2009

ROLE OF HEPATIC CHOLESTEROL ESTER HYDROLASE (CEH) IN HYDROLYZING CHOLESTEROL ESTERS (CE) DELIVERED VIA SR-BI (SCAVENGER RECEPTOR CLASS B TYPE I) AND SR-BII

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A Thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

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Richmond, Virginia
May, 2009

Acknowledgements

Being new to the world of research I did not know what to expect or what ways to conduct my research. I was hesitant at what lay ahead of me, and was afraid if I was even conducting my research properly. Luckily I have been fortunate enough to be surrounded by amazing, brilliant and very helpful people. By no means could I have accomplished my goals without their influence.

I would like to express my greatest gratitude to Dr. Shobha Ghosh. She was very supportive and patient in helping me to achieve my goals throughout this past year, and in helping me to think critically and logically; which is not only required as a successful researcher in science, but also a successful person in life. I would forever be indebted to her for her love, support, care and patience with me throughout my journey.

I would also like to thank Dr. Siddhartha Ghosh for spending countless days in helping me figure out instruments. His jovial nature always kept me upbeat when things were not going my way and his constant assurance gave me confidence that I needed. I would like to also thank Bin Zhao, Jingmei Song and Jinghua Bei for always being there to answer my questions and helping me understand many experiments.

Dr. Philip B Hylemon and Dr. Srinivasa M Karnam took time from their busy schedule to sit through my meeting and also to partake as my committee members, and for that I would like to thank them wholeheartedly. Lastly I would like to thank my family and friends for patiently dealing with me through the many tribulations that I have gone throughout this year and supporting me in all my endeavors.

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By Saurabh Ajay Bajpai, B.S.

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Virginia Commonwealth University, 2009

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Reduction of cholesterol ester (CE) from lipid burden lesion-associated macrophage foam cells has been shown to reduce plaque volumes. Hydrolysis of CE to free cholesterol (FC) in macrophages is an essential step for removal of CE from the macrophage and its transport to the liver by high density lipoprotein (HDL) for further metabolism. Since CE must again be hydrolyzed into FC in the liver catalyzing this hydrolysis, it becomes imperative to find the identity of these enzymes. In this study the role of key enzyme in catalyzing the hydrolysis of CE to FC, neutral cholesterol ester hydrolase (CEH) was

evaluated. Further, ability of this CEH to hydrolyze CE delivered via scavenger receptor BI (SR-BI) or SR-BII was also monitored. CE hydrolysis and FC efflux were monitored from cells transfected with CEH expression vector. No significant difference was noted in either the intracellular CEH activity or FC efflux between cells transfected with an empty vector or a CEH expression vector. Further no difference was seen when experiments were repeated with cells stably transfected with SR-BI or SR-BII. Future experiments with more optimization of the cells system used will be required to reach any conclusions on the role of CEH in hydrolyzing HDL-CE delivered via SR-BI/BII.

INTRODUCTION

The name Cholesterol comes from the Greek *chole-* (bile) and *stereos* (solid), and the chemical suffix –ol for an alcohol, because Francois Poulletier de la Salle first identified cholesterol in solid from gallstones in 1769, but is was named as the compound "cholesterine" in 1815 by Eugene Chevreul (1). Cholesterol is a soft, waxy alcohol that is composed of a head group which is a hydroxyl group at C-3 and nonpolar hydrocarbon body, which is the steroid nucleus and the hydrocarbon side chain at C-17. Fatty acids esterified to cholesterol can reach as long as 16-carbon. Cholesterol synthesis in vertebrates usually takes place in the liver and it's the principal sterol synthesized by animals, but small quantities may be produced by other eukaryotes; such as fungi (2). A small fraction of the cholesterol is incorporated in the membrane of the hepatocytes, but most of it usually is exported as biliary cholesterol, bile acids, or cholesteryl esters in the form of lipoproteins. Polar derivatives of cholesterol are bile acids that act as detergents in the intestine, acting as emulsifying agents; so dietary fats become more readily accessible to digestive lipases. Bile salts help in the absorption of fat soluble vitamins such as Vitamin A, D, E and K in the intestine. All growing cells need cholesterol for new membrane synthesis, where it contributes to the level of rigidity and permeability of the membrane. The hydroxyl group on cholesterol interacts with the polar head groups of

the membrane phospholipids and sphingolipids, while the hydrocarbon chain and bulky steroid are embedded in the membrane; alongside the nonpolar fatty acid chain of other lipids, this allows the permeability of the plasma membrane to hydrogen and sodium ions. Apart from serving as membrane constituents, cholesterol can also be used in steroid hormone synthesis such as cortisol and aldosterone; which are potent hormones that regulate gene expression. Cholesterol also functions as a precursor to sex hormones such as progesterone, estrogens and testosterone. Cholesterol also functions in intracellular transport, cell signaling and nerve conduction. The myelin sheath, which are rich in cholesterol provide insulation for more efficient conduction of action potential within neurons. Cholesterol and cholesteryl esters, just like triacylglycerols and phospholipids, are hydrophobic. However they have to be moved from tissue of origin to the tissues in which they will be stored or utilized. Cholesterol is carried through the blood with a combination of phospholipids, cholesterol, cholesteryl esters, and triacylglycerol and plasma lipoproteins. Apolipoprotein combines with lipids to form a complex with a hydrophobic center, which stores the lipids and a hydrophilic outer surface made of amino acid side chains to facilitate its movement through the aqueous environment. There are many types of lipoproteins and each type has a specific function, which can be determined by its origin of synthesis, reaction of specific antibodies and its contents.

WHOLE BODY CHOLESTEROL HOMEOSTASIS

Organisms obtain their cholesterol need from three sources: fats such as a complex mixture of triglycerides, cholesterol, sterols, monoglycerides, diglycerides and phospholipids consumed in diet, Cholesterol esters stored in cells as lipid droplets, and cholesterol synthesized and packaged into lipoprotein in an organ to export to another. Different species will range from using only one method to utilizing all three for their cholesterol needs. Plants for example utilize the fats such as triglyceride stored in seeds during germination; otherwise they do not depend on triglycerides for energy. Vertebrates obtain fat from their diet, such as meat, poultry, fish, egg, butter, cheese and whole milk, while producing cholesterol from the liver and storing cholesterol esters within cells. Food from plant based sources like fruits, vegetables and cereals do not contain fats, except for nuts and seed. Foods with saturated fats also cause the body to make more cholesterol. Vertebrates also utilize fats stored in tissues (adipose) and, the liver converts excess carbohydrates to fats for export to other tissues. In vertebrates, before the ingested triacylglycerols are absorbed through the intestinal wall, they have to be converted from macroscopic insoluble fat particles to finely dispersed microscopic micelles. This solubilization of ingested triacylglycerols is carried via bile salts, which are synthesized from the cholesterol in the liver. Bile salts are amphiphatic compounds which act as detergents, helping in the breakdown of triacylglycerols into mixed micelles of bile salts, cholesterol and fatty acids. Bile salt formation starts when bile acids are produced in the liver and secreted in the intestine via the gall bladder, where they are stored. Bile acids are oxidation products of cholesterol; they are conjugated with taurine or the amino acid

glycine. Hydrophobic/toxic bile acids are conjugated with sulfate or a glucuronide. Taurocholic acid and glycoholic acid (derivatives of cholic acid) represent approximately forty percent of all bile acids in humans. Once the micelle is formed via the assist of bile acids, it exponentially increases the fraction of lipid molecules, which will be accessible to the action of hydrophilic lipases in the intestine. The products of the lipases diffuse across the epithelial lining the intestinal surface, where they will be reconverted back into triacylglycerols and be incorporated with cholesterol and apolipoproteins, into chylomicrons. Chylomicrons which contain apolipoprotein C-II (apoC-II) move from the intestinal surface (intestinal mucosa) into the lymphatic system, and then enter the blood, from there they are carried to the muscles or tissues where their contents are utilized. In the capillaries of the tissue the extracellular enzyme lipoprotein lipase, which is activated by apoC-II hydrolyzes the triacylglycerols into fatty acids and glycerol, which are taken up by the cells in the target tissues. The chylomicrons which are now mostly depleted of their triacylglycerols still contain cholesterol and apolipoproteins. Chylomicron reminants are taken up via the liver through endocytosis, mediated by receptors for their apolipoproteins. When the diet contains more fatty acid then is needed for immediate fuel for metabolic regulation in the organism or as precursor for membranes or signaling molecules, the liver converts them to triacylglycerols, which are packaged into very low density lipoprotein (VLDL); the liver synthesizes triacylglycerols as well. Excess carbohydrates in the body can also be converted to triacylglycerols and exported via VLDLs. VLDLs also contain cholesterol and cholesteryl esters, with various lipoproteins such as apoC-II and apoB, apoB being the main apo-protein. As the VLDL moves

