EFFECTS OF CARNOSINE AND RELATED COMPOUNDS ON THE STABILITY AND MORPHOLOGY OF ERYTHROCYTES FROM ALCOHOLICS

V. D. PROKOPIEVA1, N. A. BOHAN1, P. JOHNSON2*, H. ABE3 and A. A. BOLDYREV4

1Mental Health Research Institute, Medical Academy of Sciences of Russia, Tomsk, Russia, 2Department of Chemistry and Biochemistry and Department of Biomedical Sciences, Ohio University, Athens, OH 45701, USA, 3Laboratory of Marine Biochemistry, University of Tokyo, Bunkyo-ky, Tokyo 113–8567, Japan and 4Department of Biochemistry, International Biotechnological Center of M. V. Lomonosov Moscow State University, 199899 Moscow, Russia

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Abstract — The effects of carnosine and related compounds on erythrocytes from alcoholics were studied. In their presence, erythrocytes showed an increased ability to resist haemolysis and showed a more normal morphology, with carnosine and N-acetyl-carnosine being the most effective compounds. These beneficial properties of the dipeptides do not appear to be directly related to their antioxidant or buffering properties.

INTRODUCTION

One of the consequences of alcoholism is an elevated level of erythrocyte haemolysis and chronic anaemia (Chi and Wu, 1991; Bizzaro et al., 1993). The alcoholic haemolysis appears to be related to the effects of ethanol on erythrocyte membrane ion transport systems (Stibler et al., 1984; Gastaldi et al., 1988; Benaim et al., 1994) and to changes in the fluidity of the erythrocyte membrane caused by ethanol consumption (Beaure et al., 1985, 1988; Benedetti et al., 1986). Such changes to the properties of the membrane may be caused by direct effects of ethanol on the membrane (Goldstein, 1986) or by the formation of reactive metabolites of ethanol which can damage membrane components oxidatively (Goldstein and Chin, 1981; Sozmen et al., 1994; Grattagliano et al., 1995; Soszynski and Schuessler, 1998).

Carnosine (β-alanyl-L-histidine) is a natural hydrophilic antioxidant (Boldyrev et al., 1988; Kohen et al., 1988) found in animal tissues (Boldyrev and Severin, 1990), which can decrease the levels of free radicals through its direct interaction with reactive oxygen species (Boldyrev, 1993; Klebanov et al., 1997). In addition to carnosine, some animal tissues contain derivatives of this compound, such as anserine, ophidine, and N-acetyl-carnosine, which may also function as natural antioxidants (Boldyrev and Abe, 1999). Carnosine has previously been shown to have protective effects on membranes, although this may not be due solely to its antioxidant properties (Alabovsky et al., 1997; Hipkiss, 1998).

Because of the antioxidant properties of carnosine and its derivatives and their presence in animal tissues, we have examined in the present study the effects of these compounds on the properties of erythrocytes from alcoholics, as such erythrocytes are known to be oxidatively damaged. We have examined the effects of these compounds on the resistance of the erythrocytes to haemolysis and on the morphology of the cells.

MATERIALS AND METHODS

Erythrocyte sources and preparation

Blood samples from 30 male alcoholics who had withdrawn from alcohol for between 1 and 5 days prior to donation were used in this study. These subjects had only 10–20% of their erythrocytes showing normal morphology, as determined by light microscopy. For control subjects, 11 healthy non-alcoholic males were used. The average age of all subjects in the study was 40.8 ± 7.5 years. Institutional approval for this study was obtained from the Mental Health Research Institute, Medical Academy of Sciences of Russia, Tomsk, and informed consent of all donors was obtained prior to blood sample collection. Sodium citrate (final concentration 0.38%, w/v) was used to prevent coagulation of the drawn blood and erythrocyte purification was performed as previously described (Chi and Wu, 1991), with the erythrocytes being used within 1 h of collection, and with each experiment lasting no longer than 4 h.

