Apolipoprotein A-I_Milano and 1-Palmitoyl-2-oleoyl Phosphatidylcholine Complex (ETC-216) Protects the in Vivo Rabbit Heart from Regional Ischemia-Reperfusion Injury

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ABSTRACT

Ex vivo studies demonstrated that a synthetic high-density lipoprotein (HDL) comprised of a complex of recombinant apolipoprotein A-I_Milano and 1-palmitoyl-2-oleoyl phosphatidylcholine protects the isolated rabbit heart from reperfusion injury. Therefore, we sought to determine whether a pharmaceutical preparation of this complex, ETC-216, was cardioprotective in an in vivo model of left anterior descending artery (LAD) occlusion and reperfusion. Initially, ETC-216 (100 mg/kg) was tested in acute (one-treatment) and chronic (two-treatment) i.v. administrations. ETC-216-treated rabbits developed smaller infarcts expressed as percentage of area at risk (p < 0.01) compared with vehicle treatments. No differences were noted between chronic and acute administration. Therefore, ETC-216 (10, 3, or 1 mg/kg) or equivalent vehicle volumes were acutely infused. Compared with vehicle, ETC-216 reduced infarct size as a percentage of the area at risk at 10 (p < 0.0005) and 3 mg/kg (p < 0.05). No significant differences occurred at 1 mg/kg. To determine whether ETC-216 could protect the heart after initiation of ischemia, the synthetic HDL (10 mg/kg) was infused intravenously beginning 5 min before the end of 30 min of LAD occlusion. Infarct size as percentage of the area at risk was 31.6 ± 3.0 (ETC-216) versus 49.5 ± 2.5 (vehicle) (p < 0.001), and as percentage of left ventricle was 19.7 ± 1.6 (ETC-216) versus 34.1 ± 2.3 (vehicle) (p < 0.0005). Electron microscopy demonstrated that ETC-216 prevented irreversible cardiac damage as assessed by mitochondrial granulation and sarcomere contraction band formation. These findings suggest ETC-216 reduces reperfusion injury and may have utility for coronary artery revascularization procedures.

Several studies have established that plasma high-density lipoprotein cholesterol (HDL-C) levels are inversely related to the incidence of primary cardiac events (Wilson et al., 1988; Frick et al., 1990). High-density lipoproteins (HDLs) retard formation of lipid-rich arterial lesions and may prevent plaque rupture and coronary events (Gordon and Rifkind, 1989; Badimon et al., 1992). Besides being a strong independent predictor of the occurrence of primary coronary events, a low plasma HDL-C level is also associated with unfavorable prognosis in patients who have had a myocardial infarction (Berge et al., 1982). A low HDL-C level adversely influences postinfarct left ventricular function in patients with a first myocardial infarction, independent of the severity of coronary atherosclerosis (Wang et al., 1998), and is an independent predictor of left ventricular dysfunction in angina patients with normal coronary angiograms (Wang et al., 1999).

Apolipoprotein A-I_Milano (apoA-I_Milano), a natural variant of human apolipoprotein A-I, confers to carriers a significant protection against cardiovascular disease (Sirtori et al., 2001). This unusual form of apoA-I is associated with markedly reduced serum HDL-C levels, mildly elevated triglycerides, and protection from cardiovascular disease. HDL formed in the carriers is smaller than normal; contains a greater ratio of surface to core components; is phospholipid enriched; and is structurally constrained, thereby preventing maturation into the large spherical HDL. Carrier HDL perhaps more closely mimics prebeta or “nascent” HDL and

ABBREVIATIONS: HDL-C, high-density lipoprotein cholesterol; HDL, high-density lipoprotein; ApoA-I_Milano, apolipoprotein A-I_Milano; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; ETC-216, ApoA-I_Milano complexed with 1-palmitoyl-2-oleoyl phosphatidylcholine; LAD, left anterior descending artery; TTC, triphenyltetrazolium chloride; AAR, area at risk; LV, left ventricle; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; RPP, rate-pressure product.
therefore may be more efficient as reverse lipid transport agents (Franceschini et al., 1990; Calabresi et al., 1997). More recently, intravenous infusion of a synthetic HDL (ETC-216) containing apoA-I_Milano complexed with 1-palmitoyl-2-oleyl phosphatidylcholine (POPC) has been shown to cause a rapid and significant regression of the percentage of coronary atheroma volume in a double-blind, randomized, placebo-controlled multicenter human pilot trial as measured by intravascular ultrasound (Nissen et al., 2003).

