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Advanced Glycation End Products Promote Pro-Atherogenic Changes in Cholesterol Transport: A Possible Mechanism for Cardiovascular Risk in Diabetes

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Abstract

Hyperglycemia is the major cause of diabetic vascular complications. Advanced glycation end products (AGEs) accumulate under hyperglycemic conditions and contribute to atherosclerosis. ATP binding cassette transporters (ABC) A1, ABCG1, and cholesterol 27-hydroxylase are reverse cholesterol transport proteins (RCT) that facilitate cholesterol removal from macrophages and constitute a first line of defense against atherosclerosis. ABCA1 and ABCG1 are known to be suppressed by AGEs. Here we investigate the effects of AGEs on the expression of RCT proteins and scavenger receptors in THP-1 human macrophages and peripheral blood mononuclear cells (HPBMC). Adherent THP-1 macrophages and HPBMC were incubated in the presence or absence of 50 μg/ml carboxymethyl lysine-human serum albumin over a time course. Following incubation, RNA and protein were isolated and subjected to quantitative real-time PCR with specific primers for the 27-hydroxylase, ABCA1, ABCG1, CD36, lectin-like oxidized low density lipoprotein receptor (LOX) 1, scavenger receptor (SR)A1 and chemokine CXC ligand 16 (CXCL16). PCR results were confirmed by Western blot. Expression of ABCA1 and ABCG1 were diminished in the presence of AGEs in both cell lines. For the first time, we demonstrate that AGEs decrease message and protein level for 27-hydroxylase (by 54.5 ± 2.9% and 48.7 ± 9.23%, respectively). In our study uptake of AGE products in THP-1 macrophages and HPBMC occurs mainly through the CD36 and CXCL16 receptors, leading to increased oxidized LDL uptake upon lipid overload and transformation of macrophages into foam cells. Expression of SRA1 and LOX-1 were not affected by introduction of AGEs. Therefore, we conclude that AGEs may contribute to accelerated atherosclerosis in diabetes through effects on both forward and reverse cholesterol movement. AGEs promote lipid overload through enhancing expression of proteins that facilitate lipid uptake (CD36 and CXCL16) and through suppressing RCT proteins 27-hydroxylase, ABCA1 and ABCG1. Our present study provides a novel atheroma-promoting effect of AGEs on lipid handling.

Keywords: Atherosclerosis; Glycation; Advanced glycation end products; Reverse cholesterol transport; Scavenger receptors; Oxidized low density lipoproteins

Abbreviations: ABC transporters: ATP-Binding Cassette transporters; CD36: Cluster of Differentiation 36; CXCL16: Chemokine CXC Ligand 16; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; HDL: High-Density Lipoprotein; HPBMC: Human Peripheral Blood Mononuclear Cells; LDL: Low-Density Lipoprotein

Introduction

Cardiovascular disease is a major cause of morbidity and mortality in patients with diabetes [1]. Non-enzymatic glycation leads to the formation of advanced glycation end products (AGEs), a heterogeneous group of compounds derived from proteins, lipids and nucleic acids [2,3]. AGEs are formed at an accelerated rate in the diabetic state and are thought to contribute to many of the deleterious vascular effects of diabetes [4]. Serum levels of AGEs are related to the degree of coronary atherosclerosis in both diabetics and non-diabetics with coronary artery disease [5-8].

The cellular and molecular mechanisms whereby diabetes accelerates cardiovascular disease and atherosclerosis are only partly understood. Abnormal lipid metabolism may contribute to the increased atherosclerosis associated with both type I and type II diabetes [9-11]. Cholesterol accumulation in arterial macrophages is a key event in atherogenesis that occurs when the balance between uptake and excretion is disrupted. This balance depends upon the expression and activity of specific proteins. Uptake is accomplished by scavenger receptors CD36, scavenger receptor (SR)-A1, lectin-like oxidized LDL receptor (LOX)-1 and chemokine CXC ligand 16 (CXCL16) [12-14]. AGEs were shown to bind to CD36, SR-A1 and LOX-1 and were able to induce gene expression of scavenger receptors CD36 and LOX-1 in diabetic rats [15]. Cholesterol efflux to extracellular acceptors involves ATP binding cassette transporters (ABC) A1 and ABCG1 [16,17] as well as conversion to polar oxysterols by the cholesterol P450 27-hydroxylase [18,19]. Previous studies demonstrate that AGEs suppress ABCA1 and elevate CD36 in human macrophages [20,21].

