Atherosclerosis and Ischaemic Disease

Treatment with the GPR55 antagonist CID16020046 increases neutrophil activation in mouse atherogenesis

Fabrizio Montecucco1,2; Alexander I. Bondarenko3; Sébastien Lenglet4; Fabienne Burger4; Fabiana Piscitelli5; Federico Carbone3; Aline Roth1; Luca Liberale1; Franco Dallegri1; Karim J. Brandt6; Rodrigo A. Fraga-Silva6; Nikolaos Stergiopulos4; Vincenzo Di Marzo5*; François Mach4

1First Clinic of Internal Medicine, Department of Internal Medicine, University of Genoa, Genoa, Italy; 2IRCCE AOU San Martino - IST, Genova, Genoa, Italy; 3Circulatory Physiology Department, Bogomolets Institute of Physiology, Kiev, Ukraine; 4Division of Cardiology, Foundation for Medical Researches, Department of Medical Specialties, University of Geneva, Geneva, Switzerland; 5Endocannabinoid Research Group, Institute of Biomolecular Chemistry, Consiglio Nazionale delle Ricerche, Naples, Italy; 6Institute of Bioengineering, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

Summary
Endocannabinoids modulate atherogenesis by triggering different receptors. Recently, orphan G protein-coupled receptors (GPRs) were suggested to be activated by endocannabinoids, possibly regulating vasorelaxation. Here, we investigated whether GPR55 antagonism with CID16020046 would impact on atherosclerotic size and inflammation in two mouse models of early and more advanced atherogenesis. Eleven-week old ApoE–/– mice were fed either a normal diet (ND) for 16 weeks or a high-cholesterol diet (HD) for 11 weeks, resulting in different degrees of hypercholesterolaemia and size of atherosclerosis. CID16020046 (0.5 mg/kg) or vehicle were intraperitoneally administrated five times per week in the last three weeks before euthanasia. Treatment with CID16020046 was well-tolerated, but failed to affect atherosclerotic plaque and necrotic core size, fibrous cap thickness, macrophage and smooth muscle cell content as well as Th cell polarisation. In ND mice, treatment with CID16020046 was associated with increased chemokine production, neutrophil and MMP-9 intraplaque content as well as reduced collagen as compared to vehicle-treated animals. In HD mice, CID1602004 increased intraplaque MMP-9 and abrogated collagen content without affecting neutrophils. In vitro, serum from CID1602004-treated ND mice increased mouse neutrophil chemotaxis towards CXCL2 as compared to serum from vehicle-treated animals. CID1602004 dose-dependently induced neutrophil degranulation that was reverted by co-incubation with the GPR55 agonist ABA-CBD. In supernatants from degranulation experiments, increased levels of the endocannabinoid and putative GPR55 ligand anandamide (AEA) were found, suggesting its possible autocrine control of neutrophil activity. These results indicate that GPR55 is critical for the negative control of neutrophil activation in different phases of atherogenesis.

Keywords
Atherosclerosis, endocannabinoids, receptors, neutrophil

Introduction
Circulating lipids and lipoproteins are widely associated with plaque formation and maturation (1, 2). It is well-established that high serum levels of LDL-cholesterol increases atherosclerotic risk, while the “good” HDL-cholesterol is considered to play a protective molecule (3). More recently, other lipids such as endocannabinoids, lysophospholipids and sphingolipids were suggested to be differentially associated with athero-progression and plaque vulnerability (4, 5). Increases in endocannabinoids may have different effects in acute and chronic settings of atherosclerosis. Acute increases may stimulate a vasorelaxation (6), while chronic exposure of increased endocannabinoids, such as obesity may rather cause coronary endothelial dysfunction (7). The potential receptors of these lipid compounds have been only partially identified. For instance, lysophosphatidylglycerol (LPG), endocannabinoids (i.e. anandamide [AEA]), and 2-arachidonoylglycerol [2-AG]) or endocannabinoid-like compounds (i.e. palmitoylethanolamide [PEA]) were demonstrated to trigger intracellular phosphorylation cascades as well as intracellular calcium in cells expressing the orphan G protein-coupled receptor 55 (GPR55) (8–11). In vitro experiments with vascular and inflammatory cells demonstrated that
LPI was intracellularly generated (12–16). Subsequent studies have contradicted the role of anandamide and 2-AG as GPR55 ligands (17). Thus, the activation of GPR55 by these lipid mediators remains elusive. GPR55 was suggested as a potential receptor for plant cannabinoids in mouse models of inflammatory diseases (18–21). Not only the activation, but also the antagonism of GPR55 (leading to interference with other transmembrane receptors) might modulate inflammatory cell functions (16) and potentially endothelium-dependent vasorelaxation (22). We tested whether treatment with the GPR55 antagonist CID16020046 would modulate atherosclerotic plaque size, lipid profile and parameters of atherosclerotic plaque vulnerability in mouse models of early (ApoE+/− mice fed with normal chow diet for 16 weeks [ND]) and more advanced atherogenesis (ApoE+/− mice fed with high-cholesterol diet for 11 weeks [HD]). Then, we tested if CID16020046 and agonists (i.e. AM251 and Abn-CBD) would interfere in vitro with pro-atherosclerotic functions of mouse inflammatory cells, such as neutrophils.

