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Elevations in Plasma Angiotensin II with Prolonged Ethanol Treatment in Rats[1]

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WRIGHT, J W , S L MORSETH, R H ABHOLD AND J W HARDING *Elevations in plasma angiotensin II with prolonged ethanol treatment in rats* PHARMACOL BIOCHEM BEHAV 24(4) 813–818, 1986 —Chronic alcohol consumption frequently leads to hypertension in humans While previous reports have implicated the renin-angiotensin system as a potential mediator of this effect, plasma angiotensin II (AII) levels were either not measured or yielded negative results The present investigation noted significant elevations in circulating AII in rats intubated daily with ethanol (4 g/kg) for 50 days Animals administered ethanol only once evidenced AII concentrations equivalent with water intubated controls Radioligand binding assay data indicated no differences in the number or affinity of Sar^1,Ile^8-AII binding sites in the thalamus, septum-anterior ventral third ventrical region or adrenal gland comparing those groups chronically treated with ethanol to water intubated controls These results may support a role for the vasoconstrictive hormone AII in the etiology of alcohol-induced hypertension

Ethanol treatment Hypovolemia Water consumption Hematocrit Plasma volume Plasma angiotensin II and III Receptor binding Rats

ALTHOUGH the effects of ethanol on blood pressure are reasonably well established, no comprehensive hypothesis has been proposed to explain its mechanism of action. Acute ethanol intake has a marked hypotensive effect both in humans [7] and rodent species [19,31], while chronic consumption frequently leads to heart disease and hypertension as verified by several epidemiological studies in humans [2, 4, 5, 8, 11, 28, 33] Of major interest to the present investigation is the observation that renin and aldosterone levels are increased during recovery from alcohol ingestion [18]. These investigators have further indicated that plasma renin activity (PRA) is significantly elevated in genetically derived alcohol preferring rats as compared with alcohol avoiding rats one hour after gavage [19] A similar elevation in PRA has been noted in human alcoholic patients with cirrhosis of the liver [35] Dehydration resulting from ethanol diuresis has been considered to be at least partially responsible for the alterations in PRA [17, 18, 22]. Elevations in PRA in turn result in the increased synthesis of angiotensin II (AII) which may elevate arterial blood pressure in several ways including: (1) direct action on arteriolar smooth muscle, (2) indirectly by stimulating the peripheral sympathoadrenal system which also leads to vasoconstriction, and (3) by stimulating the central nervous system via the circumventricular organs (CVOs) resulting in neurogenically controlled elevations in peripheral resistance and cardiac output [9,25]. Angiotensin stimulation of CVOs in nonalcohol treated animals has been shown to increment the release of antidiuretic hormone (ADH) from the neurohypophysis [9]. Although alcohol treatment initially results in body dehydration [3,36] presumably because of decreased release of ADH [34,37], this state of dehydration disappears following continued intake [6,36] with an accompanying return of normal circulating ADH levels in laboratory animals [37], and in human alcoholic patients [14, 15, 23]

Given that AII plays a prominent role in the control of blood pressure and body water balance via direct action and also by influencing catecholamine release from adrenal glands and ADH release from the neurohypophysis [9,27], it is reasonable to hypothesize that the complex influence that ethanol has on vascular resistance and body water balance may be at least in part mediated by AII [16]. Thus, the present investigation specifically examined the potential role of AII in mediating the alterations in vascular volume observed to accompany acute and chronic ethanol consumption. We predicted that acute ethanol treatment would result in a decrease in circulating AII levels to account for the hypotensive effect with elevated levels following chronic ethanol administration thus providing a potential explanation for the occurrence of hypertension. We further hypothesized alter-

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TABLE 1

MEAN (±SEM) DAILY FLUID CONSUMPTION IN ml/100 g BODY WEIGHT FOR GROUPS CHRONICALLY INTUBATED WITH WATER OR ETHANOL ON THE FIRST AND LAST TEN DAYS OF TREATMENT

