

## ROLE OF ACETALDEHYDE IN THE INDUCTION OF HEART LEFT VENTRICULAR ATRIAL NATRIURETIC PEPTIDE GENE EXPRESSION IN RATS

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**Abstract** — We studied the effects of ethanol and acetaldehyde on myocardial gene expression of atrial natriuretic peptide (ANP) and growth of rats. Combined ethanol and calcium carbimide treatment increased blood-acetaldehyde levels and ANP mRNA levels by 40–60% in 2–8 day experiments, compared to the controls. The results suggest a role for acetaldehyde in the development of alcoholic heart dysfunction.

### INTRODUCTION

Several studies have shown that the total lifetime dose of ethanol correlates positively with the development of alcoholic heart disease (Regan, 1971; Urbano-Marquez *et al.*, 1989), but the precise mechanism and the factors involved are still unidentified. Heart failure usually develops slowly and is preceded by periods of myocardial dysfunction during which overall cardiac pump function may be maintained by neuro-humoral regulatory mechanisms (Capasso *et al.*, 1991). Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are antagonists of a family of vasodilatory, natriuretic, and growth-inhibitory peptides (Levin, 1997). In the normal adult mammalian heart, ANP is synthesized predominantly in the cardiac atrioocytes and minor quantities of ANP mRNA have been detected in normal adult ventriculocytes. BNP mRNA is constitutively expressed in the adult rat and human left ventricular myocardium. It is widely documented that the gene expression of ANP in the ventricle is markedly induced during the process of cardiac hypertrophy upon ventricular overload (Ruskoaho, 1992). BNP secretion is stimulated by ventricular dilatation and sustained increases of intramural ventricular pressure (Yoshimura *et al.*, 1993).

Acetaldehyde, the first metabolite of ethanol, has been shown to have significant cardiovascular effects (James and Bear, 1967) and has been shown to interfere with protein synthesis in heart muscle (Siddiq *et al.*, 1993). Therefore, in the present study models of elevating the ethanol-derived blood-acetaldehyde levels by the aldehyde dehydrogenase inhibitor, calcium carbimide, were used and compared with effects of ethanol alone. Ventricular ANP and BNP gene expression, blood-ANP concentration as well as heart and body weights were studied in ethanol- and/or calcium carbimide-treated rats.

### MATERIALS AND METHODS

#### *Experimental animals*

In a preliminary study, left ventricular samples were taken from a limited number of rats participating in an experiment, in which the dose effect of calcium carbimide on alcohol

drinking was studied (Eriksson *et al.*, 1997). Three-month-old male (alcohol-preferring (AA) rats were exposed to ethanol *ad libitum* for 6 weeks ( $n = 3$ ). The other animals ( $n = 4$ ) had been drinking ethanol for 3 weeks prior to adding calcium carbimide to their diets (25 mg/kg body wt or 100 mg/kg of diet) in addition to drinking ethanol *ad libitum* for the remaining 3 weeks. Average alcohol consumptions were similar in all groups (4 g/kg/day). One day after stopping drinking (4–24 h before decapitation), all animals received an additional dose of ethanol (1 g/kg, i.p.). Blood samples for ethanol and acetaldehyde measurement were taken from the tail vein 2 days before decapitation.

