Cyclodextrin Reduces Cholesterol Crystal–Induced Inflammation by Modulating Complement Activation

Siril S. Bakke,*† Marie H. Aune,*† Nathalie Niyonzima,*‡ Katrine Pilely,*‡‡ Liv Ryan,* Mona Skjelland,§ Peter Garred,¶¶ Pål Aukrust,¶¶¶ Bente Halvorsen,¶¶¶¶ Eicke Latz,*†† Jan K. Damás,* Tom E. Mollnes,*¶¶¶¶¶ Terje Espevik*

Cholesterol crystals (CC) are abundant in atherosclerotic plaques and promote inflammatory responses via the complement system and inflammasome activation. Cyclic oligosaccharide 2-hydroxypropyl-β-cyclodextrin (BCD) is a compound that solubilizes lipophilic substances. Recently, we have shown that BCD has an anti-inflammatory effect on CC via suppression of the inflammasome and liver X receptor activation. The putative effects of BCD on CC-induced complement activation remain unknown. In this study, we found that BCD bound to CC and reduced deposition of Igs, pattern recognition molecules, and complement factors on CC in human plasma. Furthermore, BCD decreased complement activation as measured by terminal complement complex and lowered the expression of complement receptors on monocytes in whole blood in response to CC exposure. In line with this, BCD also reduced reactive oxygen species formation caused by CC in whole blood. Furthermore, BCD attenuated the CC-induced proinflammatory cytokine responses (e.g., IL-1α, MIP-1α, TNF, IL-6, and IL-8) as well as regulated a range of CC-induced genes in human PBMC. BCD also regulated complement-related genes in human carotid plaques treated ex vivo. Formation of terminal complement complex on other complement-activating structures such as monosodium urate crystals and zymosan was not affected by BCD. These data demonstrate that BCD inhibits CC-induced inflammatory responses, which may be explained by BCD-mediated attenuation of complement activation. Thus, these findings support the potential for using BCD in treatment of atherosclerosis.

The Journal of Immunology, 2017, 199: 2910–2920.

Therapies for atherosclerosis are characterized by a bidirectional interaction between lipids and inflammatory mechanisms that in some degree could be modulated by statins (1). However, statins may fail to improve cardiovascular outcome in some patients (2–4), and it is a global priority to find new, efficient, and cheap treatments for atherosclerotic disorders. Atherosclerosis is also considered a chronic or nonresolving inflammatory reaction where the mechanisms behind triggers of plaque inflammation have not yet been fully elucidated. Studies during the last decade, however, establish cholesterol crystals (CC) as an important trigger of inflammatory responses during development of atherosclerosis. It is thought that CC contribute to pathogenicity by fueling chronic inflammation in plaques (5) through activation of the NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome (6–8). Thus, one strategy for treatment and prevention of atherosclerosis is to inhibit the early inflammatory response to CC, which constitutes a characteristic hallmark of atherosclerosis (9).

The complement system plays a critical role in the development of atherosclerosis (10), including its ability to mediate CC-induced inflammation (8). The pattern recognition molecule C1q is the initiator of the classical pathway (CP) of complement and binds to the CC surface, resulting in downstream complement activation, opsonization, and formation of the terminal complement complex (TCC) (8). Reactive oxygen species (ROS) and proinflammatory cytokines are generated from monocytes and granulocytes that

Copyright © 2017 by The American Association of Immunologists, Inc. 0022-1767/17/$35.00

Received for publication February 24, 2017. Accepted for publication August 9, 2017.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1700302
have phagocytosed CC (8). Pattern recognition molecules of the lectin pathway (LP) include mannose-binding lectin (MBL) and the ficolins. Recently, we found that CC can activate both the CP and LP through C1q, MBL, and ficolin-2 (11). The CP, LP, and alternative complement pathways merge at C3, leading to cleavage of C3 to C3a and C3b, with subsequent cleavage of C5 to C5a and C5b, with the latter leading to the assembly of the TCC complex (C5b–C9). Additionally, C3a and C5a are themselves potent anaphylatoxins that induce potent inflammatory responses (12, 13).

The cyclic oligosaccharide 2-hydroxypropyl-β-cyclodextrin (BCD) is commonly used for drug delivery to improve solubility, bioavailability, and stability (14). Additionally, it is used as treatment of the lysosomal storage disease Niemann–Pick disease type C (15–17) and is therefore Food and Drug Administration approved and has been shown to be safe in several species (18–20). Recently we showed that BCD is effective in both preventing and treating atherosclerosis in a mouse model (21). The beneficial effects of BCD on atherogenesis include decreasing lesion size, lowering the CC burden, promoting plaque regression, increasing reverse cholesterol transport, and decreasing systemic inflammation. The mechanism proposed is that BCD initiates production of oxysterols in cells and hence activates the liver X receptor, and this was shown in mouse macrophages and in human carotid plaques. This reprograms cells to an anti-inflammatory state together with a more active cholesterol efflux resulting in less free cholesterol in the cells (21). As BCD is reported to bind to CC (21), we hypothesized that BCD may also inhibit CC-induced inflammatory responses by inhibiting complement activation.