through the blood stream, apoC-II activates extracellular lipoprotein lipase, which causes the release of free fatty acids from VLDL. The loss of triacylglycerol converts VLDL to VLDL remnant, also known as low-density lipoprotein (LDL). LDLs are very rich in cholesterol, cholesteryl esters and apoB-100 lipoprotein. As LDLs move through the blood stream, tissues that have specific plasma membrane receptors that recognize apoB-100 take up the cholesterol and cholesterol esters. Free cholesterol (FC) is utilized in the tissues and the tissues store excess FC as cholesterol ester (CE), which is esterified by acyl-CoA: cholesterol acyltransferase 2 (ACAT2) in the liver and intestine, and ACAT-1 in all other tissues. Once the tissues have fulfilled their needs for the cholesterol and cholesterol esters, they down regulate the expression of LDL receptor and LDL is then taken up via the liver through the LDL receptor and it re-enters hepatic lipid metabolism (3). The process of LDL returning back to the liver is known as the classic cycle. When in excess the LDL becomes modified and cannot be taken up by tissues, it is then taken up via arterial wall-associated macrophages due to their expression of scavenger receptors (SR-A or CD-36) (4). The uptake of modified LDL leads to formation of foam cells, accumulation of which in the lumen leads to coronary artery disease such as atherosclerosis (5). Removal of CE from foam cells is possible through a process known as reverse cholesterol transport (RCT).

IMPORTANCE OF LIVER

Once the modified LDL is taken up via macrophages, the LDL-CE are hydrolyzed in the lysosomes and the resulting FC is transferred out of the lysosome; the excess FC is re-

esterified by ACAT-1; this is known as the futile cycle and it helps maintain proper FC concentration (6). One of the ways to retrieve the cholesterol ester (CE) from within the macrophage and the only way to get rid of it through the body is done via a process known as “Reverse Cholesterol Transport”, which plays a role in even delaying or reversing early stages of atherosclerosis; in which the liver plays a major role. The liver is the central organ in cholesterol manufacturing and processing, and its inherent ability to dispose of excess cholesterol through production of biliary cholesterol and bile acid, which help in digestion of fats and can also be excreted through the feces. The liver manufactures Apolipoprotien A-I (ApoA-I) which is necessary for the in vivo formation of HDL (7). The premise of HDL and Apo-AI being protective against development of aortic lesions coronary disease has been supported by expressing human Apo-AI in transgenic mice (8). Also the decrease of atherosclerosis is shown in apo-AI transgenic mice (9). Increased plasma levels of HDL and Apo-AI have also been shown to be inversely proportional to the risk of developing coronary heart disease (10,11). ApoA-I protein in turn accepts the FC from the foam cells, which is mediated by the ATP-binding cassette protein A1 (ABCA1) and ABCG1/ABCG4. ABCA1 and ABCG1/ABCG4 mediates the transfer of cellular FC, which is CE hydrolyzed by neutral cholesterol ester hydrolase (CEH) within the cell and phospholipids to Apo-AI, to form HDL (12, 13). The FC is esterified to CE by plasma lecithin cholesterol acyltransferase (LCAT) and then transferred inside the core of HDL. The HDL moves through the blood stream and is taken up via the liver through Scavenger receptor BI (SR-BI) or minor form SR-BII (14). The CE then is hydrolyzed via neutral CEH into FC. Conversions of FC to bile acids and

direct secretion of FC into bile are the two major mechanisms for cholesterol elimination from the body. In humans there is a 50:50 ratio, of conversion of FC to bile acid and direct secretion into bile (15). Since there is only a small proportion (5-20%) of biliary acid which is actually made de novo (16, 17), most of the cholesterol has to be supplied by hepatic uptake of lipoprotein (18). The main lipoprotein that provides cholesterol for the liver is HDL which is taken up via SR-BI/BII receptor. Goodman and Lequire showed that after the uptake of HDL-CE there is a significant amount of tracer associated with FC, thus proving involvement of an efficient hydrolysis (19). Shimada et al. showed that this hydrolysis is extra lysosomal and catalyzed by a neutral CEH (20). Thus, neutral CEH plays an important role in CE hydrolysis in the first and last step of RCT.

CHOLESTROL ESTER HYDROLASE

Reverse cholesterol transport is the primary mechanism for removal of cholesterol from foam cells and the rate limiting step is the intracellular hydrolysis of CE to FC by neutral CEH (21, 22). In the liver, CEH is also responsible for hydrolyzing CE to FC for use in biliary cholesterol secretion into bile. CEH thus plays a major role in the first and last step of RCT. Compared to ACAT which differs from macrophage form (ACAT-1) (23) and hepatic form (ACAT-2) (24), and is coded by different genes (25). Human liver and macrophage CEH are highly homologous and coded by a single gene on human chromosome 16 (26). Initially CEH was not regarded as the enzyme responsible for hydrolysis of CE in macrophage and hepatic cells, even with observed inverse correlation of CEH expression to atherosclerosis in animal species (27,28,29). Many thought that

cholesterol ester hydrolase in murine macrophages to be similar to hormone sensitive lipase (HSL), which are present in adipose and steroidogenic tissues (30, 31, 32). The idea of HSL being the cholesterol ester hydrolase were proven to be moot with the finding, that overexpression of HSL in transgenic mice macrophages showed increased atherosclerosis (33). Dr. Ghosh has identified and reported the cloning of human macrophage CEH cDNA (34). Over expressing this neutral CEH has been shown to decrease the amount of CE within macrophages (34), while RNA silencing of neutral CEH has shown a decrease in CE hydrolysis and overexpression has shown inhibition of CE levels in macrophages (35). All evidence point towards the likely candidate for CE hydrolysis in macrophage and hepatic cell to be neutral CEH. Even though the liver still synthesizes a small amount of cholesterol de novo, it must still take up CE from HDL-CE for majority of the CE it hydrolyzes. The primary source of CE is from HDL-CE via SR-BI/BII, which provides the liver with the CE necessary for hydrolysis into FC; which is used for bile acids synthesis and direct secretion into bile.

SCAVENGER RECEPTOR- BI/BII

Reverse cholesterol transport plays a key role in extracting CE from foam cells and inserting them in the core of the HDL to be hydrolyzed within the liver and converted to bile acids, or directly secreted into the bile. This whole process requires scavenger receptor BI/BII (SR-BI/BII). SR-BI/BII are members of the scavenger receptor super family of proteins, they bind to a variety of ligands and seem to have a high affinity for HDL; showing selective lipid uptakes by tissues (36, 37). Many forms of lipids can be

taken up via these receptors, but highest uptake constants have been for CE and FC, while low constant rates have been observed for phospholipids and triglycerides (38). SR-BI/BII are splice variant of the same gene and are bonafide HDL receptors. SR-BI is mostly localized at the surface of cells, while SR-BII is expressed intracellularly. SR-BI mediates uptake of HDL at the cell membrane level, while SR-BII mediates it through endocytosis; showing that they both contain a distinct mechanism (38). Pulse chase experiment shows that SR-BII facilitates higher uptake of HDL-CE than that of SR-BI, due to it being endocytosed; while SR-BI maintained expression at the surface of cells (39). SR-BI/BII are highly expressed in the liver, adrenal gland and ovary for HDL metabolism. The SR receptors play a major role in providing the cholesterol that these tissues need for bile acids (liver) and hormones (adrenal gland). Absence of SR-BI receptors in knockout mice has shown depletion of internal store of cholesterol in the adrenal glands after the mice fasted, also showing a substantial increase of CE in wild type mice adrenal gland and lowering of CE in knockout mice by 44%; showing the importance of SR-BI receptors in accepting cholesterol through its hydrophobic core for the adrenal gland to utilize it for synthesizing hormones (40). SR-BI suppression in hyperglycemia has also been linked to accelerated atherosclerosis in diabetics (41). SR-BI expression has also been seen in small intestine, but its role remains unclear. SR-BI/BII plays a vital role in the uptake of CE from HDL for various purposes, but they also play an important role in reducing plaque volumes in atherosclerosis. SR-BI/BII's expression on the macrophages allows HDL to take up FC from the macrophage and deliver it to the liver. The liver in turn takes up the FC from the HDL-CE via SR-BI/BII

receptors. By removing CE from within the macrophages, the cell is prevented from bursting and depositing the oxidized cholesterol in the lumen; leading to fatty streaks which promotes atherosclerosis. It has been clearly shown that over expressing SR-BI/BII reduces atherosclerosis in mice and human (42, 43).