The term “reperfusion injury” is defined as the conversion of reversibly injured cells to a state of irreversible injury resulting from reintroduction of oxygenated blood to an ischemic area (Park and Lucchesi, 1999). Reperfusion, after a period of ischemia, is associated with oxygen radical formation (Zweier et al., 1987), oxidized glutathione release (Tritto et al., 1998), and conjugated diene formation (Ambrosio et al., 1991), which are the “signatures” of oxidative attack.

The impetus for these studies was derived from a preliminary examination of synthetic HDL complex comprised of recombinant apoA-I_Milano and POPC (in about a 1:1 ratio by weight) in the isolated rabbit heart. This complex had no effect on baseline contractile function or hemodynamic parameters. However, treatment with the HDL complex significantly improved the functional outcome compared with vehicle-treated hearts that developed irreversible contractile dysfunction (Marchesi, E. A. Booth, K. R. Hill, C. L. Bissigera, and B. R. Lucchesi, unpublished observation). Due to these results, we sought to determine whether the cardioprotective effects could be observed in an in vivo model of regional ischemia-reperfusion injury in the rabbit heart.

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 of 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, Department of Health, Education, and Welfare Publ. No. (National Institutes of Health) 86-23.

Surgical Preparation. We used an experimental model of cardiac regional ischemia to test the cardioprotective effects of ETC-216 (Kilgore et al., 1998). Briefly, male New Zealand White rabbits (2.2–2.6 kg) were anesthetized with a mixture of xylazine (3.0 mg/kg) and ketamine (35 mg/kg) intramuscularly followed by i.v. sodium pentobarbital (30 mg/kg). Anesthesia was maintained with i.v. sodium pentobarbital. A cuffed endotracheal tube was inserted and the animals were placed on positive-pressure ventilation with room air. The right jugular vein was cannulated for test agent administration. The right carotid artery was instrumented with a micro-tip pressure transducer (Millar Instruments Inc., Houston, TX) immediately above the aortic valves to monitor aortic blood pressure. A lead II electrocardiogram was monitored throughout the experiment. A left thoracotomy and pericardiectomy were performed, exposing the left anterior descending artery (LAD). A silk suture (3.0; Deknatel, Fall River, MA) was passed behind the artery and tied around a short length of polyethylene tube lying adjacent to the vessel. Downward pressure on the polyethylene tube while exerting upward tension on the free ends of the suture compressed and occluded the LAD, resulting in regional myocardial ischemia. After 30 min, the polyethylene tube was withdrawn to allow reperfusion. Regional myocardial ischemia was verified by discoloration in the area of distribution of the occluded vessel and by electrocardiogram changes consistent with transmural regional myocardial ischemia (ST-segment elevation).

Treatment Protocol. Different dosing regimens were used in the three protocols described below (Fig. 1).

First protocol. This protocol was to determine the effect of an acute or chronic treatment on ischemia-reperfusion injury. Twenty-four rabbits (six rabbits per group) received 100 mg/kg ETC-216 or an equivalent volume of vehicle (~17 ml of sucrose-mannitol), either as chronic treatment (two doses given 1 day before and also at the time of the surgical procedure; Fig. 1A) before the onset of regional ischemia or as an acute treatment at the time of surgery (for 60 min beginning 15 min before 30 min of LAD occlusion and extending 15 min into the 4-h reperfusion period; Fig. 1B).

Second protocol. Thirty rabbits were used in this protocol. They received acute doses of 10 mg/kg ETC-216 (n = 6) or a matched vehicle (~1.7 ml; n = 6); 3 mg/kg ETC-216 (n = 6) or a matched vehicle (~0.5 ml; n = 6); or 1 mg/kg ETC-216 (n = 3) or a matched vehicle (~0.1 ml; n = 3) (Fig. 1B). Each rabbit was administered an intravenous infusion for 60 min beginning 15 min before 30 min of LAD occlusion and extending 15 min into the 4-h reperfusion period.

