JPET Fast Forward. Published on November 27, 2007 as DOI: 10.1124/jpet.107.129411

ANTI-INFLAMMATORY AND CARDIOPROTECTIVE ACTIVITIES OF SYNTHETIC HDL CONTAINING APOLIPOPROTEIN A-I MIMETIC PEPTIDES

Monica Gomaraschi, Laura Calabresi, Giuseppe Rossoni, Stefania Iametti, Guido Franceschini, John A. Stonik, and Alan T. Remaley

Center E. Grossi Paoletti, Department of Pharmacological Sciences, University of Milano, Italy (MG, LC, GF), and Molecular Disease Section

Department of Pharmacology, Chemotherapy and Medical Toxicology, University of Milan, Italy (GR)

Department of Molecular and Agroalimentary Sciences, University of Milano, Italy (SI)

Lipoprotein Metabolism Section, Vascular Medicine Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA (JAS, ATR).

CARDIOPROTECTIVE EFFECTS OF APOLIPOPROTEIN A-I MIMETIC PEPTIDES

Corresponding author:

Alan T. Remaley
National Institutes of Health
10 Center Dr.
Bldg. 10/Rm. 2C-433
Bethesda, MD 20892
Tel:301-402-9796
Fax:301-402-1885
aremaley@nih.gov

Number of text pages: 15

Number of tables: 3

Number of figures: 5

Number of references: 40

Number of words in Abstract: 250

Number of words in Introduction: 590

Number of words in Discussion: 1,323

Non-standard abbreviations: synthetic HDL (sHDL), ischemia/reperfusion (I/R)

ABSTRACT

Apolipoprotein A-I (apoA-I) mimetic peptides may represent an alternative to apoA-I for large-scale production of synthetic high density lipoproteins (sHDL) as a therapeutic agent. In this study, the cardioprotective activity of sHDL made with either L37pA peptide or its D-stereoisomer, D37pA, was compared to sHDL made with apoA-I. The peptides were reconstituted with palmitoyl-oleoyl-phosphatidylcholine (POPC), which yielded sHDL particles comparable to apoA-I sHDL in diameter, molecular weight, and α-helical content. Pre-treatment of endothelial cells with either peptide sHDL reduced TNFα stimulated VCAM-1 expression to the same extent as apoA-I sHDL. In an isolated rat heart model of ischemia/reperfusion (I/R) injury, L37pA and D37pA sHDL significantly reduced post-ischemic cardiac contractile dysfunction compared to the saline control, as indicated by a 49.7±6.4% (L37pA; $P<0.001$) and 53.0±9.1% (D37pA; $P<0.001$) increase of left ventricular developed pressure (LVDP) after reperfusion, and by a 45.4±3.4% (L37pA; $P<0.001$) and 49.6±2.6% (D37pA; $P<0.001$) decrease of creatine kinase (CK) release. These effects were similar to the 51.3±3.0% ($P<0.001$) increase of LVDP and 51.3±3.0 ($P<0.001$) reduction of CK release induced by apoA-I sHDL. Consistent with their cardioprotective effects, all three types of sHDL particles mediated an approximate 20% ($P<0.001$) reduction of cardiac TNFα content and stimulated an approximate 35% ($P<0.05$) increase in post-ischemic release of prostacyclin. In summary, L37pA and D37pA peptides can form sHDL particles that retain a similar level of protective activity as apoA-I sHDL on the endothelium and the heart, and thus apoA-I mimetic peptides may be useful therapeutic agents for the prevention of cardiac I/R injury.

INTRODUCTION

Plasma-derived and synthetic high density lipoproteins (HDL) exert a diverse set of atheroprotective effects, ranging from stimulation of reverse cholesterol transport to prevention of endothelial dysfunction and reduction of oxidative stress (Calabresi et al., 2006). Early studies in cholesterol-fed rabbits showed that only a few injections of homologous plasma-derived HDL can induce regression of established lesions (Badimon et al., 1990), thus providing a rationale for a new therapeutic approach aimed at atherosclerosis regression through the administration of synthetic HDL (sHDL), a complex of purified apolipoprotein A-I (apoA-I) with phospholipids. Indeed, a number of preclinical investigations in different animal models of human atherosclerosis have demonstrated that sHDL treatment can bring about significant changes in arterial lipid and cell dynamics, leading to both plaque stabilization and regression (Calabresi et al., 2006). sHDL-induced cholesterol mobilization from the arterial wall is likely to play an integral role in atherosclerosis stabilization/regression, as indicated by the enhanced cholesterol efflux-promoting capacity of plasma (Shah et al., 2001), and by the rapid and striking increase of unesterified cholesterol in the plasma HDL fraction (Chiesa et al., 2002) in sHDL-treated animals. The consistent positive findings in preclinical studies have provided the basis for the clinical development of sHDL containing the apolipoprotein A-I_{Milano} (apoA-I_M) variant (Nissen et al., 2003) or wild-type apoA-I (Tardif et al., 2007), as a novel treatment for promoting atherosclerosis regression in coronary patients.

The growing interest in sHDL as a therapeutic agent has prompted us to also test its cardioprotective effect against myocardial ischemia/reperfusion (I/R) injury in isolated rat hearts (Rossoni et al., 2004). Increasing doses of sHDL were given either immediately before ischemia or during the first minutes of reperfusion. In both instances, treatment caused a dose-dependent improvement in the recovery of left ventricle contractile capacity

at reperfusion, associated with a reduction of myocyte necrosis (Rossoni et al., 2004). The two constituents of sHDL, apoA-I and phosphatidylcholine (PC), given separately did not show any improvement of post-ischemic cardiac function (Rossoni et al., 2004). To establish that sHDL can exert a cardioprotective effect against I/R injury *in vivo*, sHDL made with the apoA-I_M variant was also examined in rabbits undergoing ligation and reopening of the left anterior descending artery (Marchesi et al., 2004). Rabbits treated intravenously with sHDL indeed developed significantly smaller infarcts than vehicle-treated animals (Marchesi et al., 2004). Because most of the pathogenic mechanisms responsible for I/R injury are shared by all organs, it is not surprising that sHDL was also found to be protective against cerebral, renal, and intestinal I/R injury (Thiemermann et al., 2003;Paterno et al., 2004;Cuzzocrea et al., 2004).

Except for its globular N-terminal domain, apoA-I is a tandem array of amphipathic helices (Sethi et al., 2007). Synthetic apoA-I mimetic peptides, which are typically much shorter than apoA-I, retain the amphipathic helical structure of apoA-I and can, like apoA-I, associate with lipids to form HDL-like particles. They also mimic most of the atheroprotective properties of apoA-I, and therefore, represent an attractive alternative to recombinant or purified human apoA-I for low-cost, large-scale production of sHDL (Sethi et al., 2007). The L37pA peptide and the its D-stereoisomer, D37pA, are bi-helical peptides that *in vitro* can promote the efflux of cholesterol and phospholipid from cells by the ABCA1 transporter (Remaley et al., 2003), one of the key cellular proteins in the reverse cholesterol transport pathway. Because previous studies on cardiac I/R injury showed that apoA-I had to be recombined with phospholipids to get a beneficial effect (Rossoni et al., 2004), the L37pA and D37pA peptides used in this study, were also combined with phospholipid, thus enabling these peptide-lipid complexes to both mediate lipid uptake and potentially other hydrophobic substances from cells, as well as possibly

be a donor of lipids and proteins to cells. The aim of the present study was, therefore, (i) to prepare and characterize 37pA-containing sHDL, (ii) to evaluate the anti-inflammatory ability of these sHDL particles on endothelial cells, and (iii) to examine their ability to protect against cardiac I/R injury in isolated perfused hearts.