ATHEROSCLEROSIS

The name atherosclerosis generates from the Greek words athero (gruel or paste) and sclerosis (hardness). Atherosclerosis is popularly thought of as a modern disease, but evidence of fatty streaks in ancient Egyptian mummies has proven otherwise. Atherosclerosis is a syndrome that affects the arterial blood vessels when they harden due to plaque formation, also causing reduced circulation, which leads to death of progressive tissues attached to the arteries. It is a chronic inflammatory response in the wall of the arteries, which is due to the accumulation of macrophages and is promoted especially by small particle LDLs and further advanced with inadequate removal of cholesterol from macrophages by HDL. Atherogenesis starts when LDL particles become oxidized by oxygen free radicals (ROS) (44, 45, 46), due to ROS atherosclerosis usually develops in arteries where oxygen levels are high versus that of veins. Once LDL has become modified, it causes damage to arterial wall and the immune system responds by sending in monocytes to take up the modified LDL. The monocytes differentiate into macrophages, which take up the modified LDL causing them to have a foamy appearance leading to their nomenclature of “foam cells”. Once the macrophages have taken up the modified LDL they are not able to metabolize it, resulting in their growth and rupture.

Rupturing of the macrophage causes deposits of oxidized cholesterol in the artery wall, resulting in recruitment of more macrophages to continue the cycle of taking up oxidized cholesterol from previously ruptured macrophages; this continuous cycle causes the arteries to become inflamed. The ruptured macrophages with lymphocytes, platelets and localized smooth cells lead to formation of so called “fatty streaks”. Eventually smooth muscle cells migrate from the tunica media to intima; responding to the cytokines secreted by damaged endothelial cells and macrophages. Micro calcification forms within the smooth muscles that are surrounding the damaged endothelial cells. As time passes and cells die, they are filled with extracellular calcium deposits between the muscular wall and outer portion of the atheromatous plaques. The capped atheromas produce enzymes which cause the artery to enlarge, as long as the arteries are enlarged enough to compensate for the thickness of the atheroma, no stenosis occurs. Eventually over time if calcification continues and the atheroma becomes thicker than the compensated size of the artery, an aneurysm is created. Though atherosclerosis a slow progressing disease, which can span decades and remain asymptomatic; once the atheroma obstructs the bloodstream in the artery it could lead to death of the tissue to which it was supplying nutrients. In most cases even when an artery ruptures and lipid matrix breaks through the thinning collagen, causing the lipid to come into contact with the blood; causing clotting. Platelet adhesion causes the clotting cascade to form a thrombus. The thrombus has the ability to move throughout the blood stream and become lodged in areas that are narrow, causing block of blood flow. Even though any arteries of the body can be involved, only critical ones come up in terms of clinical relevance; such as in the heart. Major plaque

ruptures in major arteries around the heart could lead to complete block of the artery, leading to myocardial infarction. Many factors are known to aid in increasing the chance of acquiring atherosclerosis, some are controllable such as lipid intake, sedentary lifestyle, high carbohydrate intake, intake of trans fat and while others include advanced age, being a male gender and genetic factors. The only mechanism for plaque regression is through the removal of CE from the foam cells. CEH must hydrolyze the CE within the foam cells to FC before HDL is able to uptake the cholesterol, The FC is esterified into CE by LCAT for storage as FC is toxic to the body at higher levels. Once HDL delivers the CE to the liver, through SR-BI/BII receptor, it must again be hydrolyzed into FC by CEH before it can be utilized in formation of biliary cholesterol, bile acid or direct secretion into bile to be excreted through the body in feces. The goal of the experiment is to evaluate the role of CEH by monitoring CE hydrolysis and evaluate SR-BI/BII receptors in taking up CE by monitoring FC efflux from the cells. CHO cells expressing no ldl receptors (ldl-A7) or stably expressing SR-BI/BII receptors (ldl-A7 SR-BI/BII) will be used in the experiments. Each type of cell will be transfected with CEH expression vector or control pcmv vector. HDL-[^{3}H]-CE will be added to the medium of the cells and respectively counted as described in methods and materials. The data will be evaluated to formulate a conclusion to our goal for the experiment.

MATERIALS AND METHODS

MATERIALS

Hexanes, Acetic Acid (HPLC grade), and $CHCL_3$ were purchased from Fisher Scientific. Diethyl ether was purchased from Sigma-Aldrich. Ham's F-12 Nutrient Mixture 1 X [+] L-Glutamine, Geneticin, Penicillin/Streptomycin and 0.05% Trypsin-EDTA were purchased from Invitrogen. Effectene transfection reagent, Enhancer and EC buffer were purchased from QIAGEN. TLC plates were purchased from ANAL TECH. Recombinant Cholesterol ester transfer protein (CETP) was obtained from Cardiovascular Targets. [^{3}H]- cholesteryl oleate was purchased from PerkinElmer Life and Analytical Sciences. Scintillation Cocktail was purchased from ALDRICH. Human HDL protein was purchased from INTRACEL.

CELL LINES:

Cell lines derived from Chinese Hamster Ovary (CHO) cell line were used. CHO cells lacking LDL receptor (ldl-A7) were generated in Dr. Monty Krieger's laboratory. These cells were stably transfected with either SR-BI (ldlA7-SR-BI) or SR-BII (ldlA7-SR-BII) expression vectors in Dr. Deneys van der Westhuysen's laboratory and all these three cell lines were obtained from Dr. Westhuysen's laboratory. ldlA7 cells were cultured in a T75 (75cm^2) tissue culture flasks in Ham F-12 Nutrient Mixture supplemented with 10%

FBS + P/S at 37ºC under 5% CO_2 in a humidified incubator. When the cell culture was ~90%-100% confluent, the cells were dissociated with 0.05% Trypsin-EDTA and passaged into a T75 flask at a split ratio of 1:3. On the day of the experiment the cells were dissociated with 0.05% trypsin EDTA, counted and were plated in a 24 well plate with $2x10^5$ cells in 1ml per well. The cells were then incubated at 37ºC under 5% CO_2 in a humidified incubator for 24 hours. ldl A7 SR-BI and ldlA7-SR-BII cells were also maintained as described above except that Geneticin ™ was added at a final concentration of 250µg /1ml of media to the SR-BI/BII cells, to maintain the stable expression of SR-BI/BII in these cells.

PLASMIDS USED FOR OVER-EXPRESSION

Eukaryotic expression vector for human CEH (pCMV-CEH) developed in Dr. Ghosh's laboratory was used for all studies. Empty vector (pCMV) was used as a control for these studies.

TRANSFECTIONS

Cells were transfected in 24-well plates after 24 h incubation at 37ºC and 5% CO_2 in a humidified incubator. The optimized conditions used for transfection were: 400ng DNA in TEDA, 50µl EC buffer (6 mM Tris-HCl [pH 7.6], 1 M NaCl), 3.2µl Enhancer, 4µl Effectene and 350µl media per well. Before any experiment, the numbers of wells to be transfected were determined and amount of DNA, Enhancer, Effectene and media needed was calculated. DNA was first mixed with EC buffer, then enhancer added and the

mixture vortexed for 15 sec and incubated at room temperature for 10 min. Effectene reagent was added after 10 min incubation, the mixture was then vortexed and incubated at room temperature for an additional 10 min. Growth media was then added to above mixture. During one of the 10 min interval, old media were removed from the plates and replaced with fresh 350µl of media per well. DNA complexes (410µl) were then added to each well containing 350µl of fresh media. The cells were then incubated for 24-72 hour at 37ºC in a 5% CO_2 humidified incubator.

