Molecular and Cellular Biochemistry **272:** 221–227, 2005.

Acute and chronic administration of disodium disuccinate astaxanthin (Cardax^{TM}) produces marked cardioprotection in dog hearts

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Received: 4 November 2004; accepted: 13 December 2004

Abstract

Previous results from our laboratory have shown that a novel carotenoid derivative (disodium disuccinate astaxanthin; Cardax^{TM}) produced dose-related reductions in myocardial infarct size (IS) in Sprague–Dawley rats when it was administered at any of three doses (25, 50 and 75 mg/kg, iv) on four consecutive days, followed by the acute infarct size study on day 5. Maximum salvage occurred at the highest dose (75 mg/kg) tested, and was shown as a 56% reduction in IS. In the present follow-up study, we used a more relevant large animal model, the dog, and looked at the effect of administering Cardax^{TM} iv either acutely 2 h prior to occlusion ($N = 8$) or for 4 days at 50 mg/kg iv as previously done in the rat model ($N = 6$). The results were compared to a saline vehicle-treated group ($N = 10$). In all groups, dogs were subjected to 60 min of left anterior descending (LAD) coronary artery occlusion and 3 h of reperfusion. IS was determined using a triphenyltetrazolium chloride (TTZ) histochemical stain and was expressed as a percent of the area at risk (IS/AAR). IS/AAR was 20.9 ± 1.6 % (mean $\pm$ S.E.M.) in controls and was reduced to 11.0 ± 1.7% (47.3% salvage; $p < 0.01$) in dogs treated only once iv at 2 h prior to occlusion, and 6.6 ± 2.8% (68.4% salvage; $p < 0.001$) in dogs treated for 4 days. In the chronic treatment group, two of the three dogs with plasma concentrations of non-esterified astaxanthin above 1 μM had 0% IS/AAR (100% cardioprotection). These results suggest that Cardax^{TM} has marked cardioprotective properties in both rodents and canines. Thus, Cardax^{TM} may be a novel and powerful new means to prevent myocardial injury and/or necrosis associated with elective and/or urgent cardiac surgical interventions such as coronary angioplasty and stenting, as well as coronary artery bypass surgery (CABG). (Mol Cell Biochem **272:** 221–227, 2005)

Key words: disodium disuccinate astaxanthin, astaxanthin, Cardax^{TM}, carotenoids, cardioprotection, myocardial salvage, infarct size, canine infarct model

Introduction

Carotenoids are a group of naturally occurring pigments, now numbering around 750 described compounds, which possess antioxidant properties related to their physicochemical structures [1, 2]. The carotenoids that occur naturally are efficient antioxidants and are known to be excellent physical quenchers of singlet oxygen, as well as chain-terminators of lipid peroxidation in cell membranes and intracellular membrane structures [3]. In contrast to vitamin E, carotenoids are effective at low physiologic concentrations and low oxygen tension [4]. The major limitation to the use of the majority of these agents therapeutically is related to their poor aqueous solubility, which limits their utility as parenterally effective agents which could directly scavenge free radicals such as superoxide anion [5, 6]. Recently, Hawaii Biotech, Inc. (HBI) synthesized a novel synthetic carotenoid derivative, disodium

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disuccinate astaxanthin (Cardax^{TM}) at multi-gram scale [7]. This compound demonstrated water dispersibility of 8.64 mg/ml, was an effective direct scavenger of superoxide anion in the aqueous phase [5], and showed desirable plasma protein binding characteristics *in vitro* [8]. These characteristics were suggestive of potential efficacy as a myocardial salvage agent in mammals.

