Species Difference in the Inhibition of Alcoholdehydrogenase by Carnitine and Acetylcarnitine

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Abstract

Acetylcarnitine, a metabolite of carnitine, has been proven to be a potent inhibitor of ethanol oxidation in hepatocytes. It inhibits the activity of alcohol dehydrogenase (ADH), but not the microsomal ethanol oxidizing system, which was significantly inhibited by acetylcarnitine at NAD:acetylcarnitine ≤ 1. The main objectives of this study were to ascertain the interaction between acetylcarnitine and NAD on ADH activity and to elucidate whether different species have different effects. The post-microsomal supernatant (PMS) was prepared from normal rat, guinea pig, mouse and broilers by differential centrifugation. Horse and yeast ADH were purchased from the Sigma Chemical Co. Prepared and purchased ADH are used for determination of ADH activity in the presence or absence of carnitine and acetylcarnitine. Binding studies showed that acetylcarnitine did bind to ADH in a dose related manner when low NAD:acetylcarnitine ratio was provided. It was found that the inhibition of ADH activity occurred only when NAD concentration was less than the inhibitor concentration. Crystalline and crude ADH preparations from different vertebrate species were inhibited by acetylcarnitine, whereas the yeast ADH was not affected by acetylcarnitine.

Key words: acoholdehydrogenase, carnitine, acetylcarnitine, NAD

INTRODUCTION

Ethanol has a wide variety of short- and long-term effects on human physiology (1). Hepatic ethanol oxidation is catalyzed primarily by alcohol dehydrogenase (ADH) and to a limited extent by MEOS and catalase under normal conditions (2,3). In mammals, ADH occurs in highest concentrations in the liver, where it is found in the cytoplasmic fraction (4). They are polymorphic and the isozymes can be classified based on their function and physicochemical properties. It has been found that isozyme ADH_3 is the enzyme responsible for ethanol oxidation in rat liver (5).

Carnitine (3-hydroxy-4-N-trimethylammonium butyrate) is widely distributed in living organisms and is biosynthesized in mammals. Food of animal origin especially, milk and meats are rich dietary sources of carnitine. Carnitine is an essential cofactor for facilitating fatty acid transport into the mitochondrial matrix where β-oxidation takes place (6). Without carnitine, most of the ingested fatty acids would not be fully metabollized for energy and the body would be forced to store the fatty acids as triglycerides in adipose tissue (7). Supplemtary carnitine has been shown to retard ethanol clearance and attenuate ethanol metabolism in intact animals under both acute and chronic conditions of ethanol administration (8,9). The effect is dose related (10) and specific to carnitine (11). Oxidation of orally administered [1-^{14}C]-ethanol to $^{14}CO_2$ is significantly reduced 4~12 hours after ethanol administration in the carnitine-supplemented intact rats. Further, in freshly prepared rat hepatocytes, while carnitine inhibits ethanol oxidation to a small degree, the inhibition by acetylcarnitine is severe and immediate (12). The activity of ADH, not MEOS, was significantly inhibited by acetylcarnitine at NAD: acetylcarnitine ≤ 1 (13,14).

The objective of this study was to ascertain the interaction between acetylcarnitine and NAD on ADH activity and whether their effects are species dependent.

MATERIALS AND METHODS

Enzyme preparation

Livers of rat, guinea pig, mouse and broilers were surgically cannulated and perfused with cold 0.15 M KCl in 0.01 mol/L phosphate buffer, pH 7.4. The liver was homogenized in ten volumes of KCl-phosphate buffer, pH 7.4, in a teflon/glass homogenizer. The homogenate was centrifuged at 600×g for 10 minutes at 2°C in a Beckman J2-21M induction drive centrifuge (Beckman Instruments, Inc. Palo Alto, CA). The 600×g supernatant (SN) was carefully transferred to another centrifuge tube and centrfuged at 10,000×g for 10 min. The 10,000 ×g SN was centrifuged further at 100,000×g for 1 hour. at 4°C in a L5-50 Ultracentrifuge. An aliquot of the post microsomal superntant was then saved. Protein concnetration in the PMS and microsomes was determined using Folin and ciocalteu's phenol reagent (15).