We found that BCD decreased deposition of IgA, IgM, and complement factors on the CC surface and reduced complement activation. In accordance with this, BCD reduced CC-induced ROS and proinflammatory cytokine release from human PBMC. Moreover, BCD was a critical regulator of inflammation and complement-related gene expression in PBMC and in human carotid plaques. These observations suggest that BCD affects upstream complement activation that may attenuate inflammation in atherosclerosis.

Materials and Methods

Reagents

Cells were isolated with Polymorphprep (Axis-Shield). Lepirudin (Refudan; Celgene) before inhibitors/stimuli diluted in PBS were added and incubated at 37°C under constant rotation. Samples added included CC (1 mg/ml), BCD (10 mM), compstatin (20μM), or HSA/PBS unless otherwise stated. Plasma was isolated by centrifugation from untreated lepirudin whole blood and stored at −20°C before use in experiments. Plasma from a patient with common variable immunodeficiency (CVID) had the following values for the Igs: IgG, 3.6 mg/ml (8–16 mg/ml); IgA, 0.5 mg/ml (0.6–3.5 mg/ml); and IgM, <0.1 mg/ml (0.4–3 mg/ml). For the serum samples, venous blood from healthy donors was collected in dry glass vials with no additive and left for 2 h at room temperature for coagulation. Serum was collected by centrifugation (2500 × g for 15 min) and stored at −80°C until use. Human PBMC were isolated from whole blood using Polymorphprep according to the manufacturer’s instructions. PBMC were kept in 50% autologous plasma and pretreated with CC (10 mM) or PBS for 1 h before adding CC (2 mg/ml) or HSA/PBS for 5 or 0.5 h. Cells were lysed in RLT buffer with 1% 2-ME for RNA isolation, and supernatant was collected for multiplex cytokine assay measurement.

Human atherosclerotic carotid plaques

Data used were reanalyzed from already published results (21). In short, patients with high-grade internal carotid stenosis (≥70%) and ischemic stroke within the last month or 3 mo ago were recruited at the Department of Neurology, Oslo University Hospital, Rikshospitalet. Biopsies from atherosclerotic carotid plaques, obtained from patients, were placed in DMEM (D-MEM/F12; Life Technologies) enriched with 30 mg/ml endothoxin-free and fatty acid–free BSA (Sigma-Aldrich). The biopsies containing atherosclerotic plaques of each patient were split into macroscopically equal pieces and incubated for 16 h with 10 mM BCD or PBS and placed in RNAlater (Qiagen) for RNA analysis. Homogenization was performed with a FastPrep 24 instrument (~6 m/s; MD Biomedicals) three times for 40 s with zirconium oxide beads (Bertin Technologies) (six 2.8-mm beads and 0.8-g 1.4-mm beads per sample) in Isol-RNA lysis reagent (5Prime; VWR International). The aqueous phase was isolated after adding chloroform and centrifugation (13,000 rpm, 15 min, 4°C), and RNA was isolated further with an RNeasy micro kit (Qiagen).

Complement activation assessment

Plasma from whole blood of healthy donors was diluted 6× in PBS and incubated with BCD (5 and 10 mM) or PBS for 30 min at 37°C in the presence of HSA/PBS, CC (0.5–1 mg/ml), or monosodium urate (MSU; 0.25 mg/ml). Plasma from an immunodeficient patient was diluted 6× and incubated with BCD (10 mM) or PBS for 30 min at 37°C in the presence of CC (1 mg/ml) or HSA/PBS. A mixture of zymosan (10 mg/ml) and heat-aggregated human IgG (10 mg/ml; Octapharma) was used as a positive control. Plasma was diluted in PBS and incubated with HSA/PBS or CC (1 mg/ml), or incubated with the manufacturer’s instructions. Plasma was diluted in PBS and incubated with HSA/PBS or CC (1 mg/ml), or incubated with a 10 mM compstatin and 20 mM BCD mixture for 1 h before adding plasma to the CC. Plasma samples were incubated with HSA/PBS or CC (1 mg/ml) or HSA/PBS for 5 or 0.5 h. Cells were lysed in RLT buffer with 1% 2-ME for RNA isolation, and supernatant was collected for multiplex cytokine assay measurement.
gated using the autogating function in FlowJo (at least 80% of the events present). Control for IgM, IgA, and IgGAM experiments were FITC goat anti-mouse IgGAM, and for the rest of the experiments isotype controls were used.

