Disodium Disuccinate Astaxanthin (Cardax) Attenuates Complement Activation and Reduces Myocardial Injury following Ischemia/Reperfusion

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ABSTRACT

Carotenoids are a naturally occurring group of compounds that possess antioxidant properties. Most natural carotenoids display poor aqueous solubility and tend to form aggregates in solution. Disodium disuccinate astaxanthin (DDA; Cardax) is a water-dispersible synthetic carotenoid that rapidly and preferentially associates with serum albumin, thereby preventing the formation of supramolecular complexes and facilitating its efficacy after parenteral administration. This study investigated the ability of DDA to reduce inflammation and myocardial injury in a rabbit model of ischemia/reperfusion. DDA (50 mg/kg/day) or saline was administered i.v. for 4 consecutive days before the initiation of the protocol for induction of myocardial ischemia/reperfusion. On the 5th day, rabbits underwent 30 min of coronary artery occlusion, followed by a 3-h reperfusion period. Myocardial infarct size, as a percentage of the area at risk, was calculated for both groups. Infarct size was 52.5 ± 7.5% in the vehicle-treated (n = 9) and 25.8 ± 4.7% in the DDA-treated (n = 9) animals (p < 0.01 versus vehicle; mean myocardial salvage = 51%). To evaluate the anti-inflammatory effects of DDA, complement activity was assessed at the end of reperfusion using a red blood cell lysis assay. DDA administration significantly reduced (p < 0.01) the activation of the complement system in the serum. The current results, coupled with the well-established antioxidant ability of carotenoids, suggest that the mechanism(s) of action by which DDA reduces the tissue damage associated with reperfusion injury may include both antioxidant and anticomplement components.

The generation of reactive oxygen species during the reintroduction of blood flow to a previously ischemic area is one of the main mechanisms underlying myocardial reperfusion injury. Damage occurs when endogenous free radical scavenging mechanisms are overwhelmed or are themselves adversely affected by the ischemic insult (Park and Lucchesi, 1999). The ability of reactive oxygen species to alter myocardial function has been shown in vitro and in vivo (Jolly et al., 1984; Bolli et al., 1989). The exposure of the heart to free radical generating systems or the addition of H2O2 to the perfusion medium is associated with alterations in cardiac function and biochemical changes similar to those observed in the damaged myocardium as a result of ischemia and reperfusion. Therefore, it has been hypothesized that administration of antioxidant compounds will attenuate myocardial damage attributed to reperfusion injury.

Carotenoids are a group of naturally occurring pigments that show antioxidant capabilities related to their physiochemical structures (Britton, 1995). These compounds are efficient antioxidants and are known to be physical quenchers of singlet oxygen, as well as chain terminators of lipid peroxidation in cell membranes and intracellular membrane structures (Devasagayam et al., 1992; Cantrell et al., 2003). The carotenoid family of compounds is divided into two groups: the hydrocarbon “carotenes” and the oxygen-substituted “xanthophylls.” Until recently, the clinical use of carotenoids as parenteral therapeutic agents has been limited primarily because of their poor aqueous solubility. Many of the early attempts to develop more soluble carotenoid derivatives resulted in compounds that shared a tendency for supramolecular assembly, a form of three-dimensional aggregation in aqueous solution that limited their ability to participate in oxidation-reduction reactions (Simonyi et al., 2003).

Cardax [disodium disuccinate astaxanthin (DDA)] is a disodium disuccinate derivative of synthetic astaxanthin (Frey et al., 2004). This derivative exhibits water “dispersibility” of...
approximately 8.64 mg/ml, allowing for parenteral injection in aqueous formulation. DDA has been well characterized as a direct scavenger of biologically produced aqueous-phase superoxide anion by electron paramagnetic resonance spectroscopy (Cardounel et al., 2003) and is carried in serum by albumin, allowing it to accumulate in tissues such as the heart after both oral and i.v. administration (Zsila et al., 2003; Gross and Lockwood, 2004, 2005; Showalter et al., 2004).