Third protocol. ETC-216 (10 mg/kg) or an equivalent vehicle volume (~1.7 ml) was given intravenously to 12 rabbits (n = 6/group) for 60 min beginning 5 min before the end of 30 min of LAD occlusion and extending 55 min into the 4-h reperfusion period (Fig. 1C).

Determination of Infarct Size. After the 4-h reperfusion period, the hearts were removed and perfused via the aorta on a Langendorff apparatus with Krebs-Henseleit buffer for 10 min at a constant flow of 22–26 ml/min to ensure that the vascular bed was blood free (Kilgore and Lucchesi, 1993). Forty-five milliliters of 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4; 37°C) was perfused through the heart. TTC demarcates the viable noninfarcted myocardium within the area at risk (AAR) by enzymatic reduction of TTC and formation of a brick-red colored formazan precipitate in viable tissue. Irreversibly injured tissue, lacking the cytosolic dehydrogenases, is unable to form the formazan precipitate and looks pale yellow. Upon completion of TTC infusion, the LAD was ligated in a location identical to the site ligated during the induction of regional myocardial ischemia. The perfusion pump was stopped and

![Figure 1](https://example.com/fig1.png)
2 ml of 0.25% Evans Blue was injected slowly through a side arm port connected to the aortic cannula for 10 s to ensure equal distribution and used to demarcate the left ventricular tissue that was not subjected to regional ischemia. The heart was removed from the apparatus and cut into three transverse sections at right angles to the vertical axis. The right ventricle, apex, and atrial tissue were discarded. Both surfaces of each transverse section were traced onto clear acetate sheets by the executor of the protocol in an unblinded manner, scanned, and downloaded into Adobe PhotoShop (Adobe Systems, Seattle, WA). The areas of the normal left ventricle (LV) nonrisk region, AAR, and infarct region were quantitated by calculating the number of pixels in each area using Adobe PhotoShop software. Measurements of infarct size were independently analyzed by two individuals blind to the treatment groups. The results represent the average of the two determinations. Total area at risk is expressed as the percentage of the LV. Infarct size is expressed as the percentage of the AAR as well as the total LV.

Electron Microscopic Analysis. Left ventricular myocardial samples from two hearts per group were subjected to electron microscopic analysis as described previously. After perfusion, the hearts were excised, mounted on a Langendorff apparatus, and fixed via the aorta for 3 min with 2.5% glutaraldehyde and 1% LaCl3 in 0.1 M sodium cacodylate buffer (pH 7.4). Tissue samples were cut into approximately 1 mm square pieces and fixed an additional 2 h at 4°C, dehydrated in an ethanol series, and embedded in EM bed-812 (Electron Microscopy Sciences, Ft. Washington, PA). Tissue blocks were sectioned with a Reichert ultramicrotome, placed on Formvar-coated copper grids, stained with 4% uranyl acetate, and examined with a Phillips CM-100 electron microscope.

Biochemical Evaluations. Blood samples were taken before, at the end of treatment, and hourly during the reperfusion period. Serum was prepared and analyzed on a Hitachi 912 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN) for total and unesterified cholesterol according to the method of Allain et al. (1974). For determination of unesterified cholesterol, the reagent was prepared without cholesterol esterase. Commercially available kits were used to determine phospholipids, triglycerides, and human apoA-I levels after administration of ETC-216. Total and unesterified lipoprotein cholesterol distribution between VLDL, LDL, and HDL were detected on-line by Superoxide 6HR gel filtration (1 × 30 cm) column chromatography as described by Kieft et al. (1991). Total and unesterified cholesterol content of lipoproteins was calculated by multiplying the independent values determined for serum total and unesterified cholesterol by the percentage of area of the lipoprotein in the respective profiles.

Materials. ETC-216 is a recombinant apoA-I/POPC complex (apoA-IPOPC) in an approximately 1:1 ratio by weight (mole ratio of apoA-I to POPC of approximately 1.36) prepared in a 6.2% sucrose, 0.9% mannitol, and 10 mM phosphate buffer, pH 7.4, at a concentration of 14 mg/ml protein. The complexes contain equal or greater than 90% of the apoA-I as dimer. Vehicle consisted of the 6.2% sucrose, 0.9% mannitol, and 10 mM phosphate buffer, pH 7.4, alone.

