SUPPLEMENTAL MATERIAL FOR THE MANUSCRIPT:

Structure-function relationships of apolipoprotein A-I mimetic peptides: implications for anti-atherogenic activities of high density lipoprotein

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Methods:

Peptide synthesis

All peptides were synthesized by a solid-phase procedure, using a Fmoc /DIC /HOBt protocol on a Biosearch 9600 peptide synthesizer and were purified to greater than 99% purity by reverse-phase HPLC on an Aquapore RP-300 column, as assessed by MALDI-TOF-MS (Bruker UltraFlex). Retention time of peptides was determined after injection of 1 mg of purified peptide on a C-18 reverse phase HPLC column and elution with a 25-85% gradient of acetonitrile, containing 0.1% TFA. The mean hydrophobic moment was calculated as the vectorial sum of all the hydrophobicity indices, divided by the number of residues using DNA Star software.

Peptides were reconstituted with palmitoyloleoyl phosphatidyl choline (POPC) or dimyrisoylphosphatidyl choline (DMPC), at a molar ratio of 1:7. Peptides and POPC or DMPC were complexed by co-lyophilization after first being dissolved in glacial acetic acid. The resultant lyophilized cakes were reconstituted with 20 mM $NaHCO_3$, 0.15 M NaCl and heated to 50°C in a water bath for 3 min and then allowed to cool at room temperature for 3 min for a total of three cycles.

Lipoproteins

LDL (1.006-1.063 g/ml) and HDL (1.083-1.21 g/ml) were isolated from human plasma (pooled plasma supplied by Red Cross) by sequential centrifugation. Apolipoprotein A-I was isolated from HDL as described previously [1].

Cholesterol efflux from THP-1 cells

THP-1 cells were maintained in RPMI medium supplemented with 10% FBS. Cells were differentiated into macrophage-like cells by incubation in RPMI supplemented with 10% FBS and 100 nMol/L phorbol 12-myristate 13-acetate (PMA) for 72 h. Cellular cholesterol was labeled by incubation in serum-containing medium with [1α,2α(n)-^{3}H]-cholesterol (GE Health-Amersham, final radioactivity 0.5 MBq/ml) for 48 h in a CO_2 incubator. Cells were then washed and incubated for 18 h at 37^{o}C in serum-free medium in the presence or absence of the Liver X Receptor (LXR) agonist TO-901317 (4 μmol/L). Cells were washed and incubated for another 4 h at 37^{o}C in serum-free medium containing indicated concentrations of the peptides or lipid-free apoA-I. The medium was collected, centrifuged for 15 min at 4^{o}C at 10,000 x g and aliquots of supernatant were counted in a β-counter. Cells were harvested and cell-associated radioactivity was counted. Cholesterol efflux was expressed as the proportion of [^{3}H]cholesterol transferred from cells to medium. Non-specific efflux (i.e. the efflux in the absence of an acceptor) was subtracted. Where indicated, cells were fixed by incubation for 20 min with paraformaldehyde (4%) prior to the efflux experiments. All experiments were done in quadruplicates; intra-assay variability was < 5%. Efflux to each peptide was assessed in 2-3 independent experiments. Inter-assay variability was assessed by including apoA-I and peptide 5A in all experiments;

this variability was up to 30% therefore the results from different experiments were combined after normalization to the efflux to apoA-I and 5A.

Cholesterol efflux from BHK cells

BHK cells stably transfected with human ABCA1 under the mifepristone-inducible promoter were a kind gift of Dr. J. Oram and were handled as described [2]. Cellular cholesterol was labeled by incubation with [^{3}H]-cholesterol for 48 h in a CO_2 incubator. Cells were then washed and incubated for 18 h at 37^{o}C in serum-free medium and then for another 24 h at 37^{o}C in serum-free medium containing 20 μMol/ml (or approximately 90 μg/ml) of the peptides. The medium was collected, centrifuged for 15 min at 4^{o}C at 10,000 x g and aliquots of supernatant were counted in a β-counter. Cells were harvested and cell-associated radioactivity was counted. Cholesterol efflux was expressed as the proportion of [^{3}H]cholesterol transferred from cells to medium. Non-specific efflux (i.e. the efflux in the absence of an acceptor) was subtracted. All peptides were tested in the one experiment done in quadruplicates and repeated twice, inter-assay variability was <10%.