The objective of the present study was to evaluate the ability of DDA to reduce the extent of myocardial damage and activation of the complement system in hearts subjected to ischemia/reperfusion injury. The rabbit in vivo myocardial ischemia/reperfusion model was used to determine whether a 4-day pretreatment regimen with DDA versus placebo could provide cardioprotection. Previous studies in rats showed a linear correlation between the plasma concentrations of nonesterified astaxanthin measured at the end of reperfusion and the extent of infarct size reduction (Gross and Lockwood, 2004); these results were confirmed in a large animal model, the mongrel dog (Gross and Lockwood, 2005). Based on this information, the current experimental protocol was designed to determine whether the cardioprotective effect of DDA involved modulation of the complement cascade and could potentially reduce the deposition of both C-reactive protein (CRP) and the membrane attack complex (MAC) in the injured myocardium.

Materials and Methods

Guidelines for Animal Research. The procedures used in this study were in agreement with the guidelines of the University of Michigan Committee on the Use and Care of Animals. The University of Michigan Unit for Laboratory Animal Medicine provides veterinary care. The University of Michigan is accredited by the American Association for Accreditation of Laboratory Animal Care, and the animal care use program conforms to the standards in The Guide for the Care and Use of Laboratory Animals, National Institutes of Health publication number 86-23.

Preparation of Stock Solutions of Cardax (DDA) and Placebo for Injection. DDA was supplied by Dr. Samuel F. Lockwood (Hawaii Biotech, Inc.) from a lot previously characterized in detail (Frey et al., 2004). The crystalline material was dissolved directly in sterile-filtered (0.2-μm filter; Millipore Corporation, Billerica, MA) deionized water. The maximum aqueous dispersibility of DDA is slightly greater than 10 mM (8.64 mg/ml). Sterile sodium chloride solution (0.9%) for injection was used as the treatment (placebo) for the control group. DDA or placebo solution was administered by slow ear vein injection using an infusion pump set at 1 ml/min.

Dosing Schedule. Male New Zealand White rabbits (2.3–2.6 kg) were assigned randomly to two separate groups. Each animal received DDA aqueous formulation (50 mg/kg) or an equal volume of sterile NaCl solution once per day i.v. The dose of DDA was selected based on the findings of previous investigations in which it was determined that a dosing regimen over 4 days produced statistically significant myocardial salvage in Sprague-Dawley rats (41% mean salvage at 50 mg/kg) and mongrel canines (68% mean salvage at 50 mg/kg) after ischemia and reperfusion (Gross and Lockwood, 2004, 2005). The animals in each group received the respective treatments on each of 4 consecutive days, with the experimental protocol being initiated on the 5th day.

Surgical Preparation and Experimental Occlusion. One day after the last treatment (DDA or placebo), rabbits were anesthetized with a combination of xylazine (3.0 mg/kg) and ketamine (35 mg/kg) administered i.m., followed by an i.v. injection of sodium pentobarbital (15 mg/kg). An endotracheal tube was inserted, and the animals were placed on a positive pressure ventilator (Harvard Apparatus Inc., Holliston, MA). The right jugular vein was cannulated for blood sampling, and the right carotid artery was instrumented with a Millar catheter microtip pressure transducer (Millar Instruments Inc., Houston, TX). The Millar catheter transducer was positioned immediately above the aortic valves to monitor aortic blood pressure. The lead II electrocardiogram was monitored throughout the protocol. A left thoracotomy and pericardiotomy were performed, followed by identification of the left anterior descending coronary artery. A silk suture (3-0; Genzyme, Cambridge, MA) was passed under the artery and around a short length of polyethylene tubing. Simultaneous downward displacement of the polyethylene tubing while applying upward traction on the suture resulted in occlusion of the coronary artery and cessation of regional blood flow. Coronary artery occlusion was maintained for 30 min, after which time reperfusion was initiated by withdrawing the polyethylene tubing. Regional myocardial ischemia was verified by the presence of a zone of cyanosis in the area of distribution of the occluded vessel and by changes in the electrocardiogram consistent with the presence of transmural regional myocardial ischemia (ST-segment elevation).

