Cardioprotection and myocardial salvage by a disodium disuccinate astaxanthin derivative (Cardax™)

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Abstract

Cardioprotection in humans by carotenoids has been inferred from observational and epidemiologic studies, however, direct studies of cardioprotection and myocardial salvage by carotenoids are lacking. In the current study, intravenous (I.V.) pre-treatment with a novel carotenoid derivative (disodium disuccinate astaxanthin; Cardax™) was evaluated as a myocardial salvage agent in a Sprague–Dawley rat infarct model. Animals were dosed once per day I.V. by tail vein injection for 4 days at one of 3 doses (25, 50, and 75 mg/kg) prior to the infarct study carried out on day 5. The results were compared with control animals treated with saline vehicle. Thirty (30) minutes of occlusion of the left anterior descending (LAD) coronary artery was followed by 2 hours of reperfusion prior to sacrifice, a regimen which resulted in a mean infarct size (IS) as a percent (%) of the area at risk (AAR) of 59 ± 3%. Area at risk was quantified by Patent blue dye injection, and infarct size (IS) was determined by triphenyltetrazolium chloride (TTC) staining. Cardax™ at 50 and 75 mg/kg for 4 days resulted in a significant mean reduction in IS/AAR to 35 ± 3% (41% salvage) and 26 ± 2% (56% salvage), respectively. Infarct size and myocardial salvage were significantly, and linearly, correlated with plasma levels of non-esterified, free astaxanthin at the end of reperfusion. These results suggest that parenteral Cardax™ may find utility in those clinical applications where pre-treatment of patients at risk for myocardial infarction is performed.

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Introduction

Ischemia-reperfusion (I/R) injury is a complex phenomenon, generally attributed to the additional damage produced by reintroduction of oxygenated blood flow to a previously ischemic area. The participation of radical, non-radical, reactive oxygen species (ROS) and reactive nitrogen species (RNS) has been well documented in experimental animals as well as in human subjects (Maxwell and Lip, 1997). Once lipid peroxidation begins in ischemic tissue, endogenous or exogenous antioxidants capable of radical chain-breaking will be required to terminate this chain reaction (Papas, 1999). Of the radical chain-breaking antioxidants, vitamin E and the carotenoids are the most well recognized. However, at low oxygen tension, only the carotenoids will likely be active as antioxidants at physiologically achievable concentrations (Beutner et al., 2001; Burton and Ingold, 1984).

Carotenoids are a related group of greater than 700 well-characterized compounds, with potent antioxidant capacity that is directly related to their physicochemical structure (Britton, 1995). The family of carotenoids is divided into hydrocarbon carotenoids (“carotenes”) and oxygen-substituted carotenoids (“xanthophylls”). The naturally-occurring carotenoids—as well as synthetic carotenoid derivatives—are excellent physical quenchers of singlet oxygen as well as lipid peroxidation chain breakers (Miki, 1991; Oliveros et al., 1994). However, the poor aqueous solubility of most carotenoids has limited their use as aqueous-phase singlet oxygen quenchers, direct radical scavengers, and lipid-peroxidation chain-breakers, and complicates parenteral delivery of these compounds (Lockwood et al., 2003). Numerous strategies have been attempted to increase the water solubility of carotenoids, including co-solvent formulations such as tetrahydrofuran (THF) (Bertram, 1999), liposomal systems (Goto et al., 2001), and complexation with cyclodextrins (Lockwood et al., 2003). In each case, either toxicity or poor overall solubility has limited the in vivo utility of these formulations. Derivatization of carotenoid compounds into novel chemical entities has been sparingly reported in the literature (Bikádi et al., 2002; Hertzberg and Liaaen-Jensen, 1985; Oliveros et al., 1994), and many derivatives also share the additional problem of supramolecular assembly, a form of 3-dimensional aggregation in aqueous solution that limits their ability to participate in oxidation-reduction reactions [reviewed in (Simonyi et al., 2003)].