In this study, we demonstrate that AGEs in THP-1 human macrophages and human peripheral blood mononuclear cells (HPBMC) elevate the level of CD36 and CXCL16 receptors, which, under lipid loading conditions, promote oxidized (ox) LDL uptake and consequent transformation of macrophages into foam cells. We reconfirm previous findings that AGEs downregulate cholesterol efflux

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proteins, while showing for the first time that AGEs affect intracellular processing of cholesterol as well, reducing expression of 27-hydroxylase and increasing accumulation of cholesterol esters. The contribution of AGEs to accelerated atherosclerosis in diabetes may involve concurrent effects on lipid overload through enhancing expression of proteins that facilitates lipid uptake (CD36 and CXCL16), down regulation of reverse cholesterol transport (RCT) proteins illustrated by disruption of intracellular cholesterol processing via 27-hydroxylase (Figure 1). Our present study provides a novel atheroma-promoting hypothesis on the effect of AGEs on lipid handling.

Materials and Methods

Cell culture and experimental conditions

Cell culture media and supplementary reagents were obtained from Invitrogen (Grand Island, NY). Carboxymethyl lysine (CML) - human serum albumin (HSA) was obtained from MBL International (Woburn, MA). THP-1 monocytes (American Type Culture Collection, Manassas, VA) and human peripheral blood mononuclear cells (HPBMC) (Lonza Inc., Allendale, NJ) were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 50 µg/ml of penicillin-streptomycin at 37°C in a 5% CO_2 atmosphere to a density of 10^6 cells per ml. Differentiation of the monocytic THP-1 cells into adherent macrophages was stimulated by 24 h exposure to 100 nM phorbol 12-myristate 13-acetate (PMA), obtained from Sigma-Aldrich (St. Louis, MO). When differentiated phenotype was achieved, the PMA-containing medium was removed, and replaced with complete RPMI 1640 supplemented with 10% FCS. The macrophages were cultured for another 24 h before treatment. HPBMC were cultured for 18 hours prior to treatment.

THP-1 macrophages and HPBMC were incubated for 30 min, 1 h, 3 h and 5 h (37°C, 5% CO_2) in the presence or absence of 50 µg/ml CML-HSA.

RNA Extraction and Real-time PCR

Immediately after the incubation period, total RNA was isolated with the Trizol reagent and dissolved in nuclease-free water. The quantity of total RNA from each condition was measured by absorption at 260 and 280 nanometer wavelengths by ultraviolet spectrophotometry (Hitachi U2010 spectrophotometer).

QRT-PCR analysis was performed using the Fast Start SYBR Green Reagents Kit according to the manufacturers' instructions on the Roche Light Cycler 480 (Roche Applied Science, Indianapolis, IN). cDNA was copied from 1 µg of total RNA using Murine Leukemia Virus reverse transcriptase primed with oligo dT. Equal amounts of cDNA were taken from each reverse transcription reaction mixture for real-time PCR amplification using gene specific primers for 27-hydroxylase, ABCA1, ABCG1, SR-A1, CD36, LOX-1 and CXCL16 as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Table 1). Each reaction was done in triplicate. To correct for differences in cDNA load among samples, the target PCRs were normalized to a reference PCR involving the endogenous housekeeping gene GAPDH. Non-template controls were included for each primer pair to check for significant levels of any contaminants.

Western blot

Cellular extracts were prepared for Western immunoblotting using radioimmunoprecipitation assay (RIPA) lysis buffer (98% PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS), supplemented with protease inhibitor cocktail (Sigma). Protein samples (20 mg/lane) were boiled for 5 min, and fractionated on 8% SDS-PAGE, and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA).