**Materials and methods**

**Mouse models of atherogenesis**

Eleven-week-old ApoE−/− C57Bl/6 mice were submitted to two validated mouse models of different severity of atherogenesis to test GPR55 antagonism by 4-[4-(3-hydroxyphenyl)-3-(4-methylphenyl)-6-oxo-1H,4H,5H,6H-pyrrolo[3,4-c]pyrazol-5-yl] benzoic acid ([CID16020046], Tocris Biosciences, Bristol, UK). Before in vivo and in vitro use, the compound was tested for LPS contamination and found below the low limit of detection (<0.01 EU per 1 µg of the compound) by the Limulus Amebocyte Lysate (LAL) method. In the early atherosclerosis protocol, mice were fed under normal chow diet (N.D.) for 16 weeks to avoid the induction of severe hypercholesterolaemia and render cholesterol levels more similar to those detected in human patients (Suppl. Figure 1A, available online at www.thrombosis-online.com) (23). In the more advanced atherosclerosis protocol, mice were fed for 11 weeks under high-cholesterol diet (H.D.) (20.1% fat, 1.25% cholesterol, Research Diets, Inc., New Brunswick, NJ, USA) in saturated picric acid for 90 minutes (min). Sections from mouse specimens (Suppl. Figure 1A, available online at www.thrombosis-online.com, and found below the low limit of detection (<0.01 EU per 1 µg of the compound) by the Limulus Amebocyte Lysate (LAL) method. In the early atherosclerosis protocol, mice were fed under normal chow diet (N.D.) for 16 weeks to avoid the induction of severe hypercholesterolaemia and render cholesterol levels more similar to those detected in human patients (Suppl. Figure 1A, available online at www.thrombosis-online.com) (23). In the more advanced atherosclerosis protocol, mice were fed for 11 weeks under high-cholesterol diet (H.D.) (20.1% fat, 1.25% cholesterol, Research Diets, Inc., New Brunswick, NJ, USA) (Suppl. Figure 1B, available online at www.thrombosis-online.com) to accelerate the disease (23). Mice were randomly assigned to receive treatments either with Vehicle (0.01 % DMSO in PBS, 200 µl) or 0.5 mg/kg CID16020046 diluted in 200 µl 0.01 % DMSO in PBS (n=8 in all mouse groups). Mice were intraperitoneally injected five times per week for the last three weeks before euthanasia in both atherosclerosis models (Suppl. Figure 1A and B, available online at www.thrombosis-online.com). No analgesia or anesthesia was required before these intraperitoneal injections. Mice tolerated these treatments well, atherosclerosis protocols and no adverse events (such as weight loss and signs of systemic toxicity) were reported. At sacrifice, haematological parameters, serum triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-c) and high-density lipoprotein cholesterol (HDL-c), free fatty acids and glucose were routinely measured and expressed in mmol/l. Animals were sacrificed by exsanguination after anaesthesia with 4% isoflurane and intraperitoneal injection of ketamine-xylazine [4 mg/0.2%]). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and was approved by the local and ethics authorities (Geneva Veterinary Office and the Ethic Commission of Animal Experimentation of the University of Geneva). This study was conformed to the “position of the American Heart Association on Research Animal Use”.

**Measurements of serum inflammatory molecule levels**

Colourimetric enzyme-linked immunosorbent assay (ELISA) kits to measure serum CXCL1, CCL2, pro-matrix metalloproteinase (MMP)-9, myeloperoxidase (MPO) and Tissue inhibitor of metalloproteinase-1 (TIMP-1) levels (all from R&D Systems Inc. Minneapolis, MN, USA), and serum MMP-8 levels (Uscn Life Science Inc., Hubei, China) were used following manufacturer’s instructions. The limit of detection was 15.6 pg/ml for CXCL1, 3.9 pg/ml for CCL2, 31.25 pg/ml for pro-MMP-9, 250 pg/ml for MPO, 37.5 pg/ml for TIMP-1, 312.5 pg/ml for MMP-8. Mean intra- and inter-assay coefficients of variation (CV) were below 6%.