Expression of CD11b on human monocytes

Resting human monocytes were isolated from blood of healthy volunteers by density centrifugation with Lymphoprep followed by Dynal Negative Monocyte Isolation kit as described previously [3]. The CD11b assay was described previously [4]. In brief, monocytes were stimulated with 1μmol/L phorbol-12 myristate 13-acetate (PMA) (Sigma, Australia) in the presence or absence of the peptides or apoA-I final (concentration 40 μg/mL) and incubated with the FITC conjugated Ab to the active epitope of CD11b (eBiosciences, USA, Clone CBRM1/5) for 15min at 37°C. Cells were then fixed with 4% paraformaldehyde. Samples were controlled for by using the isotype matched negative control (FITC-anti-mouse IgG, Serotec, USA, Clone W3/25). CD11b expression was measured by flow cytometry using FACS Calibur (Becton Dickinson). Analysis was conducted using the Cell Quest Pro software. Results were expressed as percentage of the CD11b expression compared to cells stimulated with PMA in the presence of a vehicle. Due to considerable inter-assay variability (mainly due to various levels of activation of monocytes from different donors) each peptide was tested with monocytes from at least three different donors and results were expressed relative to CD11b expression after stimulation with PMA.

Expression of VCAM-1 in mouse endothelial cells

SVEC4-10 cells, a mouse endothelial cell line [5], was stably transfected with the Pgl3 plasmid, containing the cDNA for firefly luciferase, with 2.27 kBp of the proximal promoter of the human VCAM-1 gene [6]. Cells were co-transfected with PsvNeo and selected with 100 μg/mL of G418.

Transfected SVEC4 cells were seeded into 96 well plates at the final density of $0.25x10^5$ cells per well. After 24 h cells were washed and apoA-I, HDL or apoA-I mimetic peptides were added at the final concentration of 0.75 mg/ml. After 18 h incubation cells were washed and tissue necrosis factor (TNF-α) was added in serum-free medium to the final concentration of 10 ng/ml. Cells were incubated for 5 h and luciferase activity was measured using Bright-Glo Assay (Promega). Data were

expressed per milligram of cellular protein and related to the luciferase activity in cells incubated with a vehicle instead of the peptides. All peptides were tested in the one experiment done in quadruplicates and repeated twice, inter-assay variability was <10%.

Oxidation of LDL

The capacity of the peptides to inhibit LDL oxidation was assessed as described by Kontush et al [7]. In brief, freshly isolated LDL (final concentration 100 μg/ml) was incubated at 25°C for the indicated periods of time with $CuSO_4$ (final concentration 15 μMol/L) in the presence of the peptides or apoA-I (final concentration of 100 μg/ml) in the cells of a multi-cell spectrophotometer (Beckman, DU800) and absorption was continually monitored at 234 nm. Rate of oxidation was calculated as maximum absorbance divided to the length of the lag period according to the published model of LDL oxidation [8]. Each peptide was tested twice with different batches of LDL, apoA-I and peptide 5A were included in each assay, inter-assay variability was <15%.

Statistics

All experiments were reproduced at least 2-4 times. Unless otherwise indicated, experimental groups consisted of quadruplicates; means ± SEM are presented. Differences between groups were analyzed by ANOVA with t values modified by the step down Bonferroni procedure; the differences considered statistically significant when $p<0.05$. Correlations were calculated using Pearson Product Moment Correlations or, when indicated, using Spearman Rank Order Correlations.