LABELING HDL CORE WITH [^{3}H-Cholesteryl Esters]

100µCi of [^{3}H]-Cholesteryl ester and 200 µl $CHCL_3$ was dried in a 4 ml glass scintillation vial under a stream of N_2. 4mg (200µl of 20mg/ml stock solution) HDL protein and 50µg CETP were added to the vial, final volume was brought to 1.5ml with 150mM NaCl, 10mM KPO4 (pH 7.2), in a sterile hood. A stir bar was placed into the vial and the vial placed in an incubator chamber at 37ºC and was stirred for 5 hours. After 5 hours the HDL-[^{3}H]-CE samples were loaded on Centricon 50 concentrators in the sterile hood and then centrifuged at 13,000g for 10 min to concentrate HDL to 300µl. An aliquot of 100µl of concentrated HDL was loaded on a Superose-6 FPLC column and run through FPLC using PBS. 1ml fractions were then collected and 5µl was counted from each fraction to determine radioactivity. Protein estimations were conducted on the samples by using the method of bicinchoninic acid (BCA) assay to determine the protein concentration of samples. The fractions containing the highest protein and radioactivity

were pooled and specific radioactivity determined which ranged from 3169-3732 DPMs/µg HDL-[^{3}H]-CE.

MEASUREMENT OF CELLULAR CHOLESTROL ESTER HYDROLASE ACTIVITY

Following transfection with either pCMV or pCMV-CEH, cellular cholesteryl ester hydrolysis of exogenously delivered radio labeled HDL-CE was determined as follows. 24h after transfection the medium on the cells was replaced with serum-free medium supplemented with 10% lipoprotein deficient serum (LPDS) and HDL-[^{3}H] CE (25µg/µl). ACAT inhibitor (CP-113, 1.25 µg/ml) was also added to the culture medium to prevent re-esterification of free cholesterol released following CEH mediated CE hydrolysis. After 24h the cells, the wells were rinsed once with PBS (500µl) and the plates were inverted to let dry completely. Once the plates were dry, 1ml of isopropanol was added to each well and the plates left in a humidified chamber overnight to extract the lipids. Next morning lipid extracts (in isopropanol) were collected into glass tubes and were stored at -20ºC. 100µl of 1.0 M NaOH was added to the now empty wells to allow for digestion of cell walls (3-4 hour). Protein estimation was later carried out on cell lysates using the method of bicinchoninic acid (BCA) assay. The extracted lipids were analyzed by TLC. Silica coated TLC plates were activated by heating in an oven at 110ºC for at least an hour. The lipid extracts were dried under nitrogen after adding 10µl of Standard FC & Standard CE (2mg/ml) as internal standards. Dried lipids were resuspended in small amount of $CHCl_3$ and spotted on the activated TLC plates. The plates were developed in 90:10:1::Hexane: diethyl ether: Acetic acid (v/v/v) and lipids

were then visualized by exposing to I_2 vapors. The Origin, CE and FC spots were marked and the plates were allowed to decolorize. All three spots for each sample were scraped and counted. Cellular CEH activity was calculated as percent hydrolysis (dpm in FC/dpm in FC+CE*100).

MEASUREMENT OF FC EFFLUX

The cells were transfected as described above. After 24h, the medium was replaced with serum-free medium supplemented with 10% Lipoprotein deficient serum (LPDS) and HDL-[^{3}H] CE (25µg/µl). This permitted the delivery of HDL-CE to the cells. In order to monitor the efflux of FC generated by CEH-mediated hydrolysis that reportedly occurs in close proximity to the plasma membrane, the medium was replaced after 2 h with serum free media supplemented with 10% LPDS and HDL (25µg/µl). Exogenously added unlabeled HDL act as an extra-cellular FC acceptor. The cells were then kept in the incubator chamber at 37ºC and 5% CO_2 for 24 hours. Next morning the medium was collected from the wells and centrifuged to pellet any floating cells or cellular debris. Of the supernatant 400µl was counted following the addition of 5ml of scintillation cocktail. The wells were rinsed with 500µl PBS and then 200µl of 1.0N NaOH was added to digest the cells (3-4 hours). 100µl of cell lysate was later counted from each sample to determine the cell associated radioactivity. FC efflux was calculated as percent efflux (dpm in the medium/Total dpm medium + cells*100).

RESULTS

LABELING OF HUMAN HDL WITH [^{3}H]-CHOLESTERYL OLEATE: Following labeling using CETP as described under "Methods", concentrated HDL was purified on a Superose-6 column using FPLC. As shown in Figure 1a, HDL eluted as a single peak with an elution volume of 5ml. The radioactivity associated with each fraction is shown in Figure 1b and the fractions containing the peak radioactivity and corresponding to the HDL protein peak were pooled. Protein concentration and associated radioactivity of this pooled fraction was determined to calculate specific radioactivity which 3169-3732 DPMs/µg protein. This purified HDL-[^{3}H]-CE was used for all experiments to deliver CE to the cells.

EFFECT OF CEH OVER-EXPRESSION ON THE INTRACELLULAR HYDROLYSIS OF HDL-CE DELIVERED VIA SR-BI/SR-BII:

To evaluate the role of hepatic CEH activity in hydrolyzing HDL-CE, CEH activity (expressed as percent hydrolysis) was calculated following transfection of ldl-A7, ldl-A7 SR-BI and ldl-A7 SR-BII cells as described in Methods. There was no significant difference in CEH activity between ldl-A7 cells transfected with empty vector pCMV or CEH over-expression vector pCMV-CEH (49.73 ± 9.33 vs 46.54 ± 1.34, P=0.75, Figure 2). No significant differences were noted when intracellular CEH activity was compared

in ldl-A7-SR-BI (47.09 ± 3.24 versus 46.93 ± 4.71, P= 0.96, Figure 3) or ldl-A7-SR-BII cells (40.83 ± 13.70 versus 47.45 ± 7.75, P=0.51, Figure 4). The data presented here is for n=3 wells. It should be noted that all cells express endogenous CE hydrolytic activity and over-expression by transient transfection may not have resulted in a significant increase in total cellular CEH activity. Although optimized transfection conditions were used, these experiments did not include a measure of transfection efficiency and it is likely that the results obtained reflect very low transfection efficiencies.

TO EVALUATE THE ROLE OF SR-BI OR SR-BII IN AFFECTING THE CEH-MEDIATED INTRACELLLULAR HYDROLYSIS OF HDL-CE:

To compare the relative role of SR-BI and SR-BII in delivering HDL-CE for intracellular hydrolysis by CEH, cellular CEH activity expressed as percent hydrolysis was compared between ldl-A7-SR-BI and ldl-A7-SR-BII cells. As shown in Figure 5, there was no significant difference seen in intracellular CEH activity between these two cell types (46.93 ± 4.71 versus 47.45 ± 7.75, P=0.93). However, since there was also no significant difference in CEH activity between cells transfected with empty vector and those transfected with CEH expression vector, no inference can be drawn about the relative contribution of SR-BI and SR-BII in delivering HDL-CE for intracellular hydrolysis by CEH.

EFFECT OF CEH OVEREXPRESSION ON CELLULAR CHOLESTEROL EFFLUX:

It is believed that HDL-CE are delivered via SR-BI in close proximity of the plasma membrane and are hydrolyzed at this location by intracellular CEH. If this hypothesis is true, then the FC released by CEH mediated hydrolysis should be readily available for efflux. To test this hypothesis, ldl-A7, ldl-A7-SR-BI and ldl-A7-SR-BII cells were transfected with either the empty vector pCMV or the CEH expression vector pCMV-CEH and FC efflux monitored. As shown in Figure 6, there was no significant difference in FC efflux from ldl-A7 cells transfected with pCMV or pCMV-CEH vector (71.28±11.66 versus 71.44±2.29, P=0.98). Similarly, no significant difference in FC efflux was noted between ldl-A7-SR-BI and ldl-A7-SR-BII cells transfected with pCMV-CEH (75.73 ± 6.23 versus 69.03 ± 3.67, P=0.20, Figure 7). Although these data suggest that CEH probably does not play a role in stimulating efflux of FC released from HDL-CE after intracellular hydrolysis, for reasons noted above, additional experiments are required to confirm these results and interpretations.

