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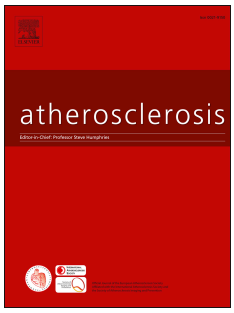

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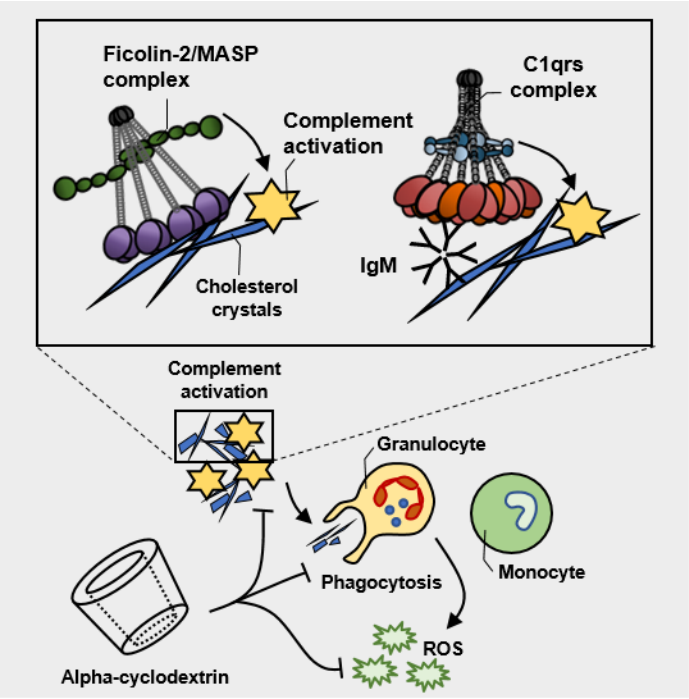

Alpha-cyclodextrin inhibits cholesterol crystal-induced complement-mediated inflammation: A potential new compound for treatment of atherosclerosis

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Abstract

Background and aims: Cholesterol crystal (CC)-induced inflammation is a critical step in the development of atherosclerosis. CCs activate the complement system and induce an inflammatory response resulting in phagocytosis of the CCs, production of reactive oxygen species (ROS) and release of cytokines. The cyclodextrin 2-hydroxypropyl-β-cyclodextrin has been found to reduce CC-induced complement activation and induce regression of established atherosclerotic plaques in a mouse model of atherosclerosis, thus inhibition of complement with cyclodextrins is a potential new strategy for treatment of inflammation during atherosclerosis. We hypothesized that other cyclodextrins, like α-cyclodextrin, may have related functions.

Methods: The effect of cyclodextrins on CC-induced complement activation, phagocytosis, and production of ROS from granulocytes and monocytes was investigated by flow cytometry and ELISA.

Results: We showed that α-cyclodextrin strongly inhibits CC-induced complement activation by inhibiting binding of the pattern recognition molecules C1q (via IgM) and ficolin-2. The reduced CC-induced complement activation mediated by α-cyclodextrin resulted in reduced phagocytosis and reduced ROS production in monocytes and granulocytes. Alpha-cyclodextrin was the most effective inhibitor of CC-induced complement activation, with the reduction in deposition of complement activation products being significantly different from the reduction induced by 2-hydroxypropyl-β-cyclodextrin. We also found that α-cyclodextrin was able to dissolve CCs.

Conclusions: This study identified α-cyclodextrin as a potential candidate in the search for therapeutics to prevent CC-induced inflammation in atherosclerosis.

Introduction

Atherosclerosis is a chronic inflammatory disease caused by accumulation of lipids, cholesterol, and inflammatory cells in the vessel wall. Lipid-lowering medications are used to reduce the risk of developing cardiovascular diseases and stroke caused by atherosclerosis, but the response to lipid-lowering medications varies widely among individuals and this leaves the patients with a considerable residual risk of death due to cardiovascular disease in spite of the treatment[1].

Cholesterol crystals (CCs), found in extracellular spaces and within macrophages in both early and late atherosclerotic plaques, are an important trigger of the inflammation in atherosclerosis[2–5]. CCs trigger an innate immune response by activation of the three complement pathways resulting in opsonization of CCs by C3b/inactivated C3b (iC3b) and production of the anaphylatoxins C3a and C5a[6–9]. This causes a downstream inflammatory response where the CCs are phagocytosed by granulocytes and monocytes and results in production of reactive oxygen species (ROS), activation of the NLRP3 inflammasome, and induction of a cytokine response[3,10–12]. *In vivo*, complement activation in atherosclerotic lesions has been observed[13–16], and we have shown that CCs are co-localized with complement pattern recognition molecules (PRMs) and activations products in atherosclerotic plaques[9]. Thus, preventing CC-induced inflammation by inhibiting complement activation or increasing removal of CCs could be a potential new treatment of atherosclerosis.