METHODS

Preparation of sHDL

ApoA-I was purified from human blood plasma (Calabresi et al., 1997); the L37pA peptide (DWLKAFYDKVAEKLKEAF-P-DWLKAFYDKVAEKLKEAF) was synthesized by a solid-phase procedure, using a Fmoc/DIC/HOBt protocol on a Biosearch 9600 peptide synthesizer with L amino acids. The D37pA peptide was synthesized with the same sequence as L37pA but with D amino acids. All peptides were purified to greater than 98% homogeneity by reverse-phase HPLC on an Aquapore RP-300 column (Remaley et al., 2003).

sHDL containing palmitoyl-oleoyl-phosphatidylcholine POPC, Sigma-Aldrich Chemie, Steinheim, Germany) and either apoA-I, L37pA or D37pA were prepared by the cholate dialysis technique (Calabresi et al., 1997), with a starting POPC:protein weight ratio of 2.5:1. Protein, peptide and phospholipid concentrations were measured as previously described (Calabresi et al., 1997). sHDL preparations were dialyzed against sterilized saline immediately before use and their concentrations are expressed as protein (peptide) concentration.

Characterization of sHDL

sHDL particle dimension and molecular weight were evaluated by fast liquid protein chromatography (FPLC). sHDL were applied to a Superose 6 10/30 column (GE Healthcare Biosciences, Uppsala, Sweden), equilibrated with phosphate buffer (150 mM

NaCl, 0.4 mM $NaH_2PO_4*H_2O$, 1.6 mM $Na_2HPO_4*2H_2O$, pH 7.4, 1 mM EDTA, 3 mM NaN_3), and eluted at a flow rate of 0.5 ml/min. Protein levels were monitored by absorbance at 280 nm. sHDL particle dimension and molecular weight were calculated on the base of the retention coefficients of ferritin (12.2 nm, 440 kDa), aldolase (9.6 nm, 232 kDa) and albumin (7.1 nm, 67 kDa) (GE Healthcare Biosciences). The number of apoA-I or 37pA molecules per sHDL particle was determined by cross-linking with dimethylsuberimidate (DMS)(Calabresi et al., 1994). Circular dichroism (CD) spectra were recorded with a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan) at the constant temperature of 25°C. All the spectra were baseline-corrected.

VCAM-1 expression in endothelial cells

Primary cultures of human umbilical vein endothelial cells (HUVEC) were purchased from PromoCell (Heidelberg, Germany) and cultured as previously described (Calabresi et al., 1997). HUVEC were incubated overnight with sHDL at 1 mg/ml, washed, and then stimulated with tumor necrosis factor alpha (TNFα, R&D Systems, Minneapolis, MN, USA) at a concentration of 10 ng/ml for 8 hours. At the end of the experiment, cells and conditioned media were immediately frozen at –80°C. VCAM-1 concentration in the conditioned media was evaluated on MaxiSorp plates (Nunc GmbH & Co, Wiesbaden, Germany), using the CytoSets™ matched antibody pairs ELISA kit (BioSource International, Camarillo, CA), according to manufacturer instructions. Endothelial VCAM-1 expression was evaluated by immunoblotting and RT-PCR analyses of total cell lysates. 30 μg of proteins were separated on a 10% SDS-PAGE and then transferred on nitrocellulose membrane. After saturation with 5% non-fat dried milk, the membrane was incubated with an antibody against human VCAM-1 (Exalpha Biologicals, Watertown, MA), and then with a HRP-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark). Bands were visualized by enhanced chemiluminescence (GE Healthcare

Biosciences). The membrane was then stripped and reprobed with an antibody against β-actin (Sigma-Aldrich). Total RNA was extracted from HUVEC with Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was prepared by reverse transcription of 1 μg of total RNA, using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA) and amplified for 25 cycles with iTaq DNA polymerase (Bio-Rad laboratories) in a MyCycler (Bio-Rad Laboratories). The following primers were used: VCAM-1 sense 5'-GAAGATG-GTCGTGATCCTTG-3', antisense 5'-ACTTGACTGTGATC-GGCTTC-3'; GAPDH sense 5'-CCACCCATGGCAAATTCCATGGCA-3', antisense 5'-TCTAGACGGCAGGTCAGGTCCACC-3'. PCR products were separated by electrophoresis on 1% agarose gel, stained with ethidium bromide, visualized by ultraviolet irradiation, and photographed with Polaroid film. Band densities were evaluated with a GS-690 Imaging Densitometer and a Multi-Analyst software (Bio-Rad Laboratories). VCAM-1 band intensities were normalized by their β-actin (for western blotting) or GAPDH (for RT-PCR) values.

I/R injury in isolated rat hearts

Male Sprague-Dawley rats (Charles River Italia) were anesthetized with sodium pentobarbital (50 mg/kg i.p.); the hearts were rapidly excised, placed in ice-cold Krebs-Henseleit buffer (K-H buffer, 118 mM NaCl, 2.8 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 5.5 mM glucose), and mounted for retrograde perfusion at 37°C through the aorta (Calabresi et al., 2003). Left ventricular developed pressure (LVDP) and coronary perfusion pressure (CPP) were continuously monitored throughout the experiment. Isolated hearts were mounted and perfused with K-H buffer at a flow rate of 15 ml/minute for 80 minutes. Hearts were equilibrated with K-H buffer at a flow rate of 15 ml/minute for 30 minutes. A moderate ischemia was induced by reducing the perfusion flow rate to 1 ml/minute for 20 minutes; the normal flow rate (15 ml/minute) was then

restored, and reperfusion continued for 30 minutes. CK release was evaluated by measuring CK activity in the coronary effluent collected every 150 seconds, during reperfusion. sHDL (1 mg/ml in K-H buffer) were administered during the 10 minutes immediately before ischemia. In separate experiments, sHDL were administered during the first 10 minutes of reperfusion at the concentration of 2 mg of protein/ml of buffer.

Hearts were snap-frozen in liquid nitrogen immediately at the end of the experiment and stored at –80°C; cardiac tissue was homogenized and solubilized as previously described (Calabresi et al., 2003). Coronary effluent was collected and immediately frozen at –80°C. The concentration of TNFα in cardiac homogenates and in coronary effluents collected during reperfusion was measured by a commercial ELISA kit specific for rat TNFα (Endogen Inc., Woburn, MA, USA) (Calabresi et al., 2003). The generation of prostacyclin (PGI_2) by cardiac tissue was assessed by measuring its stable metabolite 6-keto-$PGF_{1\alpha}$ on coronary effluent aliquots collected in the 5 minutes immediately before ischemia and during the first 10 minutes of reperfusion by a commercial ELISA kit (GE Healthcare Biosciences).

All investigational animal procedures conformed to the Guide for the Care and Use of Laboratory Animals guidelines published by the National Institutes of Health, National Institutes of Health Publication 85-23 (revised 1996).

Statistical analyses

Results are reported as mean±SEM, unless otherwise stated. Group differences were determined by analysis of variance (ANOVA); differences with a value of $p<0.05$ were considered as statistically significant. For the pre-ischemia treatment I/R experiments, a sample size of 4 per group has 90% power to detect a difference of 300 mm in LVDP-AUC, assuming a standard deviation of 93 mm (Calabresi et al., 2003), using a t-test with

a 0.025 significance level. For the post ischemia-treatment I/R experiments, a sample size of 3 per group has 90% power to detect a difference of 300 mm in LVDP-AUC, assuming a standard deviation of 65 mm (Calabresi et al., 2003), using a t-test with a 0.025 significance level. The area under the curve (AUC) calculations were determined for the entire reperfusion time period (30 minutes) and was measured by the trapezoid method, with a MicroCal Origin 3.5 program (OriginLab Corp, Northampton, MA).

RESULTS

Characterization of sHDL

The POPC:apoA-I weight ratio in sHDL made with purified apoA-I was 2.28±0.11, and the percentage of lipid-associated apoA-I was 89.6±3.5% of the starting amount. sHDL made with L37pA and D37pA peptides had a final POPC:peptide weight ratio of 3.33±0.25 and 3.43±0.19 respectively, significantly higher than that of apoA-I sHDL (p=0.013 and 0.009). The percent association with POPC was 62.5±2.5% and 63.1±4.8%, respectively. Any unassociated peptides (4.2 kDa) or phospholipids were removed by gel filtration and not present in the final sHDL preparations.