Experimental Protocol. The animals were allowed to stabilize for 15 min before beginning the protocol that involved both a vehicle control and a DDA-treated group. Cessation of coronary blood flow was maintained for 30 min, after which the ligature was removed, and the heart was allowed to reperfuse for 3 h before terminating the study.

Tetrazolium Method for Infarct Size Determination. At the completion of the 3-h reperfusion period, the hearts were removed. The aorta was cannulated, and the coronary vascular bed was perfused on a Langendorff apparatus with Krebs-Henseleit buffer at a constant flow of 30 to 32 ml/min. The hearts were perfused with buffer for 10 min to clear the vascular compartment of plasma and blood cellular elements. Fifty milliliters of a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO) in phosphate buffer (pH 7.4, 37°C) was perfused through the heart. TTC demarcates the noninfarcted myocardium within the area at risk with a brick red color, indicating the presence of a formazan precipitate resulting from reduction of TTC by dehydrogenases present in viable myocardial tissue. Irreversibly injured tissue, lacking cytosolic dehydrogenases, is unable to form the formazan precipitate and appears pale yellow. Upon completion of the TTC infusion, the left anterior descending coronary artery was ligated at the site identical to that ligated during the induction of regional myocardial ischemia. The perfusion pump was stopped, and 3 ml of a 0.25% solution of Evans blue dye was injected slowly through a sidearm port connected to the aortic cannula. The dye was passed through the heart for 15 s to ensure its uniform tissue distribution. The presence of Evans blue dye was used to demarcate the left ventricular tissue that was not subjected to regional ischemia, as opposed to the risk region. The heart was removed from the perfusion apparatus and cut into transverse sections at right angles to the vertical axis. The right ventricle, apex, and atrial tissue were discarded. Both surfaces of each tissue section were traced onto clear acetate sheets. The images were photocopied, enlarged, and then digitized using a flatbed scanner. The areas of the normal left ventricle nonrisk region, area at risk, and infarct region were determined by calculating the number of pixels occupying each area using Adobe Photoshop software (Adobe Systems, Mountain View, CA). Total area at risk is expressed as the percentage of the left ventricle. Infarct size is expressed as the percentage of the area at risk.

Plasma and Tissue Concentrations of Nonesterified, Free Astaxanthin. To determine the plasma and tissue concentrations of nonesterified, free astaxanthin in blood and organs, samples were taken at the end of reperfusion in selected rabbits (n = 5) treated with DDA and determined by methods previously described (Osterlie et al., 2000). Nonesterified, free astaxanthin in vivo is generated after cleavage of the water-dispersible disaccharide diester to mono-
succinate and subsequently to nonesterified, free astaxanthin by the intrinsic esterase activity of serum albumin (Curry et al., 1999) or by nonspecific esterase activity in plasma and solid organs (Jensen et al., 1999). Nonesterified, free astaxanthin then accumulates in myocardium and other tissues after plasma clearance in a dose-dependent manner after both oral (Showalter et al., 2004) and i.v. administration (Gross and Lockwood, 2004, 2005). Tissue concentrations of free astaxanthin reported in nanomolars follows the precedent set by Kurthara et al. (2002).

**Measurement of Cardiac-Specific Troponin I.** Whole blood was drawn at baseline (preischemia) and at the end of reperfusion for the determination of cardiac-specific troponin I (cTnI). Serum levels of the proteins were measured using an enzyme-linked immunosorbent assay (developed in conjunction with Dr. Chris Chadwick, Life Diagnostic, Inc., West Chester, PA). Briefly, plasma was prepared from whole blood drawn and frozen immediately in liquid nitrogen. The samples were stored at −80°C until the day of the assay, when they were thawed over ice and diluted appropriately with sample diluent supplied with each assay kit. Protein concentrations were determined using the optical density of each sample compared with a standard curve.