Recently, a member of the cyclodextrin (CD) family – 2-hydroxypropyl-β-cyclodextrin (2HPBCD) – was found to reduce atherogenesis and induce regression of established plaques in a mouse model of atherosclerosis[17]. 2HPBCD reduced CC-induced inflammation by inhibiting initiation of the complement system in human whole blood[18]. CDs are cyclic oligosaccharides, made up of six,

seven, or eight α-D-glucopyranose units corresponding to the native cyclodextrins: alpha-cyclodextrin, beta-cyclodextrin, and gamma-cyclodextrin respectively. The cyclodextrins have the shape of a truncated cone and form inclusion complexes with a wide range of molecules e.g. lipids, carbohydrates, proteins and nucleic acids[19]. To our knowledge, the effect of the other native CDs: α-cyclodextrin and γ-cyclodextrin and their hydroxy-propylated derivates on CC-induced complement activation has not previously been described.

We therefore decided to screen a panel of cyclodextrins (CDs): α-cyclodextrin (ACD); 2-hydroxypropyl-α-cyclodextrin (2HPACD); 2-hydroxypropyl-γ-cyclodextrin (2HPGCD), and to compare them to the previously described 2HPBCD[18] in relation to their ability to inhibit CC-induced inflammation. Native β-cyclodextrin and γ-cyclodextrin was not included due to their low solubility in aqueous solutions. The specific objectives were to determine if the CDs inhibited CC-induced complement activation and if this lead to changes in the downstream inflammatory response measured as phagocytosis of CCs and production of ROS in granulocytes and monocytes. Furthermore, we wanted to investigate if the CDs dissolved CCs.

Materials and methods

Reagents

Ultrapure cholesterol (C8667), 1-propanol (279544), bovine serum albumin (BSA) (A2153), human serum albumin (HSA) (A9731), alpha-cyclodextrin (C4680), 2-hydroxypropyl-alpha-cyclodextrin (390690), 2-hydroxypropyl-beta-cyclodextrin (C0926), methyl-beta-cyclodextrin (MBCD) (C4555), 2-hydroxypropyl-gamma-cyclodextrin (H125) were purchased from Sigma-Aldrich. EDTA (324503) was purchased from Calbiochem. Streptavidin-HRP was from GE Healthcare (RPN1231), TMB One was from Kem-En-Tec Diagnostics, lysing buffer was from DAKO (S2364), hirudin vials was from Roche (06675751), and PHAGOBURST™ kit was from BD Biosciences (341058).

The C3 inhibitor compstatin Cp40 was provided from Professor John D. Lambris (University of Pennsylvania)[20].

Commercial antibodies: Rabbit anti-C3c polyclonal antibody (pAb) (DAKO, A0062), rabbit anti-C4c pAb (DAKO, Q0369), rabbit anti-C1q pAb (DAKO, A136), rabbit anti-IgM pAb (DAKO, A0425), rabbit anti-IgA pAb (DAKO, A0262), mouse anti-C5b-9 monoclonal antibody (mAb) (IgG2a) (Antibody Shop, 011-01), mouse-IgG2a isotype-control (BD Bioscience, M5409), rabbit-IgG isotype-control (Invitrogen, 10500C), FITC-conjugated goat anti-rabbit pAb (Sigma-Aldrich, F1262), FITC-conjugated goat anti-mouse pAb (DAKO, F0479), HRP-conjugated donkey anti-rabbit-IgG (GE Healthcare, NA934V), anti-CD14-PE (BD, 345785), and anti-CD45-FITC/anti-CD14-PE (BD, 342408).

In house produced antibodies: mouse anti-C4c mAb clone 99-72-18, mouse anti-C3bc mAb clone BH6, mouse anti-C9 mAb clone aE11, mouse anti-C6 mAb clone 9C4, and mouse anti-ficolin-2 mAb clone FCN219.

Preparation of CCs

CCs were prepared essentially as described by Samstad *et al.* (2014)[11]. Ultrapure cholesterol (200 mg) was dissolved in 1-propanol (100 ml). Distilled water (150 ml) was added to the solution and it was left undisturbed for 15 min for the crystals to stabilize. The solution was centrifuged for 15 min at 3000*xg* and the pellet was left to dry. All steps were performed at room temperature (RT). The CCs were resuspended in PBS/0.05% HSA and stored at 4 °C in the dark.

Collection of hirudin plasma

A pool of normal human plasma was obtained by drawing venous blood from eight healthy donors (four male and four female donors) into hirudin vials. Plasma was collected by centrifugation at 2000*x*g for 15 min, pooled, and stored at -80 °C, awaiting further analysis.

Flow cytometry

Samples were analysed by flow cytometry using a Gallios flow cytometer (Beckman Coulter) and data were analysed using Kaluza software.

Complement deposition on CCs

Complement deposition on CCs from plasma was assessed by flow cytometry. CCs (1 x 10^6 particles/ml) were incubated with 10% hirudin plasma ± 2.5 mM, 5 mM or 10 mM ACD, 2HPACD, 2HPBCD, 2HPGCD or 20 µM C3 inhibitor compstatin Cp40 (30 min at 37 °C). Deposition of C4b/iC4b and C3b/iC3b was detected using 0.1 µg/ml rabbit anti-C4c or rabbit anti-C3c (30 min at 4 °C), followed by 2 µg/ml FITC conjugated goat anti-rabbit (20 min at 4 °C). C5b-9 deposition was detected using 2 µg/ml mouse anti-C5b-9 (30 min at 4 °C), followed by 2.5 µg/ml FITC conjugated goat anti-mouse (20 min at 4 °C). CCs were washed in barbital buffer (5 mM barbital sodium, 145 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, [pH 7.4])/0.5% heat inactivated fetal calf serum (HI-FCS) after each step.

