Investigating the kinetics of apolipoprotein A-I exchange between its high-density-lipoprotein-bound and lipid-free states

Emma Ka[1], Dr. Mark Borja[2]
[1]Amador Valley High School, Pleasanton, California
[2]California State University East Bay, Department of Chemistry and Biochemistry, Hayward, California

Previous research has shown that risk for cardiovascular disease (CVD) is inversely associated with high-density lipoprotein (HDL) functionality. HDL possesses certain cardioprotective properties enabling it to promote the efflux of cholesterol, which then prevents cholesterol buildup on the artery walls. Apolipoprotein A-I (apoA-I) is the major protein component of HDL. However, apoA-I's transition between its lipid-free and HDL-bound states, a property indicative of HDL functionality, remains poorly understood. This study set out to characterize this transition through kinetic analysis of HDL-apolipoprotein-A-I-exchange (HAE) assays using reconstituted HDL (rHDL). Our findings support our hypothesis that smaller rHDL particle size and use of an apoA-I genetic variant, apoA-I milano, produce greater rates of exchange and thus enhanced HDL functionality.

I. Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide, stemming from the build-up of cholesterol in the inner artery walls [1]. High-density lipoprotein (HDL), commonly known as the "good cholesterol," has been associated with lower CVD risk, as it circulates in the blood and plays a significant role in removing excess cholesterol from the body through reverse cholesterol transport (RCT) [1]. In this pathway, HDL is first synthesized by the intestine and liver before entering the bloodstream and circulating to arteries in the peripheral tissues [2]. There, HDL interacts with the receptors of various cell types, including macrophages, to bind to cholesterol and carry it to the liver, where it will be distributed to other tissues or removed from the body [3]. However, merely raising HDL quantity with treatments like niacin has proven to be ineffective in reducing CVD risk [4]. In comparison, HDL functionality, the rate at which it performs cholesterol efflux, is a more reliable metric of cardiovascular health, and can be measured with HDL-apolipoprotein-A-I-exchange (HAE) assays. These assays quantify the ability of HDL's principal protein, apolipoprotein A-I (apoA-I), to exchange HDL [3]. The dynamic nature of apoA-I stems from its amphipathic α-helices, giving the molecules the flexibility to adapt to the surface of HDL particles in a double-belt conformation [5]. This binding and unbinding mechanism of HAE is a vital step in RCT, as it enables the formation of both lipid-free (off HDL) and lipid-bound (on HDL) apoA-I. Lipid-free apoA-I is the preferred substrate of the transporter ATP-binding cassette A1 (ABCA1) mediating cholesterol efflux [6]. Under the conditions of CVD, apoA-I exchange rate is significantly reduced, suggesting that kinetic analysis of HAE could provide relevant insight into HDL functionality in removing cholesterol and serves as a biomarker of CVD risk to consider [1]. Despite its importance, only 5-10% of apoA-I in human plasma is in its lipid-free form, and the mechanisms of the conformational change between its HDL-bound and lipid-free states remain poorly understood [5].

The present research set out to analyze the kinetics of apoA-I exchange using fluorescent-labeled apoA-I variants and recombinant HDL. The lack of cysteine residues on wild type apoA-I allows us to attach molecular probes such as fluorophores and spin-labels through substitutions at amino acid sites [6] — in this case, a cysteine near the N-terminus (V19C). Since HAE can occur in a range of sizes for HDL particles (from 7-13 nm in diameter), we used two different sizes, 9.6 nm and 17 nm, to test the hypothesis that greater size impairs apoA-I exchange and in turn, HDL functionality. To further examine the relationship between the structure and function of apoA-I, we investigated the effects of genetic variants on the rate of exchange, using the naturally occurring mutant apoA-I milano (R173C). Despite its association with low HDL levels typically indicative of high CVD risk (27.0 ± 1.4 mg/dL when it should be over 40 mg/dL), individuals carrying apoA-I milano do not display an increase in risk or symptoms [7]. This condition may be due to apoA-I milano's structure — the replacement of arginine at the 173rd position with a cysteine residue causes a loss of positive charge and the formation of disulfide bonds, which in turn may reduce the milano's ability to interact with lipid particle surfaces in its N-terminal domain [8]. This confers a propensity toward the formation of lipid-free apoA-I. In fact, studies have found that synthetic apoA-I milano (ETC-216) administered intravenously in patients with acute coronary syndromes produced significant regression in atherosclerosis, or the buildup of cholesterol in the arteries [9]. Consequently, this study aims to investigate apoA-I milano alongside wild type (wt) apoA-I in hopes of uncovering enhanced cardioprotective properties and as such, potential use as a coronary treatment. This study also aims to show that the mutant apoA-I milano would result in increased rates of apoA-I exchange.