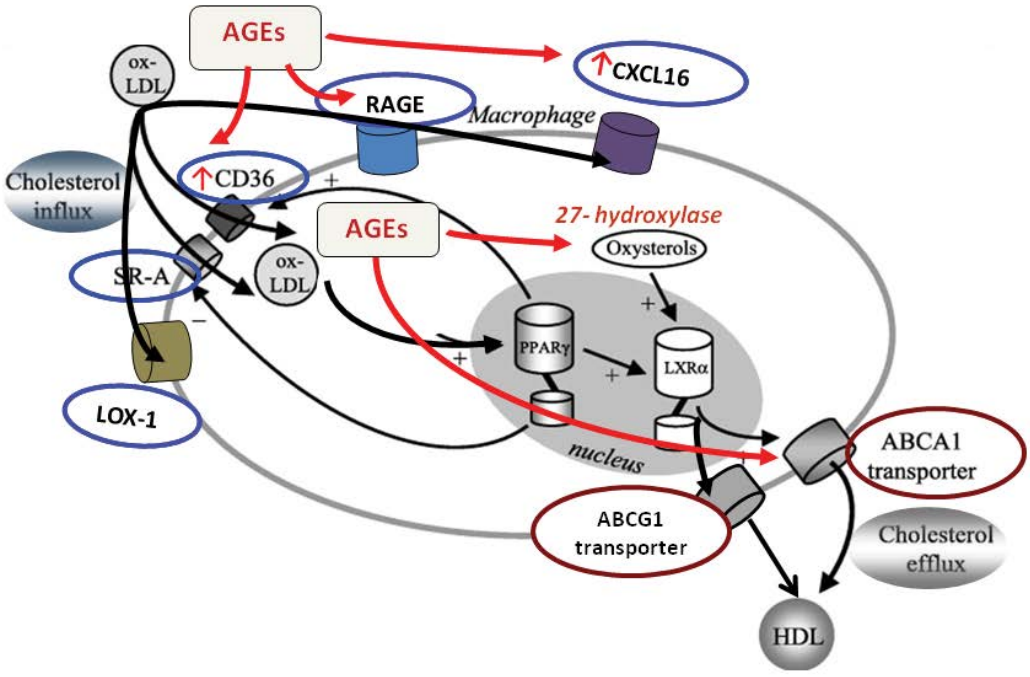

Figure 1: Scheme for atheroma-promoting effect of AGEs on lipid handling in monocytes/macrophages. AGEs increase expression of scavenger receptors while depressing RCT proteins leading to uptake of oxLDL with compromised outflow of cholesterol. This creates an imbalance in lipid processing that enhances foam cell formation, a crucial early event in atheroma development.

Gene	Primer
ABCA1	F 5'-GAAGTACATCAGAACATGGGC-3' R 5'-GATCAAAGCCATGGCTGTAG-3'
ABCG1	F 5'-CAGGAAGATTAGACACTGTGG-3' R 5'-GAAAGGGGAATGGAGAGAAG-3'
27-hydroxylase	F 5'-AAGCGATACCTGGATGGTTG-3' R 5'-TGTTGGATGTCGTGTCCACT-3'
CD36	F 5'-GAGAACTGTTATGGGGCTAT-3' R 5'-TTCAACTGGAGAG-GCAAAGG-3'
LOX-1	F 5'-TTACTCTCCATGGTGGTGCC-3' R 5'-AGCTTCTTCTGCTTGTTGCC-3'
ScR-A1	F 5'-CTCGTGTTTGCAGTTCTCA-3' R 5'-CCATGTTGCTCATGTGTTCC-3'
CXCL16	F 5'- ACTACACGACGTTCCAGCTCC -3' R 5'- CTTTGTCCGAGGACAGTGATC -3'
GAPDH	F 5'- ACCATCATCCCTGCCTCTAC-3' R 5'-CCTGTTGCTGTAGCCAAAT-3'

Table 1: The list of specific primers used for QRT-PCR.

The membrane was blocked for 1h at room temperature in blocking solution (3% nonfat dry milk (Bio-Rad) in 1X Tris-buffered saline/1% Tween20 [TTBS]) and then immersed in a 1:500 dilution of primary antibody overnight at 4°C. Anti-cholesterol 27-hydroxylase antibody is an affinity-purified rabbit polyclonal antibody raised against residues 15-28 of the cholesterol 27-hydoxylase protein. Rabbit anti-human ABCA1 (sc-20794) (Santa Cruz, CA) and ABCG1 (ab-36969) (Abcam Inc., Cambridge, MA) were used for detection of ABCA1 and ABCG1, respectively. Rabbit anti-human LOX-1 (ab60178), CD36 (ab64014), SR-A1 (ab36625), and CXCL16 (ab101404) were purchased from Abcam Inc. (Cambridge, MA). As a loading control, on the same transferred membrane, β-actin was detected using mouse anti-human β-actin antibody (ab8227, Abcam Inc.).