The various sHDL preparations were comparable in terms of particle diameter and molecular weight, as shown in Table 1. Cross-linking of apoA-I in sHDL particles resulted in two bands corresponding to monomeric (28 kDa) and dimeric (56 kDa) apoA-I; thus, one sHDL particle (189 kDa) contained up to two molecules of apoA-I and 175 molecules of POPC (Mw=760 Da, Table 1). Cross-linking of the 37pA peptides in sHDL resulted in ten bands, ranging from a monomer (4.2 kDa) to a decamer (42.5 kDa); thus, one sHDL particle (194-214 kDa) contained up to ten molecules of the 37pA peptide and approximately 198 to 225 molecules of POPC (Table 1). Because apoA-I is approximately 6.5 times larger in molecular weight compared to the peptides, the ratio of the mass of the

peptides to phospholipids is approximately 40% lower than that for apoA-I sHDL. The far-UV CD spectra of lipidated apoA-I and L37pA are each indicative of a highly α-helical structure, with negative troughs at 208 and 222 nm; as expected, D37pA gave an inverted but completely symmetrical profile to that of L37pA (Figure 1). The overall percentage of α-helix in all three sHDL preparations was similar (Table 1).

Anti-inflammatory effects of sHDL on endothelial cells

sHDL made with the 37pA peptides were tested for their capacity to inhibit VCAM-1 expression in comparison to apoA-I sHDL. HUVECs were pre-treated overnight with sHDL before stimulation with TNFα, and the concentration of soluble VCAM-1 in the conditioned media was then measured by ELISA. Stimulation of HUVEC with TNFα caused a marked increase in the release of soluble VCAM-1 protein in cell culture medium compared to unstimulated cells (47.2±3.4 ng/ml vs 2.0±0.5 ng/ml, respectively; $p<0.001$); consistent with our previous findings (Calabresi et al., 1997). Treatment of HUVEC with apoA-I sHDL before TNFα stimulation caused a significant 67.2% reduction of VCAM-1 concentration in the medium (15.5±6.3 ng/ml, $p=0.002$) (Figure 2). The concentration of VCAM-1 in conditioned medium positively correlated with endothelial expression of VCAM-1 protein, as evaluated by western blotting analysis of total cell lysate. VCAM-1 was undetectable in unstimulated cells, whereas a marked signal was visible after TNFα stimulation. Pre-incubation with sHDL reduced VCAM-1 protein expression in cells (Figure 3, panel B). The inhibition occurred at the transcriptional level, as indicated by the sHDL-mediated reduction of VCAM-1 mRNA levels in endothelial cells treated with sHDL before stimulation with TNFα (Figure 3, panel A).

sHDL made with both L and D 37pA peptides had a similar anti-inflammatory activity as apoA-I sHDL (Figure 2). VCAM-1 concentration in the conditioned medium of cells treated with L37pA sHDL was 13.8±2.0 ng/ml, with a 70.8% reduction compared to untreated TNFα-stimulated cells (p=0.002); likewise, VCAM-1 concentration in the conditioned medium of cells treated with D37pA sHDL was 16.6±7.3 ng/ml, a 64.8% reduction compared to the control (p=0.002). As demonstrated for apoA-I sHDL, the modulation of VCAM-1 concentration occurred at the level of transcriptional (Figure 3).

Cardioprotective effects of sHDL against I/R

The 20 minute low-flow ischemia caused a marked cardiac dysfunction and necrosis at reperfusion, as demonstrated by the partial recovery of LVDP after reperfusion, the elevation of CPP and the release of CK in the coronary effluent of saline-treated hearts (Table 2). The pre-ischemic administration of 37pA sHDL improved the post-ischemic LVDP recovery, and attenuated the CPP elevation and CK release (Figure 4). The cardioprotective effect of the three sHDL preparations was compared by calculating the area under the curve (AUC) described by LVDP, CPP and CK, during the 30 minutes of reperfusion. No difference was detected among the cardioprotective effect of sHDL containing L37pA, D37pA, or apoA-I, (Table 2). L37pA sHDL and D37pA sHDL improved LVDP recovery by 49.7% and 53.0% and reduced CPP at reperfusion by 42.3% and 40.4%, respectively; CK release was also reduced by 45.4% and 49.6%, respectively. These effects were comparable to the 51.3% increase of LVDP, the 40.4% reduction of CPP and the 50.3% reduction of CK release induced by apoA-I sHDL. A significant cardioprotection was also observed when L37pA sHDL were given during the first 10 minutes of reperfusion (Fig. 5); the response was somewhat lower than that of apoA-I sHDL, but the difference between the two preparations did not reach statistical significance (p=0.091 for LVDP, p=0.118 for CPP and p=0.065 for CK) (Table 2).

The cardioprotective effect of apoA-I sHDL was previously shown to be mediated, at least in part, by an improvement of post-ischemic prostaglandin release and by the ability of sHDL to bind and remove TNFα from cardiac tissue (Calabresi et al., 2003; Tardif et al., 2007). L37pA sHDL and D37pA sHDL, given either before or after ischemia, significantly and equally reduced cardiac TNFα content compared to saline (Table 3), and as expected resulted in a concomitant increase of TNFα concentration in the coronary effluent was observed (Table 3). No difference was observed when 37pA sHDL were compared to apoA-I sHDL, indicating that sHDL made with the 37pA peptide can act as scavenger of TNFα as well as apoA-I sHDL (Table 3).

The pre-ischemic administration of 37pA-containing sHDL also caused a significant increase of pre-ischemic and post-ischemic concentration of 6-keto-PGF_1,a stable metabolite of the potent vasodilator PGI_2 , in the coronary effluent.(Table 3). When given at reperfusion, sHDL also enhanced post-ischemic 6-keto-$PGF_{1\alpha}$ release, with no effect on pre-ischemic effluent concentrations (Table 3). Again, no major difference was detected between sHDL made with the 37pA peptides or apoA-I (Table 3).

DISCUSSION

HDL has been found in numerous epidemiologic studies to be one of the main anti-atherogenic constituents of plasma (Linsel-Nitschke and Tall, 2005; Ader, 2006). The ability of HDL to promote reverse cholesterol transport was believed to largely account for its atheroprotective effect, but recently HDL has been recognized to also have a multitude of other salutary effects on endothelial cells and inflammatory cells in preventing atherosclerosis (Mineo et al., 2006; Navab et al., 2006). Several epidemiologic studies have also shown that HDL may have a direct cardioprotective effect beyond its ability to prevent atherosclerosis (Berge et al., 1982;Goldbourt et al., 1986;Wang et al., 1998).

Interestingly, patients with a myocardial infarction have improved cardiac function and a better prognosis, if they have elevated HDL at the time of infarction (Berge et al., 1982; Goldbourt et al., 1986; Wang et al., 1998). These findings have prompted this and other recent studies on the use of HDL as a potential therapeutic agent for preventing cardiac I/R injury. The main finding from this study is that sHDL made with apoA-I mimetic peptides can also reduce cardiac I/R injury, which suggests that such peptides may be useful as therapeutic agents for not only reversing atherosclerosis in patients with acute coronary syndrome (Navab et al., 2006; Sethi et al., 2007), but also in the acute treatment of patients with myocardial infarction.

The mechanism for the cardioprotective effect of apoA-I mimetic peptides is not known, but these peptides have been shown to mediate a wide range of biological effects, such as anti-oxidant, anti-inflammatory, and pro-vasodilatory effects (Navab et al., 2006; Sethi et al., 2007), all of which have been proposed to explain their ability to block the progression of atherosclerosis. HDL can potentially mediate these various cellular effects either by removing substances from cells, such as lipids, by acting as a donor to cells, or by triggering cell signalling events. Some of these cellular effects may also be relevant to their ability to protect against I/R injury. For example, apoA-I, as well as L37pA and D37pA, can mediate the efflux of excess cholesterol from cells by the ABCA1 transporter (Remaley et al., 2003). Because of the rapidity of the events related to cardiac I/R injury, it is not likely, however, that increased efflux of cholesterol by sHDL is relevant to the protection observed in this study. Besides cholesterol, however, other lipids, including oxidized lipids, can also be removed and sequestered by HDL, as well as catabolized by HDL-associated enzymes, such as paraoxonase (Navab et al., 2004). Oxidized lipids rapidly form during reperfusion after ischemia and can have acute effects on vascular cells by a several different signaling pathways (Carney et al., 1996; Buffon et al., 2000; Carden

and Granger, 2000). The anti-oxidant properties of apoA-I mimetic peptides is thus one possible explanation for the observed benefit of these peptides on cardiac IR injury.