Binding of C1q, ficolin-2, IgM, and IgA to CCs

Binding of C1q, ficolin-2, IgM, and IgA to CCs from plasma was assessed by flow cytometry. CCs (1 x 10^6 particles/ml) were incubated with 5% hirudin plasma ± 10 mM ACD, 10 mM 2HPBCD, or 20 µM C3 inhibitor compstatin Cp40 (30 min at 37 °C). Binding of C1q, IgM, and IgA was

detected using 0.5 µg/ml rabbit anti-C1q, 1 µg/ml rabbit anti-IgM, or 1 µg/ml rabbit anti-IgA (30 min at 4 °C), followed by 2 µg/ml FITC conjugated goat anti-rabbit (20 min at 4 °C). Ficolin-2 binding was detected using 5 µg/ml mouse anti-ficolin-2 (30 min at 4 °C), followed by 2.5 µg/ml FITC conjugated goat anti-mouse (20 min at 4 °C). CCs were washed in barbital buffer/0.5% HI-FCS after each step.

Phagocytosis

Hirudin whole blood (100 µl) ± 10 mM ACD, 10 mM 2HPBCD, or 20 µM C3 inhibitor compstatin Cp40 was incubated for 30 min at 37 °C with PBS or CCs (6 x 10^6 particles/ml). Cells were stained with anti-CD45-FITC/anti-CD14-PE (30 min at 4 °C) and red blood cells were lysed. Samples were washed in PBS and run on the flow cytometer. Granulocytes (CD14-medium) and monocytes (CD14-high) were gated based on CD45/CD14 expression. Phagocytosis was determined as a shift in side scatter induced by ingestion of CC and quantified as percentage of phagocytic cells in a gate.

ROS production

ROS were detected using PHAGOBURST™ kit following the manufacturer's protocol, with some modifications. Hirudin whole blood (100 µl) ± 10 mM ACD, 10 mM 2HPBCD, or 20 µM C3 inhibitor compstatin Cp40 was incubated for 10 min at 37 °C with PBS or CCs (6 x 10^6 particles/ml). Samples were stained with 20 µl dihydrorhodamine-123 (DHR-123) substrate and incubated for 10 min at 37 °C. Red blood cells were lysed and cells were fixed using the PHAGOBURST™ kit reagent F. Cells were washed in PHAGOBURST™ kit reagent A, stained with anti-CD14-PE for 15 min at RT, washed in PHAGOBURST™ kit reagent A and run on the flow cytometer. Granulocytes (CD14 medium) and monocytes (CD14 high) were gated based on

CD14 expression. Gating was performed on CD14/side scatter dot plots. ROS production was quantified as: percentage of phagocytic cells producing ROS (able to convert DHR-123 into rhodamine-123 (R-123)) or enzymatic activity (MFI, amount of R-123 per cell).

Dissolution of CC

CCs (2 x 10^6 particles/ml) were incubated with barbital buffer/50% hirudin plasma ± 10 mM ACD, 2HPBCD, or MBCD for 0-48 h at 37 °C, shaking. The CCs were washed in barbital buffer/0.5% BSA and run on the flow cytometer. The number of CCs dissolved by the different CDs were assessed as percentage in a gate compared to the whole sample. The gate was determined from a sample containing no CCs.

ELISA

Fluid phase complement activation products C4c, C3bc, and soluble C5b-9

Fluid phase complement activation products C4c, C3bc, and soluble C5b-9 (sC5b-9) were measured in hirudin plasma after incubation with CCs. 50% hirudin plasma was incubated with PBS or CCs (4.0 x 10^6 particles/ml) ± 10 mM ACD, 10 mM 2HPBCD, or 20 µM C3 inhibitor compstatin Cp40 and incubated 30 min at 37 °C. The samples were then centrifuged for 5 min at 2000*x*g and the supernatants were stored at -80 °C until analysis. The levels of complement activation products were measured in previously described sandwich ELISAs[21–23].

Statistical analysis

GraphPad Prism version 7 was used for statistical analysis. Data are expressed as mean ± SEM. Fig. 1 and 5: Statistical analysis was performed on three independent experiments using 2-way ANOVA with Bonferroni's multiple comparison correction. $p < 0.05$ was considered statistically significant.

Multiplicity adjusted p-values: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. Fig. 2 and 4: Statistical analysis was performed on three independent experiments using paired t-test. $P < 0.05$ was considered statistically significant. * $p \leq 0.05$; ** $p \leq 0.01$.

Ethical approval

The study was approved by the regional health ethics committee in the Capital Region of Denmark (reference no. H2-2011-133) and conducted in accordance with the principles of the Declaration of Helsinki. All participants signed a written informed consent.