References

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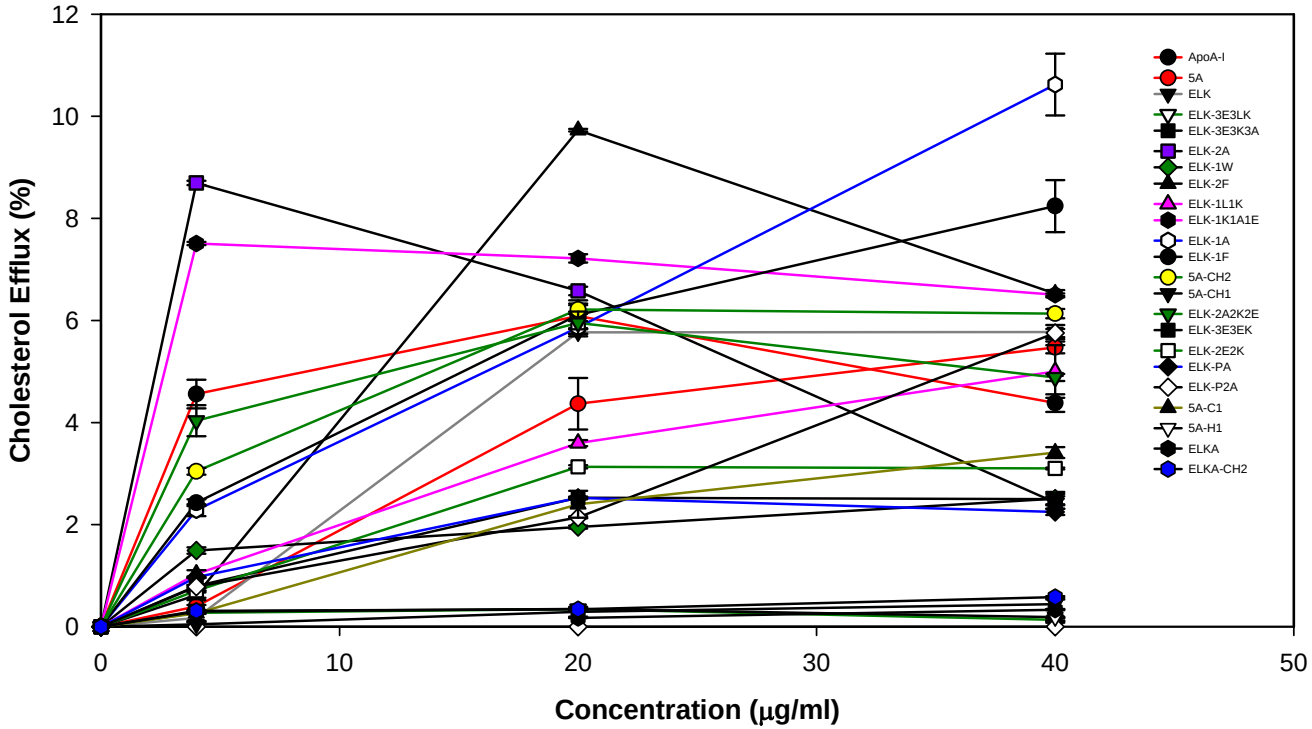
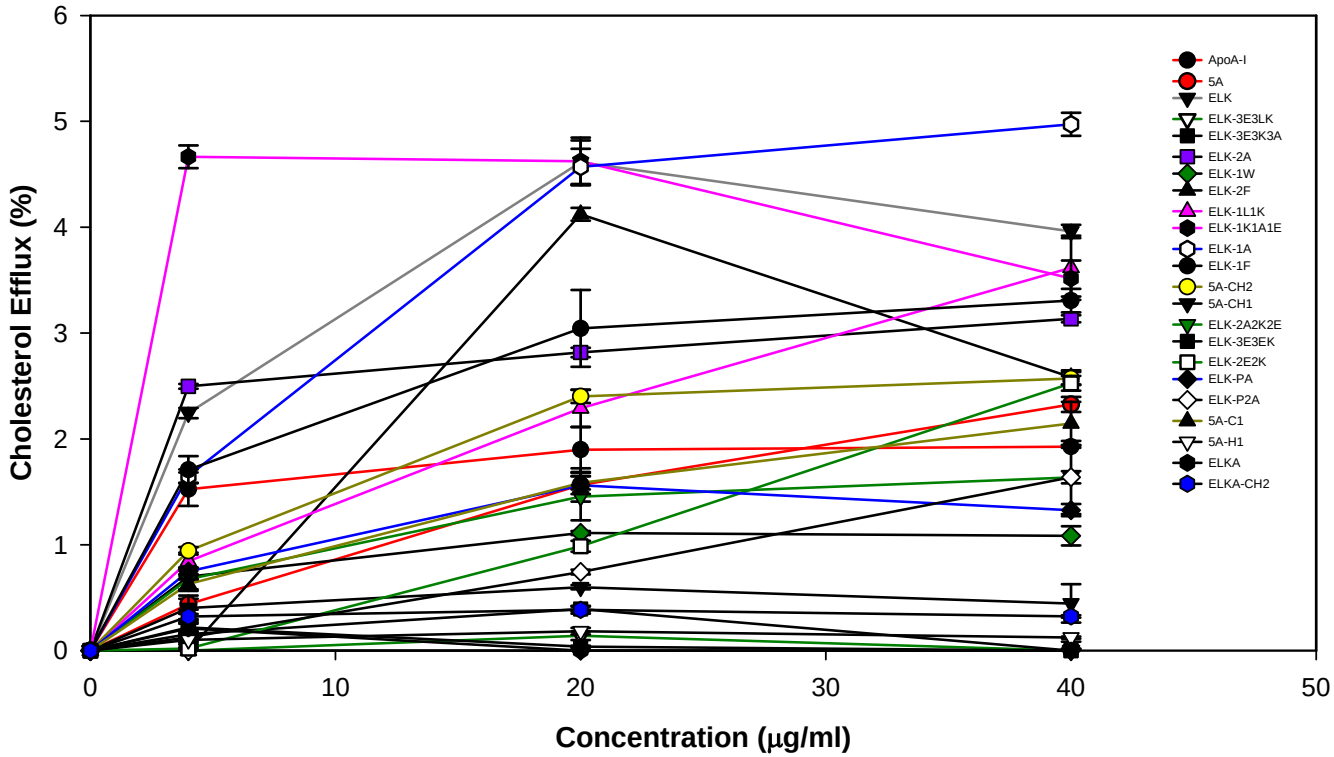