Acid haemolysis of erythrocytes

The stability of erythrocytes was determined by the time course of erythrocyte haemolysis in the presence of 2 mM HCl. In this procedure, erythrocytes were suspended in a 0.9% (w/v) NaCl solution (pH 5.5) at 23 ± 0.5°C, and haemolysis was estimated from the change in optical density at 670 nm following addition of HCl (Stusj, 1994). The compounds tested were added to the incubation medium 5 min prior to HCl addition at concentrations between 1 and 10 mM. These concentrations were chosen based on the biological effectiveness of carnosine and related compounds, which is in the millimolar range (Boldyrev and Abe, 1999) and on their average concentrations in brain and muscle tissues of 2–10 mM (Boldyrev and Severin, 1990).

Treatment of erythrocytes with carnosine and related dipeptides

Stock solutions of carnosine and its derivatives anserine (N-1-methyl-carnosine), ophidine (N-3-methyl-carnosine) and N-acetyl-carnosine as well as histidine and N-acetyl-histidine were prepared in a 0.9% NaCl solution adjusted to pH 5.5 and were added to erythrocyte preparations 5 min prior to haemolysis to give appropriate final concentrations (noted in

*Author to whom correspondence should be addressed.
the legends to the figures and tables). Equal concentrations of PIPES [piperazine-N-N-bis (2-ethane sulphonate)] in a solution of the same pH were added to control samples in order to control for the buffering capacity of these imidazole compounds.

Erythrocyte morphology determination

The size and shape of erythrocytes were characterized by light microscopy using an MBI-15-2 microscope (LOMO, Russia). Microscope pictures were taken at a 1400× magnification after staining of the erythrocytes with Romanovsky’s dye (Britton, 1969; Zolotnitskaya, 1987).

Statistical analysis

Haemolysis data in Figs 1 and 2 and morphological analyses in Table 1 are presented as the means ± SD. Haemolysis curves were judged to be significantly different from each other when ANOVA indicated that significant differences (P < 0.05) existed between each data set on the curves from 3 min to 6 min of haemolysis time. Morphological data were analysed by ANOVA and a P < 0.05 was used as evidence of a significant difference between data sets.

RESULTS

In preliminary experiments, the effects of anserine, ophidine, histidine, and N-acetyl-histidine (all at 2 mM) on acid haemolysis of erythrocytes from alcoholic subjects were examined (results not shown). With the exception of histidine, all caused an increase in resistance to acid haemolysis with the following order of efficiency: N-acetyl-histidine < anserine = ophidine < carnosine < N-acetyl-carnosine. Because N-acetyl-carnosine and carnosine were the most effective compounds in increasing resistance to haemolysis, further studies were focused on these two compounds.

Figure 1 shows haemolytic curves of erythrocytes from normal (non-alcoholic) individuals. Neither 2 mM carnosine nor 2 mM PIPES had a significant effect on the resistance of the cells to acidic haemolysis, but the results of duplicate experiments with 2 mM N-acetyl-carnosine showed a right shift of the haemolysis curve, which is suggestive of enhanced resistance to haemolysis.

Figure 2 shows the haemolytic curve for erythrocytes from normal individuals and the curves for erythrocytes from alcoholics in the absence and presence of carnosine and N-acetyl-carnosine. It was found that erythrocytes from alcoholic subjects have a significantly decreased resistance to haemolysis in comparison to the cells from normal subjects as evidenced by the left shift of the curve. For erythrocytes from alcoholics, both carnosine and N-acetyl-carnosine significantly increased the resistance to haemolysis to greater levels than those found for untreated erythrocytes from non-alcoholic subjects. These results also show that N-acetyl-carnosine was significantly more effective than carnosine in increasing resistance to haemolysis, as seen from the more pronounced right shift of the curve in the presence of N-acetyl-carnosine.