**Immunohistochemistry in atherosclerotic plaques**

Mouse aortic sinus was serially cut in 5 µm transversal sections, as previously described (23, 24). Sections from mouse specimens were fixed in acetone and immunostained with specific antibodies anti-mouse CD68 (macrophages, ABD Serotec, Düsseldorf, Germany), anti-mouse Ly-6G (neutrophils, BD Pharmingen™, San Jose, CA, USA), anti-mouse MMP-9 (R&D Systems), anti-mouse actin smooth muscle (Thermo Fisher Scientific Inc., Waltham, MA, USA). Quantifications were performed using the MetaMorph software. Results for other parameters were calculated as percentages of stained area on total lesion area or number of infiltrating cells per mm² of lesion area.

**Oil Red O staining for lipid content**

Five sections per mouse aortic sinus were stained with Oil Red O, as previously described (23, 24). Sections and aortas were counterstained with Mayer's hemalun and rinsed in distilled water. Quantifications were performed using the MetaMorph software. Data were calculated as ratios of stained area on total lesion area.

**Sirius red staining for collagen content**

Five sections per mouse aortic sinus were rinsed with water and incubated with 0.1% Sirius red (Sigma Chemical Co, St Louis, MO, USA) in saturated picric acid for 90 minutes (min). Sections were rinsed twice with 0.01 N HCl for 1 min and then immersed in water. After dehydration with ethanol for 30 seconds and covering the sections were photographed with identical exposure settings under ordinary polychromatic or polarised light microscopy. Total collagen content, fibrous cap thickness and necrotic
core were evaluated under polychromatic light (25, 26). Interstitial collagen subtypes were evaluated using polarised light illumination; under this condition thicker type I collagen fibres appeared orange or red, whereas thinner type III collagen fibres were yellow or green (27). Quantifications were performed with MetaMorph software. Data were calculated as percentages of stained area on total lesion area.

Real-time RT-PCR

Total mRNA was isolated with Tri-reagent (MRC Inc.) from lymphnodes, spleen and abdominal aortas of ApoE−/− mice. Reverse transcription was performed using the iProm-II Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Real-time PCR (StepOne Plus, Applied Biosystems, Waltham, MA, USA) was performed with the ABIolux™ QPCR Mix (ABgene by Thermo Scientific, Hudson, NH, USA).

Specific primers and probes (Suppl. Table 1, available online at www.thrombosis-online.com) were used to determine the mRNA expression of Gpr55, chemokines known to attract neutrophils (Cxcl1 and Cxcl2), proteases (Mpo, Mmp-8 and Mmp-9) and markers of different T helper (Th) CD4+ lymphocyte subsets (Th1: Tim3, Ifng; Th2: Gata-3, Il4; Treg: Foxp3, Il17), Tgf-β1 and Hprt (housekeeping gene) (19). The fold change of mRNA levels was calculated by the comparative Ct method. The resultant Ct values were first normalised to the internal control. This was achieved by calculating a delta Ct (ΔCt) by subtracting the internal control Ct values from the Hprt Ct value. A delta delta Ct (ΔΔCt) was calculated by subtracting the designated control ΔCt value from the other ΔCt values. The ΔΔCt was then plotted as a relative fold change with the following formula: 2-ΔΔCt.

Mouse peritoneal neutrophil isolation and migration assay

ApoE−/− mice (8–12 weeks of age, n=4) were used to obtain neutrophils from the peritoneal lavage fluids 3 hours after intraperitoneal injection of 4 % thioglycollate solution (28, 29). The purity (around 97 %) of the isolated cells was morphologically checked on stained cytopreparations. Then, neutrophils were resuspended at a density of 10⁶ cells/ml in HBSS containing 1 mM CaCl2, 2 mM MgCl2 (ICN Biomed, Irvine, CA, USA) and 0.2 % BSA (Sigma Chemicals) (mouse chemotaxis medium). Mouse neutrophil chemotaxis was assessed in a 48-well microchemotaxis chamber using a 5 µm pore (mouse chemotaxis medium). Mouse neutrophils were seeded in the upper well, while control medium or recombinant mouse CXCL2 (ICN Biomed, Irvine, CA, USA) and 0.2 % BSA (Sigma Chemicals) (mouse chemotaxis medium alone. Mouse neutrophil degranulation assay