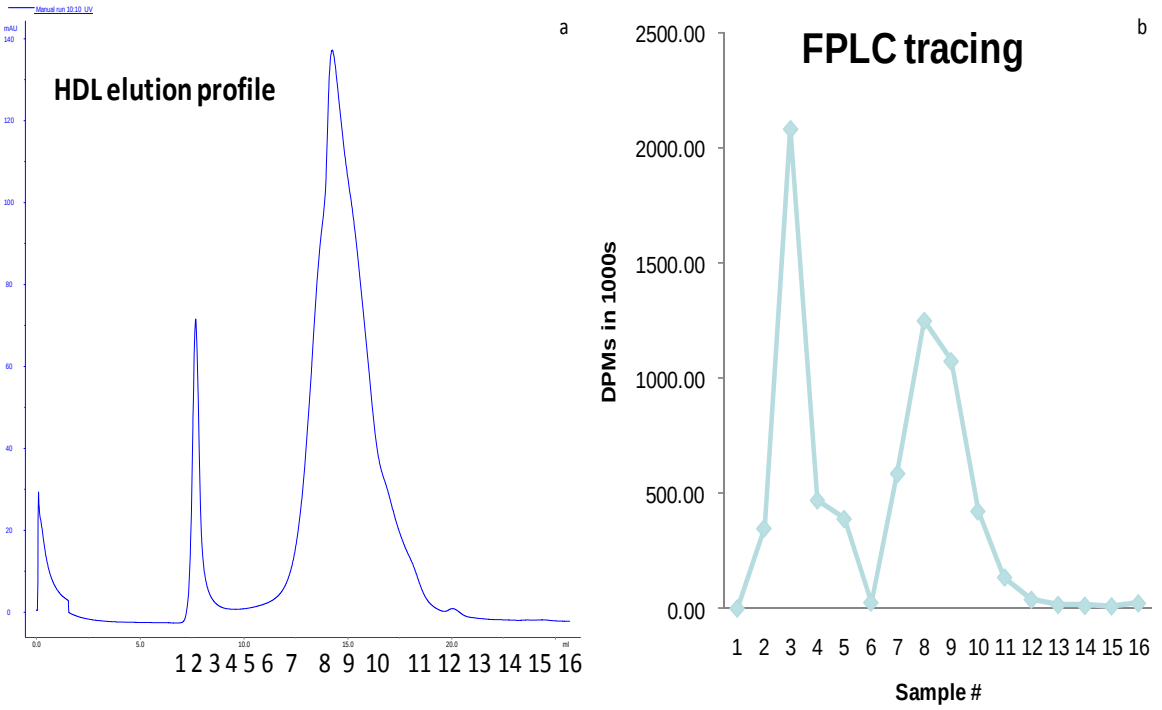

FIGURE 1: <u>a. Separation of HDL-[^{3}H]-CE with Superose -6 FPLC column.</u> Radioactive cholesterol esters were reacted with HDL in presence of CETP to generate HDL-[^{3}H]-CE. The generated HDL-[^{3}H]-CE was separated from the mixture using Superose-6 FPLC column in PBS buffer. The flow rate was 0.5ml/min and the fractions were collected at 2 min intervals. **<u>b. FPLC tracing of HDL-[^{3}H]-CE fractions.</u>** 100µl of each fraction was counted, and the total counts for each fraction are shown on the Y-axis. As shown on the X-axis HDL-[^{3}H]-CE eluted between fractions 6-12.

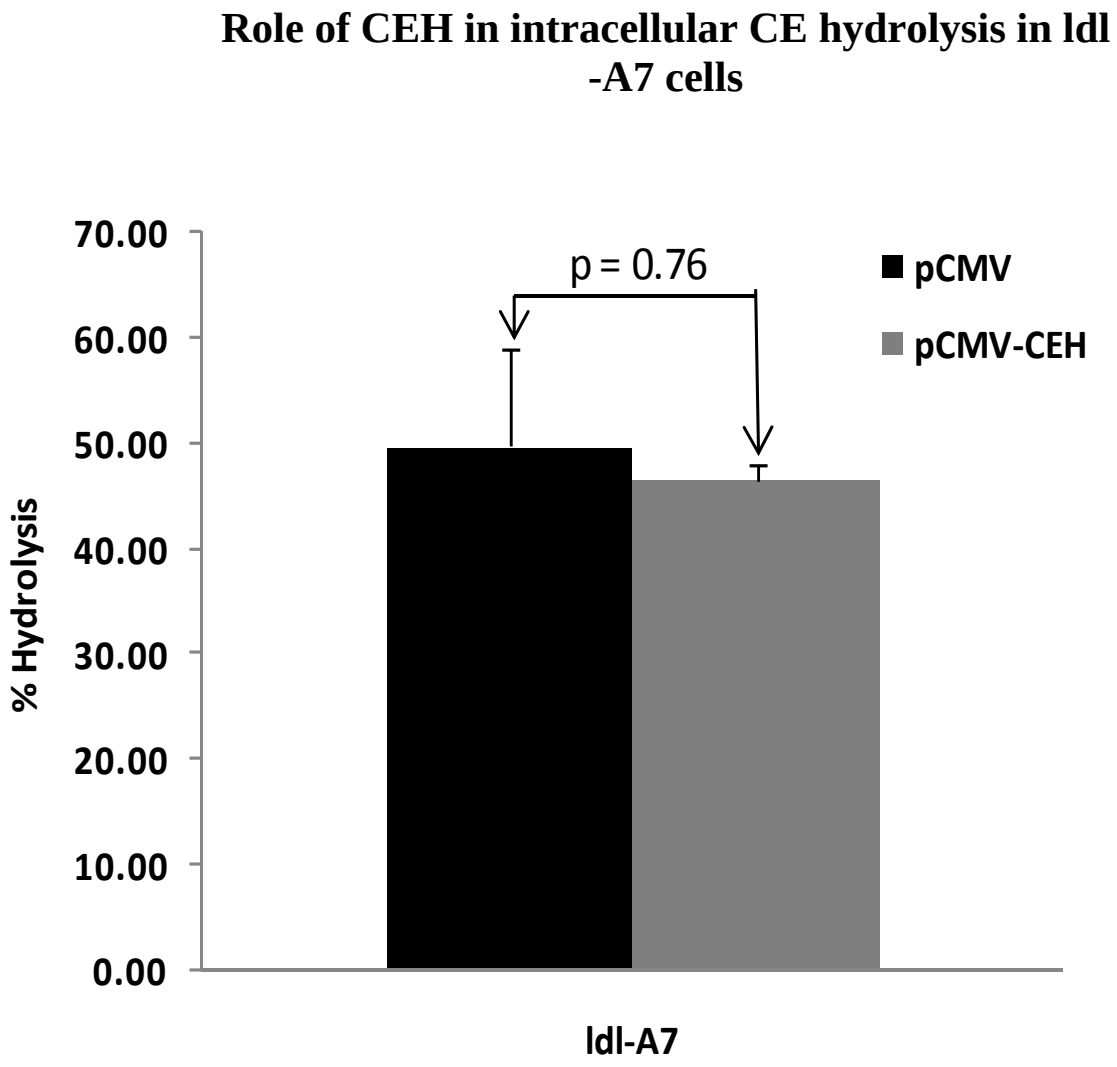

FIGURE 2: <u>Effect of CEH over expression on hydrolysis of HDL-CE in ldl-A7 cells.</u> Cells were plated and transfected as described in "Methods". Total lipids were extracted 72 hours after transfection and FC/CE separated by TLC and associated radioactivity determined. Data are expressed as % hydrolysis (Mean ± SD).