Treatment group	N	Day of Treatment									
		1	2	3	4	5	6	7	8	9	10
Water	30	7 7 ± 0 5	6 3 ± 0 5	5 7 ± 0 5	5 7 ± 0 4	6 2 ± 0 3	6 5 ± 0 5	6 1 ± 0 4	6 1 ± 0 4	5 9 ± 0 4	6 1 ± 0 5
Ethanol	30	6 4 ± 0 4	5 8 ± 0 3	4 8 ± 0 3	5 2 ± 0 3	6 2 ± 0 3	5 9 ± 0 4	5 7 ± 0 3	6 0 ± 0 3	5 7 ± 0 3	5 4 ± 0 3
		Day of Treatment									
		41	42	43	44	45	46	47	48	49	50
Water		6 7 ± 0 4	5 0 ± 0 4	5 9 ± 0 4	6 4 ± 0 5	6 5 ± 0 5	6 1 ± 0 4	6 2 ± 0 5	6 1 ± 0 4	6 1 ± 0 4	6 3 ± 0 6
Ethanol		6 0 ± 0 3	5 0 ± 0 4	5 5 ± 0 3	6 3 ± 0 4	5 0 ± 0 3	5 7 ± 0 3	5 8 ± 0 4	5 9 ± 0 4	5 5 ± 0 4	5 9 ± 0 4

TABLE 2

ETHANOL INFLUENCES UPON HEMATOCRIT AND ESTIMATED PLASMA VOLUME FOR EACH TREATMENT GROUP FOLLOWING INTUBATION

Treatment	Post-Intubation (h)				
	1/4	1/2	1	2	4
Acute-water					
Hematocrit (% RBC)	47 6 ± 0 7*	49 2 ± 0 5	47 0 ± 0 7	49 5 ± 1 1	50 4 ± 1 5
Plasma vol (ml/100 g)	3 04†	2 83	3 12	2 79	2 68
Chronic-Water					
Hematocrit	49 4 ± 1 2	51 0 ± 0 9	51 6 ± 1 2	53 3 ± 0 9	47 7 ± 0 8
Plasma vol	2 81	2 60	2 52	2 30	3 03
Acute-Ethanol					
Hematocrit	51 7 ± 0 4	50 4 ± 1 0	51 3 ± 1 0	51 3 ± 1 1	50 0 ± 1 2
Plasma vol	2 51	2 68	2 56	2 56	2 73
Chronic-Ethanol					
Hematocrit	57 6 ± 2 1	49 7 ± 1 6	53 8 ± 0 7	51 3 ± 1 4	50 2 ± 1 9
Plasma vol	1 75	2 77	2 24	2 56	2 70

*Mean ± SEM
†Derived from regression equation, PV=9 18–0 129 (Hct) [36]

ations in AII receptor numbers and/or affinity in peripheral and central tissues

METHOD

Animals

One hundred twenty-six male Sprague-Dawley rats weighing from 390–500 g at the beginning of the study were individually housed in hanging cages at 21–23°C on a 12–12 light-dark photo-period initiated at 0700 hr. Purina lab chow was available ad lib to all animals

Procedure

The animals were randomly divided into four major groups of 30 each Two of these groups were designated chronic treated and received either ethanol (4 g/kg brought to a total volume of 4 ml with distilled water) or distilled water (4 ml) once daily at 0930–1100 hr by gavage (16 g intubation needle, Popper & Sons) Twenty-four hr water consumptions were measured daily at 0900–0930 hr by the use of 100 ml graduated cylinders fitted with stainless steel spouts Body weights were recorded every other day On treatment days 50 or 51 each member of the four major groups was further randomly assigned to one of five post-gavage time points, 6 animals at each point, and a single blood sample was taken by cardiac puncture This yielded 5 subgroups of chronic ethanol and 5 subgroups of chronic water treated animals with each subgroup assigned to one of 5 time points following gavage 15, 30, 60, 120 and 240 min Members of the other two major groups, designated for acute treatment, were intubated only once with ethanol or distilled water and independent sets of 6 animals were assigned to the time points indicated above and were similarly blood sampled In

addition, blood was taken from 6 naive animals matched for age with the other animals. Two days were required to complete blood sampling with time points 15, 60 and 240 min conducted on treatment day 50, and time points 30, 120 and the naive control group on day 51

A single blood sample was taken from each animal under light ether anesthesia and the procedure was completed within 90 sec of removal of the animal from its home cage. Microhematocrit tubes were prepared in triplicate and the plasma was immediately separated at 2,000 g for 15 min at 4°C and stored at −20°C until assayed for angiotensin concentration by radioimmunoassay

Upon completion of the 4 hr post-inbutation data collection period, 8 animals were randomly selected from each of the two chronic treatment groups and were killed by decapitation and the brains and adrenal glands were removed The brains were dissected over ice and kept moist with ice-cold 0.15 M NaCl The thalamus and septum-anterior ventral third ventricle (AV3V) regions were retained for AII radioligand binding assay.