**Phagocytosis, complement receptor 3, and complement receptor 1 expression**

CC (1 mg/ml) or PBS was preincubated with BCD (10 mM), compstatin CP40 (20 μM), or PBS for 15 min and then incubated with whole blood for 30 min at 37°C. Cells were fixed and RBCs lysed with lysing solution for 15 min in room temperature, and then stained with anti-CD14-FITC, anti-CD11b-Brilliant Violet 605, and anti-CD35-PE for 15 min in room temperature. The samples were run on an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo (version 10.1; Tree Star). Gating was performed on scatter-CD14 flow cytometric dot plots where the monocyte population was defined as CD14+ (high) and the granulocyte population was defined as CD14+ (medium). Phagocytosis was determined based on shift in side scatter induced by CC ingestion, and negative control was gated so that <1–3% of the events are positive. Expression of complement receptor 3 (CR3; CD11b) and complement receptor 1 (CR1; CD35) was measured as mean fluorescence intensity.

**ROS production**

ROS were detected using the oxidative burst test Phagoburst, following the manufacturer’s protocol with some modifications. CC (1 mg/ml) or PBS were preincubated with BCD (10 mM), compstatin CP40 (20 μM), or PBS for 15 min and then incubated with whole blood for 10 min at 37°C, after which dihydrorhodamine 123 substrate was added for 10 min. RBCs were lysed with FACS lysing solution for 15 min at room temperature, and the cells were washed and incubated with anti–CD14-FITC for 15 min at room temperature. The samples were run on an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo (version 10.1; Tree Star). Gating was performed on scatter-CD14 flow cytometric dot plots where the monocyte population was defined as CD14+ (high) and the granulocyte population was defined as CD14+ (medium).

**Caspase-1 activation detection**

A FAM FLICA in vitro caspase-1 detection kit was used. CC (1 mg/ml) or PBS was preincubated with BCD (10 mM), compstatin CP40 (20 μM), or PBS for 15 min and then incubated with whole blood for 4 h and incubated for 2 h with FLICA probes for caspase-1 detection. Blood was stained with anti–CD14-PE for 15 min at room temperature before RBC lysis with erythrocyte-lysing reagent for 20 min at room temperature. Analysis was performed on a BD FACSCanto II (BD Biosciences). Data were analyzed with FlowJo (version 10.1; Tree Star). Gating was performed on scatter-CD14 flow cytometric dot plots where the monocyte population was defined as CD14+ (high) and the granulocyte population was defined as CD14+ (medium).

**Gene expression and bioinformatic analysis**

RNA expression analysis was run on the nCounter analysis system, running 12 samples at a time (one strip). The procedure was performed according to the manufacturer’s instructions, applying ~100 ng mRNA. The kit used for PBMC was a fixed codeset for mRNA analysis with genes involved in human immunology nCounter GX human immunology kit v1 and v2 (NanoString Technologies). The kit used for plaques was a fixed codeset for mRNA analysis, nCounter GX human immunology kit v2 (NanoString Technologies), spiked with another 30 probes (nCounter panel plus) (21). The number of mRNA molecules per gene was accounted for detection level (mean negative controls + 2 SD of negative controls) and normalized against instrument variations (positive controls) and housekeeping genes.
found to be stable (for PBMC, G6PD, OAZ1, RPL10, POLR2A, and HPRT1; for plaques, RPL19, EEF1G, TUBB, OAZ1, GAPDH, POLR2A, G6PD, and HPRT1) using nSolver analysis software 2.5.34 (NanoString Technologies). The data were imported into Partek Genomics Suite 6.6, and the data were 2 log transformed and batch corrected for the donor variations (PBMC) and for the interaction strip and patient (plaques). For PBMC, a gene list was prepared merging genes involved in the cytokine–cytokine receptor interaction (kegg map 04060) and chemokine signaling pathway (kegg map 04062), and the BCD effect on the CC-induced genes was presented as a volcano plot with fold change (FC) and \( p \) values obtained from the ANOVA. Pathway enrichment analysis was performed in Partek Pathway for the plaque data (Fisher exact test), and gene expression of genes relevant for complement cascade was illustrated in Adobe Illustrator with 18.0.0 FCs obtained from the ANOVA.

**Measurement of cytokine release**

Supernatants from PBMC were analyzed according to the manufacturer’s instructions by multiplex cytokine assay (Bio-Plex; Bio-Rad Laboratories) for IL-1\( \alpha \), IL-1\( \beta \), IL-6, IL-8/CXCL8, MCP-1/CCL2, MIP-1\( \alpha \)/CCL3, and TNF.