Neutrophils were obtained from ApoE−/− mice after intraperitoneal injection of 4 % thioglycollate solution, as previously described (28, 29). The peritoneal lavage fluids of mice (n=16) was used. Neutrophils were resuspended in culture medium (serum-free RPMI 1640 medium containing 25 mmol/l Hepes) and cultured at 5×10⁵ cells per well in the presence or absence of control medium, 10 ng/ml phorbol-12-myristate-13-acetate (PMA, positive control from Sigma-Aldrich, Buchs, Switzerland), or different doses (0.01–10 µM) of CID16020046, 10 µM AM251, 10 µM Abn-CBD or CID16020046 vehicle (0.01 % DMSO). After an incubation of 30 min at 37°C in a humidified atmosphere 5 % CO2 in polystyrene plates, supernatants were collected for being tested for degranulation of pro-MMP-9, MPO and TIMP-1. Levels of pro-MMP-9, MPO and TIMP-1 in neutrophil supernatants were assessed by the same ELISA kits from R&D systems that were used to detect these molecules also in mouse serum.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from mouse neutrophils and atherosclerotic aorta with Trizol reagent (Ambion, Carlsbad, CA, USA), following the manufacturer’s instructions. The concentration and purity of RNA were determined by spectrophotometry analysis and cDNA was prepared from 1000 ng total RNA using the iProm-II Reverse Transcription System (Promega), as suggested by the manufacturer. As a negative control, reverse transcription experiments were performed by omitting the reverse transcriptase enzyme from the reactions (RT-). PCR was performed using primer pairs specific for mouse Gpr55 based on the nucleotide sequences available in GenBank (forward primer: 5′-ggacacgagggcaacat-3′ and reverse primer: 5′-acctgtcaggctagaga-3′). The house-keeping gene hypoxanthine-guanine phosphoribosyl transferase (Hprt) was used as a positive control (forward primer: 5′-ttgatagccaggactgtt-3′ and reverse primer: 5′-tataaggcaacattacagag-3′). The PCR products were separated by electrophoresis in a 2 % agarose gel and visualised by UV illumination in the presence of SYBR Safe (Invitrogen Corporation, Carlsbad, CA, USA).

Measurement of endocannabinoids (AEA and 2-AG) and endocannabinoid-like mediators (PEA and oleylethanolamide [OEA])

The extraction, purification, and quantification of endocannabinoids from mouse neutrophil supernatants was performed as on the basis of previous studies (28). After incubation (60 min, 37°C), the filters were removed from the chambers, washed and stained with Diff-Quick (Baxter, Rome, Italy). The cells of five random oil-immersion fields were counted and the chemotaxis index (C.I.) was calculated by dividing the number of cells migrated towards chemoattractants through the number of cells migrated to chemotaxis medium alone.

© Schattauer 2016

Thrombosis and Haemostasis 116:5/2016
previously described for other cell types and tissues (30). Briefly, supernatants were extracted with chloroform/methanol/Tris–HCl 50 mmol/l pH 7.5 (2: 1: 1, vol/vol) containing internal standards ([2H]8 AEA 5 pmol; [2H]5 2-AG, [2H]3 PEA and [2H]4 OEA 10 pmol each). The lipid-containing organic phase was dried down, weighed, and pre-purified by open-bed chromatography on silica gel mini-columns. Fractions were obtained by eluting the column with 99: 1, 90: 10 and 50: 50 (v/v) chloroform/methanol. The 90: 10 fraction was used for AEA, 2-AG, PEA and OEA quantification by liquid chromatography–atmospheric pressure chemical ionisation–mass spectrometry by using a Shimadzu high-performance liquid chromatography apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2020) quadrupole mass spectrometry via a Shimadzu atmospheric pressure chemical ionisation interface as previously described (30). LC analysis was performed in the isocratic mode using a Discovery C18 column (15 cm × 4.6 mm, 5 µm) and methanol/water/acetic acid (85: 15: 1 by vol.) as mobile phase with a flow rate of 1 ml/min. The amounts of endocannabinoids in supernatants, quantified by isotope dilution with the abovementioned deuterated standards, were normalised to milligram of total lipid extract.

Statistical analysis
Statistical analyses were performed with IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA). Continuous variables are presented as mean (standard deviation [SD]) or median (interquartile range [IQR]) as appropriate. Two group comparisons were then performed by two-tailed t-test (paired and unpaired) or non-parametric Mann-Whitney U test when the normality assumption of variable distribution was violated. Finally, one-way ANOVA was used for multiple group comparison. A two-sided p-value <0.05 was considered statistically significant.
Results

Treatment with CID16020046 does not affect atherosclerotic plaque size in ApoE\(^{-/-}\) mice under ND or HD

In order to investigate potential direct effects on atherogenesis of GPR55, an investigation of the expression of GPR55 in mouse aortic tissues was performed. GPR55 mRNA levels were well-detectable in aortic tissues from both adult wild-type and ApoE\(^{-/-}\) mice under HD (data not shown). No statistically significant upregulation of GPR55 mRNA levels in wild-type mouse aorta as compared to ApoE\(^{-/-}\) mice was observed (Suppl. Figure 2, available online at www.thrombosis-online.com).