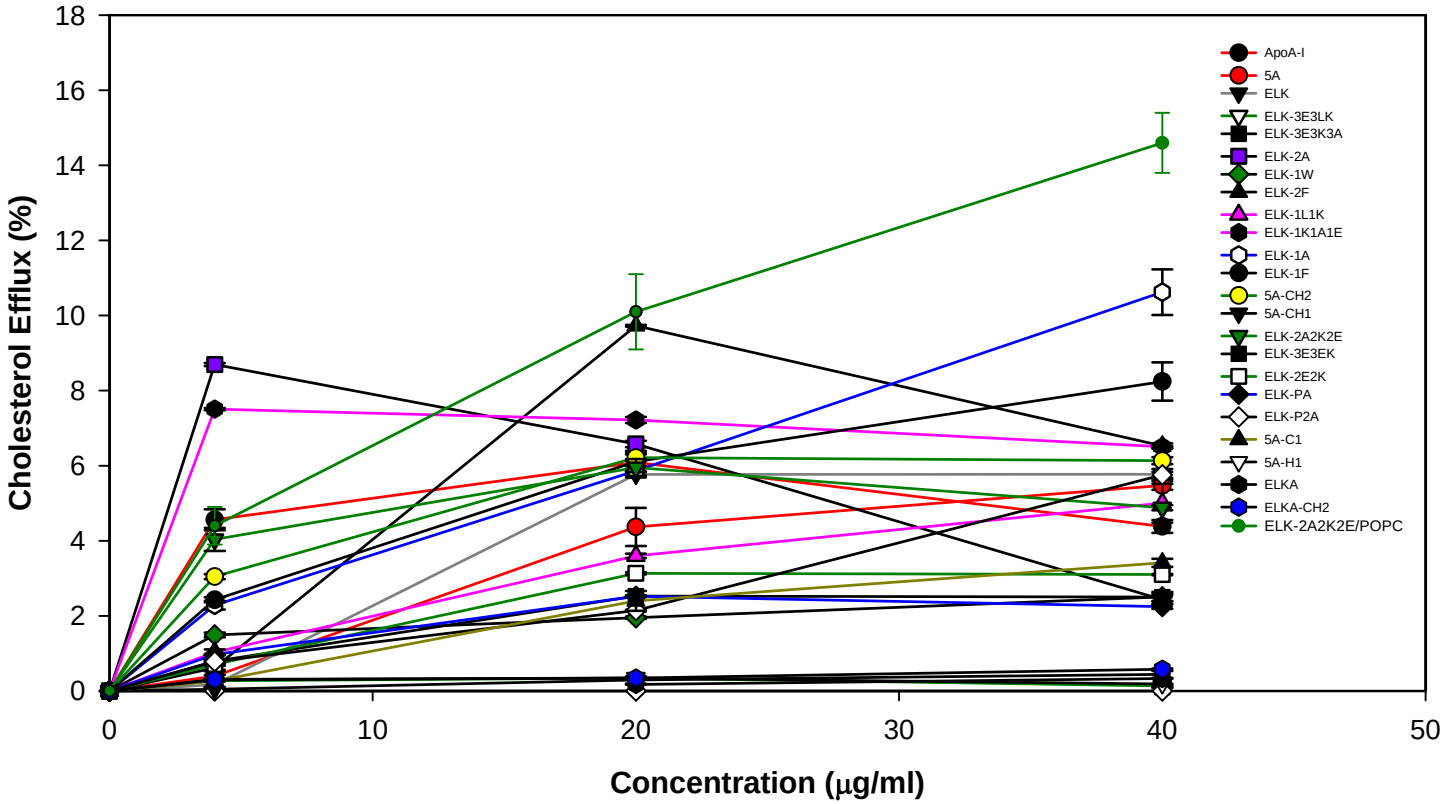
***Supplementary Figure I*. Dose-dependence of cholesterol efflux from THP-1 cells activated with LXR agonist**