As was shown for HDL (Calabresi et al., 1994), sHDL made with apoA-I mimetic peptides was able to remove the pro-inflammatory cytokine TNFα from the heart (Table 3). The production of TNFα during reperfusion is known to mediate some of the detrimental effects of I/R injury on the heart (Meldrum, 1998; Moe et al., 2004). L37pA and D37pA sHDL were shown, in this study, to be equally effective as apoA-I sHDL in removing TNFα; thus the anti-infllammatory property of the apoA-I mimetic peptides could also be contributing to the protection of sHDL against cardiac I/R injury.

Decreased tissue perfusion after ischemia also plays a central role in the pathogenesis of cardiac injury after a myocardial infarction (Carden and Granger, 2000). Like apoA-I sHDL, sHDL made with L37pA and D37pA were equally effective in increasing the concentration of 6-keto-$PGF_{1\alpha}$, a stable catabolite of the potent vasodilator PGI_2 (Tanaka et al., 2004). Thus increased production of PGI_2 and increased vasodilation by 37pA sHDL could also account for the observed decrease in CPP after reperfusion of the rat hearts (Table 2). HDL has previously been shown to increase PGI_2 by several different mechanisms (Mineo et al., 2006). HDL can induce the expression of COX-2 in endothelial (Norata et al., 2004) and smooth muscle cells (Vinals et al., 2004), although this is unlikely the mechanism in this study given the relatively short time frame of the reperfusion study. HDL can also increase PGI_2 by providing cells a source of arachidonate (Pomerantz et al., 1985) for production by COX-1 and by stabilizing PGI_2 against degradation (Pirich et al., 1993), which could both possibly contribute to the observed provasodilatory effect in this study for sHDL made with L37pA and D37pA.

Another novel finding from this study was the observation that L37pA and D37pA sHDL, like apoAI sHDL, can inhibit the expression of VCAM-1 by endothelial cells (Figs. 2-3). VCAM-1 is one of the main adhesion proteins on endothelial cells, and thus blocking the attachment of circulating neutrophils and monocytes to the endothelium by HDL and their subsequent infiltration into cardiac tissue could reduce I/R injury. No blood cells, however, were present in the isolated heart perfusion system, thus precluding this as a possible explanation for the experimental results in this study, although this is likely an important *in vivo* factor for the vascular protection afforded by HDL (Nicholls et al., 2005). HDL is known to block VCAM-1 expression by blocking the activation of the transcription factor NF-Kappa B (Calabresi et al., 1997; Robbesyn et al., 2003). NF-Kappa B activation can lead to numerous other gene and cellular changes besides VCAM-1, such as increased cytokine production, induction of iNOS, increased MCP-1 (De Martin et al., 2000), all of which all can possibly contribute to cardiac I/R injury and activation of endothelial cells. Inhibition of NF-kappa B activation in the heart has already been shown to markedly decrease I/R injury (Yeh et al., 2005) , thus the inhibition of NF-Kappa B activation in endothelial cells and possibly in cardiac myocytes could be another mechanism for the cardioprotective effects of sHDL, which warrants future studies.

Finally, HDL is also known to be the main transporter of sphingosine-1-phosphate (S1P) in plasma (Nofer and Assmann, 2005), and S1P has been shown to protect against cardiac IR injury (Theilmeier et al., 2006). S1P is a potent bioactive lipid that mediates a wide range of effects on endothelial, as well as on cardiac cells, via specific S1P receptors (Nofer and Assmann, 2005). ApoA-I sHDL and 37pA sHDL used in this study, however, were synthesized in absence of any S1P, so it is unlikely that S1P accounts for any of their observed effects, unless sHDL in the perfusate was able to scavenge S1P from the cardiac tissue and was then able to deliver it back to cells in such a way that it still

mediated signaling via the S1P receptor. Even if S1P does not account for any of the cardioprotective benefits in this study, it is possible that the addition of S1P during the reconstitution of the apoA-I mimetic peptides with phospholipids could further enhance their cardioprotective effects.

The main approach for treating patients with a myocardial infarction is to restore blood flow as soon as possible with stents or thrombolytic therapy. It would be very valuable, if such therapies could be coupled to a drug to also prevent any subsequent I/R injury after cardiac blood flow is restored. Many different agents have been shown to prevent I/R injury in various animal models, but none have been found so far to be useful in clinical trials. Much further work, therefore, has to be done to determine whether infusion of sHDL containing apoA-I mimetic peptides will be a useful approach. The fact that one can duplicate the benefit of HDL with apoA-I mimetic peptides when given after ischemia does raise the possibility, however, of using these peptides as therapeutic agents. Compared to full length apoA-I, apoAI mimetic peptides are relatively short and can be readily synthesized, which would facilitate their manufacturing and the ability to do future structure-function studies to optimize their effect and to better understand their mechanism of action. Results from this study showing that lack of stereoselectivity in any of the anti-inflammatory or cardioprotective effects of L37pA and D37pA sHDL have already revealed that the molecular interaction accounting for their biological properties does not likely involve a classic receptor-ligand type interaction. This is further supported by the fact that the sequence of the 37pA peptide has no homology to apoA-I or to the other apolipoproteins, but simply contains an amphipathic helix (Remaley et al., 2003), thus indicating that this is the main protein structural motif that is necessary for their cardioprotective effect. Finally, the main finding of this study that apoA-I mimetic peptides protect against cardiac I/R and the potential mechanisms uncovered may provide

new insights into their atheroprotective properties and to the ongoing work in developing these agents for the treatment of atherosclerosis (Navab et al., 2004; Sethi et al., 2007).

REFERENCES

Ader DJ (2006) Molecular regulation of HDL metabolism and function: implications for novel therapies. *J Clin Invest* **116**:3090-3100.

Badimon JJ, Badimon L and Fuster V (1990) Regression of atherosclerotic lesions by high density lipoprotein plasma fraction in the cholesterol-fed rabbit. *J Clin Invest* **85**:1234-1241.

Berge KG, Canner PL and Hainline A (1982) High-density lipoprotein cholesterol and prognosis after myocardial infarction. *Circulation* **66**:1176-1178.

Buffon A, Santini SA, Ramazzotti V, Rigattieri S, Liuzzo G, Biasucci LM, Crea F, Giardina B and Maseri A (2000) Large, sustained cardiac lipid peroxidation and reduced antioxidant capacity in the coronary circulation after brief episodes of myocardial ischemia. *J Am Coll Cardiol* **35**:633-639.

Calabresi L, Vecchio G, Longhi R, Gianazza E, Palm G, Wadensten H, Hammerstrom A, Olsson A, Karlstrom A, Sejlitz T et al (1994) Molecular characterization of native and recombinant apolipoprotein A-I_{Milano} dimer. The introduction of an interchain disulfide bridge remarkably alters the physicochemical properties of apolipoprotein A-I. *J Biol Chem* **269**:32168-32174.

Calabresi L, Franceschini G, Sirtori CR, De Palma A, Saresella M, Ferrante P, Taramelli D (1997) Inhibition of VCAM-1 expression in endothelial cells by reconstituted high density lipoproteins. *Biochem Biophys Res Commun* **238**:61-65.

Calabresi L, Rossoni G, Gomaraschi M, Sisto F, Berti F and Franceschini G (2003) High-density lipoproteins protect isolated rat hearts from ischemia-reperfusion injury by reducing cardiac tumor necrosis factor-alpha content and enhancing prostaglandin release. *Circ Res* **92**:330-337.

Calabresi L, Gomaraschi M, Rossoni G and Franceschini G (2006) Synthetic high density lipoproteins for the treatment of myocardial ischemia/reperfusion injury. *Pharmacol Ther* **111**:836-854.

Carden DL and Granger DN (2000) Pathophysiology of ischaemia-reperfusion injury. *J Pathol* **190**:255-266.

Carney JM, Hall NC, Cheng M, Wu J and Butterfield DA (1996) Protein and lipid oxidation following ischemia/reperfusion injury, the role of polyamines: an electron paramagnetic resonance analysis. *Adv Neurol* **71**:259-268.

Chiesa G, Monteggia E , Marchesi M, Lorenzon P, Laucello M, Lorusso V, Di Mario C, Karvouni E, Newton RS, Bisqaier CL et al (2002) Recombinant apolipoprotein A-I_{Milano} infusion into rabbit carotid artery rapidly removes lipid from fatty streaks. *Circ Res* **90**:974-980.