**Immunofluorescent Detection of the MAC and CRP.** The immunofluorescent method for detection of CRP was based on protocols previously developed in our laboratory (Lauver et al., 2005). Briefly, tissue samples used for infarct size determination were fixed in 10% buffered formalin immediately after the completion of the experimental protocol. The tissue samples were embedded in paraffin blocks and cut into 2-μm-thick sections, which were then mounted on glass slides. Two consecutive sections (mirror images) from a single heart slice were mounted on each slide. The slides were deparaffinized and subjected to antigen unmasking (Vector Laboratories, Burlingame, CA). After blocking for 30 min, primary antibodies were incubated at room temperature in a humidity chamber for 45 min. One section per slide was incubated with a chicken anti-rabbit MAC antibody (final dilution, 1:2500; developed solutions, Newark, DE), and the other section was incubated with a rabbit CRP antibody (final concentration, 5).

Images were analyzed using IP Lab (Scanalytics, Fairfax, VA) software to determine mean fluorescence intensity per heart section. The sections were normalized to the amount of background on each slide. The mean intensities for three hearts in each treatment group were averaged and compared.

**Assessment of Complement Inhibition.** A red blood cell (RBC) lysis assay was used to determine whether the pretreatment with DDA compared with placebo-treated animals was able to inhibit the rabbit complement system. The ex vivo analysis of complement activity is based on the C5b-9-dependent lysis of human red blood cells on exposure to rabbit plasma. Complement-mediated RBC hemolysis was assessed by a turbidimetric method described previously (Pascual et al., 1990). The hemolysis assay is an accepted method of assessing the complement titer of plasma or serum samples (Whaley, 1985). Rabbit plasma was obtained from whole blood samples drawn from rabbits that were pretreated with DDA (50 mg/kg, 4 days, n = 5) or sterile 0.9% sodium chloride solution (4 days, n = 5). After obtaining informed consent, human whole blood for the isolation of red blood cells was obtained by venipuncture of the forearm vein of a healthy male donor who had not been exposed to any medication for the past 7 days. The cells were washed three times in 10 ml of phosphate-buffered saline (pH 7.4) and diluted in phosphate-buffered saline to achieve a final RBC concentration of 1 × 10^8 cells/ml.

The assay was initiated by the addition of 15 μl of diluted human RBCs to 185 μl of rabbit plasma, and the light transmittance was monitored for 5 min. The final assay volume was 200 μl. Light transmittance (100%) was set with RBCs lysed with a 1:1 mixture of rabbit plasma and deionized H2O.

Fig. 1. Effects of DDA on myocardial infarct size after 30 min of left anterior descending coronary artery occlusion and 3 h of reperfusion compared with vehicle. DDA (50 mg/kg) was administered daily for 4 days before the experimental occlusion on day 5. The mean areas at risk were similar between groups, indicating that the degree of the ischemic insult was similar. Infarct size after ischemia/reperfusion is expressed as a percentage of the area at risk. The infarct region is decreased significantly in the group treated with DDA compared with vehicle. Values are expressed as mean ± S.E.M.; saline group, n = 9 (white bars); DDA group, n = 9 (black bars); **, p < 0.01 versus saline.
Statistical Analysis. Results are expressed as the mean values ± S.E.M. Parameters between the two groups were compared using Student's t test for unpaired comparisons. The p values of <0.05 and <0.01 are regarded as significant.

Results

Hemodynamics. There were no differences in heart rate, blood pressure, or blood gases at baseline or throughout the experimental protocol performed on day 5 between the two groups (unpublished data).

Effect of DDA on Myocardial Infarct Size. The mean size of the area at risk expressed as a percentage of the total left ventricle was similar in both groups, indicating that both groups were subjected to similar degrees of ischemia. Rabbits treated with DDA (50 mg/kg/day) exhibited significantly smaller mean infarcts expressed as a percentage of the area at risk (25.8 ± 4.7%) compared with rabbits treated with placebo (52.5 ± 7.5%, p < 0.01) (Fig. 1). This represented mean myocardial salvage of 51%.