Determination of ADH activity

ADH activity was estimated by using a modified procedure of Bergmeyer et al. (16). Each reaction was performed in a total volume of 3 ml at 37°C. The components and final concentration in the reaction mixture were sodium pyrophosphate buffer

(55 mmol/L), pH 7.4, ethanol (20 mmol/L), NAD (0.2~2 mmol/L), reduced glycine (1.6 mmol/L), and enzyme preparation (2~3 mg SN protein). whenever necessary, deviation in the concentration of a component was made as it is indicated in the secific experiment. The reaction mixture was preincubated for 3 minutes at 37°C in a temperature controlled Backman Model 34 dual-wavelength spectrophotometer. The reaction was initiated by the addition of ethanol and absorbance at 340 nm was recorded for at least 3 minutes.

Acetylcarnitine binding assay

[1-^{14}C]-acetylcarnitine was synthesized according to the method of Murthy and Pande (17). In a 1.5 ml microcentrifuge tubes (conical bottom with attached closure) the total volumes of 0.5 ml contained sodium pyrophospate buffer, pH 7.4 (55 mmol/L), NAD(0, 1, 2, and 4 μmol/L), reduced glutathione (1.1 mmol/L), [1-^{14}C]-acetylcarnitine (2 μmol/L, specific radioactivity 123,659 dpm/nmole or 2061 Bq/nmole), and horse ADH (0.17 IU or 100 μg protein). The ratio of [NAD]:[^{14}C-acetylcarnitine] in each tube was controlled by adding different NAD concentration stated above resulting in NAD: [1-^{14}C]-acetylcarnitine ratio of 0, 0.5, 1, and 2. The reaction mixture was incubated for 5 minutes at 37°C in a Dubnoff metabolic shaking water bath (80 oscillation/min.). The reaction mixture was then transferred to an 30,000 NMWL Ultrafree-MC filter unit (Millipore, Bedford, MA) and centrifuged at the maximum speed (15,000×g) for 5 min in an Eppendof centrifuge, mode 15412 (Sybron/Brinkmann Instruments, Westbury, NY). The residue on membrane sample cup was transferred to a scintillation vial containing 5 ml scintillation fluid and the radioactivity was counted in a Beckman LS 3801 Liquid Scintillation counter.

Statistical analysis

All values are expressed as mean±SEM of triplicate determinations. The student's t-test was used to determine significance of the differences between the means of two groups at the level of p<0.05. When 2 or more treatments were compared, an ANOVA was employed followed by a Duncan's Multiple Range Test (18).

RESULTS AND DISCUSSION

Analysis of the products of ethanol oxidation by hepatocytes revealed 80% inhibition of acetaldehyde formation in the presence of acetylcarnitine suggesting that the site of inhibition must be at the enzymatic level (14). Since ADH is the main enzyme of ethanol oxidation, it was considered to be the target enzyme for studying the effect of carnitine and acetylcarnitine. A previous study showed that acetylcarnitine inhibited ADH only when the ratio of NAD:acetylcarnitine was low (13). The inhibition was minimum (7%) at the ratio of 1:1 and maximum (45%) at ratio of 1:8 (13,14). When this ratio was higher than 1:1, there was no effect of acetylcarnitine on ADH activity. On the other hand, at any ratio of NAD:carnitine, the inhibition of ADH activity was no more than 10% (13). Another previous

study (9) also confirmed that carnitine had little or no inhibitory effect on ADH activity. In an intact system, it would be difficult to maintain a low ratio of NAD:acetylcarnitine because NAD is being constantly regenerated and acetylcarnitine is being excreated in urine. However, the picture may be quite different in the chronic alcoholic condition.

