with vehicle "control group" was 27.25 ± 5.283 seconds which was highly significantly lower than losartan (74.125 ± 18.828 sec) treated group. In comparison, it was only significantly lower than diazepam (anxiolytic dose) (65.5 ± 9.615 sec) and captopril (66.375 ± 7.294 sec) treated groups (see figure 3).

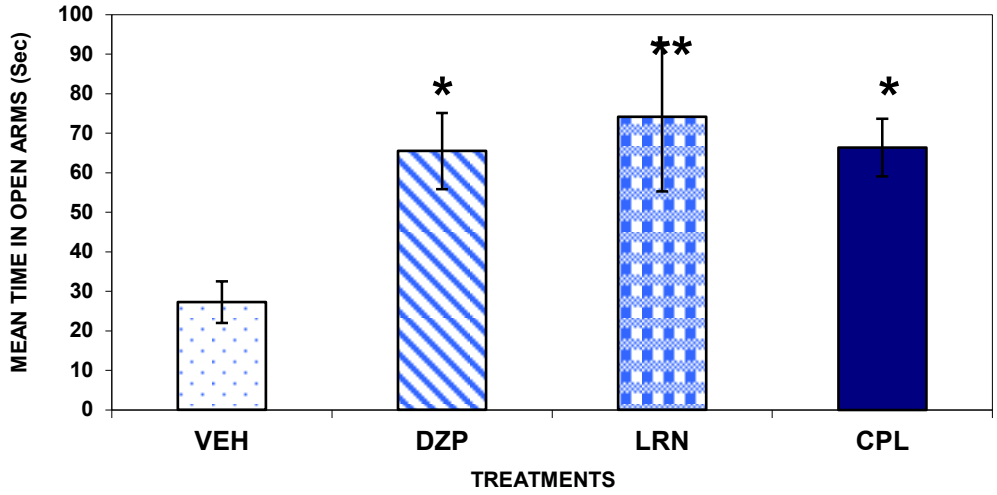

Fig. 3: Effects of losartan (LRN), captopril (CPL), diazepam (DZP) and vehicle (VEH) on the time spent in open arms in the diazepam withdrawal-induced anxiety using elevated plus-maze (n = 8 in each group).
**p<0.01 = Highly significantly different. *p<0.05 = Significantly different.
All data are mean values with (±S.E.M) and compared with control.

Acute effects of drugs on number of entries on open arms after chronic diazepam withdrawal:
The number of entries into open arms by vehicle treated group "control group" was (2.25 ± 0.25) which was highly significantly lower than losartan (7 ± 1.69) treated group and was significantly lower than diazepam (anxiolytic dose) (5.5 ± 0.779) and captopril (5.75 ± 0.920) treated groups (see figure 4).

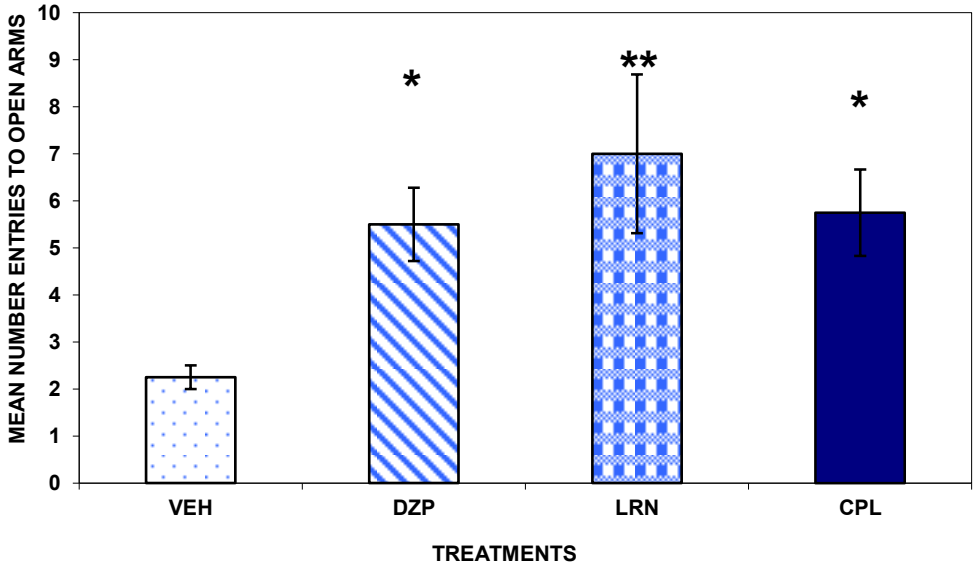

Fig. 4: Effects of losartan (LRN), captopril (CPL), diazepam (DZP) and vehicle (VEH) on the number of open arms entries in the diazepam withdrawal-induced anxiogenic action in elevated plus-maze (n = 8 in each group).
**p<0.01 = Highly significantly different. *p<0.05 = Significantly different.
All data are mean values with (±S.E.M) and compared with control.