In the experiment designed to clarify the results of our preliminary observation, 64 2-month-old male Wistar rats (Laboratory Animal Centre, University of Helsinki, Finland) were assigned randomly to eight experimental groups. Animals were housed in a temperature-controlled room with a 12 h light:12 h dark cycle and provided with chow and water *ad libitum*. The four experimental groups were as follows: controls (half of the group: saline 8.3 ml/kg i.p. once a day; half of the group no treatment); ethanol (1 g/kg body weight, i.p. as a 12% w/v solution in saline once a day); calcium carbimide (half of the group: 100 mg/kg of diet; half of the group: calcium carbimide 100 mg/kg of diet + saline, i.p.) and ethanol + calcium carbimide (ethanol 1 g/kg i.p. once a day + calcium carbimide 100 mg/kg of diet). The calcium carbimide diet started in the morning on day 1, ethanol on day 2. This way, in the 2-day experiment, rats received calcium carbimide for 2 days and ethanol for 1 day, and in the 8-day experiment rats received calcium carbimide for 8 days and ethanol for 7 days. Blood samples for ethanol and acetaldehyde measurement were taken from the tail vein 2 h after alcohol injection 1 day before decapitation and for N-terminal pro-ANP (NT-ANP) at the time of decapitation. After decapitation, hearts were removed within 60 s, weighed and the free walls of the left ventricles were carefully dissected and immediately frozen in liquid nitrogen for RNA isolation. All tissue samples were stored at  $-80^{\circ}\text{C}$  until mRNA analysis. The study protocols were approved by the National Public Health Institution Animal Care and Use Committee.

#### *Blood-ethanol and blood-acetaldehyde measurements*

Tail blood samples were haemolysed, and their ethanol and acetaldehyde contents measured with head-space gas

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chromatography on the day that the samples were collected (Eriksson *et al.*, 1977).

#### Radioimmunoassay (RIA) for plasma NT-ANP

Blood for NT-ANP assay was taken in tubes containing aprotinin (500 IU/ml) and EDTA (1 mg/ml) and immediately placed on ice. After centrifugation at 1000 *g* for 10 min at 4°C, aliquots of plasma were stored at -80°C and later subjected to RIA by a commercial kit (Biotop, Oulu, Finland). All the samples were run within the same assay.

#### Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using internal standards

RT-PCR was performed as described in our earlier study (Jänkälä *et al.*, 1997). Synthetic control RNA was produced by *in vitro* transcription, was 120 bases long and included sequences complementary for the ANP and BNP primers used (Feldman *et al.*, 1991). The cDNA was amplified in a DNA Thermal Cycler (Perkin Elmer) using 2.5 U Dynazyme DNA polymerase (Finnzymes, Finland). A trace amount of  $\gamma$ [<sup>32</sup>P]-labelled 3' primer was added to provide about  $1.5 \times 10^6$  cpm per reaction to label the DNA. The first cycle started with a 4 min denaturation at 96°C. In the following cycles, each step lasted for 1 min: the denaturation at 96°C, primer annealing at 54°C (ANP) or 58°C (BNP), and the synthesis step at 72°C. Oligonucleotide PCR primers complementary to the rat genes encoding ANP (Seidman *et al.*, 1984) and BNP (Kojima *et al.*, 1989) are the following: 5'-TCG AGC AGA TCG CAA AAG ATC-3' (ANP sense), 5'-CAC ACT AAA CCA CTC ATC TAC-3' (ANP antisense), 5'-CAG AAG ATA GAC CGG ATC g-3' (BNP sense), 5'-CAG GAT CAC TTG AGA GGT T-3' (BNP antisense). Since internal controls also contained the complementary sequence, the PCR primers amplified both sample and control cDNAs. Comparing the levels of radioactivity, the mRNA level of interest could then be calculated from the known amount of control RNA by using values from the exponential cycles (Feldman *et al.*, 1991).

#### Northern blot hybridization

Total RNA was isolated from ventricles using a modification of the acid guanidium thiocyanate/phenol/chloroform extraction (RNAzol B, Tel-Test Inc., Friedswood, TX, USA). Fifteen  $\mu$ g of total RNA were loaded onto a 1.2% (w/v)

agarose gel containing formaldehyde and, after electrophoresis, transferred onto Zeta-Probe GT membranes (BioRad, Hercules, CA, USA) by capillary transfer. The 1.0 kb bands of preproANP mRNAs were detected by hybridization with appropriate randomly primed  $\alpha$ [<sup>32</sup>P]-dCTP-labelled cDNA probe (Seidman *et al.*, 1984). The 1.6 kb glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were analysed identically to estimate the possible differences in the amounts of total RNA loaded.

#### Drugs

Calcium carbimide (Dipsan®) was purchased from Cyanamide Canada Inc., Montreal, Canada.