In order to elucidate the site of acetylcarnitine interaction with ADH, a more direct assessment was made in the present study by studying the binding [1-^{14}C]-acetylcarnitine to ADH at different ratio of NAD: [1-^{14}C]-acetylcarnitine (Table 1). The binding of [1-^{14}C]-acetylcarnitine to ADH was decreased as the ratio of NAD:acetylcarnitine was increased. Inhibitor molecules bind to enzyme protein either at the coenzyme binding site or at the substrate binding site. Three main coenzyme binding sites of ADH where inhibitors are known to bind are 1) the adenosine binding cleft for aromatic molecules, 2) the anion binding site where the pyrophosphate group of the coenzyme binds, and 3) the nicotinamide binding region (19). Acetylcarnitine appears to bind to ADH at the site of NAD binding since NAD inhibited acetylcarnitine binding to ADH in a dose dependent manner. When the ratio of NAD:acetylcarnitine was increased from 0 to 2, binding of [1-^{14}C-acetylcarnitine to ADH decreased 67% (Table 1). The affinity of ADH for binding is greater for NAD than for acetylcarnitine as indicated by the preincubation experiment shown in Table 1. However, these experiments do not inhibitorily rule out the possibility of alteration of NAD concentration by acetylcarnitine.

There are a large number of molecules which have some degree of inhibitory effect on ADH activity. Among these, pyrasol and its 40 substituted derivatives are most potent, and widely studied with regard to the inhibition of ADH activity (20,21). The precise mode of binding of pyrazole has not been elucidated. However, it has been proposed that heterocyclic ring of pyrazole forms a bridge through its two nitrogen atoms and the catalytic zinc atom and C-4 of the nicotinamide moiety of NAD (19). The mechanism of an inhibitory action of acetylcarnitine on ADH activity might be similar to that of pyrazole since it also has a quaternary nitrogen.

Table 1. Binding of [1-^{14}C]-acetylcarnitine to horse alcoholdehydrogenase at different ratio of [NAD]:[1-^{14}C]-acetylcarnitine[1,2]

NAD:[1-^{14}C] Acetylcarnitine	[^{14}C] DPM/mg Enzyme protein	% Binding
0	5051±252[a]	5.1±0.23[a]
0.5	3845±240[b]	3.9±0.23[b]
1	3499±214[c]	3.5±0.17[c]
2	1772±102[d]	1.7±0.05[d]

[1]Conditions: 0.5 ml of reaction mixture contained sodium pyrophosphate buffer (55 mmol/L), pH 7.4, NAD (0-2 nmol/L), reduced glutathion (1.1 mmol/L, [1-^{14}C]-acetylcarnitine (1 nmol/L), and horse ADH (0.17 μmol/L or mg protein). Reaction mixture was incubated for 5 min. at 37°C, filtered through millipore filter by centrifugation, and counted radioactivity in the filter.
[2]The values are means±SEM of 3 determinations.
Values bearing different superscript letters in same column are significantly different at p≤0.05.

The effect of carnitine and acetylcarnitine on the activity of ADH derived from different species is shown in Fig. 1, 2. Neither carnitine nor acetylcarnitine inhibited yeast ADH activity (A of Fig. 1). However, activities of ADH from horse, rat, guinea pig, mouse and broilers were significantly inhibited by acetylcarnitine (B of Fig. 1 and Fig. 2). The specificity may be related to different isozymes of the species (22). The ADH is not a single enzyme but a system consisting of many isozymes which are species specific to a certain degree. Yeast and mammalian ADH differ in substrate specificity and rate of catalytic activity (19). Human and rodent ADH exists as a heterogeneous group of isoenzymes that can be placed into three categories based upon structural and functional distinctions (23). There are clear analogies between human ADH classes and rat

ADH isoenzymes. There is approximatly 82% sequence homology among subunits of human and rat liver ADH (22). The kinetics and molecular properties of rat ADH₃ are similar to those of human class I isoenzymes. It is well known that ethanol is the substrate for classical human class I ADH (24) and rat ADH₃ (22) isoenzyme. Both human class I ADH and rat ADH₃ are mainly localized in the liver. Rat ADH₃ enzyme exhibits a Km of 1.4 mmol/L for ethanol, and is strongly inhibited by pyrazole, Ki=0.4 μmol/L.