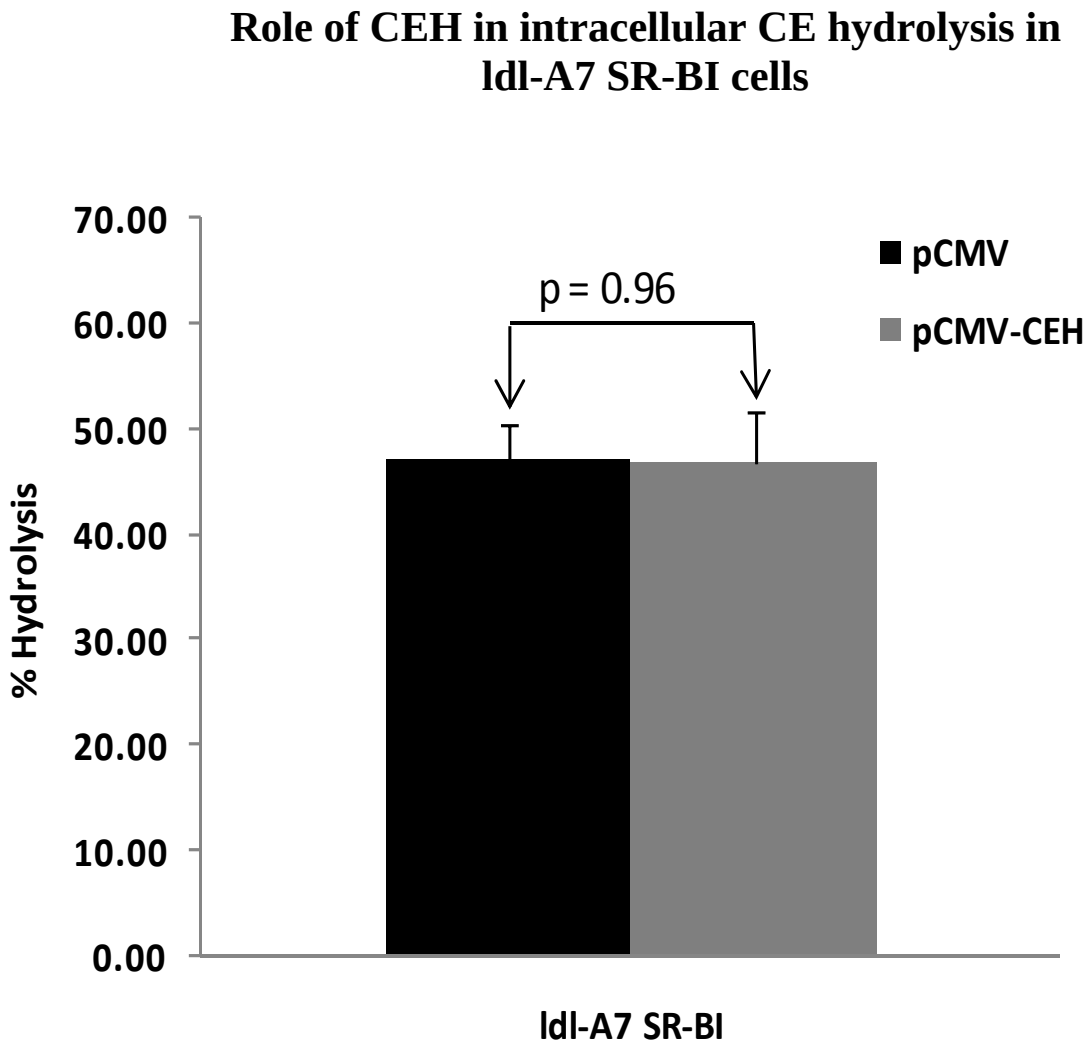

FIGURE 3: <u>Effect of CEH over expression on hydrolysis of HDL-CE in SR-BI cells</u>. Cells were plated and transfected as described in "methods". Total lipids were extracted 72 hours after transfection and FC/CE separated by TLC and associated radioactivity determined. Data are expressed as % hydrolysis (Mean ± SD).

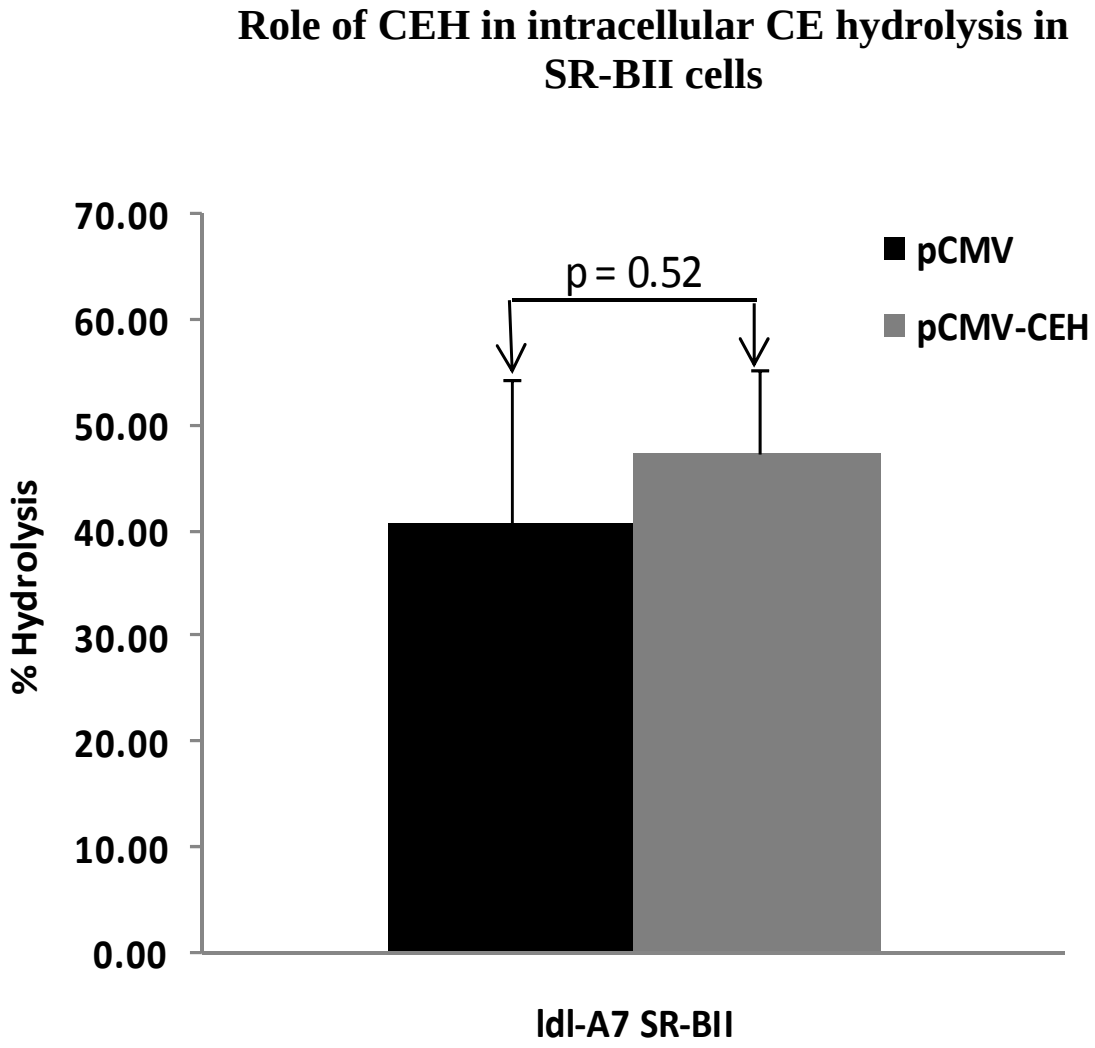

FIGURE 4: <u>Effect of CEH over expression on hydrolysis of HDL-CE in SR-BII cells.</u> Cells were plated and transfected as described in "methods". Total lipids were extracted 72 hours after transfection and FC/CE separated by TLC and associated radioactivity determined. Data are expressed as % hydrolysis (Mean ± SD).