Plasma and Tissue Levels of Nonesterified, Free Astaxanthin. The mean plasma concentration of nonesterified, free astaxanthin at the end of 3 h of perfusion is presented in Fig. 2. Pretreatment with DDA at 50 mg/kg for 4 days resulted in a mean plasma concentration of 222.1 ± 51.0 nM. However, the mean myocardial tissue concentration of DDA was several orders of magnitude greater than that observed in the plasma (10.1 ± 1.6 μM) (Fig. 2), revealing highly favorable mean myocardium/serum ratios in the rabbit after i.v. subchronic administration.

Serum Levels of cTnI. Mean serum concentrations of cTnI were similar at baseline (preischemia) in both treatment groups (0.07 ± 0.04 ng/ml in the vehicle group and 0.12 ± 0.04 ng/ml in the DDA-treated animals). DDA-treated rabbits exhibited a lower mean cTnI concentration (11.24 ± 4.16 ng/ml) at the end of reperfusion compared with vehicle controls (19.57 ± 4.56 ng/ml), although this difference was not statistically significant (p = 0.14) (Fig. 3).

Immunofluorescence. Left ventricular tissue sections used for immunofluorescence were obtained from hearts that had been subjected to 30 min of regional ischemia, followed by 3 h of reperfusion. Heart sections taken from the infarct region in animals treated with placebo showed bright fluorescence with both anti-CRP and anti-MAC antibodies, indicating the deposition of both proteins in the area of infarction. Conversely, hearts treated with DDA exhibited significantly reduced fluorescence, indicative of a reduction in the deposition of CRP and MAC in the infarct region (Fig. 4, A–D). The mean intensity of fluorescence in heart sections obtained after treatment with DDA was significantly (p < 0.05) lower in tissue sections stained for either CRP or MAC (Fig. 4E).

Inhibition of Rabbit Complement Activation. The erythrocyte hemolysis assay was used to determine the ability of DDA to inhibit the activation of the complement system (Fig. 5). Pretreatment with DDA (50 mg/kg, 4 days) significantly reduced (p < 0.01) mean rabbit plasma-induced hu-
Fig. 4. Representative fluorescent images of a heart from a saline control rabbit (A and C) and a rabbit treated with DDA (B and D) after 30 min of ischemia and 3 h of reperfusion. In the saline-treated rabbit, immunohistochemical staining for CRP (A) and MAC (C) is present in the area of infarction. In the DDA-treated hearts, little or no staining for CRP (B) or MAC (D) can be observed in areas of infarction. E, graph illustrating the comparison of mean fluorescence intensity per heart section. Values are presented as mean ± S.E.M.; saline group, n = 3 (white bars); DDA group, n = 3 (black bars); *, p < 0.05 versus vehicle control.
human erythrocyte hemolysis compared with plasma from placebo-treated rabbits.

Discussion

It is known that free radical scavengers can attenuate myocardial reperfusion injury by quenching reactive oxygen species that are released on the reintroduction of blood flow (Kilgore et al., 1994; Lucchesi, 1994). Perfusion of the rabbit isolated heart in the presence of xanthine and xanthine oxidase (reactive oxygen-generating system) results in tissue edema, disorganization of myofilament, and mitochondrial swelling in myocytes (Ytrehus et al., 1987; Lucchesi, 1994). In this study, it was observed that the superoxide anion scavenger Cardax (DDA) also reduced the deposition of CRP and the MAC in the infarct-related reperfused myocardium. The results show a significant mean reduction in the infarct size as a percentage of the area at risk in rabbits subjected to 30 min of coronary artery occlusion followed by a 3-h period of reperfusion. DDA produced a mean myocardial salvage of approximately 51% when the rabbits were dosed with 50 mg/kg daily for 4 consecutive days. This level of salvage at the 50-mg/kg subchronic i.v. dose is intermediate between that obtained in rats (41% salvage) and mongrel dogs (68%), showing appropriate pharmacokinetic scaling across these mammalian species (Gross and Lockwood, 2004, 2005). In addition, we were able to show a mean reduction in the circulating concentration of the biochemical injury marker cTnI. Although these results did not achieve statistical significance, clear evidence of a downward trend in this serum marker of irreversible myocardial tissue injury was obtained. The reduced statistical power observed in this study versus previous results obtained in our laboratory for this marker (Lauver et al., 2005) may have resulted from the curtailed period of reperfusion in the current study. There may have been insufficient time for cTnI to reach peak plasma concentrations in this study.