More recently, we tested the potency and efficacy of this new water-dispersible carotenoid in an established rat model of myocardial infarction in which we measure infarct size histochemically using a triphenyltetrazolium (TTZ) chloride stain. Cardax^{TM} was administered by intravenous (iv) tail vein injection once per day at one of three doses (25, 50 and 75 mg/kg) for four consecutive days, and the acute infarct size experiment was performed on the fifth day – 24 h after the last injection [9]. Plasma levels of non-esterified astaxanthin (generated after *in vivo* cleavage of Cardax^{TM}) were measured at the end of 30 min of occlusion and 2 h of reperfusion. As suspected from the previous *in vitro* studies, we found that Cardax^{TM} produced a statistically significant, linear, and dose-related reduction in infarct size, with mean maximal salvage of 56% obtained at the 75 mg/kg daily dose. Linear correlation was found between the plasma concentrations of non-esterified astaxanthin measured at the end of reperfusion and the magnitude of infarct size reduction. This confirmed our proof-of-principle that this derivative would have cardioprotective properties *in vivo*. With this promising data in hand, the present study was designed to further determine the cardioprotective efficacy of Cardax^{TM} in a large animal model, the dog, with heart size, anatomical and physiological parameters more closely related to those found in man. We also tested the possibility that a single acute dose of Cardax^{TM} might also possess cardioprotective properties, similar to those expected when the compound was given chronically as demonstrated in the previous rat study.

Methods

Materials

Cardax^{TM} was synthesized from commercially available crystalline astaxanthin as previously described [7]. The final product purity was 97% (as area under the curve, AUC) by HPLC analysis.

In our laboratory, Cardax^{TM} was dissolved directly in sterile-filtered deionized (DI) water. The maximum aqueous dispersibility of Cardax^{TM} is approximately 10 mM, or 8.64 mg/ml. Vehicle consisted of isotonic sterile saline. The 50 mg/kg dose was chosen for the current canine studies, the intermediate dose used in the previous rodent study [9]. At the 50 mg/kg dose in rats, 47% mean salvage was obtained. Increased salvage at this dose was predicted for the dog based on pharmacokinetic scaling factors in the larger animal (data not shown).

In the chronic dosing experiments, each dog received either Cardax^{TM} aqueous formulation (50 mg/kg) by iv leg vein infusion, or an equal volume of sterile saline solution once per day for 4 days. The acute infarction experiments were then performed on day 5. Plasma samples for HPLC analysis of non-esterified astaxanthin [10, 11] were obtained at the end of 3 h of reperfusion in the Cardax^{TM}-treated group. HPLC analysis was performed by M. Østerlie (HIST, Trondheim, Norway).

General dog preparation

All experiments conducted in this study were in accordance with the 'Position of the American Heart Association on Research and Animal Use' adopted by the American Heart Association, and the guidelines of the Biomedical Resource Center of the Medical College of Wisconsin. The Medical College of Wisconsin is accredited by the American Association of Laboratory Animal Care (AALAC).

The protocol detailing our experimental setup in dogs has been previously described in detail [12, 13]. Briefly, adult mongrel dogs of either sex weighing between 10–20 kg were fasted overnight. The dogs were anesthetized with a combination of sodium barbital (200 mg/kg) and sodium pentobarbital (15 mg/kg), and ventilated with room air supplemented with 100% oxygen. Body temperature was maintained at $38 \pm 1\,^{\circ}$C with a heating pad. Atelectasis was prevented by maintaining an end-expiratory pressure of 5–7 cm of water with a trap. Arterial blood pH, pCO_2 and pO_2 were monitored by an AVL automatic blood gas analysis system and maintained within normal physiological limits by adjusting the respiration rate and oxygen flow, or by iv administration of 1.5% sodium bicarbonate solution if necessary. A flowmeter (Statham 2202) was used to measure left anterior descending (LAD) coronary artery blood flow. A mechanical occluder was placed distal to the flow probe such that there were no branches between the flow probe and occluder. A Millar double-tipped catheter was inserted into the carotid artery and left ventricle (LV) to measure systemic arterial pressure and left ventricular pressure and its first derivative (LV dP/dt) in mm Hg/sec. All hemodynamic variables were monitored and recorded by a polygraph throughout the experiment. The left atrium was cannulated via the appendage for radioactive microsphere injections.