Results

ACD dose-dependently inhibits deposition of complement activation products C4b/iC4b, C3b/iC3b, and C5b-9 on CCs

The effect of the CDs on CC-induced complement activation was examined by flow cytometry by measuring deposition of complement activation products (C4b/iC4b, C3b/iC3b, and C5b-9) on CCs. We found that ACD significantly and dose-dependently reduced complement deposition on CCs (Fig. 1). ACD was the most effective inhibitor of CC-induced complement activation compared to the previously described 2HPBCD[18]. The effect of ACD on reducing the deposition of complement activation products was statistically significant different from the effect of 2HPBCD using 5 mM or 10 mM ACD vs. 2HPBCD measuring C4b/iC4b and C3b/iC3b deposition, and 2.5 mM and 5 mM ACD vs. 2HPBCD measuring C5b-9. 2HPACD and 2HPGCD had partial complement inhibitory effects when used in a concentration of 5 or 10 mM, but the inhibitory effects were not as good as for ACD and 2HPBCD. In the remaining experiments only ACD and 2HPBCD were used, since 10

mM ACD were most effective in reducing CC-induced complement activation and 2HPBCD was included as a basis for comparison with ACD.

ACD inhibits generation of fluid phase complement activation products C4c, C3bc, and sC5b-9 in plasma incubated with CCs

To investigate if the CDs were specific inhibitors of CC-induced complement activation and not inhibitors of general/systemic *in vitro* complement activation, we measured fluid phase complement activation products in plasma incubated with CDs with/without CCs. ACD significantly reduced CC-induced complement activation assessed as the level of complement activation products C4c, C3bc, and sC5b-9 (Fig. 2). The effect of ACD on reducing the CC-induced generation of fluid phase complement activation products was significantly different from the effect of 2HPBCD (significant difference measuring C4c and C3bc), confirming the results from Fig. 1 and suggesting that ACD is a more effective inhibitor of CC-induced complement activation than 2HPBCD. The complement inhibitory effect of ACD and 2HPBCD was CC-specific as the CDs had no complement inhibitory effects on complement activation in plasma without CCs incubated for 30 min at 37 °C (Fig. 2). As expected, the C3 inhibitor – compstatin – significantly inhibited both CC-induced complement activation (C3bc and sC5b-9) and complement activation in plasma without CCs incubated for 30 min at 37 °C (Fig. 2).

ACD inhibits binding of C1q, ficolin-2, IgM, and IgA to CCs

Complement activation on CCs is initiated by the complement PRMs C1q (mediated by IgM) and ficolin-2[6–9,18]. IgG does not bind to CCs[9], however IgA binds to CCs[18], but the role of IgA in CC-induced complement activations is still unknown. We therefore examined if ACD reduced binding of C1q, ficolin-2, IgM, and IgA to CCs. ACD and 2HPBCD similarly reduced binding of C1q,

ficolin-2, IgM, and IgA to CCs (observed as a reduction in median fluorescence intensity (X-Med) compared to buffer) (Fig. 3). As expected, the C3 inhibitor – compstatin – did not reduce the binding of complement PRMs to the CCs (Fig. 3).

ACD inhibits phagocytosis and production of ROS in granulocytes and monocytes

Next, we investigated how the CDs effect phagocytosis and ROS production in granulocytes and monocytes (Fig. 4). We found that ACD significantly reduced phagocytosis and ROS production in granulocytes and monocytes. 2HPBCD reduced ROS production from granulocytes and monocytes, and reduced phagocytosis of CCs, although the reduction in phagocytosis was not statistically significant. Phagocytosis of CCs and ROS production from granulocytes and monocytes was primarily complement dependent since the complement inhibitor compstatin inhibited both. Taken together Fig. 1-4 show that the CDs ACD and 2HPBCD inhibit CC-induced complement activation by inhibiting binding of C1q (via IgM) and ficolin-2 to CCs and thereby inhibit phagocytosis of the CCs and CC-induced ROS production.

ACD dissolves CCs over time

The effect of ACD on CCs dissolution was examined by incubating CCs with 10 mM ACD (Fig. 5). As positive controls, 70% ethanol, 2HPBCD, and MBCD, known to dissolve cholesterol[17,18,24], was included in this experiment. CCs were dissolved by incubation with ACD and 2HPBCD after 2-48 h incubation (Fig. 5E). No statistical difference between the percentage of dissolved CCs was observed between CCs incubated with ACD or 2HPBCD at the 0-24 h timepoints. At the 48 h timepoint incubation of CCs with 2HPBCD resulted in a higher percentage of dissolved CCs compared to ACD ($p < 0.01$, not noted in Fig. 5). MBCD did dissolved CCs already after 0.5 h, although the difference compared to incubation with buffer/50% hirudin plasma did not reach

statistical significance until the 2 h timepoint (Fig. 5E). Compared to ACD and 2HPBCD, MBCD dissolved CCs more effectively from the 2 h timepoint ($p < 0.0001$ at 2h, 24h, and 48h compared to ACD or 2HPBCD, not noted in Fig. 5). CCs were, as expected, significantly dissolved after 0.5 h incubation with 70% ethanol and the percentage of dissolved CCs increased over time (Fig. 5E).

Discussion

In this study we examined how CC-induced inflammation in plasma and whole blood was affected by a panel of CDs: ACD, 2HPACD, 2HPGCD, and 2HPBCD. The results showed that ACD was a strong and specific inhibitor of CC-induced complement activation. Furthermore, ACD reduced CC-induced inflammation and dissolved CCs. These are important findings that could be the first steps in exploring the use of ACD as a potential substance to inhibit CC-induced inflammation during atherosclerosis.