The immunoreactive proteins were detected using the Pierce ECL Western Blot substrate system and film development in SRX-101A (Konica Minolta Holdings, Inc., Tokyo, Japan). Stripping and reprobing of the membranes were performed according to the manufacturer's protocol (Thermo scientific, Rockford, IL). Band intensities for Western blot protein samples were quantified using Kodak Digital Science 1D, version 2.0.3, after imaging with Kodak Digital Science Electrophoresis Documentation and Analysis System 120.

oxLDL internalization assay

Differentiated macrophages were cholesterol-loaded with 50 μg/ml oxLDL (Inracel, Frederick, MD) for 24 h, and then incubated with 50 μg/ml CML-HSA in the presence of 5 μg/ml 1,1'-dioctadecyl-3,3,3',3'-tetramethylin dicarbocyaninet (DiI)-oxLDL (Inracel, Frederick, MD) for an additional 3 h. After incubation, accumulation of DiI-oxLDL in cells was determined by fluorescent intensity of accumulated DiI-oxLDL with a Nikon A1 microscopy unit with 40X magnification and photographed with a DS-Ri1 digital camera. Fluorescent intensity was quantified from at least 3 random fields (1024×1024 pixels) per slide, from 3 slides per experimental condition and graphed.

Cholesterol efflux analysis

Cholesterol efflux to HDL was analyzed when THP-1 macrophages were exposed to human HDL (Intracel, Frederick, MD) in RPMI for 6 h (20 μg/ml) in the presence/absence of CML-HSA. After incubation, both extracellular (in cell growth medium) and intracellular (in cells) total (TC) and free (FC) cholesterol were analyzed. The Amplex® Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR) was used according to the manufacturer's protocol. Cholesterol esters (CE) were calculated as the difference between TC and FC. The HDL-mediated net cholesterol efflux was calculated by subtraction of the cholesterol mass of the medium from that of the cells. CE/FC ratio was calculated.

Statistical analysis of experimental data

Statistical analysis was performed using Graph pad Prism, version 5.01. Pair wise multiple comparison was made between control and treatment conditions using unpaired t tests, two tailed 95% confidence intervals, significance $p<0.05$.

Results and Discussion

In this study we chose CML-HSA because it represents the most prevalent AGE *in vivo* [22]. Intracellular AGEs modify properties of cells of the arterial wall that are critical for vascular homeostasis. Thus, activation of the receptor for AGE (RAGE) on endothelial cells leads to hyper permeability and alters cell surface structure [23,24]. Soluble AGEs induce monocyte migration across an endothelial cell monolayer, activate monocytes, change the expression of receptors for oxLDL and stimulate foam cell formation [21,25]. Here we examined gene expression of the proteins involved in cholesterol flux in both human macrophages and monocytic cells. THP-1 human monocytes/macrophages, a well-accepted model for atherosclerosis are used to monitor changes in cholesterol metabolism and immune response by our group and others [26,27]. We included replication of the experiments with CML modified HSA in naïve primary human monocytes for confirmatory purposes. Monocytic cells are rarely evaluated although, in the circulation *in vivo*, they receive prolonged exposure to AGEs prior to migration and transformation into intimal macrophages. Moreover, monocytes are easy to obtain, have a strong role in mediating inflammatory processes and can be used as surrogates for quantitative gene analysis studies in atherosclerosis [28].

Cholesterol accumulation in arterial monocytes/macrophages and their transformation into foam cells are key events in atherogenesis. These events occur when the balance between uptake and excretion is disrupted.

As previously reported [20,21], in cultured THP-1 macrophages expression of ABCA1 and ABCG1 diminishes in the presence of CML-HSA (Figure 2A). HPBMC show a similar pattern in response to CML-HSA, but with a less dramatic effect on the ABCA1 transporter (76.5 ± 10.3% [n=3, $P<0.01$] and no significant effect on the expression of ABCG1 (Figure 2B). Protein expression analysis for cholesterol efflux proteins is presented as supplementary Figure 1. Levels of ABCG1 are very low in PBMC and gene expression pattern are known to differ between monocytes and macrophages in humans [29]. Observed differences in human monocytic cells and macrophages also might reflect differences in signaling that occur upon monocyte/macrophage transformation. In addition, expression changes in one ABC transporter may influence expression of the other [30,31]. In our experiments expression of ABC transporters in THP-1 macrophages paradoxically returns to the control levels after 5 h of incubation. It might represent non-linear changes in the expression upon exposure to CML-HSA similar to those observed upon oxLDL overload [32].