Experimental design

Dogs were randomly assigned to one of the three experimental groups: (1) a control saline-injected series ($N = 8$)

received saline once per day on each of 4 days prior to the infarct experiment performed on day 5; (2) a Cardax^{TM}-treated group (50 mg/kg, $N = 6$) received treatment drug once per day for 4 days prior to the infarct experiment performed on day 5; and (3) a series in which Cardax^{TM} (50 mg/kg, $N = 8$) was administered as a single dose once 2 h prior to the beginning of the acute infarct experiment. In all groups, hemodynamics, blood gas analyses and myocardial blood flow measurements (RA microspheres) were performed at baseline and at 30 min into the 60-min occlusion period. Hemodynamics were measured every hour during the reperfusion period and after 3 h of reperfusion; regional myocardial blood flow was then determined at the end of the reperfusion period. At the end of the experiment, the hearts were electrically fibrillated, removed and prepared for infarct size determination and regional myocardial blood flow measurements.

Infarct size determination

The methodology for infarct size determination has been previously described in detail by our laboratory [12, 13]. Briefly, at the end of 3 h of reperfusion, the LAD was again ligated and cannulated distal to the ligation site. To determine the anatomic area at risk (AAR) and the non-ischemic area, 5 ml of Patent Blue dye and 5 ml of saline were injected at equal pressure into the left atrium and LAD, respectively. The heart was then immediately fibrillated and removed. The LV was dissected and sliced into serial transverse sections 6–7 mm in width. The non-stained ischemic area and the blue-stained normal area were separated and incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) in 0.1 mol/l phosphate buffer, pH 7.4 at 37 °C for 15 min. After incubation overnight in 10% formaldehyde, the non-infarcted and infarcted tissues within the AAR were separated and determined gravimetrically. Infarct size was expressed as a percentage of the AAR (IS/AAR). Mean salvage [14, 15] was calculated as 1 − [mean IS/AAR (%) in treated animals ÷ mean IS/AAR (%) in the vehicle treated animals], as previously calculated by Gross and Lockwood [9].

Regional myocardial blood flow

Regional myocardial blood flow was measured by the radioactive microsphere technique developed in this laboratory as previously reported [16–18]. Microspheres were administered at 30 min into the 60 min occlusion period and at the end of 3 h of reperfusion. Transmural blood flows in the non-ischemic and ischemic areas were calculated as the weighted average of five pieces of the subepicardium, midmyocardium and subendocardium in each region.

Plasma concentrations of non-esterified astaxanthin

To determine the plasma concentrations of non-esterified astaxanthin in blood, samples ($N = 14$) were taken and analysed by HPLC at the end of 3 h of reperfusion in the two drug-treated groups as previously described [9, 10]. Free astaxanthin is generated following cleavage of the water-dispersible disuccinate diester *in vivo* to monosuccinate astaxanthin and subsequently to non-esterified, free astaxanthin by promiscuous esterases and the intrinsic esterase activity of serum albumin [19]. Tissue samples taken from the non-ischemic and ischemic myocardium at the end of reperfusion were also placed in liquid nitrogen and subsequently analysed for non-esterified astaxanthin, which accumulates in the myocardium after relatively rapid plasma clearance.

Statistical analyses

Statistical analyses were performed using the Prism 4 software package. Infarct size data were analyzed by a one-way ANOVA and the Bonferroni correction for treatment groups versus the control group. Linear regression analysis plotting plasma non-esterified astaxanthin (x-axis) versus IS/AAR (y-axis) was performed by a least squares analysis. A similar regression analysis was performed by plotting the IS/AAR (y-axis) versus the transmural collateral blood flow. Data for infarct size reduction and myocardial salvage for each group are reported as the mean ± S.E.M. $^{*}p < 0.01$ versus control group. $^{**}p < 0.001$ versus control group.

Results

Hemodynamic data

A total of 24 animals survived the entire protocol out of 30 initially enrolled. Three animals fibrillated in the control group, and two fibrillated in the acute Cardax^{TM}-treated group at reperfusion; one (1) dog was not successfully administered drug in the chronic Cardax^{TM}-treated group. No statistically significant differences in hemodynamic values were observed at any of the time points measured (Table 1). Similarly, no significant differences in either transmural blood flow (Table 2) or areas at risk (data not shown) were observed among the three groups during occlusion, or at 3 h of reperfusion – thus indicating that all three groups were subjected to equivalent degrees of ischemia (Table 2). Finally, arterial blood gas values also remained within the normal range, and were not significantly different among the three groups (data not shown).