II. Methods

The procedures used for expression & purification of apoA-I and preparation of rHDL are described in respective studies conducted by Ryan [10] and Cavigiolio *et al* [11].

The fluorophore Alexa 488 was coupled to V19C on apoA-I, as binding and sensing of lipids occurs at the opposite C-terminus. Coupling was performed on a Hi-Trap nickel chelating column with a protein:fluorophore ratio of

1:10. Disulfide dimers were reduced prior to coupling, with exposure to 1 mM TCEP at room temperature for 15 minutes. The fluorescent label was then dissolved in 1.4 mL of 20 mM phosphate, pH 7.4, 300 mM NaCl, 3 M guanidine, and this solution was passed through the column and allowed to incubate at room temperature for 2 hours. In order to minimize the flourophore's exposure to light, the procedure was conducted in a darkened fume hood, and the column was wrapped in aluminum foil. Elution was performed using Fast Protein Liquid Chromatography (FPLC) by passing 20 mM phosphate, pH 7.4, 150 mM NaCl, 500 mM imidazole, through the column. Fractions containing Alexa 488-labeled apoA-I were dialyzed in 2 L of 20 mM phosphate, pH 7.4, 150 mM NaCl buffer overnight at 4˚C. Protein concentration was determined by bicinchoninic acid assay (BCA) and stored at 3 mg/mL for future use.

RHDL was mixed with 20 mM phosphate, pH 7.4, 150 mM NaCl, to achieve a concentration of 0.20 mg/mL for the HDL-apoA-I exchange assay. Then, the reaction mixture was placed in a 37 ˚C heat block, and exchange was initiated by addition of Alexa 488 apoA-I to a final concentration of 0.75 mg/mL. Samples were collected at time intervals of 1, 2, 3, 6, 9, and 12 minutes and immediately placed on ice to halt the exchange process. Each tube was mixed with one volume of native sample loading dye and loaded onto a 4-20% Tris-glycine native gel. After running for 12 hours at 50 V, the gel was imaged and analyzed using ChemiDoc (Bio-Rad). Fluorescence intensity was measured by densitometry, comparing the relative percentages of fluorescent signal in the lipid-free and HDL-bound regions of the gel, thus quantifying the kinetics of exchange.

III. Results

Gel electrophoresis was used to visualize the kinetics of apoA-I exchange between its two forms, with each band representing a time interval when a sample was taken and progression of the reaction. From the fluorescent gel image of each assay, a green band of growing fluorescence intensity for HDL (top region) was observed and a band of gradual disappearance for apoA-I (bottom region) with each increasing time interval, confirming the proposition that lipid-free, HDL-unbound apoA-I can efficiently displace HDL-associated apoA-I (Fig. 4).

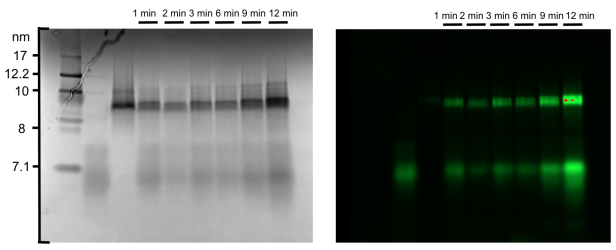

Figure 1. Fluorescent gel (left) and coomassie dye stained gel (right) showing HAE activity at time intervals from 1 min to 12 min for apoA-I wt 9.6 nm rHDL.

To investigate the effect of rHDL particle size and apoA-I genetic mutant on apoA-I exchange, we compared the kinetic rate (K), which represents the percent of apoA-I molecules binding to HDL particles per minute and is a direct measurement of HDL-apoA-I exchange (HAE). The data was fitted to a one-phase association exponential binding model. For particle size, kinetic analysis of apoA-I wt (Fig. 2A) revealed that the rate constant K of 9.6 nm rHDL (K = 0.5217 min^{-1}) was almost three-fold that of 17 nm rHDL (K = 0.1946 min^{-1}). This supports the hypothesis that smaller particle size corresponds to greater exchange rate and in turn, increased HDL functionality. Extending the study to apoA-I milano as well (Fig. 2B), similarly it is observed that there is a higher rate constant in the smaller size of 9.6 nm (K = 1.526 min^{-1}) compared to the larger size of 12.2 nm (K = 0.8175 min^{-1}). It is also noteworthy that apoA-I milano exhibits a three-fold higher exchange rate than apoA-I wt when particle size is kept constant at 9.6 nm (1.526 min^{-1} versus 0.5217 min^{-1} respectively), indicating the presence of cardioprotective features expected in the mutant (Fig. 2C).