Processes that contribute to the elimination of cholesterol from macrophages involve both outflow of cholesterol to extracellular acceptors and intracellular catabolism to polar sterols, exported more readily than cholesterol. The presence of CML-HSA repressed removal of cholesterol from THP-1 macrophages to medium in the presence/absence of HDL (Figure 3A). Thus, HDL mediated efflux was decreased and resulted in accumulation of intracellular total cholesterol (TC) (mainly through increase in CE fraction): a three-fold increase in

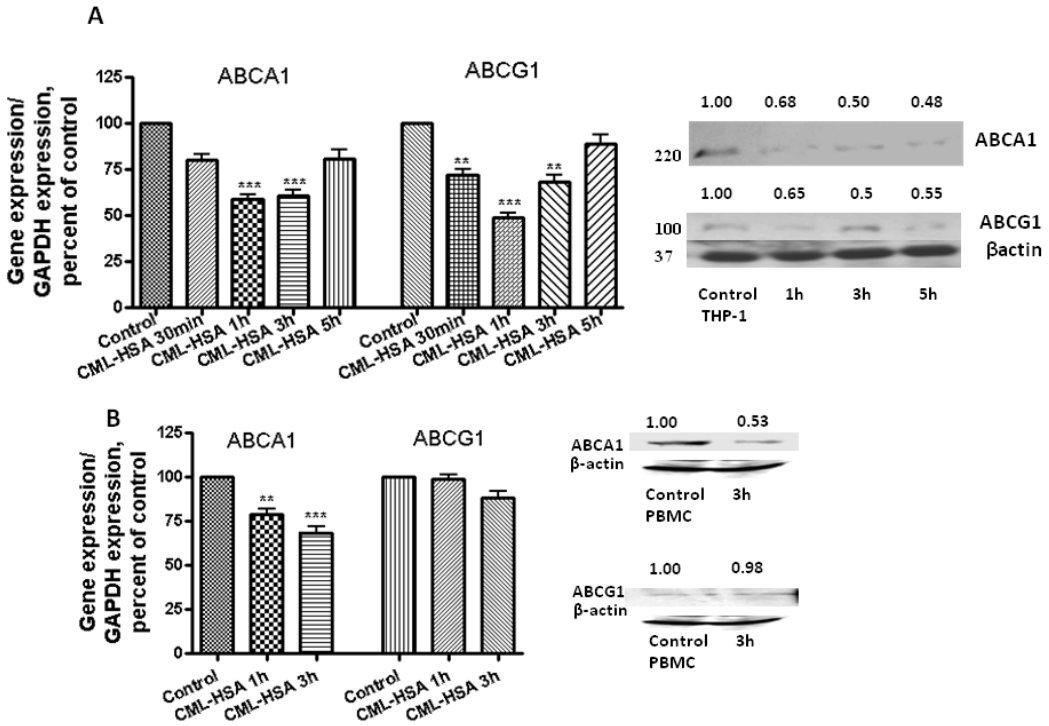

Figure 2: CML-HSA downregulates expression of RCT proteins in THP-1 human macrophages and HPBMC. Effect over time of CML-HSA on expression of the major proteins responsible for cholesterol efflux: ABC transporters A1 and G1 in THP-1 macrophages (**A**) and HPBMC (**B**). All results are expressed as mean ± SEM of three independent experiments. ** - P<0.01; *** - P<0.001 vs. control untreated cells. Results of RT-PCR were confirmed with Western Blot.