Statistics. Data are expressed as mean ± S.E.M. Differences between control and experimental groups were determined using a one-way or two-way analysis of variance for repeated measures. For rate-pressure product and biochemical analyses, two-way ANOVA was performed. Differences between groups were determined using Bonferroni’s post hoc test. A value of \( p < 0.05 \) was considered significant. Statistical analysis was performed using GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA).

Results

Hemodynamic Effects. Hemodynamic variables were obtained to determine the effects of ETC-216 or vehicle in mediating alterations in arterial blood pressure and heart rate. The rate-pressure product (RPP), defined as mean arterial blood pressure multiplied by the heart rate divided by 100, was used as an indicator of myocardial oxygen consumption. The RPP decreased in each group (vehicle and ETC-216 chronic, vehicle and ETC-216 acute) from equilibration to 30 min after treatment and then remained stable throughout the duration of the protocol (data not shown). For the second and the third protocol, RPP was similarly reduced in all groups with no significant difference between groups. The RPP remained stable throughout the duration of the protocol (data not shown).

Electrophysiological data did not demonstrate any changes due to administration of ETC-216. All animals exhibited ST-segment elevation during the induction of regional myocardial ischemia (data not shown). The ST-segment changes resolved toward baseline upon removal of the occlusive ligature. No deaths from either cardiac arrhythmias or cardiac failure were noted in any of the groups.

Infarct Size. Infarct size, expressed as the percentage of the LV or AAR, was used to measure ETC-216’s ability to protect the myocardium after reperfusion.

In the first protocol, no significant differences were noted between treatment groups or between chronic and acute administration (100 mg/kg), in the AAR expressed as the percentage of LV (Fig. 2A). This indicated that there was no bias in the tissue amount subjected to ischemia episodes in any of the treatment groups. Rabbits treated with ETC-216 developed significantly smaller infarcts \( (p < 0.01) \) expressed as a percentage of the AAR (Fig. 2B) and as a percentage of the total LV \( (p < 0.05) \) compared with rabbits treated with vehicle (Fig. 2C). Note that two treatments (chronic) had no apparent advantage over a single treatment (acute) for cardioprotection.

In the second protocol, the minimal effective dose was found using the single-treatment protocol (acute) was used in which the animals received 10, 3, or 1 mg/kg ETC-216 or vehicle. The AAR expressed as a percentage of the LV was similar in all treatment groups (Fig. 3A). Rabbits treated with 10 mg/kg ETC-216 developed smaller infarcts \( (p < 0.0005) \) expressed as a percentage of the AAR compared with vehicle-treated rabbits (Fig. 3B). A reduction in myocardial infarct size \( (p < 0.0001) \) also was observed when the data were expressed as a percentage of the LV (Fig. 3C). Similar results were observed at 3 mg/kg. Infarcts expressed as a percentage of the AAR \( (p < 0.05) \) or as a percentage of the LV \( (p < 0.05) \) were reduced with ETC-216 treatment compared with controls (Fig. 3, B and C). No significant differences were noted with a dose of 1 mg/kg between ETC-216 and vehicle in the infarct size (Fig. 3, B and C).

In the third protocol, ETC-216 was tested as a single treatment in which the animal was exposed to 10 mg/kg of the agent or an equivalent volume of vehicle during the last 5 min of regional ischemia and continued through the first 55 min of reperfusion. For both groups, the AAR as a percentage of the LV were similar (Fig. 4A). Rabbits treated with ETC-216 developed smaller infarcts \( (p < 0.001) \) as a percentage of the AAR compared with rabbits treated with vehicle (Fig. 4B). Myocardial infarct size \( (p < 0.0005) \) was also reduced as a percentage of the LV (Fig. 4C).

Electron Microscopic Analysis. Left ventricular myocardial samples were obtained from animals from all proto-
electron microscopy. Examples, obtained from animals that underwent protocol 3, are shown in Fig. 5. Hearts from vehicle-treated rabbits (Fig. 5A) that show sarcomere structural features are obliterated and contraction bands are present. The mitochondria are markedly swollen with disrupted cristae and osmophillic inclusion bodies. In the hearts from ETC-216-treated animals (Fig. 5B), the sarcomere structure is relatively normal and the mitochondria look intact with only minimal swelling. The virtual absence of contraction bands stands in marked contrast with hearts observed in the vehicle-treated rabbits. Similar aberrant ultrastructural features in the vehicle-treated rabbits were observed in protocols 1 and 2, whereas the ETC-216 treatment (i.e., 3, 10, and 100 mg/kg treatments) the hearts were protected (data not shown).