Cuzzocrea S, Dugo L, Patel NS, Di Paola, Cockerill GW, Genovese T, Thiemermann C (2004) High-density lipoproteins reduce the intestinal damage associated with ischemia/reperfusion and colitis. *Shock* **21**:342-351.

De Martin R, Hoeth M, Hofer-Warbinek R and Schmid JA (2000) The transcription factor NF-kappa B and the regulation of vascular cell function. *Arterioscler Thromb Vasc Biol* **20**:E83-88.

Goldbourt U, Cohen L and Neufeld HN.(1986) High density lipoprotein cholesterol: prognosis after myocardial infarction. The Israeli Ischemic Heart Disease Study. *Int J Epidemiol* **15**:51-55.

Gomaraschi M, Basilico N, Sisto F, Taramelli D, Eligini S, Colli S, Sirtori CR, Franceschini G, Calabresi L (2005) High-density lipoproteins attenuate interleukin-6 production in endothelial cells exposed to pro-inflammatory stimuli. *Biochim Biophys Acta* **1736**:136-143.

Linsel-Nitschke P and Tall AR(2005) HDL as a target in the treatment of atherosclerotic cardiovascular disease. *Nat Rev Drug Discov* **4**:193-205.

Marchesi M, Booth EA, Davis T, Bisgaier CL and Lucchesi BR (2004) Apolipoprotein A-IMilano and 1-palmitoyl-2-oleoyl phosphatidylcholine complex (ETC-216) protects the in vivo rabbit heart from regional ischemia-reperfusion injury. *J Pharmacol Exp Ther* **311**:1023-1031.

Meldrum DR (1998) Tumor necrosis factor in the heart. *Am J Physiol* **274**:R577-95.

Mineo C, Deguchi H, Griffin JH and Shaul PW (2006) Endothelial and antithrombotic actions of HDL. *Circ Res* **98**:1352-1364.

Moe GW, Marin-Garcia J, Konig A, Goldenthal M, Lu X and Feng Q (2004) In vivo TNF-alpha inhibition ameliorates cardiac mitochondrial dysfunction, oxidative stress, and apoptosis in experimental heart failure. *Am J Physiol Heart Circ Physiol* **287**:H1813-1820.

Navab M, Ananthramaiah GM, Reddy ST, Van Lenten BJ, Ansell BJ, Fonarow GC, Vahabzadeh K, Hama S, Hough G, Kamranpour N, Berliner JA, Lusis AJ and Fogelman AM (2004) The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL. *J Lipid Res* **45**:993-1007.

Navab M, Ananthaaramaiah GM, Reddy ST and Fogelman AM (2006)Apolipoprotein A-I mimetic peptides and their role in atherosclerosis prevention. *Nat Clin Pract Cardiovasc Med* **3**:540-547.

Nicholls SJ, Dusting GJ, Cutri B, Bao S, Drummond GR, Rye KA and Barter PJ (2005) Reconstituted high-density lipoproteins inhibit the acute pro-oxidant and proinflammatory vascular changes induced by a periarterial collar in normocholesterolemic rabbits. *Circulation* **111**:1543-1550.

Nissen SE, Tsunoda T, Tuzcu EM, Schoenhagen P, Cooper CJ, Yasin M, Eaton GM,Lauer MA, Sheldon WS, Grines CL et al (2003) Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *JAMA* **290**:2292-2300.

Nofer JR and Assmann G (2005) Atheroprotective effects of high-density lipoprotein-associated lysosphingolipids. *Trends Cardiovasc Med* **15**:265-271.

Norata GD, Callegari E, Inoue H and Catapano AL (2004) HDL3 induces cyclooxygenase-2 expression and prostacyclin release in human endothelial cells via a p38 MAPK/CRE-dependent pathway. *Arterioscler Thromb Vasc Biol* **24**:871-877.

Paterno R, Ruocco A, Postiglione A, Hubsch A, Andresen I and Lang MG (2004) Reconstituted High-Density Lipoprotein Exhibits Neuroprotection in Two Rat Models of Stroke. *Cerebrovasc Dis* **17**:204-211.

Pirich C, Banyai M, Efthimiou Y and Sinzinger H (1993) Lipoproteins and prostacyclin stability *Semin Thromb Hemost* **19**:1387-1343.

Pomerantz KB, Becherer PR, Tall AR and Cannon PJ (1985) Enrichment of endothelial cell arachidonate by lipid transfer from HDL: Relationship to prostaglandin I_2 synthesis. *Curr Med Chem Cardiovasc Hematol Agents* **26**:1269-1276.

Remaley AT, Thomas F, Stonik JA, Demosky SJ, Bark SE, Neufeld EB, Bocharov AV, Vishnyakova TG, Patterson AP, Eggerman TL et al (2003) Synthetic amphipathic helical peptides promote lipid efflux from cells by an ABCA1-dependent and an ABCA1-independent pathway. *J Lipid Res* **44**:828-836.

Robbesyn F, Garcia V, Auge N, Vieira O, Frisach MF, Salvayre R and Negre-Salvayre A (2003) HDL counterbalance the proinflammatory effect of oxidized LDL by inhibiting

intracellular reactive oxygen species rise, proteasome activation, and subsequent NF-kappaB activation in smooth muscle cells. *FASEB J* **17**:743-745.

Rossoni G, Gomaraschi M, Berti F, Sirtori CR, Franceschini G and Calabresi L (2004) Synthetic high-density lipoproteins exert cardioprotective effects in myocardial ischemia/reperfusion injury. *J Pharmacol Exp Ther* **308**:79-84.

Sethi AA, Amar M, Shamburek RD and Remaley AT(2007) Apolipoprotein AI mimetic peptides: possible new agents for the treatment of atherosclerosis. *Curr Opin Investig Drugs* **8**:201-212.

Shah PK, Yano J, Reyes O, Chyu KY, Kaul S, Bisqaier CL, Drake S, Cercek B (2001) High-dose recombinant apolipoprotein A-I_{Milano} mobilizes tissue cholesterol and rapidly reduces plaque lipid and macrophage content in apolipoprotein E-deficient mice: potential implications for acute plaque stabilization. *Circulation* **103**:3047-3050.

Tanaka Y, Yamaki F, Koike K and Toro L (2004) New insights into the intracellular mechanisms by which PGI2 analogues elicit vascular relaxation: cyclic AMP-independent, Gs-protein mediated-activation of MaxiK channel. *Curr Med Chem Cardiovasc Hematol Agents* **2**:257-265.

Tardif JC, Gregoire J, L'Allier PL, Ibrahim R, Lesprance J, Heinonen TM, Kouz S, Berry C, Basser R, Lavoie MA (2007) Effects of reconstituted high-density lipoprotein infusions on coronary atherosclerosis: a randomized controlled trial. *JAMA* **297**:1675-1682.

Theilmeier G, Schmidt C, Herrmann J, Keul P, Schafers M, Herrgott I, Mersmann J,

Larmann J, Hermann S, Stypmann J, Schober O, Hildebrand R, Schulz R, Heusch G, Haude M, von Wnuck Lipinski K, Herzog C, Schmitz M, Erbel R, Chun J and Levkau B (2006) High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the S1P3 lysophospholipid receptor. *Circulation* **114**:1403-1409.

Thiemermann C, Patel NS, Kvale EO, Cockerill GW, Brown PA, Stewart KN, Cuzzocrea S, Britti D, Mota-Filipe H, Chatterjee PK (2003) High density lipoprotein (HDL) reduces renal ischemia/reperfusion injury. *J Am Soc Nephrol* **14**:1833-1843.

Vinals M, Martinez-Gonzalez J and Badimon L (2004) Regulatory effects of HDL on smooth muscle cell prostacyclin release*. Arterioscler Thromb Vasc Biol* **19**:2405-2411.

Wang TD, Wu CC, Chen WJ, Lee CM, Chem MF, Liau CS, Sung FC and Lee YT (1998) Dyslipidemias have a detrimental effect on left ventricular systolic function in patients with a first acute myocardial infarction. *Am J Cardiol* **81**:531-537.