The beneficial effects of carnosine and N-acetyl-carnosine on erythrocytes from alcoholic subjects were also seen in morphological analysis of the erythrocytes. As shown in Fig. 3, erythrocytes obtained from non-alcoholic individuals had a normal shape with a diameter of 7.0–7.5 μm (Fig. 3A), whereas erythrocytes from alcoholics (Fig. 3B) had variable sizes (anisocytosis), and abnormal shapes (poikilocytosis and acanthocytosis). In the presence of carnosine (Fig. 3C) or N-acetyl-carnosine (Fig. 3D), the morphology of the erythrocytes was normalized, the proportion of poikilocytes was reduced, acanthocytes were absent and no evidence of anisocytosis was seen. In some cases, N-acetyl-carnosine was even found to cause a complete restoration of normal size and shape of the erythrocytes. Figure 3E shows that PIPES did not cause normalization of cell parameters, indicating that the effects of carnosine and N-acetyl-carnosine were not related to their osmotic or buffering properties.
Data for the effects of carnosine, N-acetyl-carnosine and PIPES on the morphology of erythrocytes from alcoholics were quantified and are presented in Table 1. Whereas PIPES treatment had no significant effect on morphology, the addition of carnosine or N-acetyl-carnosine resulted in large and statistically significant increases in the percentages of erythrocytes.
with normal cell morphology. Although N-acetyl-carnosine treatment gave a higher mean percentage of normal cells than did treatment with carnosine (approximately 40% compared to 30%), this difference itself was not statistically significant ($P = 0.086$).

**DISCUSSION**

The present studies reveal that, of the carnosine-related compounds tested, carnosine and N-acetyl-carnosine were the most effective in increasing the resistance to haemolysis and normalizing the morphology of erythrocytes from alcoholics. These effects, which were not seen with erythrocytes from non-alcoholic control subjects, suggest that the dipeptides may normalize membrane structure and stability in erythrocytes from alcoholics given that resistance to haemolysis and alterations in erythrocyte morphology have been related to the disordering of the normal interaction between the membrane and cytoskeleton (Chi and Wu, 1991; Sozmen et al., 1994), and suggests that the dipeptides may become integrated into the erythrocyte membrane and reverse the disordering effects (Hipkiss, 1998). There are, however, no previous reports on the integration of histidine dipeptides into membranes, although other membrane-protecting effects of these dipeptides, such as protection against osmotic shock in fibroblasts (Kantha et al., 1996) and oxidative damage in neurons (Boldyrev et al., 1998), have been reported.

Previous studies have shown that carnosine is a more effective antioxidant than N-acetyl-carnosine, as judged by the effects of these compounds on the viability of neurons under oxidative stress (Boldyrev et al., 1988; Boldyrev and Abe, 1999). However, in the present work on erythrocytes from alcoholics, N-acetyl-carnosine was found to be more effective than carnosine in increasing resistance to haemolysis and in improving erythrocyte morphology. These results indicate that the effects of these compounds on erythrocytes cannot be explained simply by their antioxidant properties, but may be related to other effects of the dipeptides, such as direct interaction with membrane components or modulation of erythrocyte enzyme activities, which are components of the membrane repair systems. The precise mechanisms by which these compounds can stabilize the erythrocyte membrane remain unknown, and further investigations will be necessary in order to understand the molecular mechanisms by which they ameliorate the pathology of erythrocytes from alcoholics.

Although carnosine and N-acetyl-carnosine are present at millimolar levels in certain tissues, such as muscle and the central nervous system (Boldyrev and Severin, 1990), the circulatory levels of these compounds in normal individuals may be only transiently increased after meat consumption or administration of carnosine into the bloodstream (Boldyrev et al., 1988). Stationary concentrations of carnosine in blood are less than 0.5 mM, because the hydrolytic enzyme carnosinase is present in the serum of many mammals including humans (Scrivener and Perry, 1988; Boldyrev, 1998). Furthermore, a previous study (Duane and Peters, 1988) has found that the level of serum carnosinase is decreased in alcoholics, and this alteration would mean that serum carnosine levels would be different in alcoholics than in normal individuals. It is however interesting to note that carnosinase exhibits a much lower catalytic effectiveness for the hydrolysis of N-acetyl-carnosine (A. Pegova et al., submitted for publication) and this may enable N-acetyl-carnosine to be used as a treatment for the improvement of erythrocyte structure in chronic alcoholics. Further studies in this area will be needed to determine the efficacy of carnosine or N-acetyl-carnosine therapy for this purpose and to establish appropriate dosages for therapy which will generate millimolar levels of these compounds in the circulatory system.

**REFERENCES**