Serum Lipid/Lipoprotein Changes. In the first protocol, where either one (acute) or two (chronic) ETC-216 doses (100 mg/kg) were compared, blood serum was assessed at intervals before and after administration of test substances for pharmacokinetic and pharmacodynamic responses (Fig. 6). For pharmacokinetic variables, blood levels of ETC-216 components, recombinant apoA-I_Milano or 1-palmitoyl-2-oleoyl phosphatidylcholine was determined as human apoA-I (only in animals receiving ETC-216) and serum phospholipid, respectively (Fig. 6, top two panels). After intravenous administration of ETC-216, as expected, serum apoA-I_Milano and phospholipid (i.e., components of the test agent) levels rose. Pharmacodynamic responses included total and unesterified cholesterol and triglycerides (Fig. 6, bottom six panels). These data demonstrate a marked and rapid elevation of serum cholesterol in response to administration of ETC-216. The rise in serum total cholesterol was predominantly detected as serum unesterified cholesterol. We also observed serum triglyceride elevation as a result of ETC-216 treatment. This likely reflects hepatic HDL-C delivery with subsequent increased VLDL production. In the chronic treatment group, triglycerides remained elevated, although cholesterol levels returned to baseline before the second ETC-216 dose.

Serum samples from protocol 1 were analyzed for the distribution of lipoprotein total (Fig. 7) and unesterified cholesterol by gel filtration chromatography. Lipoproteins separated by size showed that the total cholesterol rise was initially and largely confined to the HDL unesterified cholesterol. This occurred during acute administration and after both doses after chronic administration. We also observed a secondary rise in VLDL-cholesterol after administration of ETC-216.

Chronic treatment did not afford an increased cardioprotective advantage over the acute treatments. Therefore, subsequent experiments focused on establishing the minimal effective single ETC-216 dose allowing cardioprotection. In these experiments, we sought to determine whether serum HDL unesterified cholesterol elevation could be used as a measurable surrogate predictive of cardioprotection. Lipoprotein unesterified cholesterol profile analysis was therefore performed on samples from select animals. Shown (Fig. 8) are typical unesterified cholesterol lipoprotein profiles from select times and animals at each dose. At the 100-mg/kg dose, we saw a distinct increase in HDL unesterified cholesterol and a subsequent elevation in VLDL cholesterol (protocol 1). At 10 mg/kg, we observed HDL cholesterol increase only at 45 min, but still observed a clear elevation in VLDL unesterified cholesterol at the later time points. With the vehicle and the two lower doses (1 and 3 mg/kg), we observed no change in HDL unesterified cholesterol. We observed a small elevation in VLDL unesterified cholesterol; however, this was also seen during the control treatment.

**Discussion**

The present findings suggest that ETC-216 may provide a novel approach to inhibit ischemia-reperfusion injury. ETC-
216 exhibits a threshold dose cardioprotective effect when administered before an ischemic insult, as well as when given during the restoration of blood flow in a regional cardiac ischemic rabbit model. The present data also suggest that a substantially low ETC-216 dose effectively protected the heart from ischemia-reperfusion injury.

ApoA-IMilano is a variant of human apoA-I distinguished by the presence of a cysteine instead of an arginine at position 173 in this 243-amino acid apolipoprotein (Sirtori and Franceschini, 1982; Weisgraber et al., 1983). This variant protein was first discovered in the blood serum of a human subject living in the geographically isolated village of Limone sul Garda, Italy (Gualandri et al., 1985a). The human apoA-IMilano variants present with an extremely low level of HDL-C, mildly elevated triglycerides, and without premature cardiovascular disease (Franceschini et al., 1985, 1987). Subsequently, additional carriers, all heterozygous for apoA-IMilano, with a similar dyslipidemia were identified (Gualandri et al., 1985b). Despite low HDL-C levels, apoA-IMilano carriers have normal carotid artery intimal to medial ratios, compared with hypoalphalipoproteinemic and normal controls. In the carriers, apoA-IMilano is found associated with HDL in homodimeric, heterodimeric (with apoA-II), and in its monomeric form (Franceschini et al., 1990). In both humans and rabbits, homodimeric apoA-IMilano, has been shown to have approximately twice the half-life compared with native apoA-I or monomeric apoA-I Milano (Roma et al., 1993; Chiesa et al., 2002).