**Statistical analysis**

GraphPad Prism version 5.03 (Graphpad Software) was used for analysis, and a \( p \) value <0.05 was considered statistically significant. Data are expressed as mean ± SEM. For statistical analysis, a two-way ANOVA with a Bonferroni posttest was employed in Fig. 1A and Supplemental Fig. 2, a repeated measures ANOVA with a Dunnett multiple comparisons test was used in Fig. 2C and 2D, and a Wilcoxon matched pair signed rank test was used in Figs. 5

![Image](https://example.com/image.png)

**FIGURE 2.** BCD prevents deposition of complement recognition molecules on the CC surface. (A–D) Human plasma or serum was incubated with CC with or without 10 mM BCD for 30 min. (A and B) C1q and ficolin-2 deposition on CC with (dashed line) or without (black solid line) the presence of BCD was stained in plasma with anti-C1q and anti–ficolin-2, and the secondary Abs were PE and Alexa Fluor 488, respectively. Data shown are one representative of three independent experiments; \( n = 3 \) healthy donors. Isotype control is light gray, filled. (C and D) Human serum was incubated with CC with or without 10 mM BCD, C1q (10 \( \mu \)g/ml), or ficolin-2 (10 \( \mu \)g/ml) blocking Abs for 30 min. C1q and ficolin-2 deposition on CC was stained in plasma with anti-C1q and anti–ficolin-2, and the secondary Abs were PE and Alexa Fluor 488, respectively. Isotype control is in the open columns. Control Ab for the blocking Abs is shown as indicated. Data shown are mean ± SEM; \( n = 3 \) healthy donors. ****\( p < 0.0001 \) versus PBS or as otherwise indicated.
Gene expression and multiplex cytokine assays were analyzed with Partek Genomics Suite 6.6 using ANOVA models.

Study approval

Approval no. 2009/2245 was received from the Regional Committee for Medical and Health Research Norway for the whole-blood experiments, and approval no. 2009/2259 was received for the carotid plaques. Plasma from the immunodeficient patient was obtained and used in accordance with a protocol approved by the Regional Committee for Medical and Health Research Norway (2015/419). The Regional Health Ethics Committee in the Capital Region of Denmark (H2-2011-133) has approved the serum experiments. The study complies with the principles outlined in the Declaration of Helsinki for the use of human tissue or subjects. Signed informed consent for participation in the study was obtained from all individuals.

Results

BCD inhibits CC-induced complement activation

Previously we have shown that CC initiate an inflammatory response through activation of the complement system (8). We therefore first evaluated whether BCD affected CC-induced complement activation. The results revealed that BCD specifically and significantly decreased CC-induced complement activation as assessed by a marked decrease in TCC generation in human plasma (Fig. 1A). Moreover, deposition of TCC on CC surface was reduced with BCD treatment (Fig. 1B). Likewise, binding of C3c to CC was inhibited by BCD treatment (Fig. 1C). At 10 mM concentration, which is a subtoxic dose (21), BCD was not as effective as the C3 inhibitor compstatin (20 

It has previously been reported that a 6-h incubation of CC with BCD will dissolve the crystals (21). We did not observe dissolution of the CC by BCD at 30 min, indicating that the effects observed on complement in the early stages of response to CC in plasma are not due to crystal dissolution (Supplemental Fig. 1).

BCD prevents deposition of complement pattern recognition molecules on CC

Complement activation is initiated by binding of pattern recognition molecules to targets (8, 11). Deposition of C1q and ficolin-2 on CC was measured in plasma or serum in the presence or absence of BCD or specific inhibitory Abs for 30 min. Deposition of C1q and ficolin-2 on the surface of CC was reduced in the presence of BCD (Fig. 2A, 2B), similar to the specific inhibitory Abs to C1q or ficolin-2 (Fig. 2C, 2D). In contrast, BCD had no effect on MBL binding to CC in serum or plasma (data not shown).