Next, the effects of CID16020046 on safety and laboratory parameters were assessed at mouse euthanasia. In the early atherosclerosis protocol (ApoE\(^{-/-}\) mice under ND), treatment with CID16020046 did not induce any significant modification of mouse body and spleen weight, lipid profile and haematology (Suppl. Table 2, available online at www.thrombosis-online.com). In the advanced atherosclerosis protocol (ApoE\(^{-/-}\) mice under HD), treatment with CID16020046 was exclusively associated with a modest increase in HDL cholesterol levels (Suppl. Table 3, available online at www.thrombosis-online.com).

Finally, we investigated the amount of atherosclerotic lesions. Treatment with CID16020046 did not affect atherosclerotic lesion size in thoraco-abdominal aortas both in ApoE\(^{-/-}\) mice under either ND or HD as compared to Vehicle (Figure 1A and B). Treatment with CID16020046 was not associated with any modification of intraplaque lipid content in either models of atherogenesis as compared to vehicle-treated animals (Figure 1C and D).

Treatment with CID16020046 does not affect intraplaque necrotic core, smooth muscle cells, macrophages, and Th cell polarisation in either model of atherogenesis

In order to identify a potential role of GPR55 in the plaque structure, we tested the necrotic core and smooth muscle cell content. In addition, chronic inflammatory mechanisms regulating...
atherogenesis were investigated by measuring macrophages and T-cell subsets within atherosclerotic plaques and lymphoid organs (i.e. lymphnodes and spleen). In neither model, treatment with CID16020046 did affect intraplaque necrotic core area (Suppl. Figure 3A and B, available online at www.thrombosis-online.com), smooth muscle cell (Suppl. Figure 3C and D, available online at www.thrombosis-online.com) as well as macrophage content (Suppl. Figure 4A and B, available online at www.thrombosis-online.com) within aortic root plaques as compared to control vehicle-treated mice. In lymphnodes from ApoE−/− mice under ND, treatment with CID16020046 was associated with a weak increase in the expression of Gata3 (a genetic marker of Th2) and a reduction in Foxp3 expression (a genetic marker of Treg) as compared to vehicle-treated mice (Suppl. Table 4, available online at www.thrombosis-online.com). However, these modifications were not accompanied by any concomitant alteration in cytokines produced by these cell subsets (Suppl. Table 4, available online at www.thrombosis-online.com). No other change in Th subsets was observed in abdominal aortas and spleen (Suppl. Table 4, available online at www.thrombosis-online.com), suggesting that treatment with CID16020046 did not efficiently affect Th polarisation as compared to control vehicle. In mice under HD, treatment with CID16020046 did not affect Th cell subsets in the tissues analysed (aorta, lymphnodes and spleen) as compared to vehicle-treated mice (Suppl. Table 5, available online at www.thrombosis-online.com).

Treatment with CID16020046 increases neutrophil and MMP-9 content and reduces collagen in aortic root plaques

Treatment with CID16020046 markedly increased neutrophil intraplaque content in ND mice (Figure 2A). A non-significant (p=0.065) increase of neutrophil intraplaque content was shown in CID16020046-treated mice fed with HD as compared to vehicle (Figure 2B). Accordingly, a significant increase in intraplaque MMP-9 content was found in CID16020046-treated mice under either atherogenesis protocol as compared to vehicle (Figure 2C).

![Figure 3: Treatment with CID16020046 reduces intraplaque collagen content. A-B) On the left panel, quantification of collagen content and fibrous cap thickness (arrows). On the right panel, representative microphotographs of collagen-stained aortic root plaques from ApoE−/− mice under normal chow diet (ND). C-D) On the left, quantification of collagen content and fibrous cap thickness (arrows). On the right, representative microphotographs of collagen-stained aortic root plaques from ApoE−/− mice under high-cholesterol diet (HD). Collagen specificity is shown under bright illumination without polarisation (upper part) and under polarised light illumination (lower part). Data are expressed as median (interquartile range), n=8 per group.]
and D). A detrimental reduction in intraplaque collagen content was also demonstrated in CID16020046-treated mice compared to vehicle in both models (▶Figure 3A and C). No difference in fibrous cap thickness was shown between groups (▶Figure 3B and D). These histological features indicated that treatment with CID16020046 was associated with histological parameters of plaque vulnerability.