Hawaii Biotech, Inc. (HBI) successfully synthesized a novel carotenoid derivative, the disodium disuccinate derivative (Cardax™) of synthetic astaxanthin [from all-trans (all-E) 3S,3′S, meso (identical 3R,3′S and 3′R,3S), and 3R,3′R dihydroxy-β,β-carotene-4,4′-dione) in a 1:2:1 statistical mixture]; (Fig. 1). This novel derivative exhibits water “dispersibility” of approximately 8.64 mg/mL, allowing for parenteral injection in aqueous formulation. Cardax™ has been well characterized as a direct scavenger of biologically-produced aqueous-phase superoxide anion by electron paramagnetic resonance (EPR) spectroscopy (Cardounel et al., 2003), and is carried in serum after injection by albumin, allowing it to “piggyback” with other drugs and fatty acids to ischemic tissues such as heart (Zsila et al., 2003). Since direct scavenging of lynchpin reactive oxygen species (ROS) such as superoxide anion in the aqueous phase, lipid-peroxidation chain-breaking in the lipophilic phase, and physical quenching of singlet oxygen are potential mechanisms that may limit or completely inhibit injury due to ischemia-reperfusion (Maxwell and Lip, 1997), we tested the potency and efficacy of I.V. Cardax™ to limit infarct size (IS) in a well-validated Sprague–Dawley rodent model of experimental infarction (Ludwig et al., 2003). Three doses of Cardax™ were injected once per day I.V. by tail vein injection (25, 50, and 75 mg/kg) for 4 days prior to experimental infarction on day 5, and compared with saline vehicle-treated controls. The results demonstrate, for the first time, significant myocardial salvage (56% reduction in IS/AAR at the
highest dose tested) using a subchronic pre-treatment regimen by a synthetic parenteral carotenoid derivative.

**Methods**

**Materials**

*Cardax*™ was synthesized from crystalline astaxanthin [3R,3′R, 3R,3′S/3′R,3S (meso), 3S,3′S (1:2:1)], a mixture of stereoisomers obtained commercially (Buckton Scott, India). The all-trans (all- *E*) form of the mixture of stereoisomers 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. The disodium disuccinate derivative of synthetic astaxanthin was successfully synthesized at >97% purity by HPLC (as AUC; chemical structures of individual stereoisomers shown in Fig. 1).

**Preparation of stock solutions of Cardax™ and vehicle for injection**

*Cardax™* was dissolved directly in sterile-filtered (0.2 micron Millipore® filter) deionized (DI) water. The maximum “dispersibility” of *Cardax™* in pure aqueous formulation is slightly greater than 10 mM (8.64 mg/mL). Vehicle used was isotonic sterile saline. The maximum solubility of *Cardax™* in aqueous formulation limits the total amount of drug that can be administered by tail vein in a single I.V. injection in Sprague–Dawley rats of this size (approximately 200 grams) without introducing hemodynamic changes. Hence, the maximum dose achievable in the current study was 75 mg/kg.

**Dosing schedule**

Each animal received *Cardax™* aqueous formulation (25, 50, or 75 mg/kg), or an equal volume of sterile saline, once per day by I.V. tail vein injection. The injection was repeated for a total of 4 days, with the experimental infarction(s) conducted on day 5. Care was taken to limit extravasation of aqueous formulation into the connective tissue of the rodent tail. A total of three control animals were studied for each of the three dose groups of *Cardax™*-treated animals.

**Infarct size determination**

General surgery and determination of infarct size was performed as previously described (Patel et al., 2001). Briefly, Sprague–Dawley rats (175–200 g) were anesthetized (Inactin, 100 mg/kg) and the jugular vein and carotid artery cannulated for drug delivery and blood pressure and heart rate measurements. A tracheotomy was then performed, and rats were ventilated with an artificial ventilator (Harvard Apparatus, South Natick, MA). Blood gases were monitored periodically (AVL 995 pH/Blood Gas Analyzer). Normal values were maintained by adjusting the respiratory rate and/or the tidal volume. Body temperature was maintained at 37 °C by heating pads.

Once heart rate and blood pressure stabilized, a left thoracotomy was performed followed by a pericardiotomy. A ligature (6-0 Prolene) was passed below the left anterior descending coronary artery (LAD) to the right portion of the left ventricle. The ends of the suture were threaded through a propylene tube to form a snare. Occlusion was elicited by pulling on the snare and clamping the snare onto the epicardial surface using a hemostat.

After 2 hours of reperfusion, the coronary artery was again occluded. The area at risk (AAR) was determined by negative staining with Patent blue dye. The heart was excised, and cut into thin cross-sectional pieces. The normal area and AAR were separated and stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC) in 100 mM phosphate buffer (pH 7.4). Tissues were fixed overnight in 10% formaldehyde, and the infarcted tissue was dissected from the AAR using a dissecting microscope (Cambridge Instruments, Woburn, MA) the following day. IS was expressed as a percent of the AAR (IS/AAR, %).
Plasma concentrations of non-esterified, free astaxanthin

To determine the plasma concentrations of non-esterified, free astaxanthin in blood, samples were taken at the end of reperfusion in selected rats (N = 25) treated with one of the 3 doses of Cardax™, and determined by methods previously described (Østerlie et al., 2000). Free astaxanthin is generated after cleavage of the water-dispersible disuccinate diester in vivo to mono-succinate, and subsequently to non-esterified, free astaxanthin by the intrinsic esterase activity of serum albumin (Curry et al., 1999), or by non-specific esterase activity in serum and solid organs (Jensen et al., 1999). Non-esterified, free astaxanthin then accumulates in myocardium and other solid-organ tissues after plasma clearance in a dose-dependent manner (Showalter et al., in press).