Cellular cholesterol was labeled by incubation in serum-containing medium with [^{3}H]-cholesterol. Cells were then washed and incubated for 18 h at 37^{o}C in serum-free medium in the presence of the LXR agonist TO-901317 (4 μmol/L). Cells were washed and incubated for another 4 h at 37^{o}C in serum-free medium containing indicated concentrations of the peptides or lipid-free apoA-I. Cholesterol efflux was expressed as the proportion of [^{3}H]cholesterol transferred from cells to medium. Non-specific efflux (i.e. the efflux in the absence of an acceptor) was subtracted. Data from different experiments were normalized to the efflux to the peptide 5A, which was included in all experiments.

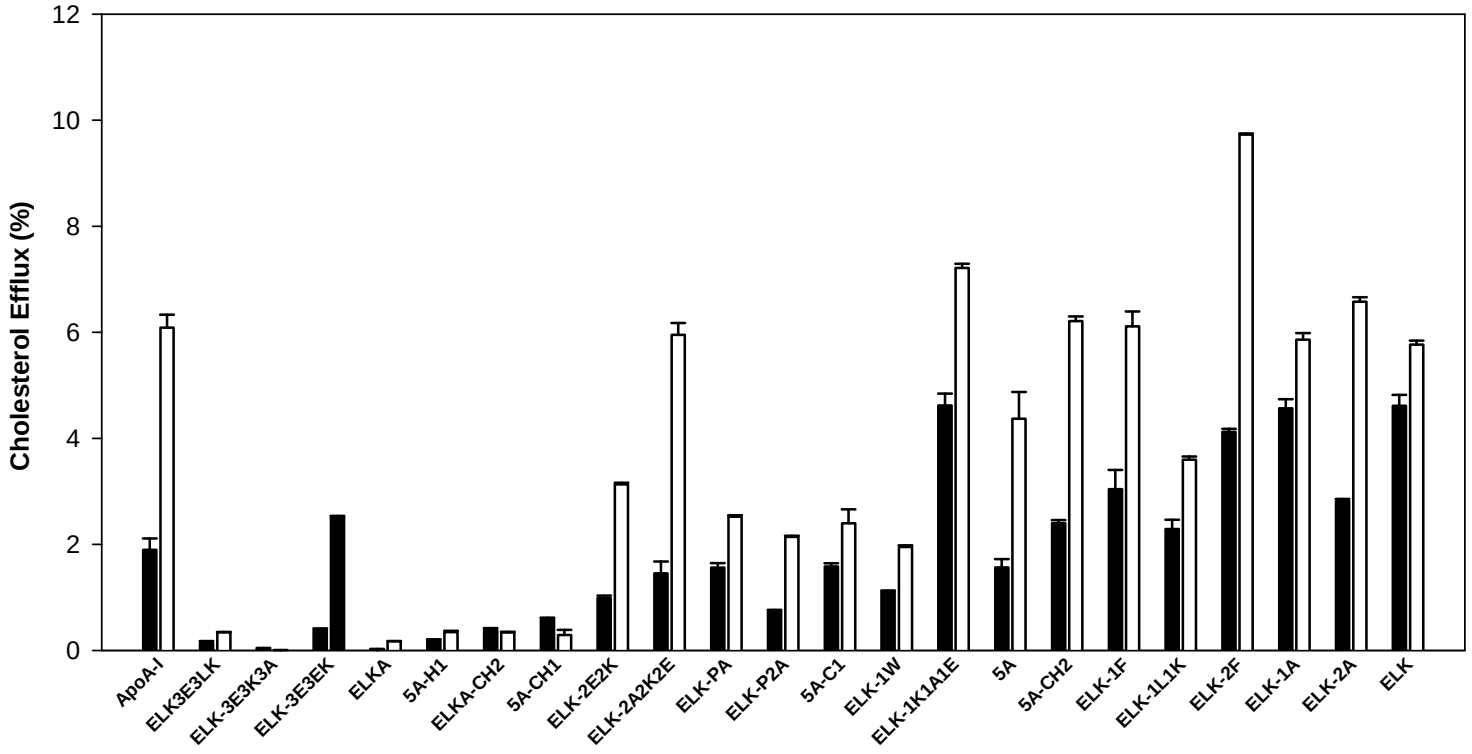