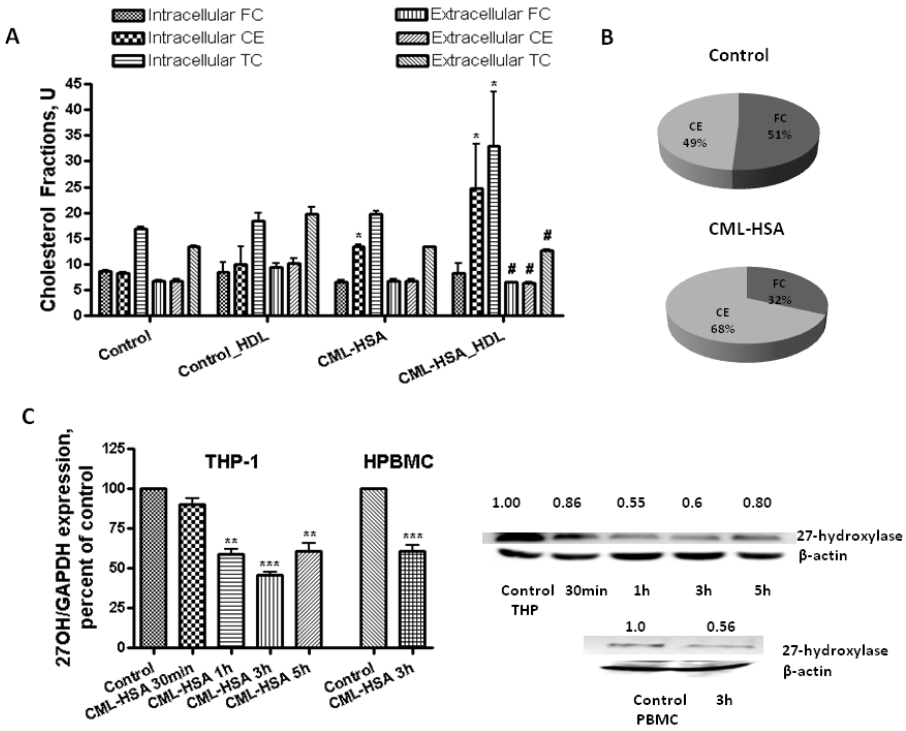

Figure 3: CML-HSA represses net cholesterol efflux and intracellular cholesterol processing in THP-1 macrophages. Net cholesterol efflux to HDL [100 μg/ml] is reduced in the presence of CML-HSA in THP-1 macrophages (**A**). CML-HSA stimulates intracellular accumulation of cholesterol esters and reduces the amount of free cholesterol (**A** and **B**). Values are mean of three independent experiments. TC- total cholesterol; FC-free cholesterol; CE- cholesterol esters. * P<0.05, ** P<0.01, versus control cells without HDL; ## P<0.01, ### P<0.001 vs. cells containing HDL in the medium. Incubation with CML-HSA significantly decreases 27-hydroxylase message and protein (**C**) over time.

the cells exposed to CML-HSA (n=3, P<0.001) vs. control cells. Moreover, in CML-HSA incubated cells we observed an increase in the intracellular ratio of CE/FC vs. control cells (Figure 3B). Cholesterol esters (CE) are much less polar than free cholesterol (FC) and are major constituents of the fatty lesions in atherosclerotic plaques. These results expand previous findings that in diabetes mellitus, AGEs impair lipid metabolism and RCT by diminishing the expression of ABCA1, ABCG1 and the activity of lecithin cholesterol acyltransferase (LCAT) [19], considered a critical enzyme behind the process of cholesterol elimination (mainly CE) from macrophages in the arterial wall for subsequent delivery to the liver. In our study we observed a decrease in extracellular cholesterol level when CML-HSA is present, indicating a delay in removal (Figure 3A). For the first time, we demonstrate that AGEs decrease message and protein level of 27-hydroxylase (by 54.5 ± 2.9% and 48.7 ± 9.23%, respectively [at 5 h]) in THP-1 macrophages (Figure 3B). The effect of CML-HSA on expression of 27-hydroxylase in HPBMC was, once again, less pronounced reducing the mRNA level by 37.2 ± 3.5% (n=3, P<0.05) (Figure 3B). Protein expression analysis for cholesterol efflux proteins is presented as supplementary Figure 1.

It has been shown in earlier studies that the class B scavenger receptors (CD36 and type I) and class A type I bind AGE ligands [21,33,34]. However, CD36 was reported not to be involved in the clearance of AGEs from the circulation, but rather in the induction of oxidative stress in the cell [35]. We demonstrate that AGE products in THP-1 macrophages and human PBMC preferably stimulate expression of CD36 and CXCL16 receptors (Figures 4A and 4B). Thus, CD36 was upregulated to 156.5 ± 5.9% for mRNA and 176.78 ± 12.2% for protein expression (n=3, P<0.01) vs. untreated THP-1 macrophages (set at 100%). mRNA and protein level of CXCL16 was two-fold amplified in THP-1 macrophages (n=3, P<0.01). In HPBMC message for CD36 was increased by 39 ± 1.69% and CXCL16 - three-fold (n=3, P<0.01) (Figure 4C). Despite the findings of other groups that AGEs increase LOX-1 in macrophages and endothelium [36,37], we did not detect significant changes in levels of LOX-1 and SR-A1 in our study. Protein expression analysis for cholesterol influx proteins is presented as supplementary Figure 2.