### Intraplaque neutrophil content in CID16020046-treated mice under ND is associated with increased levels of CXC chemokines

In order to identify the molecular mechanisms modulating neutrophil recruitment in vivo, we investigated the levels of neutrophil chemoattractants in both systemic circulation and aortas. In ND mice, treatment with CID16020046 increased the serum levels of the chemokine CXCL1 (known as potent neutrophil activator) (31) in both serum and aorta (▶Table 1). Accordingly, treatment with CID16020046 significantly upregulated Cxcl2 mRNA in mouse aorta as compared to vehicle-treated animals (▶Table 1). A borderline significant (p=0.050) increase of serum CCL2 was also observed in CID16020046-treated mice (▶Table 1). In ApoE<sup>-/-</sup> mice under HD, treatment with CID16020046 was not associated with any modification of chemokine expression levels within aorta and systemic circulation. We further analysed whether the serum from these CID16020046-treated mice would directly affect mouse neutrophil chemotaxis in vitro. We first demonstrated that mouse neutrophils express GPR55. A well-detectable mRNA expression of GPR55 was demonstrated in neutrophils isolated from eight different mouse donors (Suppl. Figure 5A, available online at www.thrombosis-online.com). The quality of mRNA samples was confirmed by the expression of housekeeping gene Hprt (Suppl. Figure 5B, available online at www.thrombosis-online.com). Then, we investigated if the serum from CID16020046-treated mice under ND (animals in which we observed the strongest neutrophil activation in vivo) might increase neutrophil locomotion in vitro towards CXCL2, a chemokine known to trigger neutrophil recruitment in atherogenesis (32). As compared to serum from vehicle-treated mice, co-incubation with serum from CID16020046-treated mice under ND increased spontaneous neutrophil migration towards CXCL2 (▶Figure 4). These data are in line with in vivo results on intraplaque neutrophil content, suggesting that increased chemokine levels following blockade of GPR55 might determine intraplaque neutrophil recruitment.

### Increased neutrophil degranulation in CID16020046-treated mice is partially associated with concomitant endocannabinoid release from these cells

Treatment with CID16020046 did not affect the mRNA expression of proteases and MPO in aortic tissues of either mouse model of atherogenesis (▶Table 2). Conversely, treatment with

#### Table 1: Chemokine levels with serum and aorta of ApoE<sup>-/-</sup> mice.

<table>
<thead>
<tr>
<th>Chemokines</th>
<th>Vehicle-treated mice (n=8)</th>
<th>CID-treated mice (n=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal diet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aorta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cxcl1, mRNA fold change</td>
<td>1.52 (0.23–3.48)</td>
<td>6.94 (5.14–18.21)</td>
<td>0.001</td>
</tr>
<tr>
<td>Cxcl2, mRNA fold change</td>
<td>1.17 (0.32–2.09)</td>
<td>10.35 (3.26–19.87)</td>
<td>0.028</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL2, pg/ml</td>
<td>57.7 (41.8–81.5)</td>
<td>127.6 (55.2–258.8)</td>
<td>0.050</td>
</tr>
<tr>
<td>CXCL1, pg/ml</td>
<td>46.9 (27.5–61.9)</td>
<td>97.6 (58.6–410.4)</td>
<td>0.021</td>
</tr>
<tr>
<td>High-cholesterol diet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aorta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cxcl1, mRNA fold change</td>
<td>0.65 (0.56–2.35)</td>
<td>1.18 (0.83–3.47)</td>
<td>0.375</td>
</tr>
<tr>
<td>Cxcl2, mRNA fold change</td>
<td>1.07 (0.72–1.43)</td>
<td>1.44 (0.96–2.91)</td>
<td>0.275</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL2, pg/ml</td>
<td>202.5 (167.7–236.8)</td>
<td>196.3 (168.3–252.1)</td>
<td>1.000</td>
</tr>
<tr>
<td>CXCL1, pg/ml</td>
<td>96.8 (65.6–129.8)</td>
<td>50.4 (16.0–84.5)</td>
<td>0.195</td>
</tr>
</tbody>
</table>

Data are expressed as median (interquartile range).

Figure 4: *In vitro* incubation with serum from CID16020046-treated mice under ND increases mouse neutrophil migration.

Mouse neutrophil migration towards 200 ng/ml recombinant CXCL2 or control medium in the presence or absence of chemotaxis medium alone (CTL) or different dilutions of serum from atherosclerotic mice (under normal chow diet (ND)) treated with vehicle or CID16020046 at sacrifice. Data are expressed mean ± SD, n=4.
CID16020046 was associated with an increase in MMP-8 levels in Apoe−/− mice fed under ND (▶Table 2). Accordingly, a non-significant increase (p=0.065) in serum MPO levels was also observed in these mice (▶Table 2). No difference between groups in mouse serum TIMP-1 levels was found in ND mice (▶Table 2). No effect on serum levels of proteases and MPO as well as TIMP-1 was induced by treatment with CID16020046 in mice under HD (▶Table 2), indicating a weak effect by the GPR55 blockade in activating circulating neutrophils as well as other cell sources of proteases in vivo when mice are fed with HD. The increase of protein amount of proteases and MPO, but not of their mRNA levels in aortic root plaques potentially indicates that treatment with CID16020046 might be associated with a degranulation of stored proteases instead of a de novo synthesis.