Discussion:

The renin angiotensin system (RAS) plays an important role through angiotensin II in the control of the balance of hydromineral and fluid volume, sympathetic efferent activity,[23] and is produced in the mammalian brain.[24,25] It exerts a wide range of physiological actions on the cardiovascular, renal and endocrine system,[26] and on the peripheral and central nervous system[27] by acting on two receptor subtypes, namely AT_1 and AT_2 receptors.[28] It is the AT_1 receptors that are the most abundant and which mediate most of the physiological responses to angiotensin. Both receptor subtypes have been identified in the brain, although AT_1 receptors account for approximately 90% of the population.[29] Combined therapy with ACE inhibitor and receptor block may enable complete blockade, but it has shown toxic symptoms in normal conditions.[30] Losartan is an orally active antihypertensive, selective AT_1 receptor antagonist.[31] Angiotensin II on intracere-broventricular (icv) administration produced anxiogenic effect in open-field behaviour, which was reversed with losartan treatment. In normal subjects losartan produced anxiolytic effect.[32] The AT_1 receptor antagonist was found to modulate the mental function and produced anxiolysis in mice.[8] Captopril is used extensively in the treatment of hypertension and heart failure.[15] It has been reported to produce anxiolytic behaviour in humans.[15,18]

In the elevated plus-maze, time spent on the open arms of the maze and the number of entries onto the open arms are taken as measures of anxiolysis.[33] The established anxiolytic agent diazepam produced a significant increase in the amount of time spent on the open arms of the maze and the number of entries onto the open arms as reported previously also.[17] Anxiolytic property of losartan has also been reported in different behavioural paradigms.[5,32] In the present study acute treatment with diazepam and losartan highly significantly (**$p<0.01$) and captopril significantly (*$p<0.05$) increased the amount of time spent on the open arms of the maze and the number of entries onto the open arms which indicates the role of angiotensin in the altered behaviour.

Drug withdrawal-induced anxiety has been used during recent years on the basis that endogenously generated anxiety is likely to provide a better and clinically acceptable animal model of anxiety.[20] It is known that drug withdrawal, after chronic administration of addictive agents, induces severe withdrawal symptoms of anxiety in rodents.[34] The following drugs have been used by others: ethanol (8% w/v, i.p.), cocaine (1 mg/kg, i.p.), nicotine (0.1 mg/kg, i.p.), diazepam (10 mg/kg, i.p.) and morphine (10 mg/kg, i.p.). The drugs were administered twice daily for 14 days (35). Anxiety is assessed 24 hr after drug withdrawal by elevated plus-maze test.[35] In this study, the anxiety induced by abrupt cessation of chronic treatment with a proven anxiolytic drug may be attributed to the escalation of RAS. The released angiotensin could precipitate anxiety, and it can also stimulate both central and peripheral sympathetic systems, including adrenal medulla.[36,37] Activation of sympathetic nervous system increases noradrenaline turnover in the brain nuclei which is involved in noradrenergic control of behaviour in normal animals.[38] Angiotensin is also shown to cause reversal of suppressed behaviour in mice due to AT_1 receptor block,[8] and to antagonize the anxiolytic action of losartan in mice[17] and of ACE inhibitors in rats.[39]

Therefore, it can be stated that the observed anxiolytic behaviour with losartan (receptor block) and captopril (ACE inhibition) may be attributed to the attenuation of the inherent anxiogenic behaviour in normal mice and to antagonizing angiotensin-mediated neurochemical alteration in anxious mice. In the present study, the effect of endogenous anxiety, generated by the withdrawal of diazepam is known to induce anxiogenesis and its antagonism by ACE inhibition or by AT_1 receptor block, provides evidence that captopril and losartan may have anxiolytic potential. This indicates anxiety as a consequence of increased RAS tone. Hence, it can be assumed that increased sympathetic activity and direct action of RAS may be responsible for the observed anxiogenesis after an abrupt cessation of a chronic treatment with diazepam and this anxiogenesis is blocked by inhibition of RAS through two different mechanisms as described above.

Conclusion:

In conclusion, RAS either by decreasing synthesis of angiotensin II (captopril) or blocking of AT_1 receptors (losartan), suppresses induction of anxiety-like behaviour. Losartan was better than captopril in this regard.

The possible mechanism may include RAS interaction with NA, Ach or GABA neurons, hormonal release, and potentiation of AT_2 receptors and possible involvement of increased bradykinin levels due to captopril. This study indicates possible involvement of AT_1 receptor. Further studies to probe into the possible involvement of above mechanisms are envisaged.

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