Both ACD and 2HPBCD specifically and dose-dependently inhibited CC-induced complement activation by preventing binding of the complement PRMs C1q (via IgM) and ficolin-2. ACD was a more effective inhibitor of complement activation than 2HPBCD, and in contrast to 2HPBCD, ACD inhibited phagocytosis of CCs as well as ROS production. The effects of 2HPBCD on CC-induced complement activation, phagocytosis, and ROS production found in this study were similar to what we have previously described[18].

In vivo, subcutaneous administration of 2HPBCD has been shown to prevent and induce regression of established atherosclerotic plaques in a mouse model of atherosclerosis where the mice were fed a cholesterol-rich diet[17]. 2HPBCD was shown to reduce CCs load in plaques, increase cholesterol transport, and decrease systemic inflammation[17]. The mechanism proposed is that 2HPBCD

activates the liver X receptor (LXR). This induces LXR-mediated transcriptional reprogramming improving cholesterol efflux and exerting anti-inflammatory effects[17].

In vivo, oral administration of ACD has been shown to reduce levels of proatherogenic lipoproteins and improve fatty acid profiles in LDLr-knock out (KO) mice fed a high-fat/high-cholesterol–containing diet[25]. Furthermore, oral administration of ACD reduced atherosclerotic lesion size, with only minimal change in plasma lipids, but was associated with potential beneficial changes in gut flora in apoE-KO mice[26]. In humans, oral intake of ACD has also been shown to have beneficial effects. In obese type II diabetic patients, oral intake of ACD was shown to increase insulin sensitivity and to reduce LDL-cholesterol in the patients that begun the study with hypertriglyceridemia[27]. In overweight patients, oral intake of ACD reduced body weight and reduced LDL-cholesterol and total serum cholesterol[28]. In healthy individuals, oral administration of ACD reduced the blood level of small-LDL particles and fasting plasma glucose concentration[29]. No reduction in total cholesterol or LDL-cholesterol was observed in the healthy individuals, suggesting that the lipid lowering effect of ACD is more effective in dyslipidemic and obese populations[29]. Thus, oral administration of ACD has been shown to have beneficial effects in mouse models of atherosclerosis and clinical studies on healthy individuals, overweight patients, and diabetic patients, but the effects of oral intake of ACD on atherosclerosis in humans remain to be investigated.

When administered orally, ACD and 2HPBCD are well tolerated and are Generally Recognized As Safe (GRAS) by FDA. However, 2HPBCD, experimentally used to treat the rare neurodegenerative disorder Niemann-Pick Disease Type C (NPC), has recently been linked to significant hearing loss[30]. Hearing loss has been shown in several species including human, and occur as a result of both central and peripheral administration[31–33]. In a fatal disease, like NPC where no alternative

treatment options are available, 2HPBCD might be a relevant treatment option. However, in atherosclerosis, where alternative treatment opportunities exist, a possible side effect like hearing loss may be a major limiting factor in future use. Thus, the *in vivo* effects and possible side effects of subcutaneously or parenteral administered ACD, as an alternative to 2HPBCD, should be investigated in animal models of atherosclerosis and potentially in clinical trials, although nephrotoxicity of ACD has been observed in one study in rats[34]. To our knowledge no studies on side effects like hearing loss has been performed in healthy or atherosclerotic patients treated with 2HPBCD or ACD. In this study we show that ACD dissolve CCs. Discoveries of CDs ability to dissolve CCs should be target for future research, because direct dissolution of CCs might be an additional beneficial effect of CDs in the treatment of atherosclerosis. Some limitations to this study should be mentioned. First, the results presented in this study were obtained from *in vitro* experiments, thus the effect of ACD *in vivo* remain to be investigated. Second, the plasma and whole blood samples used in the *in vitro* experiments were obtained from healthy individuals, but the results described are relevant to the clinical characteristics of patients with atherosclerosis. Therefore, a major limitation to the study design, is the lack of experiments using plasma and/or whole blood samples from patients with atherosclerosis or related pathologies, such as the metabolic syndrome, with clinical characteristics that could have had an impact on the results. Third, continuing treatment with a complement inhibition substance could lead to increased risk of infection, however, in our experiments ACD specifically inhibits only CC-induced complement activation and not complement in general. Thus, we regard this as a targeted approach that will not influence systemic complement function per se.

In conclusion, this study identifies ACD as an effective inhibitor of the upstream inflammatory response induced by CCs. CC-induced complement activation is a critical step in the development

of atherosclerosis, thus inhibition of complement with ACDs is a potential new substance to be used in the treatment of atherosclerosis. We showed superior complement inhibitory effects of ACD on CC-induced inflammation compared to 2HPBCD, but the anti-inflammatory effects of ACD in atherosclerosis *in vivo* remains to be elucidated and should be target for future research.

Conflict of Interest:

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

KP: study design, experimental work, data interpretation, drafting the article, and final approval. SSB, EDB, M-OS, YP: experimental work, data interpretation, critical revision of the article, and final approval. TE and PG: study design, data interpretation, critical revision of the article, and final approval.

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Fig. 1: ACD dose-dependently inhibits deposition of complement activation products C4b/iC4b, C3b/iC3b, and C5b-9 on CCs.