#### Statistical analysis

All the data are presented as means  $\pm$  SEM. The differences between study groups were analysed by the two-tailed Mann-Whitney U-test. *P* values < 0.05 were considered to be significant.

## RESULTS

#### Blood-ethanol and blood-acetaldehyde levels

In the preliminary study, morning blood-ethanol and blood-acetaldehyde samples were taken 2 days before decapitation. These represent the blood levels of ethanol and acetaldehyde during free-choice drinking and demonstrate that all the groups consumed alcohol. As evident in Fig. 1, despite this, ethanol levels were as expected, low in all groups, whereas those of acetaldehyde showed the expected increase after calcium carbimide administration.

In the 2- and 8-day experiments, blood samples for ethanol and acetaldehyde were taken 2 h after ethanol injection and thus represent peak blood concentrations. Ethanol and acetaldehyde levels are shown in Table 1. Some ethanol-treated rats displayed detectable acetaldehyde in blood, but, as expected, combined ethanol + calcium carbimide treatment induced much higher blood-acetaldehyde levels. The way saline (i.p.) was given to some of the rats had no effect on blood-ethanol or blood-acetaldehyde concentrations, neither did it affect other results.

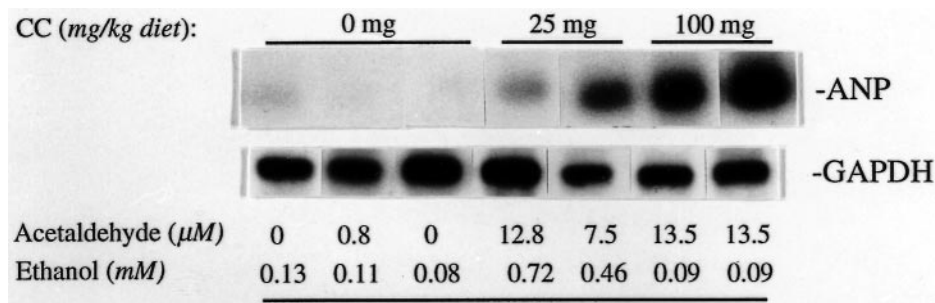


Fig. 1. Northern blot analysis of heart left ventricular mRNA, probed for atrial natriuretic peptide (ANP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes.

CC, calcium carbimide. Three rats were exposed to ethanol by drinking *ad libitum* for 6 weeks (CC = 40 mg/kg diet). Other animals had been drinking ethanol for 3 weeks prior to adding CC into their diets (25 mg/kg or 100 mg/kg of diet) in addition to ethanol *ad libitum* for the remaining 3 weeks. The size of mRNAs were 1.0 kb for ANP and 1.6 kb for GAPDH.

Table 1. Blood-ethanol and blood-acetaldehyde concentrations

Rats	Ethanol (mM)	Acetaldehyde ( $\mu$ M)
2-Day experiment		
Control	n.d.	n.d.
Ethanol	2.6 $\pm$ 0.6	2.1 $\pm$ 1.6
CC	n.d.	n.d.
Ethanol + CC	10.6 $\pm$ 0.8	205 $\pm$ 46
8-Day experiment		
Control	n.d.	n.d.
Ethanol	5.7 $\pm$ 0.7	n.d.
CC	n.d.	n.d.
Ethanol + CC	12.8 $\pm$ 0.5	418 $\pm$ 77

Blood-ethanol and blood-acetaldehyde levels were measured 2 h after ethanol injection 1 day before decapitation. Values are means  $\pm$  SEM for 8 rats/group. n.d., not detectable; CC, calcium carbimide (100 mg/kg of diet). Ethanol was given in a 1 g/kg body weight dose i.p.