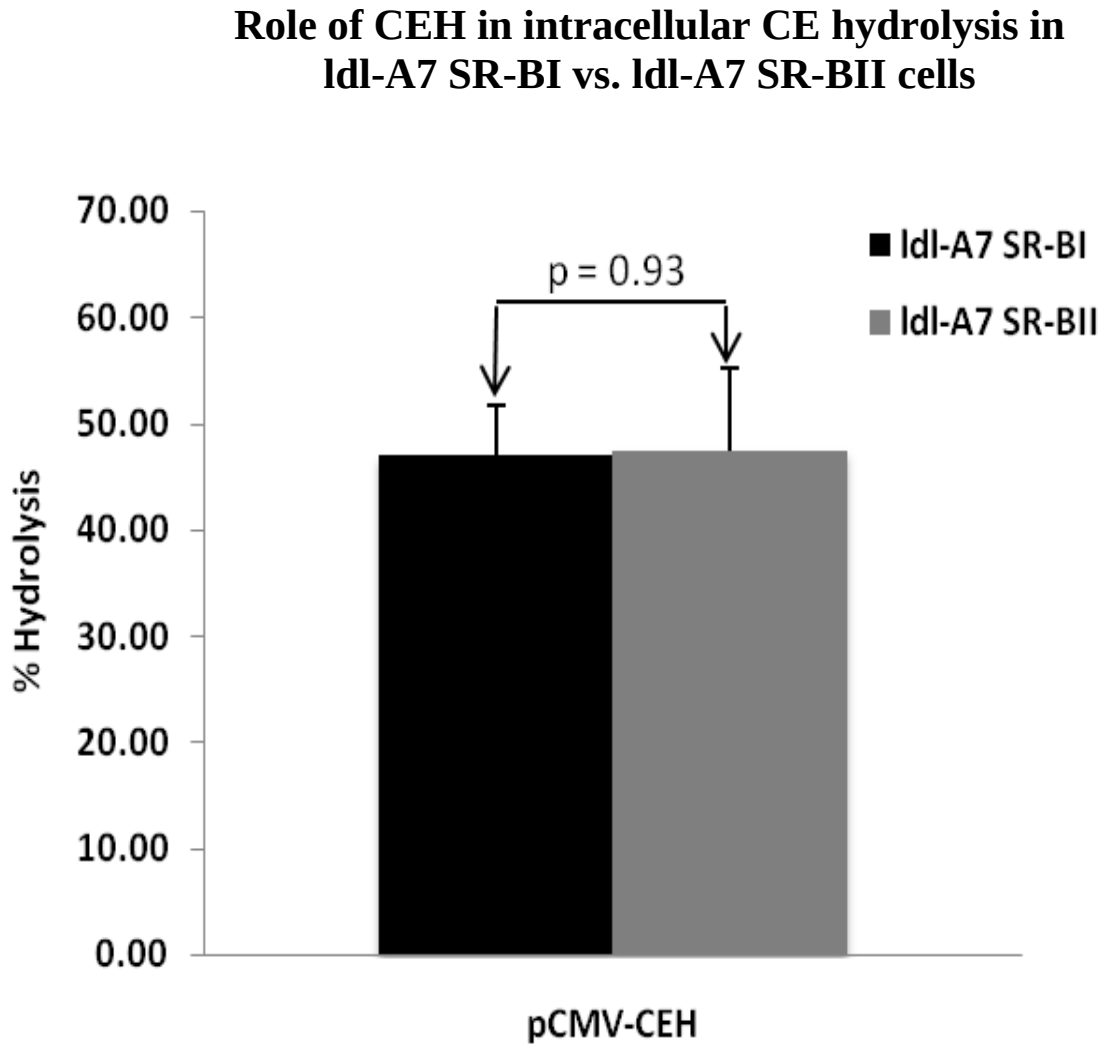

FIGURE 5: <u>Comparison of CEH mediated HDL-CE hydrolysis: SR-BI vs. SR-BII dependent uptake of HDL-CE.</u> Cells were plated and transfected as described in "methods". Total lipids were extracted 72 hours after transfection and FC/CE separated by TLC and associated radioactivity determined. Data are expressed as % hydrolysis (Mean ± SD).

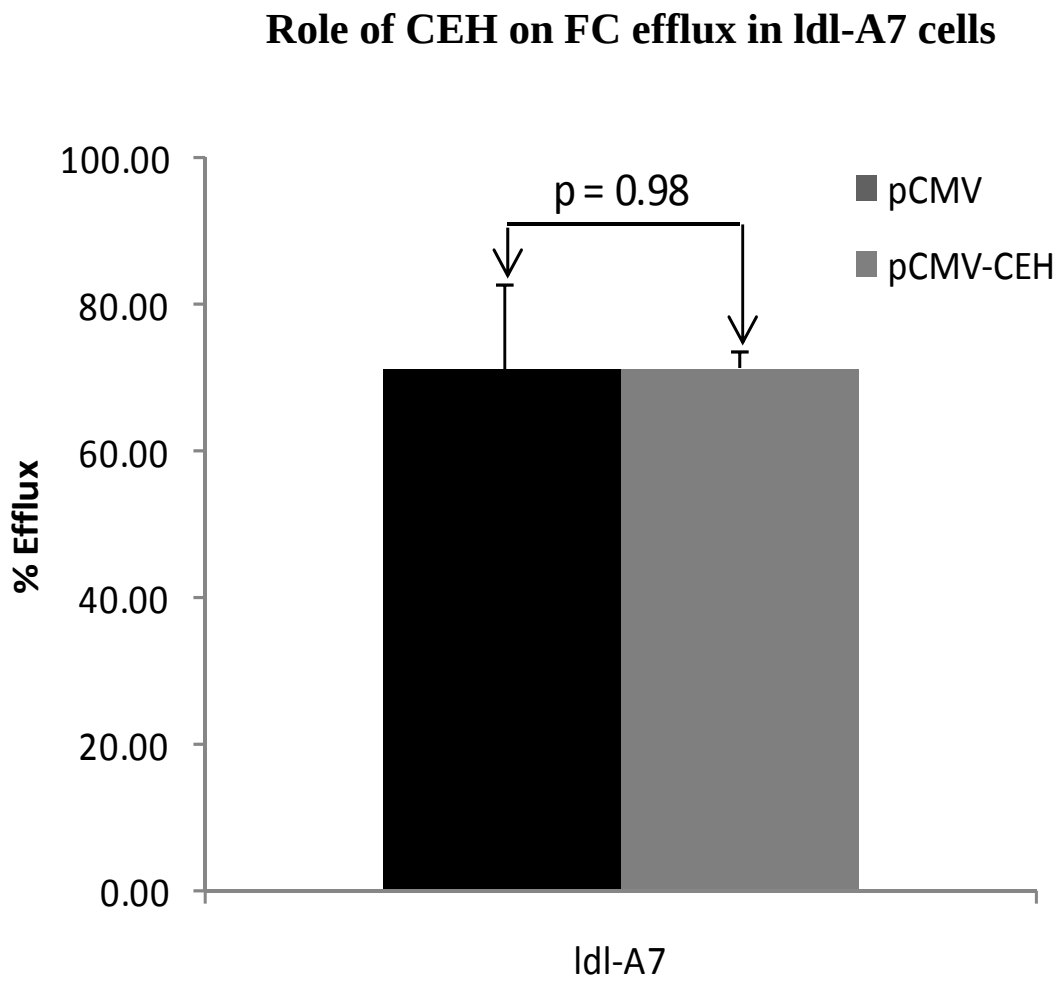

FIGURE 6: **Effect of CEH over expression on FC efflux generated by intracellular hydrolysis of HDL-CE in ldl-A7 cells**. Cells were plated, transfected and lysed as described in "methods". Cell lysis and media were counted 48 hours after transfection and associated radioactivity determined. Data are expressed as % efflux (Mean ± SD).