IgA and IgM bind to CC, and BCD prevents their depositions on the CC surface

Complement activation is also initiated by binding of Igs to target surfaces (11), most likely through interaction with the pattern recognition molecules. We first assessed the ability of native IgA,
IgG, or IgM to bind to CC. The results revealed that, in human plasma, IgM and IgA, but not IgG, were detected on the CC surface (Fig. 3A–C). Furthermore, deposition of IgA and IgM (Fig. 3D, 3E) on CC was markedly lower in plasma of a CVID patient than in the healthy donor. The binding of IgM and IgA to the CC surface (Fig. 3D, 3E) was restored by reconstitution of plasma with Pentaglobin (a mixture of IgG, IgA, and IgM) that resulted in a 2.6-fold increase in the IgM concentration in the plasma from this patient. TCC formation in response to CC and zymosan-IgG was greatly reduced in the plasma of a CVID patient in comparison with plasma from healthy individuals (Fig. 3F). This response was also restored by reconstitution of plasma with Pentaglobin (data not shown). Because BCD binds to the CC surface, we wanted to see whether the inhibitory effect of BCD on CC-induced complement was due to this compound affecting Ig deposition on CC. IgM and IgA detected on CC were reduced by BCD in human plasma (Fig. 4), but no reduction in IgM deposition was observed for compstatin (Supplemental Fig. 3E, 3F). These results demonstrate that binding of IgM and/or IgA to CC is essential for complement activation initiated by CC, and that BCD inhibits IgA and IgM deposition onto CC.

BCD reduces CC-induced increase in surface expression of phagocytic receptors on monocytes

The initiation of the complement cascade after CC exposure leads to phagocytosis of CC (8). CR3 (CD11b/CD18) recognizes mainly iC3b, whereas CR1 (CD35) recognizes C3b and C4b, and together they promote phagocytosis. Having observed a strong inhibition of complement deposition on CC by BCD, we next assessed whether BCD also reduced phagocytosis of CC. Addition of CC to whole blood resulted in phagocytosis of CC by monocytes and granulocytes; however, we found that phagocytosis of CC was not reduced by BCD (Fig. 5A, 5B). Surprisingly, BCD gave an increase in CC phagocytosis in granulocytes (Fig. 5B). Expression of CR1 and CR3 on the cell surface was increased by CC, and BCD significantly decreased both receptors in monocytes, but not in granulocytes (Fig. 5C–F). These results indicate that BCD affects phagocytic receptor expressions in monocytes in response to CC exposure.

BCD inhibits CC-induced ROS production

Our recent findings show that phagocytosis of CC leads to ROS and active caspase-1 in a complement-dependent manner (6, 8). CC-induced ROS production and caspase-1 activity were assessed in the presence or absence of BCD in granulocytes and monocytes. BCD reduced the CC-induced ROS formation in both monocytes and granulocytes (Fig. 6A, 6B). BCD had only a minimal effect on CC-induced caspase-1 activity (Fig. 6C, 6D). These results demonstrate that BCD inhibits ROS production, which may affect CC-induced inflammasome activation.

To evaluate whether any of the effects observed was due to cytotoxic responses of the substances used, whole blood was incubated with CC with or without BCD at maximum incubation time (6 h) to examine cytotoxicity. The results revealed that none of the substances in the concentrations used in this study was cytotoxic for blood cells (Supplemental Fig. 2).

BCD modifies CC-induced gene expression and reduces cytokine release in PBMC

The ability of CC to activate complement results in the release of multiple cytokines and chemokines from human blood cells (8).

![FIGURE 4](https://example.com/figure4.png)

**FIGURE 4.** BCD prevents deposition of IgA and IgM on the CC surface. (A–C) Human plasma was incubated with CC with or without BCD (10 mM) for 30 min. Deposition of IgGAM (mixture of IgG, IgA, and IgM), IgM, and IgA on the crystals with (dashed line) or without (black solid line) BCD was determined by staining with FITC-conjugated Ab against IgG, or IgM to bind to CC. The isotype control is presented as light gray, filled. Data shown are one representative of three independent experiments; n = 3 healthy donors.
Having observed a strong inhibition of BCD on CC-induced complement, we examined the effect of BCD on gene expression induced by CC in human PBMC. PBMC were isolated from whole blood, incubated with CC for 5 h in the presence or absence of BCD, and gene analysis of immunology-related genes involving cytokine–cytokine receptor interaction and chemokine signaling pathway were performed. These data revealed that BCD affected gene expressions of a range of CC-induced chemokines.

**FIGURE 5.** BCD reduces CC-induced upregulation of CR3 and CR1 in monocytes. (A–F) CC (1 mg/ml) were preincubated with BCD (10 mM), compstatin (20 μM), or PBS for 15 min, before incubation with whole blood for 30 min. (A and B) Phagocytosis of CC was determined based on side scatter signal shift after CC ingestion by monocytes and granulocytes, presented as percentage of cells with CC. Mean fluorescence intensities of CR3 (C) and (D) and CR1 (E) and (F) on monocytes and granulocytes were measured. Data shown are mean ± SEM for n = 6 healthy donors. *p < 0.05 versus CC. MFI, mean fluorescence intensity.
and cytokines and their related genes (Fig. 7A). A number of key genes that regulate inflammatory responses to CC, including the inflammasome-dependent proinflammatory cytokine IL-1β and other key genes such as IL-6 and IL-1α, were significantly reduced upon exposure to BCD. CC-induced mRNA expression of TNF was reduced by BCD but did not reach statistical significance (FC = 2.1.6, \( p = 0.06 \)). Additionally, genes related to the NLRP3 pathway, including the NLRP3 sensor (FC = 2.2.3, \( p = 0.08 \)) and caspase-1 (FC = 1.5, \( p < 0.05 \)), were also reduced by BCD treatment. Moreover, BCD significantly inhibited CC-induced mRNA expression of TNF (FC = −1.7, \( p = 0.02 \)) and NLRP3 (FC = −1.7, \( p = 0.01 \)) after 30 min of CC exposure (data not shown).