Table 2. Heart weights, heart weight: body weight ratios and increases in body weights in each experimental group

Rats	Increase in body weight (%)	Heart weight (g)	Heart: body weight ratio ( $\times 10^3$ )
2-Day experiment			
Control	5.1 $\pm$ 0.6	1.14 $\pm$ 0.03	3.84 $\pm$ 0.06
Ethanol	4.5 $\pm$ 0.4	1.13 $\pm$ 0.04	3.86 $\pm$ 0.08
CC	0.0 $\pm$ 1.6***	1.09 $\pm$ 0.04	3.87 $\pm$ 0.09
Ethanol + CC	0.8 $\pm$ 0.4***	1.08 $\pm$ 0.04	3.94 $\pm$ 0.03
8-Day experiment			
Control	14.4 $\pm$ 1.1	1.21 $\pm$ 0.04	3.51 $\pm$ 0.09
Ethanol	12.9 $\pm$ 1.8	1.21 $\pm$ 0.03	3.73 $\pm$ 0.09
CC	11.5 $\pm$ 1.3	1.00 $\pm$ 0.02**	3.35 $\pm$ 0.08
Ethanol + CC	2.6 $\pm$ 1.3***	0.93 $\pm$ 0.10*	3.53 $\pm$ 0.06

Values are expressed as means  $\pm$  SEM for 8 rats/group. CC, calcium carbimide (100 mg/kg diet); ethanol (1 g/kg body weight). \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 compared with control.

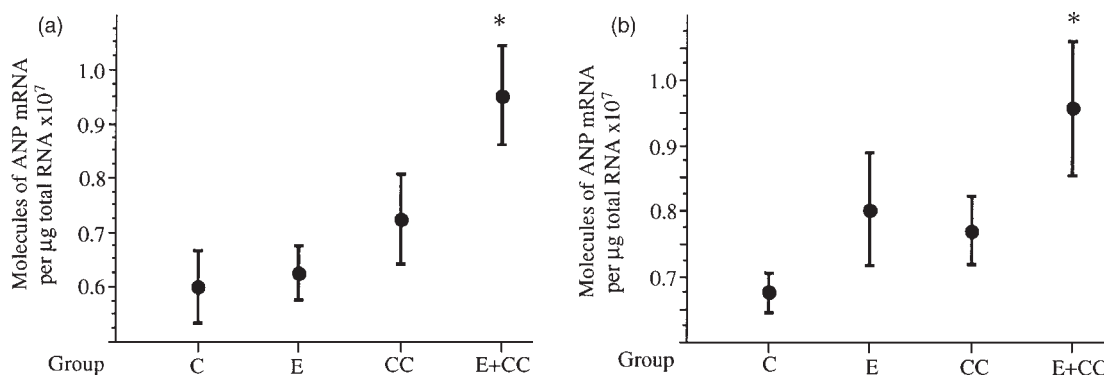


Fig. 2. Effects of ethanol, calcium carbimide and combined ethanol + calcium carbimide treatment on heart left ventricular ANP mRNA levels.

Ethanol (E, 1 g/kg) and calcium carbimide (CC, 100 mg/kg of diet) were given as described in Table 2. (a) Two-day experiment; (b) 8-day experiment. Data are shown as means  $\pm$  SEM for 8 rats in each group. \* $P$  < 0.05 as compared with the control group. Three dimensional analysis of variance performed on the combined 2- and 8-day data revealed independent effects of ethanol and calcium carbimide ( $P$  < 0.01 and  $P$  < 0.005, respectively).

### Body and heart weights

In the 2-day experiment, total body weight increased in the control group by 5.1  $\pm$  0.6%. Calcium carbimide (100 mg/kg of diet) treatment alone for 2 days, as well as combined with ethanol treatment, almost completely prevented the total body weight increase ( $P$  < 0.001 in both cases) when compared with controls (Table 2). In the 8-day experiment, total body weight increased in the control group by 14.4  $\pm$  1.1%. Calcium carbimide treatment alone had a negative effect only on heart weight, but heart weight:body weight ratio did not change significantly. Ethanol + calcium carbimide-treated rats, with elevated blood-acetaldehyde levels, demonstrated strong inhibition of the total body weight increase ( $P$  < 0.001), and showed lower total body weights (298  $\pm$  9 vs 344  $\pm$  8;  $P$  < 0.01) and heart weights (Table 2) compared with controls.