Radioimmunoassay

The plasma from each animal was analyzed for combined angiotensin II and III (AIII) levels in triplicate, 200 μl per assay The plasma was initially extracted in 750 μl of 95% ethanol by incubation at 27°C for 15 min, then at 4°C for 15 min The mixture was centrifuged at 2000 g for 10 min at 4°C, and the supernatant was dried down overnight in a speedvac Internal standards were used to assess the efficiency of extraction which averaged 84% Values were normalized based on calculated recoveries All glassware and assay tubes were coated with heat-treated 0.1% bovine serum albumin (BSA) solution (Sigma 44503, 6 hr at 56°C) and dried overnight prior to use The incubation mixture contained the following components 3 pg of [^{125}I]-AIII with a specific activity of 1,700 μCi/μg in 200 μl of buffer (10 mM $NaPO_4$, pH 7 0); 100 μl of reconstituted rabbit antiserum diluted 1 25,000 in buffer, 200 μl of standard AIII or unknown, an additional 500 μl of buffer was added to bring the total reaction volume to 1.0 ml. Following incubation for 24 hr at 4°C, 500 μl of dextran-coated charcoal (charcoal: Fischer C-170, 12 mg/ml, dextran Sigma D-1390, 1.2 mg/ml) was added to the incubation mixture The resultant suspension was vortexed, allowed to stand for 5 min, and centrifuged at 2,000 g for 10 min at 4°C, and the supernatant containing the bound angiotensin was retained for counting (Beckman Gamma 5500) A complete standard curve, nonspecific binding in the absence of antibody (blank) and zero standard binding, was determined routinely with each set of assays. The percentage of labeled AIII bound was graphed against the total AIII in the assay to form the standard curve A logit transformation of the standard curve was used to calculate the unknowns expressed as pg/100 μl of plasma The assay was sensitive to 1 5 pg of AIII The antiserum was equivalently reactive to AII and AIII as determined by displacement analyses and, as a result, reflected the total AII and AIII levels in the plasma

Binding Assay

Following dissection, the tissues were weighed and Polytron homogenized in 10 ml of ice-cold homogenization buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, and 5 mM EDTA. The whole homogenates were centrifuged at 40,000 g in a Beckman J-21C centrifuge for 30 min at 4°C. The supernatant was discarded and the pellet was rehomogenized in homogenization buffer using 1 ml of buffer for every 20 mg of original tissue weight. Final protein concentrations of the tissue homogenates varied from 1 0–2 5 mg/ml.

The binding of the angiotensin II competitive antagonist, ^{125}I-Sar^1,Ile^8-AII (^{125}I-SI-AII) was determined by a modification of the method of Sirett *et al* [29]. Two hundred microliters of homogenate containing 0.2–0.5 mg of protein were incubated with 200 μl of ^{125}I-SI-AII (spec. act =1840 μCi/μg) in 12×75 mm culture tubes giving a final concentration of 0 06 nM (70,000 cpm at 70% efficiency/assay tube). Assay buffer had the same composition as the homogenization buffer with the addition of dithiotheitol (final concentration 5 mM) and bovine serum albumin (final concentration 0.2%). In tubes where total ^{125}I-SI-AII binding was to be determined, an additional 100 μl of assay buffer was added giving a final volume of 0 5 ml. In tubes where non-specific binding was to be determined, 100 μl of 2 μM unlabelled SI-AII was added instead of the additional assay buffer giving a final SI-AII concentration of 200 nM. The final assay solution was vortexed and incubated at room temperature for 60 min. The assay suspension was then filtered through GF-B filters that had been soaked with 0 1% BSA in isotonic saline. After filtration the filter was washed 4 times with 4 ml of saline The filter was then placed in a culture tube and counted in a Beckman Gamma 5500 at 70% efficiency Assays for total and non-specific binding were performed in triplicate. Specific binding was considered the difference between total and non-specific binding The detection limits of the assay were 0 02 fmol AII bound/mg protein Protein was estimated according to Lowry *et al* [20], using bovine serum albumin as standard Kd and Bmax values were determined by the displacement method of Akera and Cheng [1] utilizing logit analysis

Statistical Analyses

Water consumption for members of each group during the initial 10 days of treatment and during the last 10 days were converted to ml/100 g body weight and each data set was submitted to a Groups × Days ANOV with repeated measures on the second factor Hematocrit ratios and plasma AII levels were each analyzed by a 4 (Treatments) × 5 (Time of blood sampling) ANOV And the levels of Sar^1, Ile^8-AII specific binding were analyzed by a 2 (Treatments) × 3 (Tissues) ANOV All post-hoc analyses were accomplished using Newman-Keuls tests at a significance level of 0.01