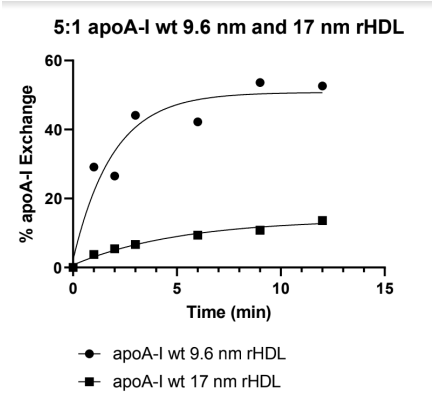

Figure 2A. Comparison of exchange for 9.6 nm and 17 nm rHDL apoA-I wt. Percentage of exchange represents fluorescence in HDL-bound region.

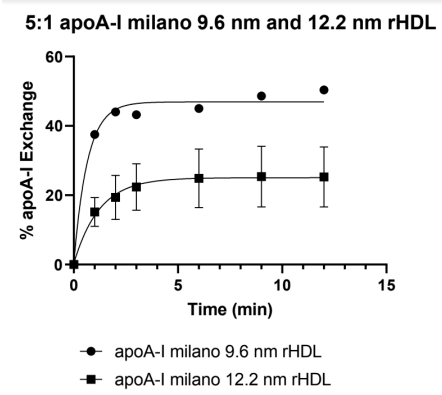

Figure 2B. Comparison of exchange for 9.6 nm rHDL apoA-I milano and

12.2 nm rHDL apoA-I milano. 95% Confidence Interval (CI) error bars for the 12.2 nm.

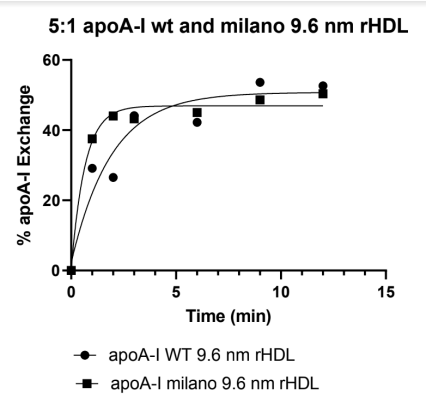

Figure 2C. Comparison of exchange for apoA-I wt and apoA-I milano using identical particle size, 9.6 nm rHDL.

IV. Discussion

Through HAE assays, we investigated the effect of rHDL particle size and genetic variant on the rate of apoA-I exchange. Kinetic analysis revealed an inverse relationship between particle size and rate constant K for both apoA-I wt and milano, which is consistent with other studies demonstrating that rHDL discs with a diameter less than 8 nm were better mediators of cell cholesterol export compared to larger discs [7]. This effect of particle size could be accounted for by the corresponding conformational changes of apoA-I. When associated with smaller rHDL particles, apoA-I experiences an increase in packing density. This results in approximately 20% more residues to form loops that serve as access channels for the incorporation of cholesterol into the lipid bilayer [11]. This in turn increases the functionality of HDL, as it is able to bind to more cholesterol molecules and transport them out of the body.

Among 9.6 nm rHDL, apoA-I milano had a threefold higher exchange rate than apoA-I wt. Additionally, we observed the same size-based trend with the larger 12.2 nm apoA-I milano rHDL particle, where apoA-I exchange rates were approximately twofold slower than the 9.6 nm particle. The higher exchange rate observed in apoA-I milano may explain previous observations of increased cholesterol efflux by apoA-I milano HDL, and may shed light as to why it is thought to exhibit enhanced cardioprotective properties. The R173C milano mutation, which replaces an arginine with a cysteine at the 173rd amino acid position, is located in the N-terminus of apoA-I. As such, the presence of a cysteine residue may affect the ability of apoA-I to interact with the lipid particle surface [12]. Previous research indicates that decreased stability of the N-terminus helix bundle in R173C apoA-I variants increases the rates of phospholipid interaction [12], which could potentially explain how the mutation results in higher cholesterol efflux through a higher affinity for binding. Overall, for both particle size and genetic variant, cholesterol efflux appeared to be dependent on the capacity of apoA-I to incorporate and retain large amounts of cholesterol.