We next tested the effect of BCD on cytokines and chemokines initiated by CC in PBMC. Addition of CC to PBMC for 5 h resulted in a significant \( (p < 0.05) \) release of proinflammatory cytokines and chemokines, including IL-1β, TNF, IL-1α, IL-6, MIP-1α, whereas IL-8 was slightly increased, but did not reach

---

**FIGURE 6.** BCD inhibits CC-induced ROS formation and reduces caspase-1 activation. CC (1 mg/ml) or PBS was preincubated with BCD (10 mM), compstatin (20 μM), or PBS for 15 min before incubation with whole blood for 10 min. (A and B) ROS production is shown as a percentage of dihydrorhodamine 123 in monocytes or granulocytes in whole blood. (C and D) Activation of caspase-1 was detected as percentage of FLICA in monocytes or granulocytes in whole blood. Data shown are mean ± SEM for \( n = 9 \) healthy donors. *\( p < 0.05 \) versus PBS or as otherwise indicated.
Atherosclerotic plaques are known to contain CC as a part of their inflammatory milieu, and CC in very early atherosclerotic lesions are suggested to fuel inflammation in the plaques (5). Data were reanalyzed from already published results (21) where human carotid plaques were incubated ex vivo in the presence or absence of BCD, and mRNA was isolated and gene profiling in immunology-related genes was performed. When taking into consideration genes changed with BCD treatment, a pathway analysis revealed a significant enrichment for the pathway “complement and coagulation cascades” (kegg map 04610, p < 0.05). Visualizing this pathway revealed that many of the genes involved in the complement system were affected by BCD to a lesser or higher degree, in particular the increase in C3 and decrease in C5 expressions (Fig. 8). The reanalysis of these data (21) suggests that BCD may affect the development of atherosclerosis in human carotid plaques through modulating complement activation.

**Discussion**

BCD is an oligosaccharide that solubilizes lipophilic substances and is commonly used in pharmaceuticals (18–20). We have previously shown that CC, abundant in atherosclerotic plaques, initiate an inflammatory response via complement and NLRP3 inflammasome activation (8). Recently we have shown that BCD has an anti-inflammatory effect on CC in atherosclerotic plaques (21). In this study, we have found that initiation of complement activation on the CC surface starts with IgM, ficolin-2, and C1q that bind to CC within 30 min, in agreement with previous findings (8, 11). Additionally, depletion of C1q was previously shown to reduce CC-induced TCC formation, indicating a strong role for the CP (8, 11). C1q has been observed to bind directly and indirectly via IgM to the CC surface (11); however, whether C1q also can bind via IgA remains unknown. Based on previous data from Pilely et al. (11) it is clear that in the presence of IgM Abs, C1q is superior in activating complement on the CC compared with the LP. Ficolin-2 may exert its main role when Abs are not present and may function as an opsonin for phagocytosis independent of complement activation. Furthermore, we found that also IgA bound to the CC in human plasma, and the ability of IgA to activate the complement system was originally thought to occur mainly via the alternative pathway of complement. However, more recent studies indicate that the LP is the initiating key event (27), but the biological importance is unknown and requires further investigations. In this study, we show that addition of BCD inhibited the deposition of C1q, ficolin-2, and C3c on the CC surface, resulting in decreased generation of TCC in plasma, most likely due to competitive binding of BCD to the CC surface (11); however, whether C1q also can bind via IgA remains unknown. Based on previous data from Pilely et al. (11) it is clear that in the presence of IgM Abs, C1q is superior in activating complement on the CC compared with the LP. Ficolin-2 may exert its main role when Abs are not present and may function as an opsonin for phagocytosis independent of complement activation.

Atherosclerotic plaques are known to contain CC as a part of their inflammatory milieu, and CC in very early atherosclerotic lesions are suggested to fuel inflammation in the plaques (5). Data were reanalyzed from already published results (21) where human carotid plaques were incubated ex vivo in the presence or absence of BCD, and mRNA was isolated and gene profiling in immunology-related genes was performed. When taking into consideration genes changed with BCD treatment, a pathway analysis revealed a significant enrichment for the pathway “complement and coagulation cascades” (kegg map 04610, p < 0.05). Visualizing this pathway revealed that many of the genes involved in the complement system were affected by BCD to a lesser or higher degree, in particular the increase in C3 and decrease in C5 expressions (Fig. 8). The reanalysis of these data (21) suggests that BCD may affect the development of atherosclerosis in human carotid plaques through modulating complement activation.