RESULTS

During the initial 10 days of treatment there was no Groups effect in water intake, however, there was a Days effect, $F(9,450)=8.80$ $p<0$ 0001, with greater consumption during the first day of treatment (mean=7.05 ml/100 g) as compared with all other days (overall mean=5.84 ml/100 g). The interaction was not significant. During the last 10 days of treatment the groups did not differ nor were there differences in consumption over days Therefore, Table 1 presents the daily mean water consumptions for the initial and last 10 days of treatment collapsed across subsets of animals chronically intubated with water or alcohol

Mean hematocrit ratios for each major group are presented in Table 2. There was a treatment effect, $F(3,100)=7$ 95, $p<0$ 001, with the chronic ethanol groups evidencing a significantly higher hematocrit level than the other treatment conditions, which did not differ among themselves. The time

TABLE 3

SAR[1],Ile[8]-ANGIOTENSIN II SPECIFIC BINDING AND AFFINITY IN BRAIN AND ADRENAL GLANDS OF CHRONIC ETHANOL AND WATER INTUBATED RATS

Treatment	N	Tissue: Adrenal gland	Thalamus	Septum-AV3V
Control (water)				
Kd (nM)	8	1 07 ± 0 25*	1 4 ± 0 18	0 83 ± 0 23
Bmax (fmol/mg protein)	8	525 65 ± 132 66	15 74 ± 1 43	10 53 ± 1 72
Ethanol				
Kd (nM)	8	1 11 ± 0 23	1 03 ± 0 24	0 76 ± 0 09
Bmax (fmol/mg protein)	8	550 49 ± 129 62	13 06 ± 2 69	12 65 ± 1 52

*Mean ± SD

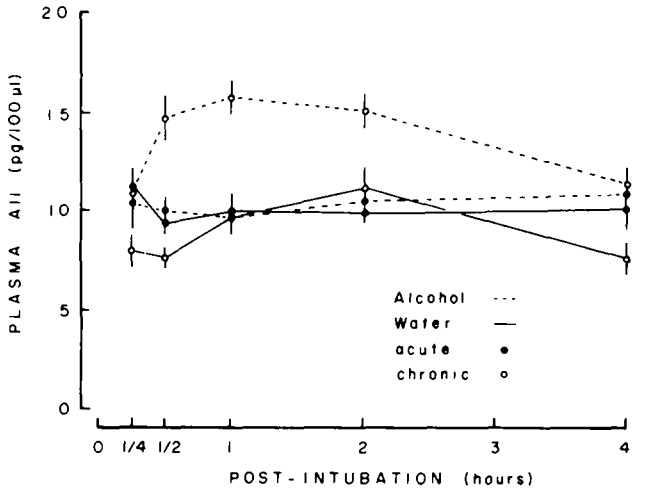

FIG 1 Mean (±SEM) plasma angiotensin II concentrations presented in pg/100 µl for each group following intubation of ethanol or distilled water

of blood sampling was not significant, however, the interaction was, $F(12,100)=3\ 24$, $p<0\ 001$ Post-hoc analyses indicated that the chronic ethanol group, blood sampled at 15 min following intubation, was different from the other 15 min groups The chronic ethanol group sampled at 60 min was different only from the acute water group at 60 min Utilizing the regression equation, Plasma volume=9 18–0 129 (hematocrit) [36] it was possible to estimate plasma volume and these mean values are also provided in Table 2 The naive control group evidences a mean (±SEM) hematocrit level of 46 6% yielding an estimated plasma volume of 3 17 ml/100 g body weight The chronic ethanol group sampled at 15 min following intubation revealed a 42 4% decrease in plasma volume as compared with the acute water control group sampled at 15 min, a 37 7% decrease compared with the chronic water control group, and a 44 8% decrease compared with the naive control group

Mean plasma angiotensin levels are provided in Fig 1 and there were significant differences due to treatments, $F(3,100)=11\ 03$, $p<0.0001$. Once again the chronic ethanol treated groups indicated elevations over the other groups which did not differ among themselves. Plasma angiotensin was significantly elevated in the chronic ethanol treated groups at 30 min, 1 and 2 hours following intubation as compared with the other groups at each sample time. The mean (±SEM) angiotensin level of the naive control group was 8 4±0 7 pg/100 µl plasma