However, limitations exist. Analysis was performed using synthesized rHDL particles rather than human HDL, which may not fully replicate HDL's natural behavior due to differences in shape and composition. For example, rHDL particles are disc-shaped whereas HDL from plasma is mostly spherical; rHDL is also controlled to contain only one kind of lipid (POPC) and cholesterol while human HDL contains a variety of lipids and proteins. Future studies may investigate whether the inverse trend between HDL particle size and apoA-I exchange rate holds true for human HDL subclasses. Additionally, this study was unable to conduct parallel cholesterol efflux studies for the HAE trials, which measure HDL functionality through their mobilization of cholesterol from macrophages. Although previous studies demonstrate the strong correlation between HAE and cholesterol efflux capacity [13], it is still important to confirm before we can extend our observed differences in exchange rate to inferred differences in HDL functionality and in turn, CVD risk.

This study provides insight into the effect of rHDL particle size and apoA-I genetic variant on the kinetic rate of apoA-I exchange. A greater understanding of these interactions could direct future drug design efforts to enhance the protective properties of HDL in order to treat cardiovascular disease — particularly, through increasing the concentration of small HDL particles or introducing apoA-I milano *in vivo*. Our findings also open further investigation to the milano mutation specifically, as it was found to increase exchange rate. However, the mechanism by which apoA-I milano prevents atherosclerosis remains unclear, and many studies disagree on whether it creates more efficient HDL, increases ABCA1 binding affinity, or promotes efflux in other ways [14]. Therefore, further research could be conducted to assess apoA-I milano's properties over a wider range of structures, including its monomeric form which is conformationally similar to the wildtype, as well as different particle sizes (17 nm) and shapes (discoidal, spherical, etc).

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References

[1] Borja M., et al. (2013). HDL-apoA-I Exchange: Rapid Detection and Association with Atherosclerosis. *PLoS One, vol. 8*.
[2] Marques, L., et al. (2018). Reverse Cholesterol Transport: Molecular Mechanisms and the Non-medical Approach to Enhance HDL Cholesterol. *Frontiers in physiology, vol. 9,* pp. 526.
[3] Cavigiolio G., Geier E., Shao B., Oda. M. (2010). Exchange of Apolipoprotein A-I between Lipid-associated and Lipid-free States. *Journal of Biological Chemistry, vol. 285*, pp. 18847–18857.
[4] Niacin in Patients with Low HDL Cholesterol Levels Receiving Intensive Statin Therapy. *New England Journal of Medicine*. (2011).
[5] Handa D., et al. (2015). Kinetic and Thermodynamic Analyses of

Spontaneous Exchange. *Biochemistry, vol. 54*, pp. 1123–1131.
[6] Oda, M.N., et al. (2011). Cysteine substitutions in apolipoprotein A-I primary structure modulate paraoxonase activity. *Biochemistry, vol. 40*, pp. 1710-1780.
[7] Phillips M. (2013). New insights into determination of HDL structure by apolipoproteins. *Journal of Lipid Research, vol. 54*, pp. 2034-2048.
[8] Calabresi, L., et al. (1999). Cell cholesterol efflux to reconstituted high-density lipoproteins containing the apolipoprotein A-I Milano dimer. *Biochemistry, vol. 38,* no. 49, pp. 16307-16314.
[9] Nissen S., et al. (2003). Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *JAMA, vol. 290,* no.17, pp. 2292-2300.
[10] Ryan R., Forte T., Oda M. (2002). Optimized bacterial expression of human apolipoprotein A-I. *Science Direct, vol. 27*, pp. 98–103.
[11] Cavigiolio G., et al. (2008). The Interplay between Size, Morphology, Stability, and Functionality of High-Density Lipoprotein Subclasses. *Biochemistry, vol. 47*, pp. 4770-4779.
[12] Alexander E., et al. (2009). Structural and functional consequences of the Milano mutation. *Journal of lipid research, vol. 50*, pp. 1409-1419.
[13] Borja M., et al. (2015). HDL-apolipoprotein A-I exchange is independently associated with cholesterol efflux capacity. *Journal of lipid research, vol. 56,* no. 10, pp. 2002-2009.
[14] Weibel G., et al. (2007). Wild-Type ApoA-I and the Milano Variant Have Similar Abilities to Stimulate Cellular Lipid Mobilization and Efflux. *Arteriosclerosis, Thrombosis, and Vascular Biology, vol. 27*, no. 9, pp. 2022-2029.