Table 1. Mean hemodynamic values obtained during coronary occlusion and reperfusion among the three test groups in the experimental canine infarct model

	CON	Cardax or VEH	OCC (30)	REP (1)	REP (2)	REP (3)
Heart rate (bpm)						
Control	154 ± 3	155 ± 3	161 ± 4	166 ± 6	165 ± 5	168 ± 6
Cardax acute	150 ± 4	155 ± 5	156 ± 6	159 ± 7	161 ± 8	163 ± 8
Cardax chronic	161 ± 5	160 ± 6	161 ± 5	158 ± 4	160 ± 5	163 ± 7
Mean BP (mmHg)						
Control	106 ± 7	101 ± 4	102 ± 4	105 ± 5	106 ± 4	108 ± 4
Cardax acute	101 ± 5	88 ± 7	89 ± 7	90 ± 6	96 ± 4	94 ± 6
Cardax chronic	111 ± 7	106 ± 7	111 ± 9	105 ± 6	114 ± 8	114 ± 8
Rate pressure product (mmHg/min/1000)						
Control	18.0 ± 1.4	17.3 ± 0.8	18.4 ± 1.0	19.3 ± 1.4	19.3 ± 1.0	19.5 ± 1.0
Cardax acute	17.4 ± 1.0	15.6 ± 1.3	16.1 ± 1.4	16.2 ± 1.5	17.7 ± 1.2	17.4 ± 1.3
Cardax chronic	21.3 ± 1.6	20.2 ± 1.8	21.1 ± 1.9	18.9 ± 1.3	20.4 ± 1.4	20.1 ± 2.3
Left ventricular dP/dt (mmHg/sec)						
Control	1650 ± 118	1545 ± 85	1665 ± 81	1575 ± 84	1620 ± 89	1590 ± 78
Cardax acute	1631 ± 87	1785 ± 125	1650 ± 85	1594 ± 136	1569 ± 150	1438 ± 151
Cardax chronic	1942 ± 137	1842 ± 135	1933 ± 157	1608 ± 47	1642 ± 55	1617 ± 72

All values are the mean ± S.E.M. ($N = 6$ to 10 dogs). There were no significant differences among groups throughout the experiments by ANOVA followed by the Newman–Keuls posthoc test.
Abbreviations: CON: control; OCC: occlusion; REP: reperfusion; VEH: vehicle.

Table 2. Mean transmural blood flow values (ml/min/gm) determined in the ischemic and non-ischemic cardiac regions among the three test groups in the experimental canine infarct model

	Nonischemic region		Ischemic region	
Group	30 min OCC	3 h REP	30 min OCC	3 h REP
Control	0.78 ± 0.15	0.93 ± 0.15	0.06 ± 0.01	0.69 ± 0.10
Cardax acute	0.75 ± 0.09	0.89 ± 0.07	0.09 ± 0.01	0.90 ± 0.10
Cardax chronic	0.73 ± 0.15	0.69 ± 0.14	0.07 ± 0.01	0.72 ± 0.18

All values are the mean ± S.E.M. ($N = 6$ to 10 dogs).
Abbreviations: TRANS: transmural; OCC: occlusion; REP: reperfusion.

Infarct size data

The infarct size data for the three groups are summarized in Fig. 1. Acute administration of Cardax™ as a single iv dose 2 h prior to coronary occlusion resulted in a significant mean reduction in IS, expressed as a percent of the AAR, from 20.9 ± 1.6 to 11.0 ± 1.7% (47.3% salvage; $p < 0.01$). Following chronic administration of the same dose of Cardax™ (50 mg/kg) for four consecutive days, there was a further mean reduction in IS/AAR to 6.6 ± 2.8% (68.4% salvage; $p < 0.001$). The data shown in Fig. 2 indicated that there was a significant inverse linear correlation between IS/AAR and transmural coronary collateral blood flow in each group, with Pearson's r values ranging from 0.73 to 0.90.