Results from the tissue binding assay are presented in Table 3 The ANOV indicated no overall difference between the treatment groups or among the tissues examined with respect to Kd which represent the relative affinity of a receptor for its ligand However, there was a significant difference among tissues, collapsing across groups, $F(2,43)=58.44$, $p<0.001$ with respect to Bmax, i e , the number of receptors per unit protein Post-hoc analyses indicated that the adrenal glands had significantly greater numbers of SI-AII binding sites than the thalamus and septum-AV3V which did not differ But, there was no difference between groups collapsing across tissues

DISCUSSION

Failure to find a persistent difference in the patterns of water consumption by the groups was somewhat surprising given that alcohol preferring animals have been reported to consume significantly more water than alcohol avoiding rats [19]. However, the comparison of subgroups of alcohol avoiding rats administered either ethanol or water by gavage would appear to be more comparable to the present experimental protocol and these data are not available

As expected there was an ethanol induced hypovolemia following gavage in the chronic ethanol treated groups with recovery during the four hour post-treatment period There was a similar trend (not statistically significant) demonstrated by the acute ethanol treated groups And there was a significant elevation in plasma AII levels in the chronic ethanol treated groups that peaked at one hour post-treatment and returned to control values by four hours following the ethanol load We failed to measure a decrease in plasma AII levels in the acute ethanol treated groups, thus no explanation is available from the present results to account for the hypotensive effect frequently observed to accompany acute alcohol ingestion [7, 19, 31] In fact the trend toward hypovolemia noted in the acute ethanol treated groups may indicate that with a larger dose of ethanol the hypovolemia could be exaggerated and approximate that seen in the chronic treated groups thus indicating greater

synthesis of AII, not reduced synthesis as predicted. This possibility awaits further inquiry.

The tissue binding data indicated no differences in the number or affinity of AII receptors comparing chronically ethanol treated animals with the controls. This was surprising given the significant elevations in plasma AII levels evidenced by members of the chronic ethanol treated group With hindsight it appears that we may have misjudged the rapidity of AII receptor turnover and by delaying tissue collection until four hours following intubation we could have missed any potential tissue differences, thus representing a major shortcoming of this investigation Recently we have determined that brain AII metabolism is very rapid with only 28% intact at 45 seconds following intracerebroventricular injection of [^{125}I]AII as measured by HPLC [13]. This may indicate that tissue AII receptor occupancy and turnover is also much more dynamic than previously envisioned. It will be necessary to repeat this study with sampling at the peak of plasma AII elevations in order to test this possibility. The elevated AII receptor numbers in adrenal gland relative to brain tissues has been reported elsewhere [12].

The previously reported increase in PRA [17] coupled with the presently measured elevations in plasma AII appear to occur as a result of the ethanol induced body dehydration that is evidenced as hypovolemia [35,36] The present hypovolemia appears to be of sufficient magnitude to serve as an adequate stimulus for the release of renin [9, 10, 25] which acts upon circulating angiotensinogen to form the decapeptide angiotensin I which in turn is converted to the octapeptide, AII, by a carboxypeptidase found primarily in the pulmonary vasculature [25,30]. Thus, chronic ethanol consumption could elevate circulating AII levels and would therefore serve as a means of directly increasing smooth muscle vasoconstriction and to promote the release of catecholamines from adrenal glands which would further contribute to elevations in blood pressure Therefore, these data support the notion that the renin-angiotensin system mediates hypertension resulting from chronic alcohol consumption as postulated by others [18, 19, 32, 35]. However, a second major shortcoming of the present investigation was our failure to measure blood pressures in members of the acute and chronic ethanol treated groups. As previously cited there are several reports correlating chronic alcohol consumption with hypertension and heart disease in humans [2, 4, 5, 8, 11, 28, 33]. To our knowledge no experimentally controlled investigation of the laboratory rat has indicated a causal relationship between alcohol consumption and hypertension although modest elevations in pressure have been reported in dogs following 9–22 months of 3.1 g ethanol/kg/day [26], and the oral consumption of 400 ml of 25% ethanol by volume/day for 14 weeks [24]. In both of these studies alcohol treatment was terminated 1–2 days before blood pressure measurements were taken encouraging the possibility that these elevations in blood pressure were due to alcohol withdrawal rather than the direct effect of ethanol And in an earlier report, utilizing the laboratory rat, mean aortic blood pressure actually decreased when measured at the conclusion of 4 months of forced intake of 25% ethanol by volume in water provided ad lib [21].

In conclusion, future efforts must be devoted to determining whether alcohol-induced AII receptor changes are indeed dynamic requiring immediate assaying, and blood pressures must be measured concomitant with significant elevations in circulating angiotensin levels to determine whether the laboratory rat can serve as an animal model of alcohol-induced hypertensin

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