Yeh CH, Lin YM, Wu YC and Lin PJ (2005) Inhibition of NF-kappa B activation can attenuate ischemia/reperfusion-induced contractility impairment via decreasing cardiomyocytic proinflammatory gene up-regulation and matrix metalloproteinase expression. *J Cardiovasc Pharmacol* **45**:301-309.

FOOTNOTES

Work done by A.T.R. and J.A.S. was supported by intramural research funds from NHLBI, NIH in Bethesda, MD, USA.

Legends for Figures

Figure 1. Circular dichroism spectra of sHDL

Far-UV CD spectra of sHDL made with L37pA (black solid line), D37pA (black dashed line), or apoA-I (grey solid line), at a concentration of 0.1 mg of protein (peptide)/ml, were recorded at the constant temperature of 25°C.

Figure 2. VCAM-1 concentration in endothelial cell media

VCAM-1 concentration was evaluated by ELISA in conditioned media from unstimulated cells (white bar) and from TNFα-stimulated cells with (filled bars) or without (striped bar) pre-treatment with sHDL. Concentrations of sHDL are expressed as protein (peptide) content. Data are mean±SEM (n=3). * $p<0.05$ vs TNFα.

Figure 3. VCAM-1 protein and steady-state mRNA levels in endothelial cells

Panel A. RT-PCR analysis for VCAM-1 mRNA levels in unstimulated cells (Control) and in TNFα-stimulated cells pre-treated with or without sHDL (1 mg of protein (peptide)/ml). VCAM-1 mRNA band intensities were normalized by GAPDH values. Panel B. Western blotting analysis of VCAM-1 protein levels in unstimulated cells (Control) and in TNFα-stimulated cells pre-treated with or without sHDL (1 mg of protein (peptide)/ml). VCAM-1 protein band intensities were normalized by β-actin values. Results are expressed as mean±SEM of 3 separate experiments; *$P<0.05$ vs TNFα.

Figure 4. Cardiac function and CK release in isolated hearts undergoing I/R

Hearts were perfused with either saline (circles) (n=12), or with sHDL containing the L37pA peptide (triangles) (n=4) or with the D37pA peptide (inverted triangles) (n=4),

during the 10 minutes immediately before ischemia. sHDL were administered at 1 mg of protein (peptide)/ml of buffer. For all time points 1 min after the reperfusion, there was statistically significant ($P<0.01$) difference between the control and treatment groups in LVDP and CK. For all time points 3 min after the reperfusion, there was statistically significant ($P<0.05$) difference between the control and treatment groups in CPP. Data are mean±SEM for each treatment group (LVDP, left ventricular developed pressure; CPP, coronary perfusion pressure; CK, creatine kinase).

Figure 5. Cardiac function and CK release in isolated hearts undergoing I/R

Hearts were perfused with either saline (circles) (n=12), or with sHDL containing apoA-I (squares) (n=6) or with the L37pA peptide (triangles) (n=3), during the first 10 minutes of reperfusion. sHDL were administered at 2 mg of protein (peptide)/ml of buffer. For all time points 1 min after the reperfusion, there was statistically significant ($P<0.01$) difference between the control and treatment groups in LVDP and CK. There was a statistically significant ($P<0.05$) difference in CPP between the control versus apoA-I or L37pA treatment groups for all time points after 3 min and 10 min after reperfusion, respectively. Data are mean±SEM for each treatment group. LVDP, left ventricular developed pressure; CPP, coronary perfusion pressure; CK, creatine kinase.

Table 1. Characterization of sHDL particles

		apoA-I	L37pA	D37pA
FPLC	Diameter	9.4 nm	9.5 nm	9.7 nm
	Molecular weight	189 kDa	194 kDa	214 kDa
# Molecules/particle	Protein or peptide	2	10	10
# Molecules/particle	POPC	175	199	225
Circular dichroism	α-helix (%)	73%	71%	72%

Molecular weights used for calculations were 28 kDa for apoA-I, 4255 Da for 37pA and 760 Da for POPC. Percentage of α-helix was calculated at 222 nm. FPLC (fast protein liquid chromatography); POPC (palmitoyl-oleoyl-phosphatidylcholine).

Table 2. Effect of sHDL on cardiac function

		n	LVDP	CPP	CK
Saline		12	905±35	1563±70	5594±213
sHDL 1mg/ml pre-ischemia	apoA-I	8	1369±65***	932±51***	2781±167***
	L37pA	4	1355±58***	902±53***	3054±193***
	D37pA	4	1385±83***	932±56***	2820±146***
sHDL 2mg/ml at reperfusion	apoA-I	6	1508±65***	816±61***	2868±143***
	L37pA	3	1203±67**	1120±83***	3679±180***

Data are expressed as area under the curve estimated accordingly to the trapezoid method from data recorded during the 30 minutes of reperfusion, mean±SEM. Concentrations of sHDL are expressed as mg of protein (peptide)/ml of buffer. LVDP, left ventricular developed pressure; CPP, coronary perfusion pressure; CK, creatine kinase. ** $p<0.01$ vs Saline, *** $p<0.001$ vs Saline.

JPET Fast Forward. Published on November 27, 2007 as DOI: 10.1124/jpet.107.129411
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3. Effect of sHDL on TNFα and 6-keto-$PGF_{1\alpha}$ concentrations

		TNFα		6-keto-$PGF_{1\alpha}$	
		Cardiac tissue	Coronary effluent	Pre-ischemia	At reperfusion
Saline		83.3±2.0	2.8±0.2	2.0±0.1	8.8±0.5
sHDL 1 mg/ml pre-ischemia	apoA-I	65.3±1.8***	9.0±1.4***	2.6±0.2**	11.9±0.5**
	L37pA	67.7±3.5***	9.2±0.5***	2.5±0.2*	11.0±0.7*
	D37pA	64.3±6.7***	9.0±1.2***	3.6±0.3***	12.6±1.4**
sHDL 2 mg/ml at reperfusion	apoA-I	64.0±2.0***	9.5±0.6***	1.8±0.1	12.1±0.2**
	L37pA	66.7±2.8**	7.9±0.6**	1.9±0.2	11.2±0.7*

Data are expressed as mean±SEM (n=4 for pre-ischemic treatments, n=3 for treatments at reperfusion). Concentrations of sHDL are expressed as mg of protein (peptide)/ml of buffer. TNFα concentrations are expressed as pg/mg of protein for cardiac tissue and as pg/ml for coronary effluent; the concentrations of 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) were measured in the coronary effluent and expressed as ng/ml. * $p<0.05$ vs Saline, ** $p<0.01$ vs Saline, *** $p<0.001$ vs Saline.

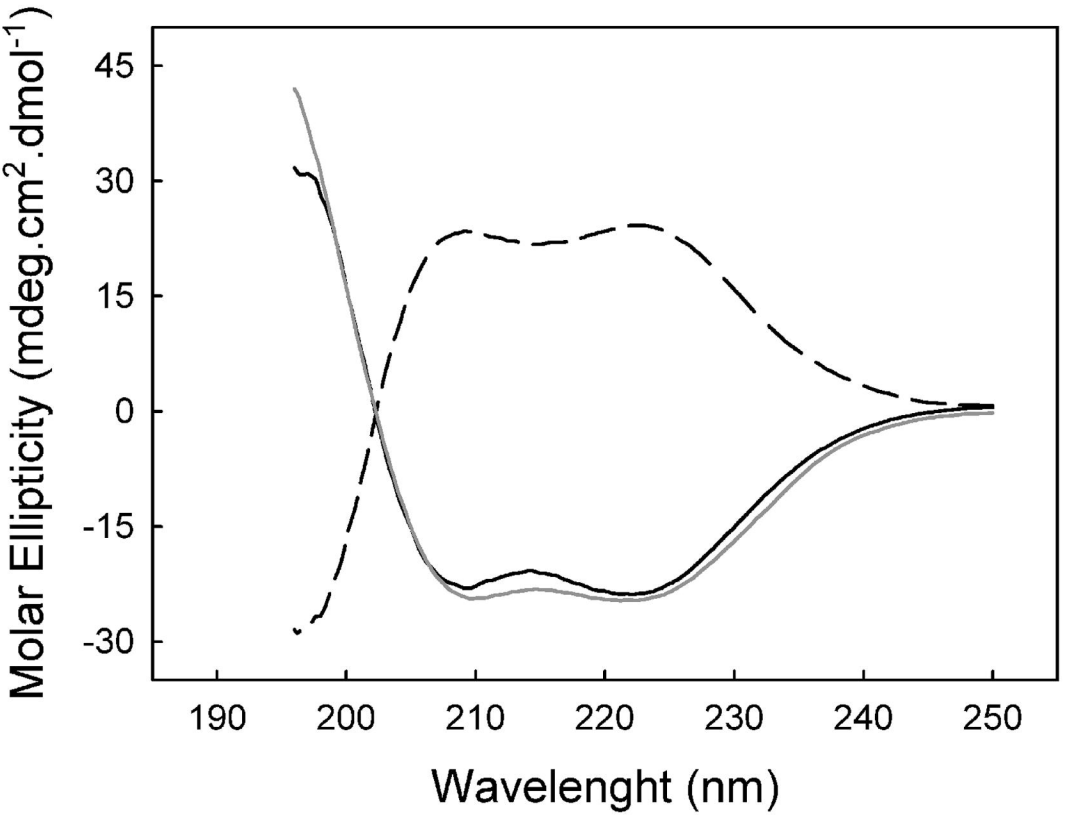
Molar Ellipticity (mdeg.cm2.dmol-1)
45
30
15
0
-15
-30
190
200
210
220
230
240
250
Wavelenght (nm)