***Supplementary Figure II*. Dose-dependence of cholesterol efflux from THP-1 cells not activated with LXR agonist**

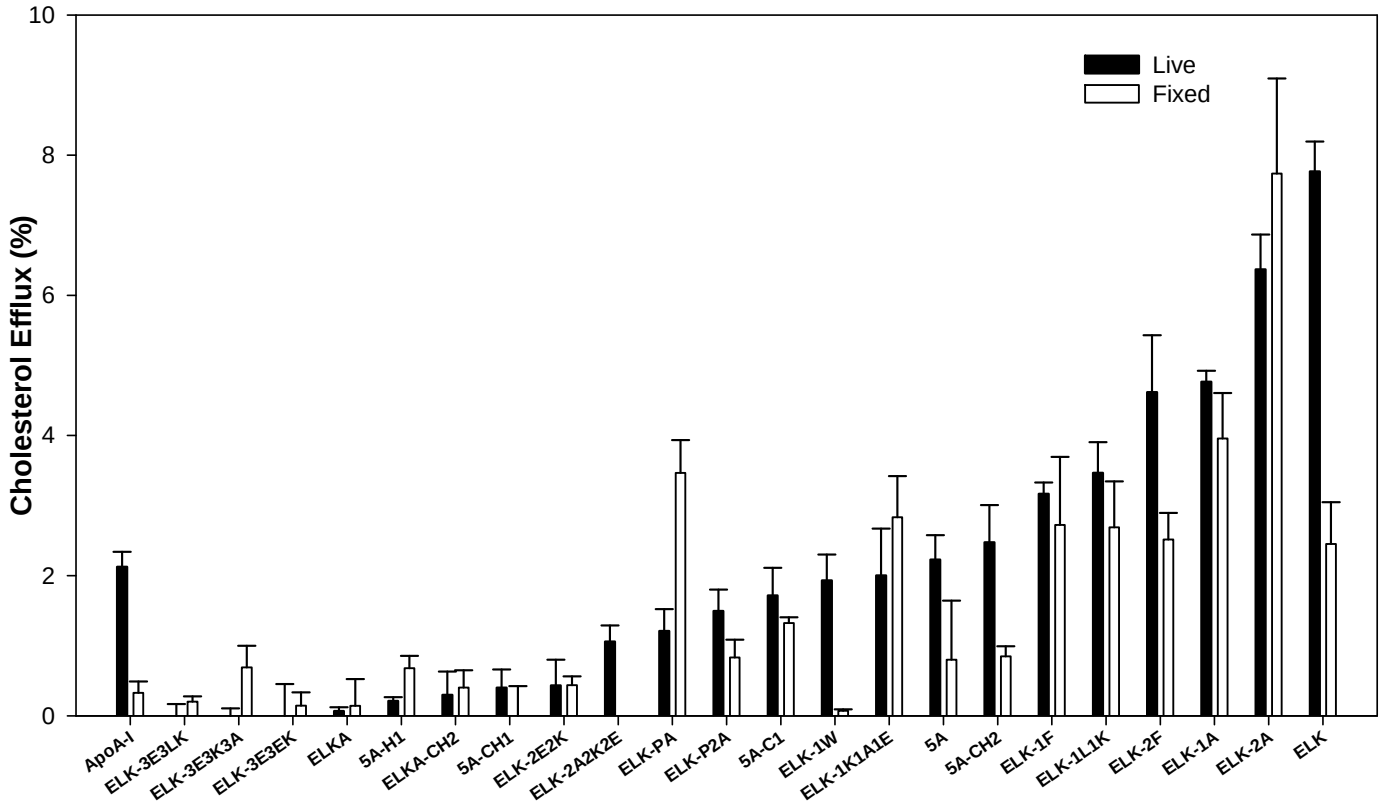
Cellular cholesterol was labeled by incubation in serum-containing medium with [^{3}H]-cholesterol. Cells were then washed and incubated for 18 h at 37^{o}C in serum-free medium, washed and incubated for another 4 h at 37^{o}C in serum-free medium containing indicated concentrations of the peptides or lipid-free apoA-I. Cholesterol efflux was expressed as the proportion of [^{3}H]cholesterol transferred from cells to medium. Non-specific efflux (i.e. the efflux in the absence of an acceptor) was subtracted. Data from different experiments were normalized to the efflux to the peptide 5A, which was included in all experiments.