The described changes in the expression of scavenger receptors could provide an explanation for elevated accumulation of oxLDL in the presence of CML-HSA (Figure 5). We demonstrate a three-fold increase in accumulated DiI-oxLDL early, at 1 h of incubation. The CML-HSA did not display fluorescent background itself at any point during incubation.

Our studies indicate a direct effect of AGEs on cholesterol homeostasis in human monocytes/ macrophages that may accelerate atherosclerosis. Future studies may reveal whether lowering AGE levels is atheroprotective in diabetes. It may be possible to target AGE levels directly with specific treatment options. Both pioglitazone and metformin reduce AGE levels in type 2 diabetic subjects [38]. There is

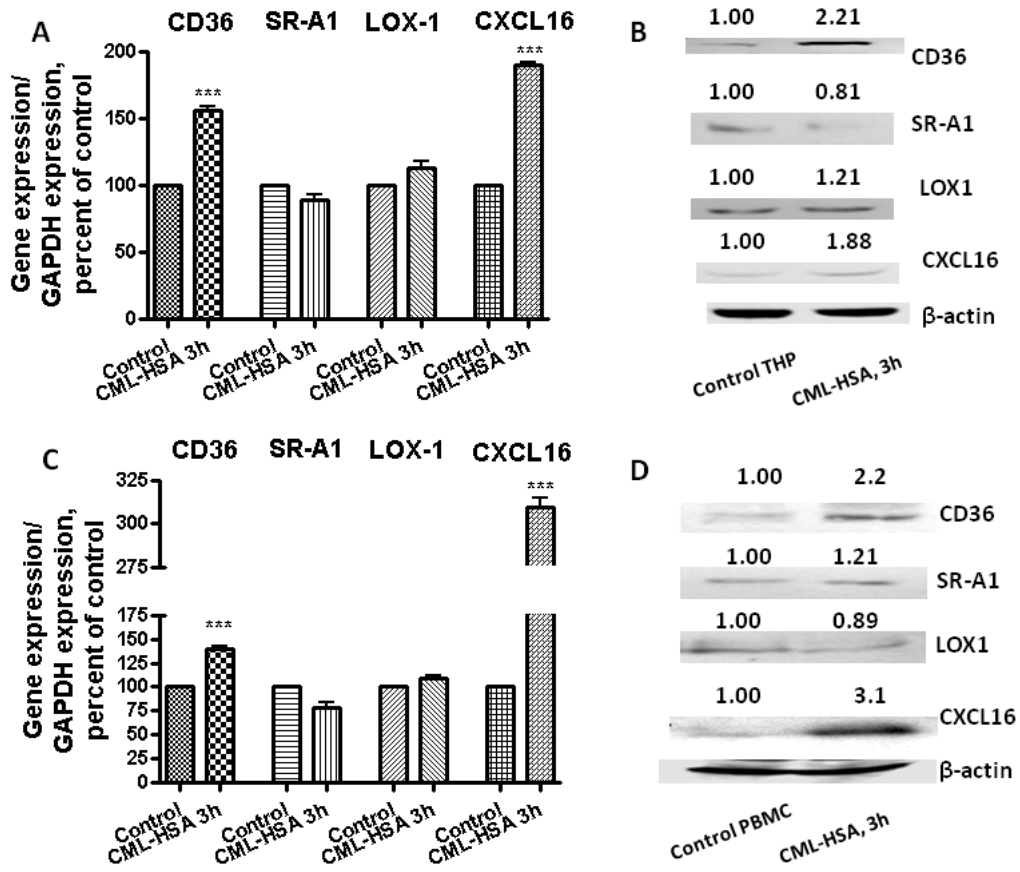

Figure 4: CML-HSA upregulates expression of scavenger receptors in THP-1 human macrophages and HPBMC. Effect of CML-HSA on the expression of major proteins responsible for lipid uptake: CD36, lectin-like oxidized low density lipoprotein receptor (LOX) 1, scavenger receptor (SR) A1 and chemokine CXC ligand 16 (CXCL16) in THP-1 macrophages (message **[A]** and protein **[B]**) and HPBMC (message **[C]** and protein **[D]**). All results are expressed as mean ± SEM of three independent experiments. ** - P<0.01; *** - P<0.001 vs. control untreated cells. Results of RT-PCR were confirmed with Western Blot.