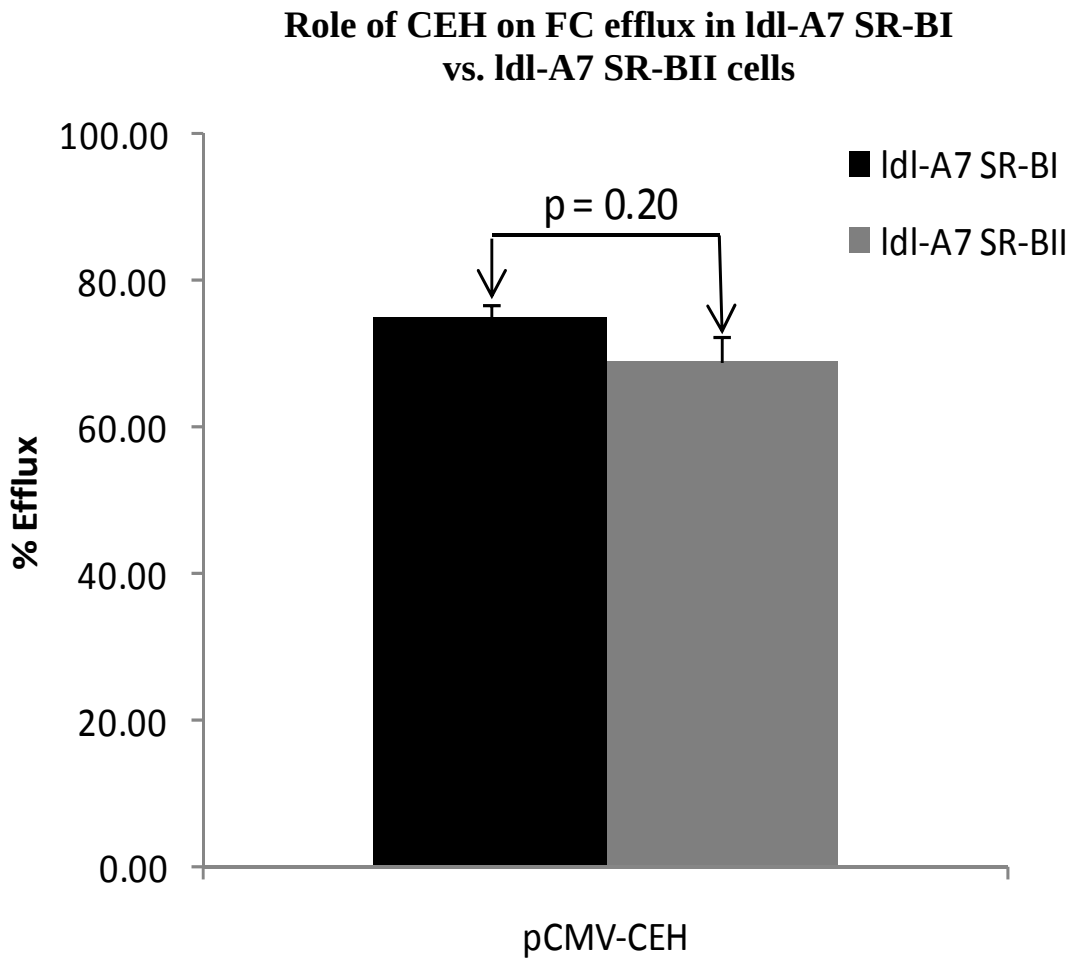

FIGURE 7: **Effect of CEH over expression on FC efflux generated by intracellular hydrolysis of HDL-CE between ldl-A7 SR-BI vs. ldl-A7 SR-BII cells.** Cells were plated, transfected and lysed as described in "methods". Cell lysis and media were counted 48 hours after transfection and associated radioactivity determined. Data are expressed as % efflux (Mean ± SD).

DISCUSSION

In order to examine the role of CEH in hydrolyzing HDL-CE delivered via SR-BI or SR-BII, we monitored the effects of CEH over expression in hydrolyzing CE delivered from the core of HDL in ldl-A7 cells (CHO cell derivative) over-expressing either SR-BI or SR-BII. Initially when intracellular CE hydrolysis was compared between ldl-A7 cells transfected with an empty pCMV vector or a CEH expression vector (pCMV-CEH), no significant difference in CE hydrolysis was noted. Since this CEH expression vector has been shown to enhance intracellular CE hydrolysis in earlier experiments (34), it is inferred that in the present study other factors contributed to the observed lack of increase in CEH activity in cells over-expressing CEH. One of the most significant factors contributing to these results could be inefficient transfection. Since no other measure of transfection efficiency was used, it is not possible to exclude the possibility that the data simply reflects poor transfection efficiency. Future studies should include measurement of mRNA to establish successful transfection.

The same cell culture system and transfection protocol was, however, used to examine the role of CEH in hydrolyzing SR-BI/SR-BII delivered HDL-CE. No significant difference was noted between intracellular CE hydrolysis in control cells transfected with pCMV and cells transfected with pCMV-CEH. While one reason could

be poor transfection efficiencies as described above, it should be pointed out that all cells possess significantly high CE hydrolytic activity and detection of an increase above and beyond the endogenous levels may be a limitation. Other investigators namely Drs. David Williams and Margery Connelly had observed similar issues and had unsuccessfully attempted to optimize a cell culture system with low levels of CE hydrolytic activity suitable for these studies (Personal communication to Dr. Ghosh). Initial experiments in Dr. Ghosh's laboratory had successfully demonstrated small but reproducible increases in CEH activity using this cell culture system and those data formed the basis for using this system for the current studies. However, the experiments performed during the period of this project were not successful. Future studies may require further optimization of the cell culture system or may be even developing a new cell line based on HEK293 cells that possess much lower CE hydrolytic activity.

It has been demonstrated that HDL-CE delivered via SR-BI are readily hydrolyzed presumably in close proximity of the plasma membrane (20). This led us to speculate that if CEH catalyzes the hydrolysis of HDL-CE, then the FC released by such hydrolysis should be readily available for efflux. However, in the present study no significant difference in FC efflux was noted between cells transfected with pCMV or pCMV-CEH. Once again, in the absence of any direct measure of transfection efficiencies, these results cannot rule out the possibility that lack of successful transfection is probably the reason. While this interpretation is favored since earlier work in Dr. Ghosh's laboratory did show a small but reproducible difference in FC efflux in

similar experiments, it is also possible that this CEH does not play a role in the hydrolysis of HDL-CE delivered via SR-BI.

The identity of the enzyme responsible for the hydrolysis of SR-BI delivered CE is not known (47). A number of enzymes have the capability to hydrolyze CE. In adrenal glands where SR-BI delivered HDL-CE is the major source of cholesterol for steroid hormone synthesis, hormone sensitive lipase is thought to be the enzyme that catalyzes this reaction (48). Liver does not express hormone sensitive lipase suggesting that some other CE hydrolase is responsible for this hydrolysis. The CEH cloned and characterized in Dr. Ghosh's laboratory is one of these enzymes. This CEH belongs to a carboxylesterase family, several members of which are highly expressed in liver. The subcellular localization of these enzymes is controversial with reports suggesting it to be present in the cytoplasm as well as endoplasmic reticulum (49). If SR-BI delivered CEs are delivered near the plasma membrane or are associated with cytoplasmic lipid droplets, then it is more likely that an enzyme present in the cytoplasm will be more likely to be involved in the hydrolysis of these CE. Further, since HDL-CE delivered via SR-BI are through a selective uptake pathway, only the CE enters the cell. This precludes the involvement of acid or lysosomal CEH that is involved in the hydrolysis of LDL associated CE (3). HDL-CE delivered via SR-BII are thought to be predominantly via endocytosis (39) and selective uptake of CE by this receptor is considered inefficient, it is possible that some of HDL-CE may be hydrolyzed in the lysosomal compartment. While the aim of this study was to establish the role of CEH characterized in Dr. Ghosh's laboratory in hydrolyzing HDL-CE delivered to the liver via SR-BI/BII, it is equally

important to establish the identity of the enzyme that is responsible for this hydrolysis in order to better understand the complete process of reverse cholesterol transport. This knowledge is central to understanding the steps that may rate limit or regulate the final elimination of cholesterol from the body.

Another aim of this study was also to determine whether CEH differentially regulates the hydrolysis of HDL-CE delivered via SR-BI or SR-BII. SR-BII is an alternative mRNA splicing variant of SR-BI. SR-BI which is mainly (~70%) localized at the surface of transfected CHO cells, the majority of SR-BII (~80-90%) was expressed intracellularly (39). Experiments showed that SR-BII rapidly internalized HDL protein, where as for SR-BI most HDL protein remained surface bound. Despite the fact that SR-BII mediates cell-association of HDL at 37ºC in similar amounts as compared to SR-BI, the selective uptake capacity of SR-BII is less than SR-BI (39). Our present data was inconclusive and future studies are required to establish these differences.

In conclusion, the data presented here could not establish the role of CEH in hydrolyzing HDL-CE delivered via SR-BI or SR-BII. Several experimental limitations accounted for the lack of conclusive data and future studies need to be designed giving due consideration to these limitations. Further, additional studies should be included to verify transfections to permit interpretations.

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