MMP-9 and MPO levels were tested in supernatants of degranulating neutrophils in response to different concentration of GPR55 agonists and antagonists. Incubation with 10 µM CID16020046 induced neutrophil degranulation of both MMP-9 and MPO in an amount comparable to those induced by the positive control PMA (▶Figure 5 A and B). Co-incubation with the selective GPR55 agonist Abn-CBD, but not AM251, was able to abrogate CID16020046-mediated degranulation. No effect on neutrophil degranulation was shown in presence of GPR55 agonists alone or CID16020046 vehicle (0.01% DMSO) (▶Figure 5 A and B). Finally, we evaluated whether CID16020046-induced degranulation might be associated with concomitant release of endocannabinoids (AEA and 2-AG) and endocannabinoid-like compounds (PEA and OEA) potentially activating GPR55. Therefore, these compounds were measured in supernatants of degranulating neutrophils. Neutrophil degranulation (in the response to the positive control PMA) was associated with increased levels of AEA (a previously reported GPR55 agonist) in the cell supernatants (▶Figure 6 A). CID16020046-induced degranulation was accompanied by increased levels of AEA, but also of 2-AG and OEA (▶Figure 6 A-C), although not of PEA (previously demonstrated as a GPR55 ligand) (▶Figure 6 D). These results suggest that AEA, as well as 2-AG and OEA, are produced as an adaptive response to degranulation induced (either by PMA, or as a result of the blockade of the action of other unidentified GPR55 ligands). Since the antagonist, unlike PMA, elevates the levels of 2-AG and OEA, it is possible that GPR55 tonically inhibits the biosynthesis of these two latter compounds, which inhibits degranulation.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Vehicle-treated mice (n=8)</th>
<th>CID-treated mice (n=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal diet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aorta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mmp-8, mRNA fold change</td>
<td>0.82 (0.71–2.09)</td>
<td>1.26 (1.00–1.95)</td>
<td>0.130</td>
</tr>
<tr>
<td>Mmp-9, mRNA fold change</td>
<td>1.15 (0.76–1.43)</td>
<td>1.39 (0.68–2.06)</td>
<td>0.375</td>
</tr>
<tr>
<td>Mpo, mRNA fold change</td>
<td>0.52 (0.33–8.56)</td>
<td>3.23 (0.85–7.14)</td>
<td>0.159</td>
</tr>
<tr>
<td>Tgf-b1, mRNA fold change</td>
<td>0.93 (0.87–1.26)</td>
<td>1.01 (0.88–1.18)</td>
<td>0.720</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro-MMP-9, ng/ml</td>
<td>55.7 (38.8–75.8)</td>
<td>94.2 (56.5–142.6)</td>
<td>0.130</td>
</tr>
<tr>
<td>MMP-8, ng/ml</td>
<td>75.3 (67.8–83.3)</td>
<td>100.8 (88.0–155.1)</td>
<td>0.001</td>
</tr>
<tr>
<td>MPO, ng/ml</td>
<td>71.9 (58.2–92.7)</td>
<td>114.1 (75.1–171.3)</td>
<td>0.065</td>
</tr>
<tr>
<td>TIMP-1, ng/ml</td>
<td>1.3 (1.0–1.5)</td>
<td>2.1 (1.2–3.0)</td>
<td>0.328</td>
</tr>
<tr>
<td>High-cholesterol diet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aorta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mmp-8, mRNA fold change</td>
<td>1.13 (0.63–1.55)</td>
<td>0.62 (0.38–1.07)</td>
<td>0.083</td>
</tr>
<tr>
<td>Mmp-9, mRNA fold change</td>
<td>0.75 (0.58–2.37)</td>
<td>0.88 (0.81–1.85)</td>
<td>0.560</td>
</tr>
<tr>
<td>Mpo, mRNA fold change</td>
<td>0.74 (0.39–2.98)</td>
<td>0.98 (0.90–1.33)</td>
<td>0.446</td>
</tr>
<tr>
<td>Tgf-b1, mRNA fold change</td>
<td>1.02 (0.92–1.07)</td>
<td>0.98 (0.86–1.09)</td>
<td>0.713</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro-MMP-9, ng/ml</td>
<td>122.3 (76.2–239.4)</td>
<td>179.9 (136.8–329.1)</td>
<td>0.105</td>
</tr>
<tr>
<td>MMP-8, ng/ml</td>
<td>134.6 (120.1–151.2)</td>
<td>198.2 (119.6–317.2)</td>
<td>0.234</td>
</tr>
<tr>
<td>MPO, ng/ml</td>
<td>284.6 (179.4–329.2)</td>
<td>397.9 (273.5–458.8)</td>
<td>0.105</td>
</tr>
<tr>
<td>TIMP-1, ng/ml</td>
<td>2.6 (2.2–3.8)</td>
<td>2.1 (1.9–2.8)</td>
<td>0.130</td>
</tr>
</tbody>
</table>