**Discussion**

BCD is an oligosaccharide that solubilizes lipophilic substances and is commonly used in pharmaceuticals (18–20). We have previously shown that CC, abundant in atherosclerotic plaques, initiate an inflammatory response via complement and NLRP3 inflammasome activation (8). Recently we have shown that BCD has an anti-inflammatory effect on CC in atherosclerotic plaques (21). In this study, we have found that initiation of complement activation on the CC surface starts with IgM, ficolin-2, and C1q that bind to CC within 30 min, in agreement with previous findings (8, 11). Additionally, depletion of C1q was previously shown to reduce CC-induced TCC formation, indicating a strong role for the CP (8, 11). C1q has been observed to bind directly and indirectly via IgM to the CC surface (11); however, whether C1q also can bind via IgA remains unknown. Based on previous data from Pilely et al. (11) it is clear that in the presence of IgM Abs, C1q is superior in activating complement on the CC compared with the LP. Ficolin-2 may exert its main role when Abs are not present and may function as an opsonin for phagocytosis independent of complement activation. Furthermore, we found that also IgA bound to the CC in human plasma, and the ability of IgA to activate the complement system was originally thought to occur mainly via the alternative pathway of complement. However, more recent studies indicate that the LP is the initiating key event (27), but the biological importance is unknown and requires further investigations. In this study, we show that addition of BCD inhibited the deposition of C1q, ficolin-2, and C3c on the CC surface, resulting in decreased generation of TCC in plasma, most likely due to competitive binding of BCD to the CC surface (11), thereby preventing complement activation. In plasma from an immunodeficient patient with low IgM and IgA concentrations, the deposition of IgA and IgM on CC was reduced, along with an abolished TCC formation in response to CC. This indicates an important role for one or both Igs in complement activation by CC, and, indeed, BCD inhibited the deposition of IgM and IgA to the CC surface. When comparing BCD and compstatin for inhibition of IgM deposition on CC, we found that BCD takes IgM deposition down to background levels, whereas no inhibitory effect was observed with compstatin. Because IgM seems crucial for CC-induced complement activation (Fig. 3F) (11), we suggest that BCD primarily affects CC-induced complement activation by reducing IgM deposition on CC. Moreover, BCD did not affect formation of TCC by either MSU crystals nor zymosan. These data suggest that BCD is a specific inhibitor of CC-induced complement activation.
BCD treatment of human carotid plaques revealed a complex regulation of complement gene expressions (21). The most prominent is the downregulation of mRNA expression of C5 and upregulation of C3, the most central factors of the complement cascade. C3 knockout mice have been observed to have an enhanced atherosclerotic development and a less beneficial lipid profile than do wild-type mice (28), and C3a receptor knockout mice are more prone to severe sepsis development (29), indicating a potential anti-inflammatory role for C3 and its cleavage products and receptors (reviewed in Ref. 30). In contrast, the cleavage product C5a is a potent effector molecule in CC-mediated inflammatory responses (8). Additionally, BCD also downregulated MBL serine protease-1, C1q A and B chain, and factor B, which together represent all three pathways indicated to be involved in CC-induced complement activation (8, 11). In line with this, BCD upregulates C1 inhibitor (SERPING1), which inhibits the C1 complex and MBL serine protease-1 and -2 (Fig. 8) (21). Taken together, these results indicate a beneficial role of BCD in regulating functions of the complement system in human carotid plaques that may result in reduced inflammation.

Following complement activation, CC are phagocytosed, but in comparison with compstatin, BCD had no reducing effect on phagocytosis of CC. This result suggests that BCD and compstatin inhibit CC-induced complement activation by different mechanisms. When comparing C3c and TCC depositions on CC, BCD was less effective than compstatin in suppressing the deposition of both of these complement factors. Thus, complement opsonins may still remain in the presence of BCD that can contribute to phagocytosis of CC. Despite that BCD did not change phagocytosis of CC, it strongly reduced the CC-induced ROS formation in monocytes and granulocytes. BCD also lowered the surface expression of CR3 and CR1 on monocytes in response to CC incubation. This was not the case in granulocytes, as is in line with low cytokine release in response to CC in these cells (8). ROS formation, caspase-1 activation, NLRP3 and IL-1β mRNA expression, and IL-1β release are events coupled to NLRP3 inflammasome activation by CC (6–8). We observed a reducing effect on CC-induced IL-1β and caspase-1 mRNA expression in PBMC and reduced ROS formation in whole blood. There was also a small, but not significant, reduction in IL-1β release and NLRP3 mRNA expression in PBMC in the presence of BCD. However, the BCD effect was more evident at earlier time points. The weak effect of BCD on NLRP3 mRNA expression may be due to a high basal expression in monocytes compared with macrophages (31), and only a weak upregulation by CC is observed in primed monocytes (8). Additionally, BCD by itself significantly reduced caspase-1 activation; however, it only weakly attenuated the CC-induced caspase-1 activation. Furthermore, BCD reduced CC-induced proinflammatory cytokine release from PBMC. Gene expression of a range of chemokines and cytokines in PBMC revealed the same pattern, with downregulation of the CC-induced proinflammatory cytokines such as IL-1α,