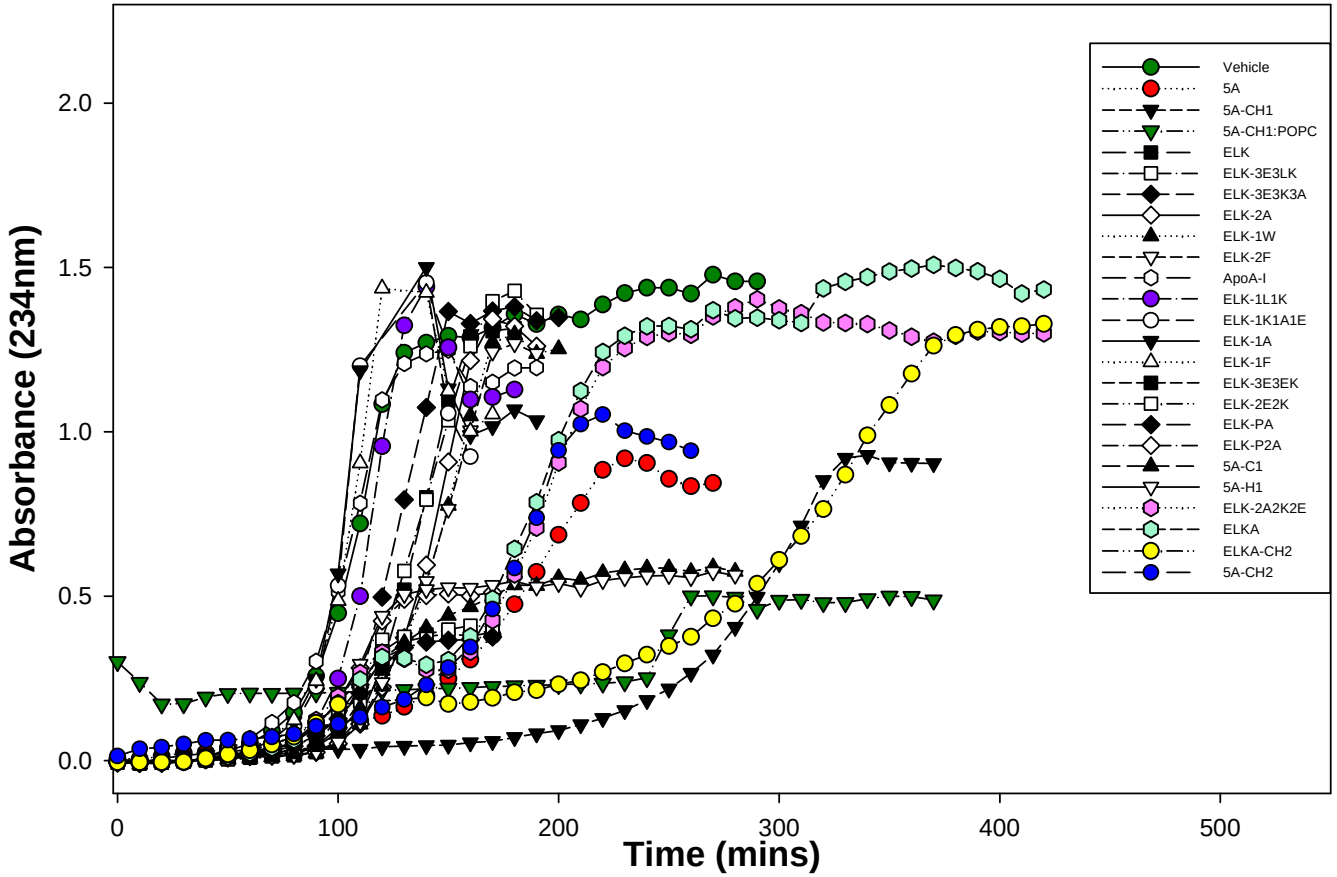
***Supplementary Figure III*. Dose-dependence of ABCA1-dependent cholesterol efflux from THP-1.**
Data presented in this figure are a difference between values presented in Supplementary Figure I and Supplementary Figure II, calculated for each data point. When calculations gave negative values they were interpreted as "0" value.



Supplementary Figure IV: **Contribution of ABCA1-dependent efflux**
Data presented in this figure show efflux from activated and non-activated THP-1 cells (data taken from Supplementary Figure I and Supplementary Figure II) at peptide concentration 20 μg/ml



Supplementary Figure V. **Specificity of cholesterol efflux from THP-1 cells.**
Cellular cholesterol was labeled by incubation with [^{3}H]-cholesterol for 48 h in a CO_2 incubator. Cells were then washed and incubated for 18 h at 37^{o}C in serum-free medium, and fixed or not fixed by incubation for 20 min with paraformaldehyde (4%). Cells washed and incubated for another 4 h at 37^{o}C in serum-free medium containing 80 μg/ml of the peptides or lipid-free apoA-I. Cholesterol efflux was expressed as the proportion of [^{3}H]cholesterol transferred from cells to medium. Non-specific efflux (i.e. the efflux in the absence of an acceptor) was subtracted.



Supplementary Figure VI. **Oxidation of LDL**

Freshly isolated LDL (final concentration 100 μg/ml) was incubated at 25°C for the indicated periods of time with $CuSO_4$ (final concentration 15 μMol/L) in the presence of the peptides or apoA-I (final concentration 100 μg/ml) in the cells of a multi-cell spectrophotometer continuously measuring absorption at 234 nm.