Plasma was incubated with CCs ± CDs. Deposition of complement activation products C4b/iC4b (A), C3b/iC3b (B), and C5b-9 (C) on CCs were measured by flow cytometry. Data are presented as mean + SEM, n=3. Statistical analysis was performed using 2-way ANOVA with Bonferroni's multiple comparison correction; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, **** $p \leq 0.0001$ compared to CCs in buffer or otherwise indicated. CCs, cholesterol crystals; CD, cyclodextrins; ACD, alpha-cyclodextrin; 2HPACD, 2-hydroxypropyl-alpha-cyclodextrin; 2HPBCD, 2-hydroxypropyl-beta-cyclodextrin; 2HPGCD, 2-hydroxypropyl-gamma-cyclodextrin.

Fig. 2: ACD inhibits generation of fluid phase complement activation products C4c, C3bc, and sC5b-9 in plasma incubated with CCs.

Plasma was incubated with PBS or CCs ± ACD (10 mM), 2HPBCD (10 mM), or compstatin (20 µM). Fluid phase complement activation products C4c (A), C3bc (B), and sC5b-9 (C) were measured by ELISA. Data are presented as mean ± SEM with individual data points shown as dots, n=3. Statistical analysis was performed using paired t-test; * $p \leq 0.05$; ** $p \leq 0.01$ compared to buffer incubated with CCs or otherwise indicated. CCs, cholesterol crystals; ACD, alpha-cyclodextrin; 2HPBCD, 2-hydroxypropyl-beta-cyclodextrin.

Fig. 3: ACD inhibits binding of C1q, ficolin-2, IgM, and IgA to CCs.

Plasma was incubated with CCs ± ACD (10 mM), 2HPBCD (10 mM), or compstatin (20 µM). Deposition of C1q (A), ficolin-2 (B), IgM (C) and IgA (D) on CCs were measured by flow cytometry. Data shown are one representative of three independent experiments. CCs, cholesterol

crystals; ACD, alpha-cyclodextrin; 2HPBCD, 2-hydroxypropyl-beta-cyclodextrin; X-Med, Median fluorescence intensity.

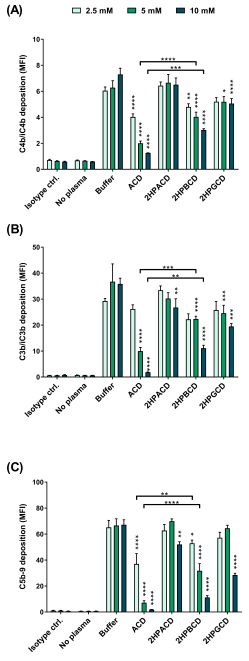
Fig. 4: ACD inhibits phagocytosis and production of ROS in granulocytes and monocytes.

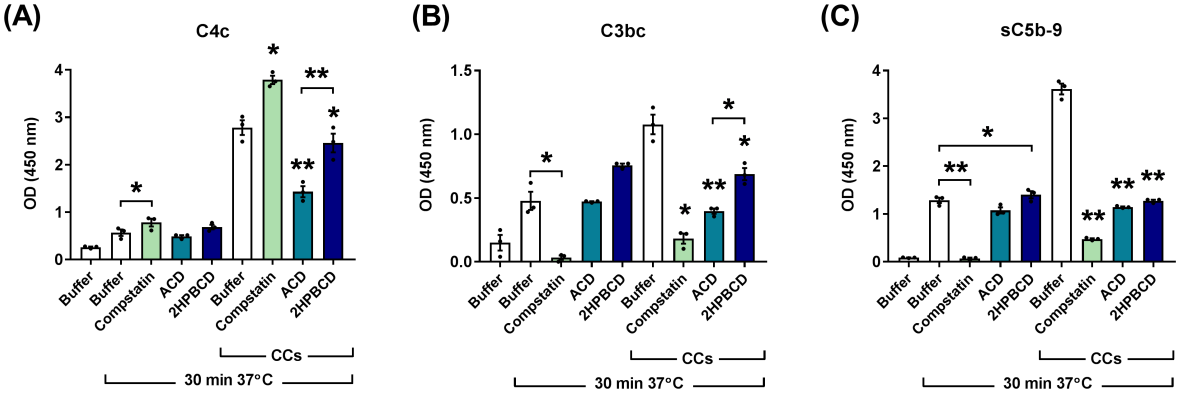
Whole blood ±ACD (10 mM), 2HPBCD (10 mM), or compstatin (20 µM) was incubated with PBS or CCs for 30 min (phagocytosis) or 10 min (ROS production) at 37 ˚C. Phagocytosis and generation of ROS was measured in granulocytes (A-C) and monocytes (D-F) by flow cytometry. ROS production was quantified as: percentage of phagocytic cells producing ROS or enzymatic activity (MFI, amount of R-123 per cell). Data are presented as mean ± SEM with individual data points shown as dots, n=3. Statistical analysis was performed using paired t-test; ns = not significant, * $p \leq 0.05$; ** $p \leq 0.01$ compared to cells in buffer incubated with CCs or otherwise indicated. CCs, cholesterol crystals; ACD, alpha-cyclodextrin; 2HPBCD, 2-hydroxypropyl-beta-cyclodextrin.