Statistical analyses

Statistical analyses were performed with the Prism 4 package (GraphPad Software Inc., San Diego, CA). One-way analysis of variance (ANOVA) on means of treatment groups versus controls was followed by Student–Newman–Keuls post hoc analysis. Regression analysis of the plasma non-esterified, free astaxanthin concentrations versus IS/AAR were performed by a least squares method. Data are reported as mean ± standard error of the mean (mean ± SEM). ** Denotes significance at the P < 0.05 level.

Fig. 2. Mean infarct size (IS) as a percentage of area at risk (AAR) in control animals (vehicle injection alone) and in animals treated with 3 doses of Cardax™ (25, 50, and 75 mg/kg). Cardax™-treated animals received the appropriate dose once daily I.V. by tail vein injection for 4 days prior to experimental infarction and IS/AAR determinations on day 5. Significant reductions in infarct size compared with control animals were seen in animals treated with 50 and 75 mg/kg (one-way ANOVA, each P < 0.001; ** denotes significance at the 0.05 level; NS = not significantly different from control).
Results

Hemodynamics

A total of 43 rats (out of 53 that began the study) survived the entire protocol. Three (3) animals fibrillated in the control group, as well as 2 each in the Cardax™-injected animals at each of the 3 doses used. Three (3) animals were eliminated because of problems with the multiple drug injections. There were no differences in heart rate, blood pressure, or blood gases at baseline or throughout the experimental protocol performed on day 5 (data not shown).

Myocardial salvage

A total of 9 animals served as vehicle-treated controls. Mean infarct size of the control animals was 59 ± 3% of the area at risk (Figs. 2 and 3). Infarct size in control animals was taken as 100% (IS = 100% in controls) and myocardial salvage in controls as 0% (salvage = 0% in controls) for the myocardial salvage calculations.

Eight (8) animals received 25 mg/kg Cardax™ once daily I.V. by tail vein injection, and the mean infarct size was 47 ± 4% of the AAR (Figs. 2 and 3). The mean infarct size was not significantly

![Graph](image-url)  

Fig. 3. Mean myocardial salvage as a percentage of infarct size/area at risk. Control IS/AAR set at 0% salvage. Control animals received vehicle injection alone; Cardax™ - treated animals received the appropriate dose once daily I.V. by tail vein injection for 4 days prior to experimental infarction and IS/AAR determinations on day 5. Myocardial salvage of 56% was achieved at the highest dose administered (75 mg/kg).
reduced relative to that in control animals (one-way ANOVA; P > 0.05, NS; Fig. 2). Mean myocardial salvage in this group was 20% (Fig. 3).

Twelve (12) animals received 50 mg/kg Cardax™ once daily I.V. by tail vein injection, and the mean infarct size was 35 ± 3% of the AAR (Fig. 2). This was a statistically significant reduction in IS/AAR

Fig. 4. The relationship between the plasma concentration (X-axis) of non-esterified, free astaxanthin determined in plasma samples obtained from rats that had been treated for 4 days with either 25, 50 or 75 mg/kg of Cardax™, versus IS/AAR (Y-axis) from the corresponding rat at each dose. There was a statistically significant (P < 0.0001**) inverse linear correlation (r² = 0.67) between drug plasma concentrations and IS/AAR utilizing the pre-treatment strategy.

Fig. 5. Mean plasma concentrations of non-esterified, free astaxanthin (nM) in rats subjected to 30 minutes of left anterior descending coronary artery (LAD) occlusion and 2 hours of reperfusion, following 4 days of Cardax™ injections given at 25, 50 and 75 mg/kg I.V. daily. The acute experiment was performed on day 5. All values are the mean ± SEM of 5, 12 and 8 experimental animals, respectively.
as compared to the control animals (one-way ANOVA; P < 0.001**; Fig. 2). Mean myocardial salvage was 41% (Fig. 3). Similarly, fourteen (14) animals received 75 mg/kg Cardax™ once daily I.V. by tail vein injection, and the mean infarct size was 26 ± 2% (Fig. 2). This was also a statistically significant reduction in IS/AAR relative to control animals (one-way ANOVA; P < 0.001**; Fig. 2). Mean myocardial salvage in this group was 56% (Fig. 3). The observed reduction in IS/AAR was significantly correlated (r² = 0.67, P < 0.0001**) with the plasma concentration of non-esterified, free astaxanthin generated by in vivo cleavage of Cardax™ (Fig. 4).

**Plasma levels of non-esterified, free astaxanthin**

The mean plasma concentrations of non-esterified, free astaxanthin at the end of 2 hours of reperfusion are depicted in Fig. 5. Non-esterified, free astaxanthin concentrations increased in a dose-dependent manner from 107 ± 24 nM at 25 mg/kg of Cardax™ to 335 ± 56 nM at 50 mg/kg, and finally to 612 ± 56 nM at the 75 mg/kg dose.