![The Complement System](image-url)
IL-1β, and IL-6, and a slight nonsignificant reduction of TNF expression after exposure to BCD. This indicates that BCD is lowering several CC-induced inflammatory responses in human PBMC.

In this study, we have focused on upstream activation processes and found that BCD is a potent inhibitor of CC-induced complement activation, which likely contributes to the observed anti-inflammatory effects of BCD, including its effect on the cytokine profile in CC exposed PBMC. BCD seems to have its main effect on reducing IgM deposition on CC, and by this reduces CC-induced complement activation. Moreover, BCD has regulatory effects on complement-related genes in cells from human atherosclerotic plaques that may reduce C5 levels, thereby lowering its cleavage product, C5a, and preventing its potent proinflammatory effect. These effects could be beneficial and important for a potential use of BCD for treatment of atherosclerosis.

Acknowledgments

We thank Kirsten Krogh Sørensen (Department of Thoracic and Cardiovascular Surgery, Oslo University Hospital Rikshospitalet, Oslo, Norway) for contributing to collection of carotid plaques and Jacob Storgaard Jensen (Storgaard Design) for help with the graphics.

Disclosures

The authors have no financial conflicts of interest.

References

1. Taylor, F. C., M. Huffman, and S. Ebrahim. 2013. Statin therapy for primary prevention of cardiovascular disease. JAMA 310: 2451–2452.
2. Diamond, D. M., and U. Ravnskov. 2015. How statistical deception created the point of no return? J. Immunol. 196: 5064–5074.
3. Barratt-Dur, A., S. E. Pischke, P. H. Nilsson, T. Espevik, and T. E. Molines. 2016. Dual inhibition of complement components C3 and C5 as a novel approach to treat inflammatory diseases—C3 or C5 emerge together with CD14 as promising targets. J. Leukoc. Biol. 101: 193–204.
4. Lofftsson, T., P. Jarho, M. Måsson, and T. Jarvinen. 2005. Cyclooxigenasins in drug development. Expert Opin. Investig. Drugs 14: 2051–2070.
5. Brady, R. O., M. R. Filling-Katz, N. W. Barton, and P. G. Pentchev. 1989. Niemann-Pick disease types C and D. Neurol. Clin. 7: 75–88.
6. Taylor, A. M., B. Liu, Y. Mari, B. Liu, and J. J. Repu. 2012. Cyclooxigenasins mediate rapid changes in lipid balance in Npc1L1−/− mice. The wirefish, carrying cholesterolemia through the bloodstream. J. Lipid Res. 53: 2331–2342.
7. Matsuo, M., T. Togawa, K. Hirabaru, S. Mochinaga, A. Narita, M. Adachi, M. Egashira, T. Irie, and K. Ohno. 2013. Effects of cyclooxigenasins in two patients with Niemann-Pick type C disease. Mol. Genet. Metab. 108: 76–81.
8. Gould, S., and R. C. Scott. 2005. 2-Hydroxypropyl-beta-cyclooxigenasins (HP-b-c): a toxicology review. Food Chem. Toxicol. 43: 1451–1459.
9. Stella, V. J., and Q. He. 2008. Cyclooxigenasins. Toxicol. Pathol. 36: 30–42.
10. Brevestor, M. E., K. S. Estes, and N. Boo. 1990. An intravenous toxicity study of 2-hydroxypropyl-β-cyclooxigenasins, a useful drug solubilizer, in rats and monkeys. Int. J. Pharm. 59: 231–243.
11. Zimmer, S., A. Grebe, S. S. Bakke, N. Bode, B. Halvorsen, T. Ulas, M. Skjelland, D. De Nardo, L. I. Labrin, A. Kerkvloë, et al. 2016. Cyclooxigenasins promotes atherogenesis regression via macrophage reprogramming. Sci. Transl. Med. 8: 333ra50.