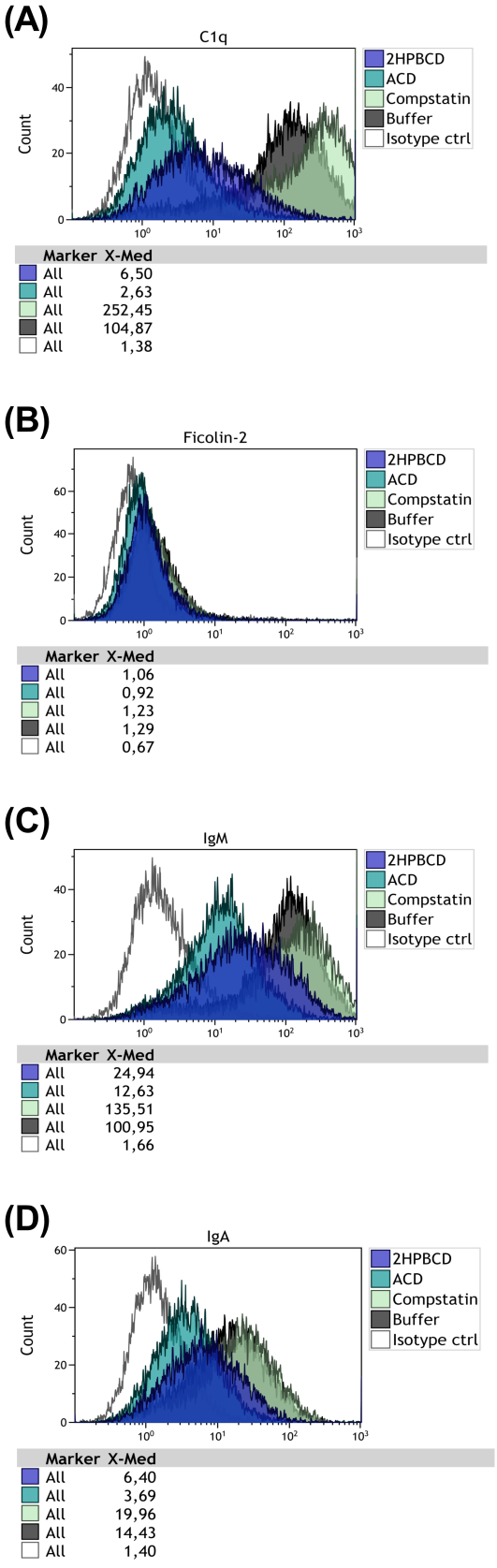
Fig. 5: ACD dissolves CCs over time.

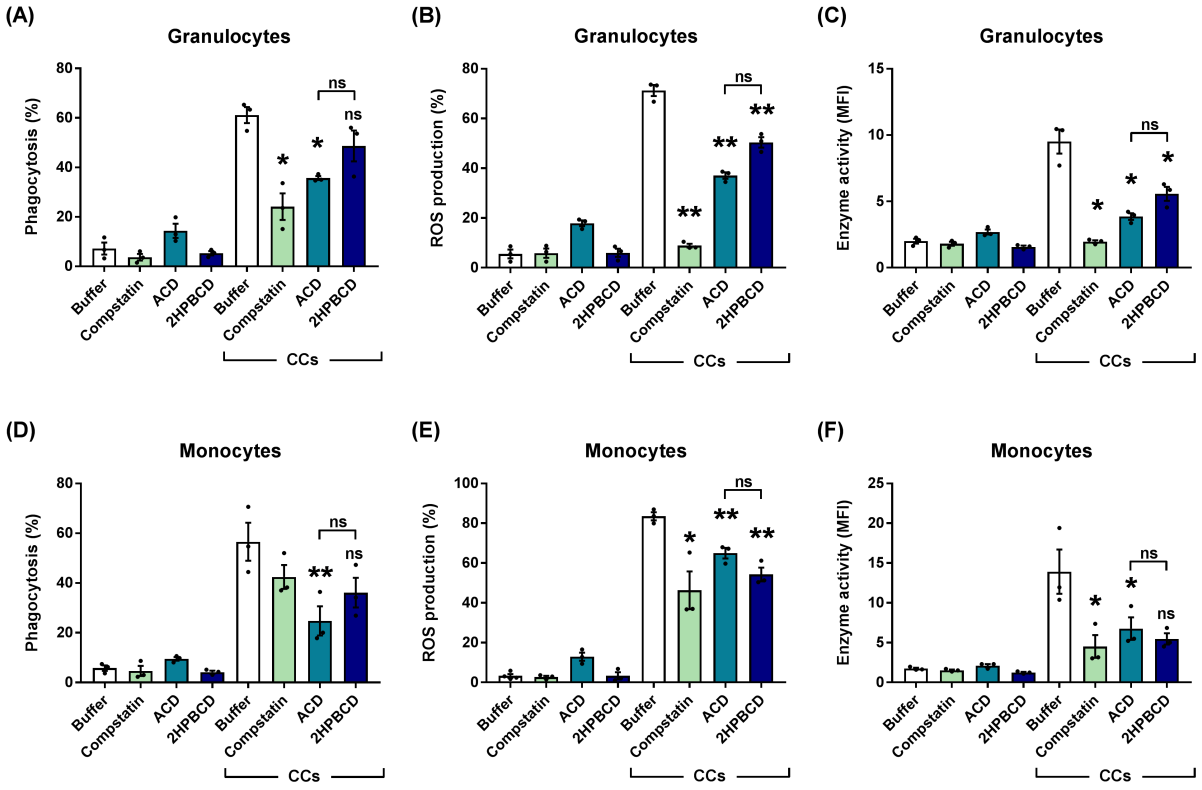
(A-D) Flow cytometer scatter plots of CCs incubated with buffer (A), 10 mM MBCD (B), or 70% EtOH (C) for 48 h. Dissolution of CCs was determined as percentage of CCs in gate A. Gate A was determined from a sample containing no CC (D). Data shown are one representative of three independent experiments. E: CCs were incubated with buffer with 50% plasma ± 10 mM CD for 0-48 h. Dissolution of CCs was determined as described in A-D. Data are presented as mean ± SEM, n=3. Statistical analysis was performed using 2-way ANOVA with Bonferroni's multiple comparison correction; ** $p \leq 0.01$; **** $p \leq 0.0001$ compared to CCs in buffer. CCs, cholesterol crystals; ACD, alpha-cyclodextrin; 2HPBCD, 2-hydroxypropyl-beta-cyclodextrin; MBCD, methyl-beta-cyclodextrin; EtOH, ethanol.