Figure 1

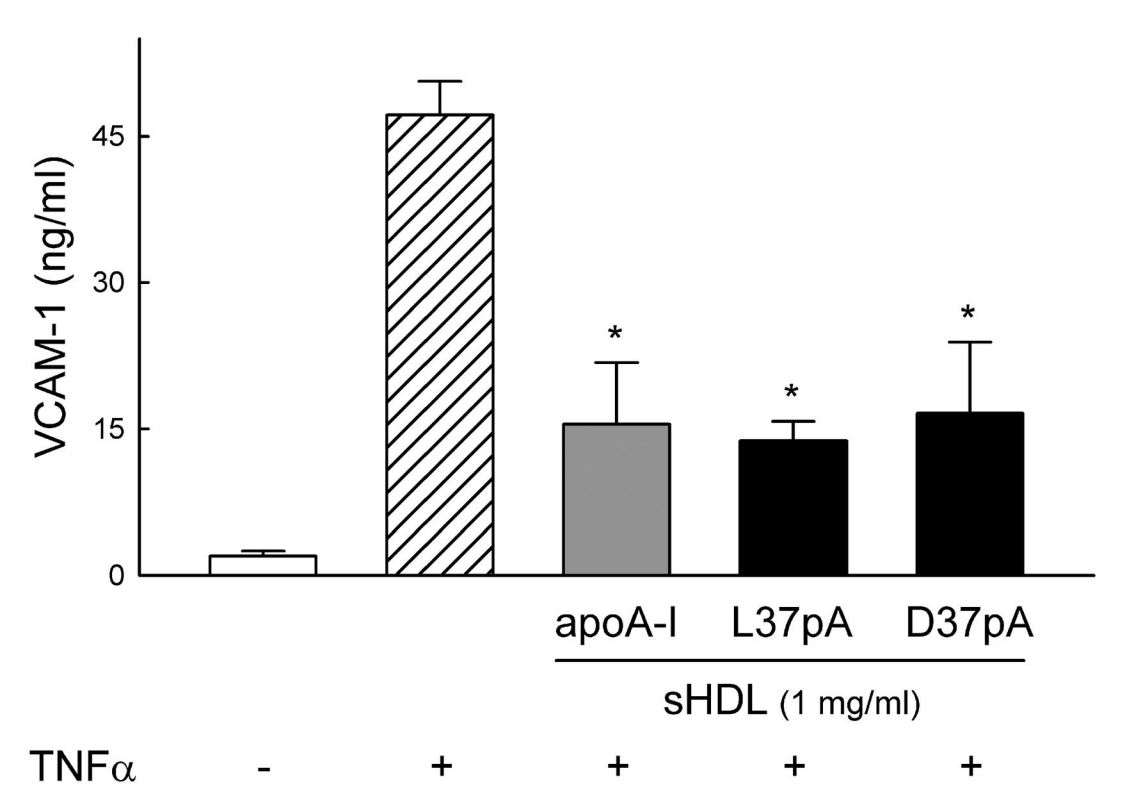

Figure 2

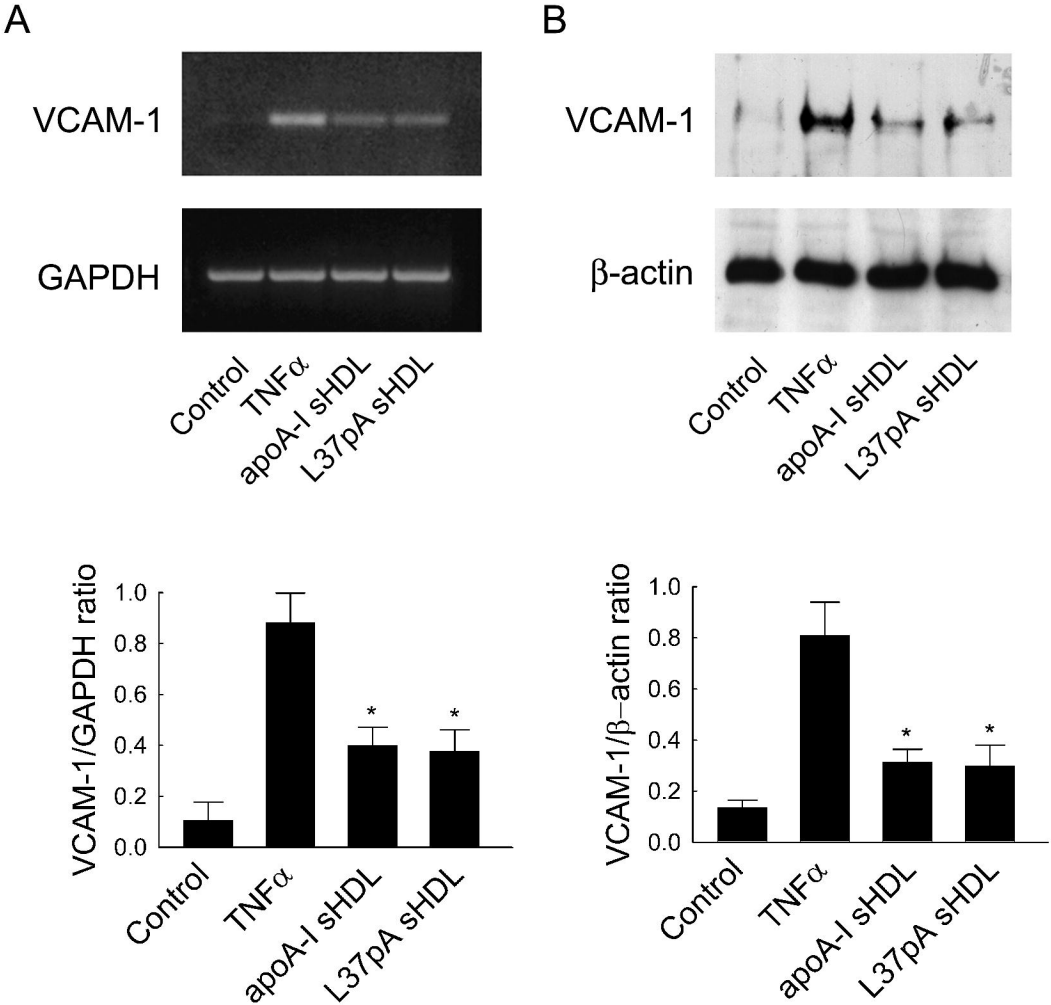
A
VCAM-1
GAPDH
Control
TNFα
apoA-I sHDL
L37pA sHDL
B
VCAM-1
β-actin
Control
TNFα
apoA-I sHDL
L37pA sHDL
VCAM-1/GAPDH ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
TNFα
apoA-I sHDL
L37pA sHDL
VCAM-1/β–actin ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
TNFα
apoA-I sHDL
L37pA sHDL