This data provides molecular explanation for carnitine mediated attenuation of ethanol metabolism in the intact rat (8,12), mouse (9) and broiler chicken (25). Similarities and differences in ethanol metabolism among species must be born in mind in the extrapolation of rat data to human.
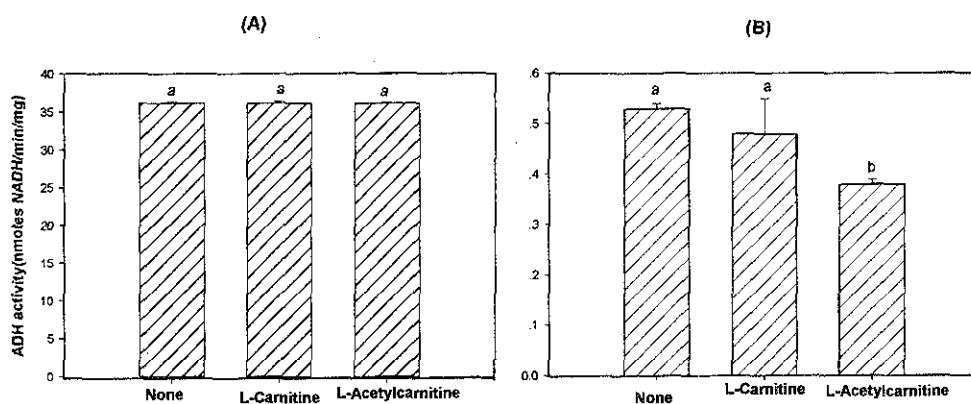
(A)   **(B)**

Fig. 1. Inhibition of alcoholdehydrogenase from Yeast (A) and Horse (B) by carnitine and acetylcarnitine. Values are the means±SEM of triplicate determinations. The Yeast and Horse ADH were purchased from Sigma chemical Co. Values with the same superscript letter are not significantly different at p<0.05.
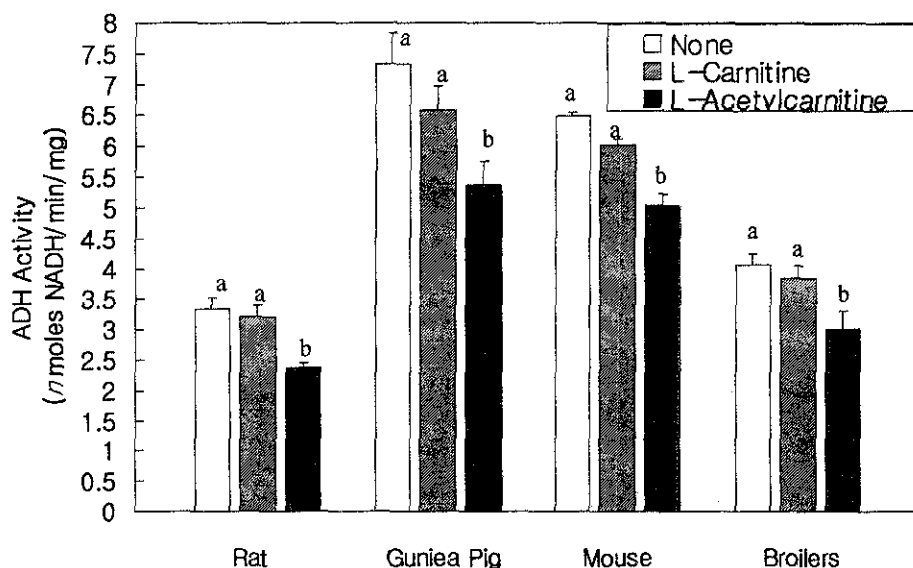

Fig. 2. Inhibition of ADH from different species by carnitine and acetylcarnitine. Values are the means±SEM of triplicate determinations. The ADH were prepared by differential centrifugation and 100,000×g supernatant were used. Values with the same superscript letter are not significantly different (p<0.05).

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