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The effects of oral Cardax™ (disodium disuccinate astaxanthin) on multiple independent oxidative stress markers in a mouse peritoneal inflammation model: influence on 5-lipoxygenase in vitro and in vivo

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Abstract

Disodium disuccinate astaxanthin (‘*rac*’-dAST; Cardax™) is a water-dispersible C40 carotenoid derivative under development for oral and parenteral administration for cardioprotection of the at-risk ischemic cardiovascular patient. In experimental infarction models in animals (rats, rabbits, and dogs), significant myocardial salvage has been obtained, up to 100% at the appropriate dose in dogs. The documented mechanism of action in vitro includes direct scavenging of biologically produced superoxide anion; in vivo in rabbits, modulation of the complement activity of serum has also been shown. A direct correlation between administration of the test compound in animals and reductions of multiple, independent markers of oxidative stress in serum was recently obtained in a rat experimental infarction model. For the current study, it was hypothesized that oral Cardax™ administration would inhibit oxidative damage of multiple relevant biological targets in a representative, well-characterized murine peritoneal inflammation model. A previously developed mass spectrometry-based (LC/ESI/MS/MS) approach was used to interrogate multiple distinct pathways of oxidation in a black mouse (C57/BL6) model system. In vivo markers of oxidant stress from peritoneal lavage samples (supernatants) were evaluated in mice on day eight (8) after treatment with either Cardax™ or vehicle (lipophilic emulsion without drug) orally by gavage at 500 mg/kg once per day for seven (7) days at five (5) time points: (1) baseline prior to treatment ($t=0$); (2) 16 h following intraperitoneal (i.p.) injection with thioglycollate to elicit a neutrophilic infiltrate; (3) 4 h following i.p. injection of yeast cell wall (zymosan; $t=16$ h/4 h thioglycollate+zymosan); (4) 72 h following i.p. injection with thioglycollate to elicit monocyte/macrophage infiltration; and (5) 72 h/4 h thioglycollate+zymosan. A statistically significant sparing effect on the arachidonic acid (AA) and linoleic acid (LA) substrates was observed at time points two and five. When normalized to the concentration of the oxidative substrates, statistically significant reductions of 8-isoprostane-$F_{2\alpha}$ (8-iso-$F_{2\alpha}$) at time point three (maximal neutrophil recruitment/activation), and 5-HETE, 5-oxo-EET, 11-HETE, 9-HODE, and $PGF_{2\alpha}$ at time point five (maximal monocyte/macrophage recruitment/activation) were observed. Subsequently, the direct interaction of the optically inactive stereoisomer of Cardax™ (*meso*-dAST) with human 5-lipoxygenase (5-LOX) was evaluated in vitro with circular dichroism (CD) and electronic absorption (UV/Vis) spectroscopy, and subsequent molecular docking calculations were made using mammalian 15-LOX as a surrogate (for which XRC data has been reported). The results suggested that the *meso*-compound was capable of interaction with, and binding to, the solvent-exposed surface of the enzyme. These preliminary studies provide the foundation for more detailed evaluation of the therapeutic effects of this compound on the 5-LOX enzyme, important in chronic diseases such as atherosclerosis, asthma, and prostate cancer in humans.

Keywords: Astaxanthin; Cardax™; Carotenoids; Disodium disuccinate astaxanthin; HETEs; HODEs; Inflammation; LC/ESI/MS/MS; 5-Lipoxygenase; Oxidative stress

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Introduction

Disodium disuccinate astaxanthin (‘*rac*’-dAST; Cardax™), a water-dispersible C40 carotenoid derivative, has demonstrated potent cardioprotective actions in vivo after both oral and parenteral administration. In 3 relevant experimental infarction model systems (rat, Gross and Lockwood, 2004; canine, Gross and Lockwood, 2005; rabbit, Lauver et al., 2005), myocardial salvage was obtained after either multiple-dose or single-dose parenteral pre-treatment regimens. The well-characterized function of non-esterified astaxanthin as an antioxidant in model systems (Britton, 1995; Miki, 1991; Shimidzu et al., 1996) has recently been supplemented with information on its anti-inflammatory activity in additional biological model systems (Aoi et al., 2003; Lee et al., 2003; Ohgami et al., 2003). In conjunction with others, we have shown that the cardioprotective activity of the novel diester was at least partially attributable to anti-complement activity in serum (Lauver et al., 2005). We originally demonstrated the potent antioxidant activity of Cardax™ against biologically-produced superoxide anion in an isolated human neutrophil assay in vitro (Cardounel et al., 2003). Documentation of in vivo antioxidant activity against multiple serum oxidative stress markers after oral administration of the compound in the Sprague–Dawley rat experimental infarction model was subsequently obtained (Gross et al., 2006).