ETC-216 is a product candidate in development for the treatment of acute coronary syndromes (Nissen et al., 2003). Preclinical and clinical investigations have shown intravascular administration of either apoA-IMilano/phospholipids complexes or ETC-216 results in reduction of lipid atherosclerotic plaques (Shah et al., 2001; Chiesa et al., 2002; Nissen et al., 2003).

Before our current work, the isolated heart model was used to assess the potential efficacy of HDL (Calabresi et al., 2003), and apoA-IMilano/POPC (M. Marchesi, E. A. Booth, K. R. Hill, C. L. Bisgaier, and B. R. Lucchesi, unpublished observations). These studies demonstrate direct cardioprotective effects of these agents and suggest an antioxidative mechanism, including reduction of tumor necrosis factor-α expression, as well as reduced lipid hydroperoxides accumulation in the hearts (Calabresi et al., 2003; M. Marchesi, E. A. Booth, K. R. Hill, C. L. Bisgaier, and B. R. Lucchesi, unpublished observations). In an isolated in vitro system, both monomeric apoA-IMilano and apoA-I have been shown to impede lipoxygenase-induced oxidation (Bielicki and Oda, 2002). These comparative studies have shown that cysteine-containing apoA-IMilano or apoA-I-paris were significantly superior to native apoA-I in preventing conjugated diene formation. Perhaps, the apoA-IMilano disulfide bridge may act in a redox manner to reduce oxidation. Alternatively, the presence of free cysteine in the apoA-IMilano-containing HDL may reduce oxidation (Bielicki and Oda, 2002). Note, the ETC-216 preparations used contain approximately 10% of apoA-IMilano is the reduced form, with the remainder dimerized.

ETC-216, apoA-IMilano/phospholipid complexes, or HDL...
preparations have also been shown to block expression of oxidation signals, reduce macrophage immunoreactivity in atherosclerotic lesions (Shah et al., 2001), and prevent inflammatory-induced intimal proliferative responses (Kaul et al., 2003) in cell-based (Navab et al., 1991) and animal studies (Shah et al., 2001; Kaul et al., 2003). These properties are in addition to HDL’s ability to regress lesions (Shah et al., 2001; Chiesa et al., 2002; Nissen et al., 2003). Collectively, these data suggest natural and synthetic HDL have the capacity to reduce oxidation and inflammation. These same properties (antioxidation, anti-inflammation, and antiproliferation) are present in agents known to be cardioprotective.

Therefore, we hypothesized that administration of apoA-I/A2/POPC complexes may potentially reduce tissue injury by a mechanism independent of enhanced lipid removal from the arteries (Chiesa et al., 2002), and examined whether ETC-216 was cardioprotective in an in vivo rabbit model of regional myocardial ischemia. We also measured serum lipid variables. One rationale for determination of serum lipoprotein cholesterol changes was to evaluate whether these variables were correlated with cardioprotection. ETC-216 (3 mg/kg, minimal effective dose) remained cardioprotective, but a measurable change in serum cholesterol was not observed, suggestive of a mechanism independent of a measurable mobilization of cholesterol to serum. Perhaps, a more subtle surrogate serum marker may be revealed upon further evaluation of the serum. At the higher dose level, the rise, then fall in serum HDL-cholesterol with a subsequent rise in VLDL-cholesterol after ETC-216 administration may reflect hepatic HDL-cholesterol delivery with enhanced VLDL production. In this regard, it is noteworthy that Stone et al. (1987) had demonstrated that intravenous administration of HDL to rats enhances hepatic VLDL production.

The exact mechanisms of action of ETC-216 will require...
additional experimentation such as potential effects on neutrophil activation, myeloperoxidase release, cellular adhesion molecule expression, and formation of oxidized lipids.