We were able to achieve plasma concentrations of nonesterified astaxanthin that were roughly equal to those previously found in other species using the same i.v. dosage regimen (Gross and Lockwood, 2004, 2005). Unlike these previous experiments, a correlation between nonesterified astaxanthin and infarct size could not be determined because only one concentration of the drug was used in the present study. We also observed a marked accumulation of nonesterified astaxanthin in the myocardium (mean, >10 μM) in the rabbits used in this study. Rapid plasma clearance of free astaxanthin and excellent myocardium and hepatic/serum ratios had previously been shown after oral administration of this compound to black mice (Showalter et al., 2004). The current results further show the favorable pharmacokinetic profile of DDA in mammalian species, suggesting that cardioprotection may be facilitated after parenteral administration in other species as well.

Along with the generation of reactive oxygen species, the activation of the complement system serves an integral role in myocardial reperfusion injury (Lucchesi, 1994). Therefore, we sought to also investigate the effects of DDA on the tissue deposition of CRP and the terminal complex (C5b-9), both of which are expressed in the process of tissue undergoing the inflammatory response after ischemia/reperfusion.

CRP is an acute phase protein known to be a highly sensitive, but nonspecific, marker of inflammation. The plasma concentration of CRP is increased in the presence of chronic inflammation, and there is a relationship between the circulating plasma concentration and subsequent cardiovascular events (de Beer et al., 1982; Yeh et al., 2001; Ridker et al., 2005). CRP is known to be involved in the local activation of the complement system (Volanakis, 1982; Diaz Padilla et al., 2003; Nijmeijer et al., 2003). Our previous studies showed that the crystalloid perfused isolated heart itself is capable of expressing mRNA and the rapid expression of complement proteins, as well as the membrane attack complex in response to ischemia/reperfusion injury (Yasojima et al., 1998). Furthermore, free radical-mediated (xanthine/xanthine oxidase) myocardial tissue injury was accompanied by the tissue expression of the MAC (Tanhehco et al., 2000). Using an immunofluorescent method to determine the presence of tissue-bound CRP and the MAC, we were able to show that DDA significantly reduced the deposition of both CRP and MAC, which were found localized within the area of infarction. We subsequently measured the complement activity in the plasma and found that DDA significantly reduced the activity of the complement system in plasma samples obtained from rabbits dosed with DDA compared with placebo-treated animals. To our knowledge, this anti-inflammatory mechanism for the novel astaxanthin diester used in the current study is the first report of such activity in the setting of ischemia/reperfusion injury.

Further investigations are warranted to more accurately define the anticomplement (and thus anti-inflammatory) effects of DDA. Previous studies have suggested a link between retinoid-like compounds and the expression of soluble complement receptor type 1 (sCR1) (Funkhouser and Vik, 1999). sCR1 is expressed primarily by erythrocytes, monocytes, neutrophils, and B cells, where it acts as a negative regulator of the complement cascade and clearance mechanism for...
immune complexes. Therefore, it is possible that alterations in sCr1 gene expression by this nonprovitamin A xanthophyll carotenoid would alter complement activity.

The mechanism(s) of action of carotenoids, in particular astaxanthin and novel astaxanthin-based esters, in cardio- protection have not been characterized completely. We report for the first time potent anticomplement effects of a novel carotenoid derivative that generates nonesterified, free astaxanthin after parenteral administration in vivo. The current results along with those from other investigators provide compelling support indicating a cardioprotective role for DDA. Furthermore, the relative safety of the primary active metabolite (nonesterified astaxanthin) (Kistler et al., 2002; Spiller and Dewell, 2003) combined with its ease of administration suggest that DDA may be worthy of further study for modulating tissue injury in a range of conditions involved with ischemia/reperfusion.

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References


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