As a result of the evidence for focused antioxidant, anti-inflammatory activity obtained in the rat ischemia–reperfusion model, a more inclusive screening of oxidative targets was chosen to further establish the utility of this compound for oxidative stress reduction. Oxidative injury is believed to participate in both generalized inflammatory as well as cardiovascular disorders such as atherosclerosis (Beckman and Ames, 1998; Jiang and Ames, 2003; Libby and Theroux, 2005; Steinberg and Witztum, 2002). In the current study, it was hypothesized that subchronic oral disodium disuccinate astaxanthin administration would inhibit oxidative damage of multiple biological targets in a representative, well-characterized murine peritoneal inflammation model (Zhang et al., 2002). We have previously developed mass spectrometry-based approaches to interrogate multiple distinct pathways of oxidation in biological systems (Shishehbor et al., 2003; Zhang et al., 2002). Our first goal was to define relevant pathways and time points for inhibition of oxidation using a tractable and readily performed model of inflammation—a murine peritonitis model—in concert with these mass spectrometry studies. Black mice were administered Cardax™ (500 mg/kg) or vehicle (lipophilic emulsion without drug) once per day by oral gavage for one week prior to performance of the inflammation model on day eight (8). In vivo measures of oxidant stress were then evaluated in peritoneal lavage samples taken from black mice (C57BL/6 strain) at the following time points: (1) baseline prior to treatment ($t=0$); (2) 16 h following intraperitoneal (i.p.) injection with thioglycollate to elicit a neutrophilic infiltrate; (3) 4 h following i.p. injection of yeast cell wall (zymosan; $t=16$ h/4 h thioglycollate+zymosan); (4) 72 h following i.p. injection with thioglycollate to elicit monocyte/macrophage infiltration; and (5) 72 h/4 h thioglycollate+zymosan. At these five time points, mice underwent peritoneal lavage, and supernatants were analyzed for multiple, distinct protein and lipid oxidation products using LC/ESI/MS/MS techniques.

Based on the data obtained in this preliminary evaluation, further studies of the in vitro interaction of the optically inactive (*meso*) form of disodium disuccinate astaxanthin (*meso*-dAST) and the human 5-lipoxygenase enzyme (5-LOX) were performed. *Meso*-dAST forms 50% of the ‘racemic’ mixture of stereoisomers in ‘*rac*’-dAST (Frey et al., 2004), and induced optical activity upon binding with protein ligands can be easily measured by using the optically inactive form of the carotenoid derivative. Circular dichroism (CD) and electronic absorption (UV/Vis) techniques developed in previous studies with relevant human protein (e.g. human serum albumin, HSA; Zsila et al., 2003, 2005) were adapted for the current study. Once an interaction was defined, the X-ray crystallographic (XRC) structure reported previously for mammalian 15-lipoxygenase was utilized to model probable binding locations for the *meso*-carotenoid ligand on 5-LOX, for which XRC data has not been reported. The results from this study demonstrate statistically significant reductions in multiple markers from peritoneal lavage samples of biologically relevant oxidative pathways in vivo, and suggest activity of this compound against, and possible interaction with, the mammalian 5-lipoxygenase enzyme, emerging as an important modulator of inflammatory activity in murine (Mehrabian et al., 2002) and human (Spanbroek et al., 2003) cardiovascular disease.

Materials and methods

Materials

Cardax™ (‘racemic’ disodium disuccinate astaxanthin; ‘*rac*’-dAST) was synthesized from crystalline astaxanthin [3*S*,3′*S*, 3*R*,3′*S*, 3*R*,3′*R* (1:2:1); Fig. 1], a statistical mixture of stereoisomers obtained commercially (Buckton-Scott, India; Frey et al., 2004). This material was utilized for oral gavage studies in mice [purity >97.0% by high performance liquid chromatography (HPLC), as area under the curve (AUC)].

The individual astaxanthin stereoisomers were also separated by HPLC as dicamphanate esters and then saponified to non-esterified astaxanthin, allowing for the synthesis of the *meso*-disodium disuccinate astaxanthin derivative (*meso*-dAST) for testing in the current study (Frey et al., 2004). The all-*trans* (all-*E*) form of the *meso* stereoisomer used was a linear, rigid molecule (bolaamphiphile) owing to the lack of *cis* (or *Z*) configuration(s) in the polyene chain of the spacer material (Fig. 2; Foss et al., 2005). The disodium disuccinate derivative of synthetic *meso*-astaxanthin was successfully synthesized at >99% purity by HPLC (as AUC).

Five hundred (500) units of human recombinant 5-lipoxygenase (5-LOX; Product No. 437996, Lot No. B60857; in 100 mM Tris containing 5 mM EDTA, pH 8.0; specific activity 18.48 units/mg protein) was obtained from Calbiochem (San Diego, CA, USA) and was used without modification. Tris HCl buffer (0.1 M, pH 8.0) and spectroscopy grade dimethyl