Plasma and tissue astaxanthin concentrations

Linear regression of the IS/AAR values against the concentration of non-esterified astaxanthin in plasma at the end of 3 h of reperfusion in the acute and chronic studies is shown in Fig. 3. A significant linear correlation was observed between these

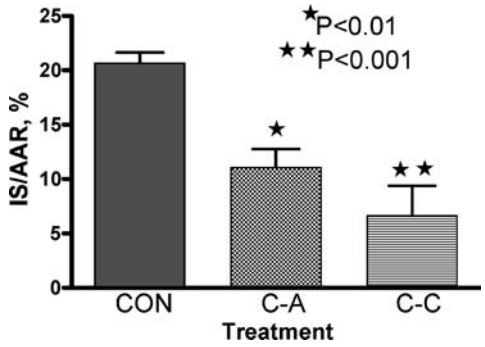

Fig. 1. Mean (± S.E.M.) infarct sizes, as a percentage of infarct size/area at risk (IS/AAR, %), observed among the three treatment groups tested in the current study. CON: control animals treated with vehicle once per day for 4 days prior to the acute infarct study conducted on day 5; C-A: Cardax™-treated animals receiving 50 mg/kg iv once 2 h prior to the acute infarct study; C-C: Cardax™-treated animals receiving 50 mg/kg iv once per day for 4 days prior to the acute infarct study conducted on day 5. Significant reductions in IS/AAR (%) vs. controls were obtained with acute (47.3% salvage) and chronic (68.4% salvage) Cardax™ treatment ($p < 0.01$ and 0.001, respectively).

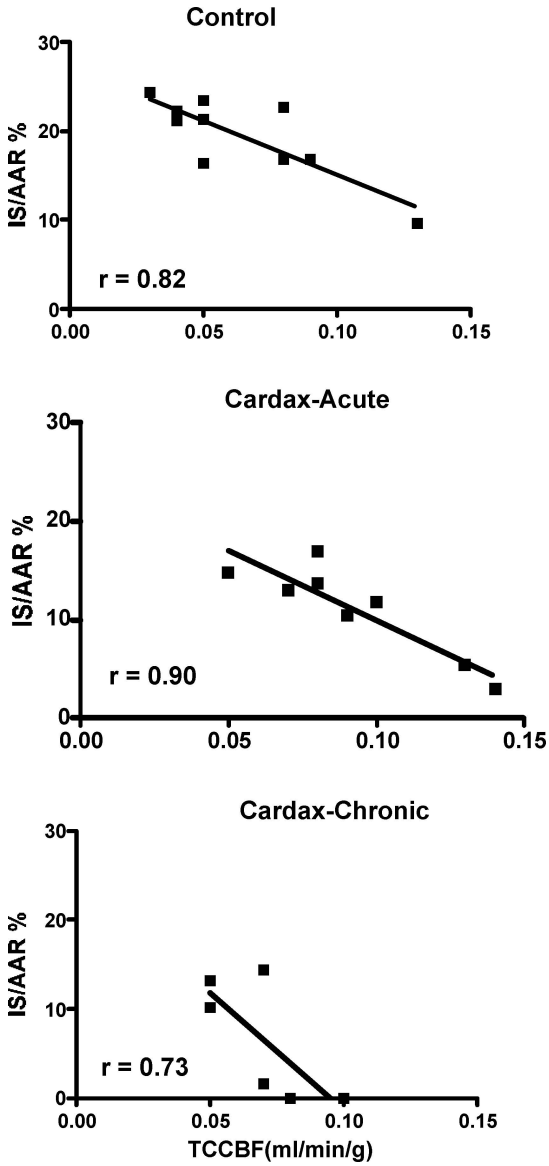

Fig. 2. Linear regression of infarct size/area at risk (IS/AAR, %; *y*-axis) vs. transmural blood flow values (TCCBF; ml/min/gm) among the three treatment groups tested in the experimental canine infarct model. Control: control animals treated with vehicle once per day for 4 days prior to the acute infarct study conducted on day 5; Cardax-acute: Cardax^{TM}-treated animals receiving 50 mg/kg iv once 2 h prior to the acute infarct study; Cardax-chronic: Cardax^{TM}-treated animals receiving 50 mg/kg iv once per day for 4 days prior to the acute infarct study conducted on day 5. As TCCBF increases, infarct size decreases linearly ($p < 0.01$). Pearson's r values range from 0.73 to 0.90.