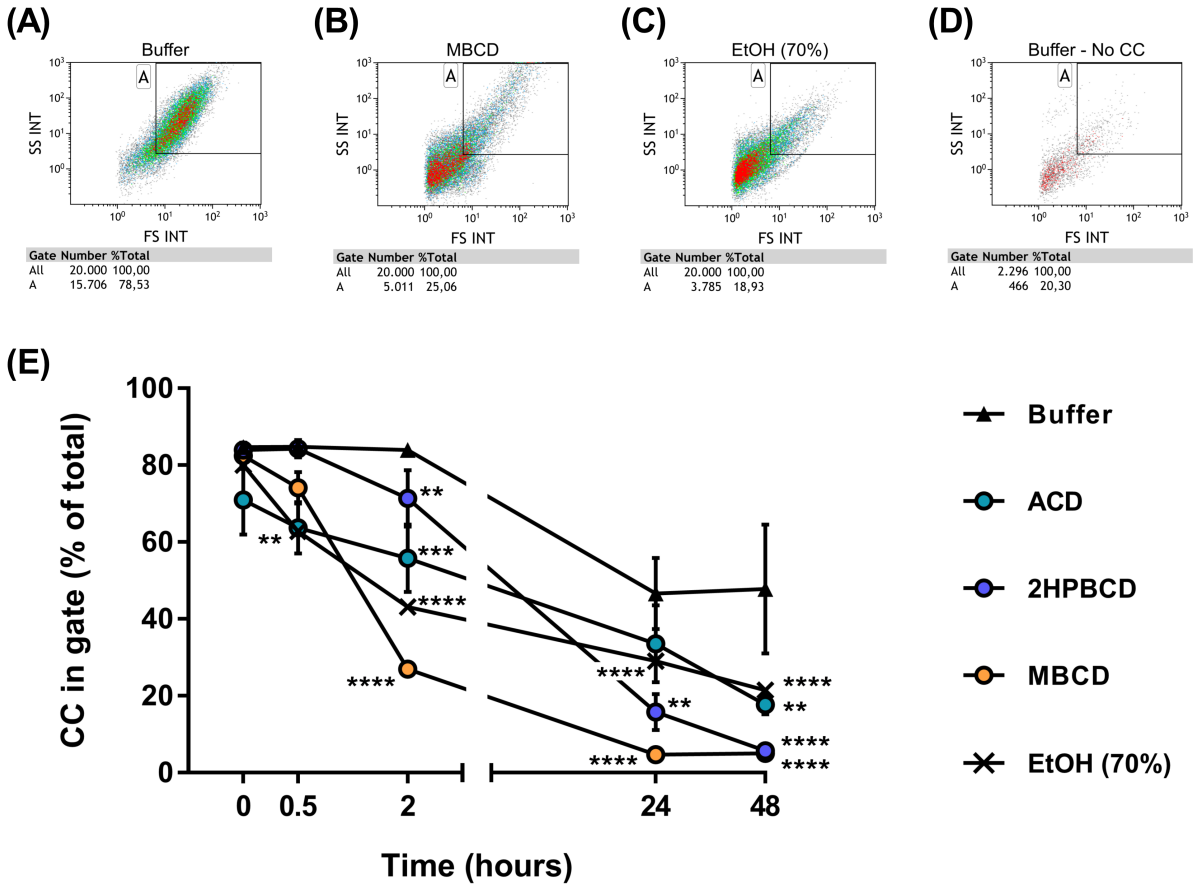
Graphical abstract: Alpha-cyclodextrin reduces cholesterol crystal-induced inflammation in plasma and whole blood, by inhibiting binding of ficolin-2 and C1q via IgM, and is a potential candidate in the search for new strategies to prevent inflammation in atherosclerosis







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Highlights

- **ACD inhibits deposition of complement activation products on cholesterol crystals**
- **ACD inhibits binding of C1q (via IgM) and ficolin-2 to cholesterol crystals**
- **ACD inhibits phagocytosis and production of ROS in granulocytes and monocytes**
- **ACD dissolves cholesterol crystals over time**

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