### ANP gene expression after ethanol ingestion for 6 weeks

There was only a minor amount of ANP gene expression demonstrable by Northern blot analysis in ethanol-treated rats, which is the expected finding in a healthy adult rat (Ruskoaho, 1992). ANP mRNA levels were, however, markedly induced in calcium carbimide + ethanol-treated rats (Fig. 1).

### ANP mRNA levels

As shown in Fig. 2(a), ethanol treatment (1 g/kg) daily increased left ventricular ANP mRNA levels by 5% and calcium carbimide treatment (100 mg/kg diet) by 22%, compared with the control group at 2 days (not significant). Combined ethanol + calcium carbimide treatment increased ANP mRNA levels by 60%, compared with the control group (0.96  $\pm$  0.09  $\times 10^7$  vs 0.60  $\pm$  0.07  $\times 10^7$ ;  $P$  < 0.05). In the 8-day experiment, the ethanol and the calcium carbimide-treated groups had 18% and 13% higher ANP mRNA levels respectively, compared with the control group (not significant). Combined ethanol + calcium carbimide treatment, however, elevated ANP mRNA levels by 41%, compared with the control group (0.96  $\pm$  0.10  $\times 10^7$  vs 0.68  $\pm$  0.03  $\times 10^7$ ;  $P$  < 0.05) (Fig. 2b).

### BNP mRNA levels

There were no statistically significant changes in BNP mRNA concentrations in the 2- or 8-day experiments. Thus, in the 2-day experiment, levels (molecules 1  $\mu$ g total RNA  $\times 10^7$  means  $\pm$  SEM for 8 rats per group) were as follows: control (1.00  $\pm$  0.10); ethanol (1.31  $\pm$  0.14); calcium carbimide (100 mg/kg diet) (1.18  $\pm$  0.20); ethanol + calcium carbimide

( $1.35 \pm 0.14$ ). The corresponding values in the 8-day experiment for the same groups were  $1.60 \pm 0.11$ ,  $1.85 \pm 0.26$ ,  $1.20 \pm 0.16$ , and  $1.60 \pm 0.17$  respectively.

#### *NT-ANP levels in plasma*

The ANP concentrations were measured in the 8-day experiment. No statistically significant differences in plasma NT-ANP between experimental groups were detected. Levels were as follows (pmol/l); controls:  $0.73 \pm 0.13$  ethanol-treated:  $0.60 \pm 0.05$ , calcium carbimide-treated:  $0.61 \pm 0.07$  and ethanol + calcium carbimide-treated:  $0.86 \pm 0.15$ .

## DISCUSSION

In this study, we have shown that treatment of rats with combined ethanol and calcium carbimide induces high blood-acetaldehyde levels and elevated preproANP mRNA concentrations in heart left ventricle wall similarly to, for example, pressure overload. The elevated expression of the ANP gene could be detected as early as after the second day of the experiment and it was still evident at 8 days of treatment. However, none of the animals studied showed any clinical signs of heart failure and the heart weight:body weight ratio was unchanged. BNP mRNA levels were not changed consistently in any of the experimental groups at the time points studied. Our results suggest that there was no sustained increase of intramural ventricular pressure, since this has been postulated to be a strong stimulus for BNP secretion (Yoshimura *et al.*, 1993). On the contrary, it has been reported that a 5 g/kg dose of ethanol had no effect on ventricular ANP mRNA levels at 30, 60 or 120 min, but BNP mRNA levels were reduced to 38% of control (Wigle *et al.*, 1995), which could not be repeated in our more prolonged experiment. In the present study, ventricular ANP mRNA production tended to be somewhat elevated in ethanol- or calcium carbimide- (for 2–8 days) treated rats. It is of note that the rats treated with ethanol alone had detectable but low acetaldehyde levels in blood. Therefore it is most likely that the high blood-acetaldehyde concentration activates the gene encoding for ANP in rat ventricular myocardium by an unknown mechanism, even without clinical evidence of heart failure or volume overload.