two parameters ($p < 0.01$). At plasma concentrations of non-esterified astaxanthin greater than 1000 nM (1 μM), we predicted based on our previous regression in the rat heart [9] that IS/AAR would be barely detectable in the canine heart; this relationship was confirmed in the current study, with two of the three dogs exhibiting these levels showing 100% cardioprotection. We also found that the mean cardiac tissue level of non-esterified astaxanthin in the six dogs treated acutely with Cardax^{TM} at 3 h of reperfusion was 632 $\pm$ 333 nM, and the IS/AAR in these same animals was 10.8 $\pm$ 2.4%, an approximately 50% mean reduction in IS/AAR from the control values (20.9 $\pm$ 1.6%). This reduction in IS/AAR would be predicted to almost match similar plasma levels of non-esterified astaxanthin as shown in Fig. 3.

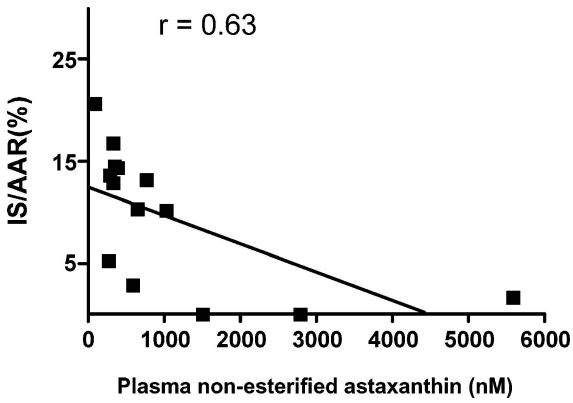

Fig. 3. Linear regression of infarct size/area at risk (IS/AAR, %; *y*-axis) vs. plasma levels of non-esterified astaxanthin (in nM; *x*-axis) obtained at the end of reperfusion in 14 animals in the two Cardax^{TM} treatment groups tested in the experimental canine infarct model. As the plasma levels of non-esterified astaxanthin increases, infarct size decreases linearly ($p < 0.01$). In two animals in which plasma levels of non-esterified astaxanthin >1000 nM (1 μM) were obtained, IS/AAR (%) was zero (100% cardioprotection).

Discussion

The results of the current study clearly demonstrated that the disodium disuccinate derivative of synthetic astaxanthin (Cardax^{TM}) produces a significant mean reduction in IS/AAR in canine hearts subjected to 60 min of LAD coronary artery occlusion followed by 3 h of reperfusion. Cardax^{TM} produced a mean reduction in IS/AAR of approximately 47% when administered as a single 50 mg/kg dose 2 h prior to occlusion, as well as a mean reduction of approximately 68% when the same dose was given consecutively for 4 days prior to the IS study performed 24 h after the last dose on day 5. Interestingly, in two of the three dogs in the chronic study in which the plasma concentration of non-esterified astaxanthin was greater than 1 μM, the IS/AAR was 0% (100% cardioprotection). These results were similar to those previously obtained in our laboratory in chronically treated rats. In the current study, however, Cardax^{TM} produced a mean of 68% salvage in the dog heart at 50 mg/kg, whereas a mean of 47% salvage was obtained at the same dose and using an identical protocol in the rat heart. It is likely that these differences may be a favourable result of differences in the pharmacokinetic

properties of Cardax^{TM} between the two species, particularly the rate of cleavage of intact Cardax^{TM} by plasma and solid organ esterases, the uptake of non-esterified astaxanthin by the heart (resulting in a favourable myocardium/serum ratio), or the level of endogenous antioxidant defence mechanisms between the two species. Additional studies are needed to determine the mechanisms responsible for these differences in efficacy observed with chronic parenteral Cardax^{TM} administration in dogs and rats.