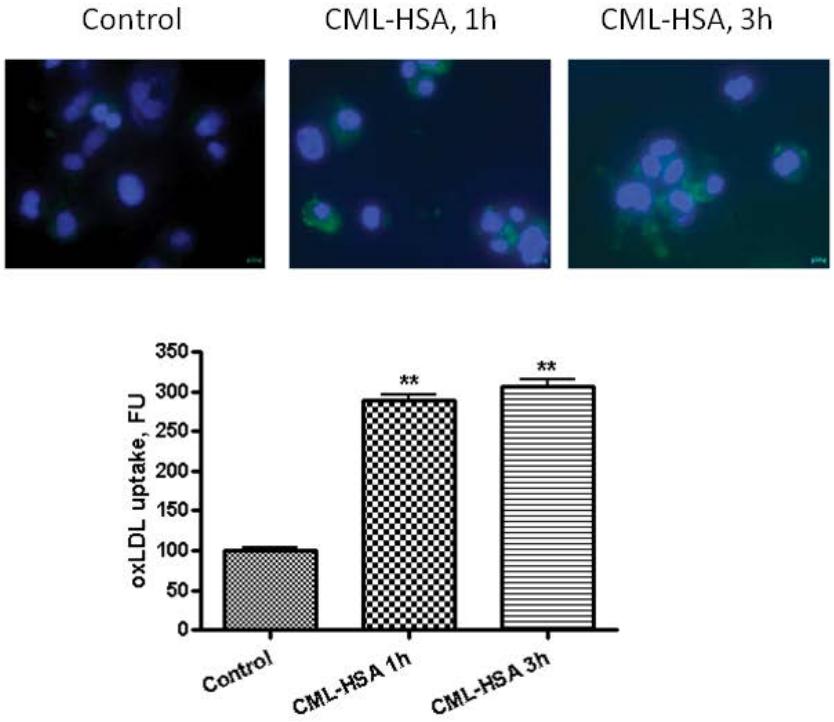

Figure 5: CML-HSA promotes oxLDL uptake in THP-1 macrophages. DiI-oxLDL internalization was measured as fluorescent intensity of integrated DiI-oxLDL and presented as a percent of control cells, set at 100%. The presence of CML-HSA significantly enhanced oxLDL uptake in THP-1 macrophages compared to control untreated cells (by 189.73 ± 11.51%, P<0.001 at 3 h and by 201.03 ± 12.6%, P<0.05 at 5 h of incubation vs. control cells set at 100%). The CML-HSA had no fluorescent background.

also some evidence that dietary supplements such as the phytoalexin resveratrol may also reduce AGE levels [39].

Conclusions

Diabetes is generally associated with lipid abnormalities and this leads to coronary heart disease, morbidity and mortality [40]. Greater understanding of the mechanisms underlying diabetes-related cardiovascular complications may broaden prevention and treatment options.

Overall, the combination of augmented lipid uptake through elevation in the expression of scavenger receptors (CD36 and CXCL16) and dampened egress via RCT proteins (ABC transporters, 27-hydroxylase) may lead to increased accumulation of lipids in monocytes and macrophages and enhanced atherogenesis in diabetics with elevated AGE levels. CD36 is a multifunctional scavenger receptor that endocytoses oxLDL and is considered a potential biomarker for macrophage activation and atherosclerosis [12]. CXCL16 promotes inflammation, lipid overload and matrix degradation [41]. ABCAI exports unesterified cholesterol and phospholipids from cells to nascent HDL and therefore has a key role in the control of the initial step in RCT. ABCA1 mRNA expression in circulating mononuclear cells from type 2 diabetic patients is significantly reduced compared with healthy subjects [42]. 27-hydroxylase is highly expressed in PBMC and THP-1 monocytes/macrophages. Cholesterol 27-hydroxylase activity provides a pathway for elimination of intracellular cholesterol by conversion to more polar metabolites that are transported to the liver for excretion. 27-hydroxycholesterol behaves like a statin, potently inhibiting HMG CoA reductase while also suppressing smooth muscle cell proliferation and diminishing macrophage foam cell formation [18].

The pro-atherogenic alteration in multiple cholesterol transport genes by AGEs warrants further study in the pursuit of better ways to predict and reduce cardiovascular disease risk in diabetes. The present work encourages exploration of approaches that normalize cholesterol transport in diabetes as a means to achieve favorable effects on individual cardiovascular risk.

Acknowledgments

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