Figure 3

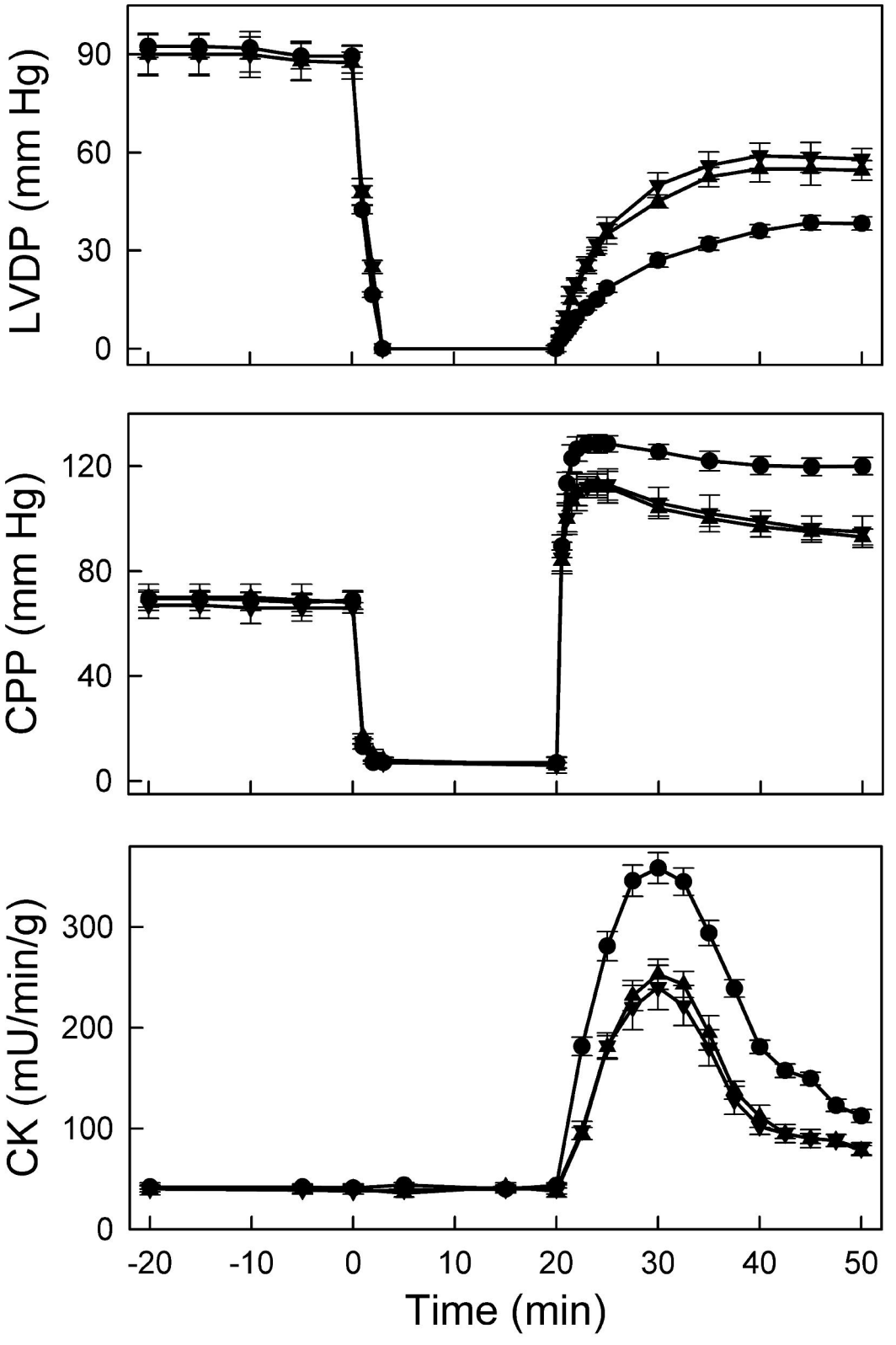
LVDP (mm Hg)
90
60
30
0
CPP (mm Hg)
120
80
40
0
CK (mU/min/g)
300
200
100
0
-20
-10
0
10
20
30
40
50
Time (min)

Figure 4

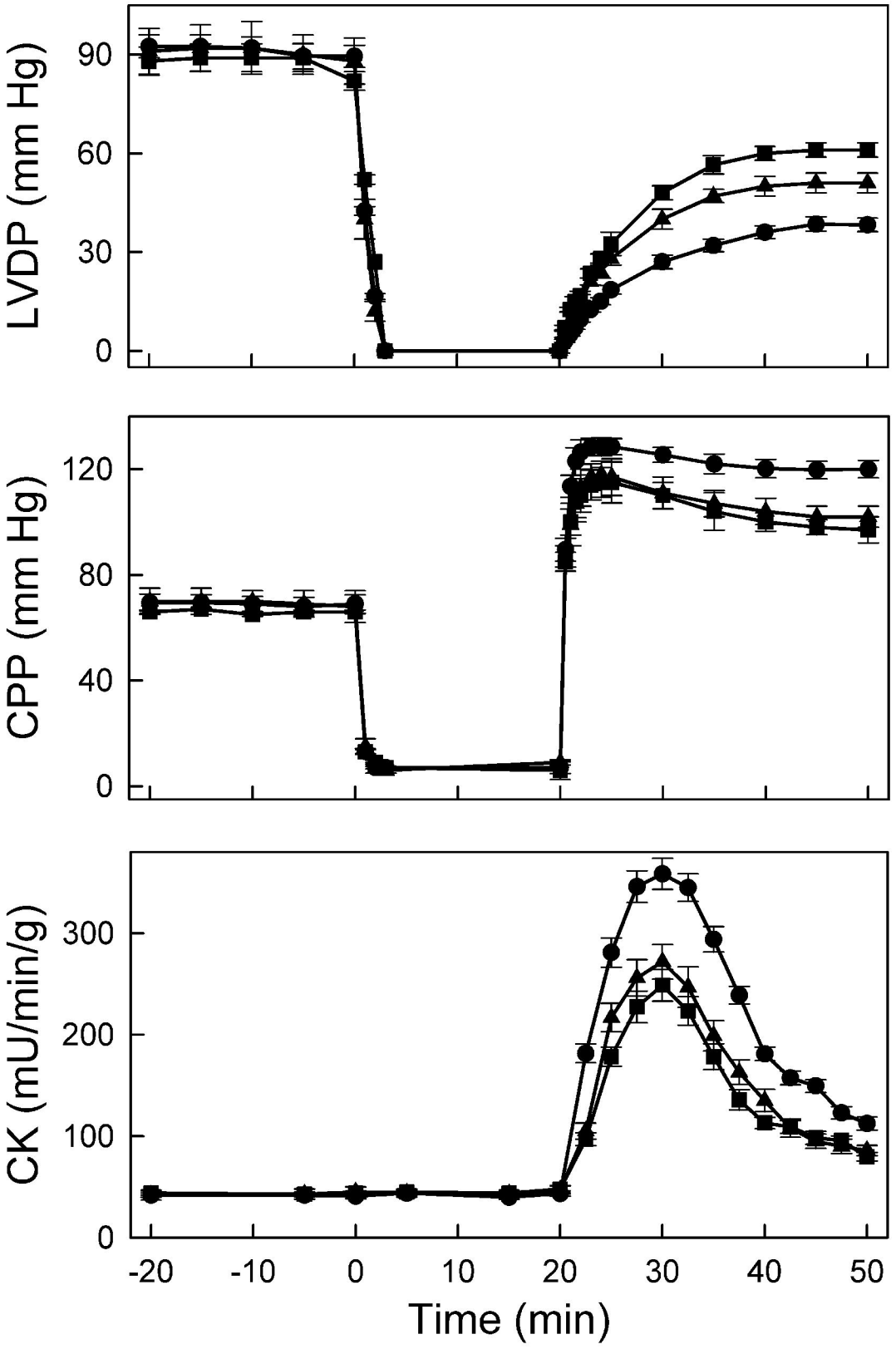
LVDP (mm Hg)
90
60
30
0
CPP (mm Hg)
120
80
40
0
CK (mU/min/g)
300
200
100
0
-20
-10
0
10
20
30
40
50
Time (min)

Figure 5