Chronic alcoholism is associated with dysfunction of the heart in as many as one-third of patients (Urbano-Marquez *et al.*, 1989). The duration of alcoholism reported by most authors is usually at least 10 years before cardiac symptoms appear (Regan, 1971). The transition from alcohol-induced injury, which is reversible, to permanent organ damage is not well understood. Clinical evidence indicates that the effects of alcohol on the myocardium are reversible if recognized and managed early and if alcohol intake is completely eliminated. Undoubtedly, there is considerable individual variation in susceptibility to alcohol and alcoholic heart disease, which is probably the result of several factors acting in concert in a susceptible person (Regan, 1971).

In earlier studies, acetaldehyde has been proposed to participate in the development of alcoholic heart disease by impairing myocardial protein synthesis (Siddiq *et al.*, 1993). This finding is significant in the light of the observation that

inhibition of myocardial protein synthesis leads to the development of myocardial failure in rabbit hearts with increased afterload (Zühlke *et al.*, 1965). It is possible that ANP could mediate the negative effect of acetaldehyde on protein synthesis, and it has been shown that ANP induces apoptosis in neonatal rat cardiac myocytes (Wu *et al.*, 1997). It is of interest that acetaldehyde has positive chronotropic and inotropic effects on the heart (Preedy *et al.*, 1994). Cardiac stimulation occurs at levels which normally develop in man after ingestion of ethanol and the stimulating effect of acetaldehyde can be blocked with propranolol, which suggests that it is due to the release of noradrenaline (James and Bear, 1967), which is known to be a stimulus for ANP synthesis (Ruskoaho, 1992). Acutely, ethanol intake increases susceptibility for arrhythmias, possibly due to sympathetic overdrive (Mäki *et al.*, 1998). Withdrawal of chronic alcohol abuse induces a period of hypersensitivity to catecholamines (Mäki *et al.*, 1990). However, there have been numerous reports on various effects of acetaldehyde on cellular and molecular levels, independent of catecholamines.

In the 2- and 8-day experiments of the present study, combined calcium carbimide + ethanol treatment had a statistically significant inhibitory effect on total body weight increase and calcium carbimide treatment alone had an inhibitory effect on body weight increase at 2 days. At 8 days, total body weights as well as heart weights of ethanol + calcium carbimide-treated rats were markedly lower, compared to values in the control group, but heart to body weight ratios, usually measured to detect clinical heart failure, were unchanged. An earlier study reported a 12% decrease in heart weights in rats, paralleling the reduction in body weight after 8 months of ethanol ingestion (Capasso *et al.*, 1991) and another group showed that 12 weeks of ethanol ingestion reduced both heart weight and heart weight:body weight ratio in rats (Brown *et al.*, 1996). In rats, combined ethanol + calcium carbimide treatment also decreases food consumption, and calcium carbimide has been shown to be anorectic *per se* (Eriksson, 1985). In isolated heart preparations, addition of acetaldehyde to the perfusate medium reduced rates of protein synthesis. An acute ethanol dose reduced protein synthesis by approximately 20%, as did treatment with both ethanol and 4-methylpyrazole. When treated with both ethanol and calcium carbimide, more marked reductions in translation rates occurred, implicating acetaldehyde as a powerful protein synthesis inhibitor (Siddiq *et al.*, 1993) and therefore a potential inducer of heart failure.

In summary, this study shows that 2 days to 3 weeks combined treatment with ethanol and calcium carbimide elevated blood-acetaldehyde concentrations and increased rat heart left ventricular ANP gene expression as typically seen in various overload and hypertrophy models of the heart. This suggests a potential role for acetaldehyde in the development of alcoholic heart disease. However, only studies performed on tissue and cellular levels will establish whether acetaldehyde is the actual triggering molecule of ANP induction and elucidate the molecular mechanisms involved. Moreover, further animal and human studies are needed to confirm whether the chronic exposure to much lower blood-acetaldehyde concentrations during prolonged alcohol abuse could be responsible for similar actions, and for alcoholic heart disease.



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