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Short communication

THE ANTIOXIDANT PROPERTIES OF CARNITINE *in vitro*

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Abstract: Many of the effects of carnitine are ascribed to its antioxidant properties. The aim of this study was to evaluate the antioxidant properties of carnitine *in vitro*. Carnitine was found to decolorize $ABTS^{\bullet+}$, and to protect fluorescein against bleaching induced by AAPH-derived peroxyl radicals and peroxynitrite, thiol groups against oxidation induced by hydrogen peroxide, peroxyl radicals, hypochlorite and peroxynitrite, and erythrocytes against hemolysis induced by peroxyl radicals and hypochlorite. These results show that carnitine has a direct antioxidant action against physiologically relevant oxidants.

Key words: Antioxidant, Carnitine, Hydrogen peroxide, Hypochlorite, Peroxyl radical, Peroxynitrite

INTRODUCTION

L-Carnitine (β-hydroxy-γ-4-*n*-trimethylaminobutyric acid) is an important metabolite that performs many functions in the body. Its main metabolic role is the transport of long-chain fatty acids into the mitochondrial matrix across the inner mitochondrial membrane. It is also a cofactor in the peroxisomal oxidation of very long-chain fatty acids, and it plays a role in the central nervous system,

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Abbreviations used: AAPH – 2,2'-azobis(2-amidinopropane) dihydrochloride; ABTS – 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate); DTNB – 5,5'-dithio-bis(2-nitrobenzoic acid); PBS – phosphate-buffered saline

mediating the transfer of acetyl groups for acetylcholine synthesis and affecting signal transduction pathways and gene expression. It intensifies respiration and peroxisome function and is often used as a food additive supposed to increase physical performance [1, 2], promote weight loss [3, 4] and increase reproductive performance [5, 6]. It has been also found to improve immunological functions [7, 8] and is suggested to be a therapeutic agent for several neurodegenerative disorders [9, 10].

The beneficial effects of carnitine appear to be mediated, at least in part, by its antioxidant properties, which include the upregulation of the level of glutathione [8, 9] and the elevation of the activities of antioxidant enzymes [8]. The *in vivo* effects may be mediated by the direct antioxidant action of carnitine or by its effects on the biosynthesis of antioxidant enzymes and other proteins. In this context, it is of interest to characterize the antioxidant properties of carnitine in well-defined systems *in vitro*, as this may contribute to the elucidation of the mechanisms of the antioxidant action of this compound. Previous studies have demonstrated that carnitine reacts with superoxide and hydrogen peroxide, has reducing properties, chelates iron, and prevents lipid peroxidation and DNA cleavage [11, 12]. The aim of this study was to further characterize the antioxidant properties of carnitine *in vitro*, particularly with regard to pathophysiologically relevant oxidants such as peroxynitrite and hypochlorite. These oxidants are not neutralized enzymatically *in vivo*, so low-molecular weight antioxidants that would protect against their action would be even more important than ones protecting against superoxide or hydrogen peroxide, which are removed enzymatically.

MATERIALS AND METHODS

Reagents

The reagents were obtained from Sigma/Aldrich (Poznań, Poland) unless stated otherwise. Peroxynitrite was synthesized from sodium azide and ozone according to the method of Pryor *et al.* [13] with minor modifications [14]. This method yields low ionic strength peroxynitrite that does not contain hydrogen peroxide as an impurity. Briefly, an ozone stream from an ozonator (75 μg/ml in oxygen, 100 ml/min) was bubbled for about 60 min through a glass-frit into 100 ml of 0.2 M sodium azide in water that had had its pH adjusted to 12 with 1 N NaOH and had been chilled to 0ºC in an ice-water mixture. The final concentration of peroxynitrite was about 70-90 mM. Stock solutions were stored at -85ºC and used within 4-6 weeks after synthesis. Before each experiment, the concentration of peroxynitrite was estimated spectrophotometrically at 302 nm in 0.1 M NaOH ($\varepsilon_M = 1670\ M^{-1}cm^{-1}$).

Reactivity with $ABTS^{\bullet+}$

We used a slight modification [15] of the methods of decolorization of the $ABTS^{\bullet+}$ radical [16]. $ABTS^{\bullet+}$ was prepared via a reaction of 19.5 mg of

ammonium 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) with 3.3 mg of potassium persulfate in 7 ml of 100 mM sodium phosphate buffer, pH 7.4, for 12 to 16 h in the dark. The thus-obtained dark blue-green solution of $ABTS^{\bullet+}$ was aliquoted and kept frozen at -20ºC until use. The aliquots were thawed and diluted with the same buffer to give an absorbance of 1.0 at 414 nm immediately before use. Aliquots of solutions of the tested compounds (10 µl) in the phosphate buffer were mixed rapidly with 990 µl of the diluted $ABTS^{\bullet+}$ solution in a spectrophotometric cuvette, and the absorbance was measured exactly 3 min after mixing the antioxidant(s) with the $ABTS^{\bullet+}$ solution.

The protection of fluorescein against oxidant-induced bleaching

Fluorescein (2 µM) in 100 mM phosphate buffer, pH 7.4, was mixed in the presence or absence of the tested antioxidants, with oxidants, namely 20 µM HOCl, $ONOO^-$ or 10 mM AAPH (final concentrations), in a final volume of 1 ml. The fluorescence of the samples was measured at the excitation/emission wavelengths of 480 nm/514 nm in a Hitachi F-2500 spectrofluorimeter after a 15-min incubation with HOCl or $ONOO^-$ at room temperature, or after a 60-min incubation with AAPH at 37ºC. The results were analyzed in a way analogous to that presented by Kohri *et al.* [17] by plotting $\Delta F/\Delta F_o - 1$ vs the concentration of the antioxidant, where ΔF is the decrease in fluorescence induced by an oxidant in the presence of a given concentration of that antioxidant, and ΔF_o is the decrease in fluorescence induced by the same amount of the oxidant in the absence of an antioxidant. The initial part of this curve is linear (Robaszkiewicz *et al.*, submitted).

The protection of thiol groups against oxidation

Glutathione solution (250 µM, final) in phosphate-buffered saline (145 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4; PBS) was mixed in the presence or absence of the tested antioxidants, with oxidants, namely 0.5 mM H_2O_2, 10 mM AAPH, 375 µM HOCl or 500 µM $ONOO^-$ (final concentrations) to make up a volume of 500 µl. After a 15-min incubation at room temperature (hypochlorite and peroxynitrite) or a 1-h incubation at 37ºC (H_2O_2, AAPH), the samples were diluted with 1 ml of 0.1 M phosphate buffer, pH 8.0, and added with 10 µl of 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). The absorbance of the thionitrobenzoic acid was measured at 412 nm.

The protection of erythrocytes against oxidant-induced hemolysis

Aliquots of erythrocyte suspensions in phosphate-buffered saline containing no oxidant or supplemented with antioxidants were added with an oxidant (30 mM AAPH or 375 µM hypochlorite) to a final volume of 200 µl. The changes in their turbidance were measured at 600 nm in an Anthos 2010 absorbance reader after a 15-min incubation at room temperature for hypochlorite, or after various times of incubation at 37ºC for AAPH. The initial concentration of erythrocytes was chosen to provide an initial turbidance for the suspension of 1.0. All the presented results are mean values from at least 3 independent experiments.

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