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In vitro co-metabolism of acetoacetate and ethanol in human hepatic mitochondrial and cytosolic fractions

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Abstract

The rate of alcohol elimination is highly resistant to acceleration in vivo in well-nourished individuals. The acceleration of ethanol elimination may be achieved by providing the conditions in which the action of alcohol dehydrogenase is not delayed by the insufficiency of the oxidized NAD form. The aim of the study was to verify the theoretically assumed mechanism of accelerating alcohol elimination by administering excessive acetoacetate (Ac-Ac) in the experimental in vitro model. Ac-Ac forming the redox system with β-hydroxybutyrate (β-HBA) is the natural acceptor of excessive protons from ethanol oxidation. Ac-Ac and β-HBA penetrate freely through the cell membranes and are easily assimilated energetic substrates. The examinations were performed using the hepatic homogenates (collected from the cadavers shortly after death) supplemented with ethanol and Ac-Ac. The ethanol levels were determined at 0, 15, 60, 90 and 150 min of the experiment. The findings showed that the equimolar addition of Ac-Ac resulted in a two- to three-fold increase in ethanol oxidation in hepatic homogenates. The biochemical system discussed above resembles the natural way of utilizing the excessive NADH, which is formed during ethanol combustion in chronic alcoholics. The results indicate that further investigations are necessary to assess the clinical importance of this metabolic system.

Keywords: Acetoacetate; Ethanol; Co-metabolism; Ketone bodies; NADH reoxidation

1. Introduction

In Poland, ethanol intoxication has constituted the highest percentage of fatal poisonings for many years. In medico-legal practice, the interactions of ethanol with various substances is extremely important because in many cases the cause of death is due to the synergistic activity of ethanol and other agents. In vitro research on human liver tissue has led to a better understanding of the interactions of ethanol with various substances which, on the one hand, alter the action of many drugs, but on the other, may result in slowing or acceleration of alcohol elimination. From a clinical point of the view, it is a very important to find methods to increase the rate of ethanol elimination in critically intoxicated individuals. In vivo studies to evaluate methods to increase alcohol elimination are difficult to perform because of obvious ethic implications. Unfortunately animal models are often unsuitable as they do not exactly replicate the human metabolism [1]. However, the effects of toxic concentrations of xenobiotics may be evaluated in vitro using tissue samples collected shortly after death.

The rate of alcohol elimination is highly resistant to acceleration in vivo in well-nourished individuals [2,3].

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The elimination of ethanol after single dose administration, as opposed to enzyme induced chronic alcoholics, is mainly by the action of alcohol dehydrogenase (ADH) in the liver. Only a small amount of alcohol is oxidized by the microsomal oxidizing system (MEOS) and extrahepatic enzymes, or excreted in the unchanged form [3]. Alcohol oxidation by ADH in the initial and late stages of alcoholaemia is characterized by kinetics similar to a first-order reaction, as even at low blood ethanol levels (approximately 2 mmol/l) ADH becomes completely saturated by the substrate and ethanol oxidation then follows the kinetics of a zero-order reaction [2].

Therefore, the acceleration of ethanol elimination may be achieved by providing the conditions in which the action of ADH is not delayed by the insufficiency of the oxidized NAD form. Based on this rationale attempts were made to accelerate ethanol elimination by altering the levels of proton acceptors which theoretically should facilitate the reconstitution of NAD from NADH [2–6].

An "ideal" NAD reactivator should be characterized by:

(a) the ability to accept protons, i.e. to form the redox system with its reduced metabolite;
(b) non-toxicity of the acceptor and its reduced form, including the capacity to excrete or metabolize their excess in the organism;
(c) easy penetration, of the acceptor and its reduced form, through the biological membranes.

Our previous studies focused our attention on acetoacetate (Ac-Ac), which by forming a redox system with β-hydroxybutyrate (β-HBA) is a natural acceptor of excessive protons from ethanol oxidation in chronic alcoholics [7]. Furthermore, the studies on the processing of ketogenesis in hypothermia revealed that ethanol strongly affects the production of ketone bodies [8,9]. Thus, reversing this process by administering excessive Ac-Ac may lead to the acceleration of ethanol elimination. Ac-Ac and β-HBA both freely penetrate through cell membranes, and are easily assimilated energy substrates which are oxidized preferentially before glucose and fatty acids. This feature becomes extremely important in starvation when most of the energy needs of glucose-dependent tissues (e.g. of the brain) are covered by combustion of those compounds. Additionally, any excess of Ac-Ac should spontaneously break down to acetone as the process is non-enzymatic [10].

Therefore, the aim of the study was to evaluate the Ac-Ac/β-HBA system as a possible mechanism to accelerate alcohol elimination in an experimental in vitro model using human hepatic mitochondrial and cytosolic fractions [11].

2. Materials and methods

The experiments were performed according to the earlier studies [12,13] using the hepatic homogenates obtained from six unselected cadavers (five men and one woman aged 17, 18, 21, 26, 52 and 80) 2–6 h after their death and frozen at −75 °C. The specimens were collected at post mortem from individuals who died due to: road accidents ($N = 3$), hanging ($N = 2$) and circulatory diseases ($N = 1$), all were sober at the time of death. The harvested liver samples showed no visible macro- or microscopic abnormalities.

The frozen hepatic samples were minced, placed in liquid nitrogen and crushed in a porcelain mortar. The obtained homogenate was diluted 1:1 with phosphate buffer 0.2 M pH = 7.4 and centrifuged at $600 \times g$ for 5 min to remove the cell nuclei and unfragmented hepatic tissue. Then the S0.6 fraction of supernatant containing cytoplasm with mitochondria was centrifuged at $9000 \times g$ for 30 min to obtain the fraction S9 without mitochondria. Both the S0.6 and S9 fractions were divided into 0.5 ml samples which were stored at −75 °C. All post mortem-derived hepatic homogenates revealed high ADH and β-hydroxybutyrate dehydrogenase (HBDH) activities.

The reaction mixtures were prepared by adding 0.4 ml of S0.6 or S9 to 40 ml of phosphate buffer and 0.2 ml of 30 mM NAD^+. The two solutions were mixed and then 0.2 ml of 0.1 M ethanol was added (the additional mixture was prepared from S9 supplemented with 0.2 ml of HBDH). Each solution was immediately divided into two 20 ml portions. 0.1 ml of 0.1 M Ac-Ac was added to the one portion and 0.1 ml of water to the other (control). 3.0 ml of each mixture was then placed in the test tubes containing 0.1 ml of 0.1% pyrazole ("0" tests). Both portions were then incubated at 30 °C. At 15, 60, 90 and 150 min after the onset of the reaction, 3.0 ml from each was collected (also to the test tubes with pyrazole which discontinued the action of ADH). For each sample, the ethanol levels were determined by gas chromatography using the headspace technique. The gas chromatograph used was Fisons 8160 with the Fisons HS-800 autosampler and capillary column Restek BAC-1. The solutions were prepared using deionized water. The reagents were from Sigma–Aldrich, except HBDH (50 mg/10 ml) which was obtained from Boehringer. The Ac-Ac concentration was controlled by gas chromatography (after thermal decarboxylation) prior to use because of its instability [10,14].