**Discussion**

In the current study, a novel water-dispersible carotenoid derivative was utilized as a myocardial salvage agent in a well-validated experimental infarction model in male Sprague–Dawley rats (Ludwig et al., 2003). A pre-treatment strategy was utilized, mimicking the intended treatment strategy in human populations at-risk for coronary events [e.g. secondary prevention of coronary heart disease (Stephens et al., 1996) and/or post-emergent treatment of unstable angina, in which coronary markers of myocardial necrosis, including cardiac troponins and creatine kinase (CK) MB fractions, are initially negative (Baldus et al., 2003)]. In the population of individuals with a diagnosis of unstable angina, those on maximum medical therapy still progress to myocardial infarction and/or sudden cardiac death at an annualized rate of 10 to 15% (R.J. Gibbons M.D., personal communication). It is postulated that the antioxidant, anti-inflammatory, and cardioprotective agent astaxanthin (generated from the soft-drug precursor Cardax™ (Buchwald and Bodor, 2002; Ohgami et al., 2003), given chronically to these at-risk patients might reduce a potential consequence of coronary occlusion (i.e. infarct size), as was demonstrated in the rodent infarct model in the current study. Administration of Cardax™ only at the time of reperfusion—modelling the clinical application in humans of post-MI treatment—was not attempted with the current compound, as hemisuccinates can be poor and/or highly variable esterase substrates in vivo (Jensen et al., 1999). Cleavage of the Cardax™ soft drug to lipophilic non-esterified, free astaxanthin during the 3 to 6 hour “golden window” of therapeutic intervention in acute MI has not been reliably achieved with this compound (Showalter et al., in press; Lockwood, unpublished results).

Plasma levels of non-esterified, free astaxanthin—obtained after 4-day pre-treatment at the end of reperfusion—were associated with linear, dose-dependent decreases in infarct size in the current study. This has potential implications for future clinical utility, as peripheral blood draws for serum and/or plasma concentration of free carotenoid will be easier to obtain than target tissue concentrations in humans (i.e. myocardial levels of free carotenoid), for which biopsy must be utilized. If an antioxidant mechanism for salvage is important in vivo, it is likely that nanomolar concentrations of non-esterified, free astaxanthin will be necessary for myocardial salvage; the ED₅₀ for astaxanthin
as an antioxidant in model systems is approximately 200 nM (Kurihara et al., 2002; Miki, 1991). Plasma levels of non-esterified, free astaxanthin in excess of 200 nM were easily achieved in this study (Fig. 4).

The mechanism(s) of action of carotenoids, and in particular astaxanthin, in cardioprotection have not been well characterized. Aoi et al. (2003) demonstrated excellent accumulation of non-esterified, free astaxanthin in rodent myocardium after chronic oral administration of esterified, natural source astaxanthin (Guerin et al., 2003). A dose-dependent cardioprotective effect was seen in their ROS-mediated strenuous exercise injury model, with antioxidant and anti-inflammatory [i.e. reduction of tissue myeloperoxidase (MPO) levels] effects observed in that seminal study. Ohgami et al. (2003) demonstrated multiple effects of intravenous astaxanthin administration in a uveitis model in rats, including anti-inflammatory [reductions in levels of nitric oxide (NO), tumor necrosis factor alpha (TNF-α), and prostaglandin E2 (PGE2)] and direct enzyme-inhibiting activity (against inducible nitric oxide synthase, iNOS). These results, coupled with the well-established role of carotenoids as modulators of connexin 43 production and maintenance and/or restoration of gap junctional communication in mammals (Bertram, 1999), suggest a multi-factorial influence of astaxanthin in cardioprotection as observed in the current study. Future studies will be directed to evaluation of the cardioprotective mechanism of action of Cardax™ in models of inflammation and experimental infarction in animals.

In summary, significant myocardial salvage is reported for the first time in the current study for a novel carotenoid derivative which generates non-esterified, free astaxanthin after parenteral administration in vivo. The current results, coupled with the results of Aoi et al. (2003), strongly suggest that cardioprotection by non-esterified, free astaxanthin will be achievable by both oral and I.V. administration, particularly in those mammalian species in which carotenoids are highly bioavailable (Clark et al., 2000). When administered as a cardioprotective pre-treatment, dose-dependent myocardial salvage was achieved up to 56% of the ischemic area at risk. The benign safety profile of non-esterified, free astaxanthin in vivo, coupled with the increased utility of injection of Cardax™, may combine to provide increased clinical utility of this novel derivative in those cardiovascular applications in humans in which pre-treatment of patients at risk for future cardiac events is practical.

References