Cardiovascular disease treatments largely focus on chronic serum LDL-C and triglyceride reduction. The use of HDL-C as a therapeutic agent to treat cardiovascular disease is a new paradigm shift. Clearly, epidemiologic evidence supports the inverse relation between HDL-C and cardiovascular disease (Abbott et al., 1988). It is not unreasonable to speculate that ETC-216 or similar agents may be

Fig. 6. Changes in serum lipid levels during acute and chronic treatments with ETC-216. Rabbits (n = 6/group) were administered 100 mg/kg ETC-216 (open symbols) or vehicle (closed symbols) once (acute treatment) or twice (chronic treatment) and subjected to regional ischemia-reperfusion (protocol 1). Time-dependent changes in serum levels of the ETC-216 components, phospholipid (open squares), and recombinant human apoA-I Milano (open triangles, measured as apoA-I) are shown in the top two panels for the acute (left) and chronic (right) treatments. Changes in serum total cholesterol, unesterified cholesterol, and triglycerides in response to acute and chronic treatments are shown in the six bottom panels. Data are expressed as the mean ± S.E.M. *p < 0.05; **p < 0.01; and ***p < 0.001 versus –30 (acute treatment) or –24 h (chronic treatment). Comparison between ETC-216 and vehicle were significant for all panels in figure.
beneficial to prevent reperfusion injury. These protective effects may also be applicable to other organs (liver, kidney, brain, lung, and spleen).

The primary objective of the present study was to determine whether ETC-216 provided a protective effect in the intact heart subjected to regional ischemia and reperfusion. The experimental procedures for induction of myocardial injury and quantification of infarct size are well established in the literature. However, aside from reducing the extent of tissue injury, it would have been informative to have had

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### Table 1

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**Fig. 7.** Lipoprotein unesterified cholesterol profiles from acute (100 mg/kg) and chronic (2 × 100 mg/kg) ETC-216 treatments. Unesterified cholesterol (FC) lipoprotein profiles of temporal serum samples from rabbits subject to acute or chronic administration of vehicle (control) or ETC-216 and regional ischemia-reperfusion (protocol 1). From left to right, the three peaks in each profile are VLDL, LDL, and HDL. Profiles were performed on two animals from each treatment group. Shown are the profiles from one of the two animals from each treatment group since both animals within each group had similar profiles.

**Fig. 8.** Lipoprotein unesterified cholesterol profiles from animals treated with various dose levels of ETC-216. Rabbits were administered vehicle alone or 10, 3, or 1 mg/kg ETC-216 and subject to regional ischemia-reperfusion (protocol 2). Blood serum was analyzed for unesterified lipoprotein cholesterol profiles from select animals. Shown are typical profiles of each treatment group at select time points. Profiles were performed on two animals from each treatment group. Shown are the profiles from one of the two animals from each treatment group since both animals within each group had similar profiles. Profiles from the acute 100 mg/kg ETC-216 treatments (protocol 1) are included on the figure for comparison to the acute treatment dose groups in protocol 2.
information on recovery of ventricular function, which could have been assessed by placement of a Millar catheter in the left ventricle to record left ventricular dP/dt. Instead, the decision was made to place the Millar catheter in the carotid artery to avoid the increased potential for development of ventricular fibrillation and loss of the experimental animals. We were also under the constraint of having to complete the study with a limited supply of ETC-216. Thus, the determination of left ventricular dP/dtmax and left ventricular end-diastolic pressure would have added significantly to assessing the cardioprotective effects of ETC-216.

An additional concern relates to the limited number of samples used for the determination of lipid profiles. The decision to examine the lipid profiles from two animals per group was in consideration of the high cost for conducting the analyses. Nevertheless, the data, albeit limited, are consistent with those published by previous investigators (Chiesa et al., 2002).

Overall, we have provided experimental evidence that ETC-216 may be a cardioprotective agent in a preclinical in vivo model. Future development of ETC-216 may have utility for both elective and nonelective procedures; however, additional testing will be required.

Acknowledgments

This work is dedicated to the memory of Laura L. Sell, who has recently passed away. Her dedication, support, and friendship were an inspiration to us all. Although she will be deeply missed, her memory will always enrich our lives. We acknowledge the contribution of Martha Humes and Janell Lutostanski (both of Esperion Therapeutics, Inc., Ann Arbor, MI) for technical assistance.

References


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