Although we are the first laboratory to demonstrate a marked cardioprotective effect of Cardax^{TM} in two species, the mechanism or mechanisms by which this compound produces its cardioprotective effects remain incompletely understood. It has been well documented that bioactive carotenoids such as astaxanthin possess potent antioxidant effects, and quench singlet oxygen [20] and inhibit lipid peroxidation of membranes both *in vitro* and *in vivo* (reviewed in [21, 22]). In this regard, Cardounel *et al*. [5] have shown that disodium disuccinate astaxanthin (Cardax^{TM}) and other similar structurally related compounds [6, 23] were potent direct scavengers of aqueous-phase superoxide anion as determined by electron paramagnetic resonance (EPR) spectroscopy using the spin trap DEPMPO in a standard isolated human neutrophil assay. Furthermore, recent evidence also suggested that carotenoids such as astaxanthin as well as Cardax^{TM} induced an upregulation of the gap junction protein connexin 43 (Cx43), as well as increased functional gap junctional intercellular communication (GJIC) *in vitro* [24, 25]. The maintenance and/or increase in Cx43 and subsequently GJIC – if it occurred in the ischemic heart *in vivo* – could prevent the loss of this important functional communication between cells, and potentially prevent the spread of myocardial injury to adjacent normal cells and therefore reduce infarct size. It has been shown clearly that this effect of carotenoids on gap junctions occurs independently of the compounds' antioxidant effects [21], which suggests that multiple independent (e.g. antioxidant and gene regulation) mechanisms may co-exist to help explain the powerful cardioprotective effects of carotenoids.

Additionally, other effects of astaxanthin on inflammatory cells and inflammation in general may also be potentially important in mediating the effects of non-esterified astaxanthin on ischemia-reperfusion injury. Lee *et al*. [26] demonstrated that astaxanthin inhibited the expression of a number of pro-inflammatory mediators (such as nitric oxide (NO), prostaglandin E_2 (PGE_2), inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), TNF-α, and IL-1β) in LPS-stimulated RAW 264.7 cells and primary macrophages in an *in vitro* murine inflammation model. Similarly, astaxanthin was shown to block the increases in the serum levels of these same inflammatory mediators in LPS-treated mice *in vivo*, and was also shown to block the activation of NF-κB by suppressing IKK activity and IκB-α degradation. Ohgami *et al*. [27] confirmed these results in a murine uveitis model, demonstrating an anti-inflammatory effect of astaxanthin on par with that of prednisolone on a mg/kg dosage basis. Most recently, astaxanthin was shown to inhibit macrophage infiltration and leukocyte apoptosis in atherosclerotic plaques formed in arteries of hyperlipidemic WHHL rabbits [28]. These authors suggested that the observed effects might promote atherosclerotic plaque stability and prevent plaque rupture by decreasing the expression of matrix metalloproteinase three (MMP3) and collagen distribution to smooth muscle cells. Similar effects have been previously observed with lipid-lowering compounds such as fluvastatin and lovastatin [29], anti-inflammatory effects independent of their lipid-lowering properties.

The previously published data from rats [9] and dogs (from the current study) complement the data of Aoi *et al*. [30], in which myocardial oxidative stress was significantly reduced by statanthine in an exercising mouse model. All three studies suggest that astaxanthin (and the soft-drug parenteral precursor Cardax^{TM}) have powerful antioxidant and anti-inflammatory properties that are most likely responsible for their potent cardioprotective properties in *in vivo* models of ischemia-reperfusion injury. Additional mechanistic studies will be needed to identify the most important properties of these compounds that are essential for their beneficial effects observed in these animal models. These preliminary studies suggest that Cardax^{TM} and similar compounds may find utility in those applications in which pre-treatment of at-risk patients is possible in the clinical setting. The current study extends previous findings in that the window of opportunity for therapeutic treatment has been expanded to include patients within 2 h of a potential ischemic procedural coronary insult.

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