Data are expressed as median (interquartile range).

Table 2: Levels of MMPs, MPO, TIMP-1 and TGFβ-1 in ApoE−/− mice.
Figure 5: CID16020046 increases mouse neutrophil degranulation by antagonism of GPR55. Mouse neutrophil supernatants in the presence or absence of control medium (CTL), 10 ng/ml phorbol-12-myristate-13-acetate (PMA, positive control), different concentrations of CID16020046, GPR55 agonists (10 µM AM251 or 10 µM Abn-CBD) or CID vehicle (0.01 % DMSO) were tested for Pro-MMP-9 (A) and MPO (B) levels. Data are expressed as mean ± SD, n=16.

Figure 6: Treatment with CID16020046 increases the release from neutrophils of AEA, 2-AG and OEA, but not of PEA. Mouse neutrophil supernatants stimulated in the presence or absence of control medium (CTL), 10 ng/ml phorbol-12-myristate-13-acetate (PMA, positive control for degranulation), and increasing concentrations of CID16020046 were tested for degranulation of AEA (A), 2-AG (B), OEA (C), PEA (D) levels. Data are expressed as mean ± SD, n=5.
Discussion

**In vivo study**

We assessed if treatment with the GPR55 antagonist CID16020046 for three weeks before mouse euthanasia might impact atherosclerotic outcomes and inflammation in two mouse models characterised by early and more advanced severity of the disease (Suppl. Figure 1, available online at www.thrombosis-online.com).

The strategy of this investigation was to inhibit GPR55 activation on immune and vascular cells by endogenous ligands that are upregulated in hyperlipidaemic mice developing atherogenesis, such as the endocannabinoid 2-AG and the two endocannabinoid-like molecules PEA and OEA (23). In both models, treatment with CID16020046 was well tolerated, as demonstrated by progressive body weight gain, haematology and serum metabolism and lipid parameters. Interestingly, no effects were observed on macrophage pathophysiology and T-cell polarisation, suggesting this drug does not interfere with chronic inflammatory processes and/or to induce immunosuppression. Treatment with CID16020046 failed to affect atherosclerotic lesion size in mouse thoraco-abdominal aortas. These results might be due to reasons related to the protocol used. In fact, in order to reduce mouse stress and discomfort due to repeated injections, we were allowed to inject animals only five days per week and for a limited time period (three weeks). Since our hypothesis that antagonism of GPR55 could be associated with increased atherogenesis, a prolonged treatment approach was judged too heavy for mice. Therefore, our study was limited and we might only speculate on the effects of different treatment schedules. Also, we were unable to assess GPR55 expression (both mRNA and protein levels) in mouse aortas at different phases of atherogenesis (baseline vs early and advanced atherogenesis). Therefore, we might only hypothesise that genetic deficiency of both ApoE and GPR55 might be even more appropriate than a chronic pharmacological inhibition. Additional studies are needed to better clarify the potential impact of GPR55 activation/expression on atherogenesis. Considering the stronger relevance of plaque structure/quality rather than size/quantity (34) as a predictor of clinical outcomes, we focused on intraplaque neutrophil content and activation. Indeed, neutrophils, by releasing proteases and other toxic mediators at inflamed tissues, are known to be recruited within plaques and responsive to cannabinoids (27).

Interestingly, in ApoE<sup>-/-</sup> mice developing a limited amount of atherosclerosis (under ND), GPR55 antagonism was associated with an increase of intraplaque content of these cells, of proteases and chemokines as well as a reduction in the protective collagen (known to stabilise atherosclerotic plaques) (27, 35). All these findings suggest that antagonism of GPR55 is associated with a more unstable plaque phenotype due to neutrophil recruitment and degranulation in response to increased levels of chemokines and partially to a direct effect on the drug. Interestingly, in more advanced atherogenesis model (mice fed with HD), treatment with CID16020046 was associated with increased neutrophil degranulation, but did not affect chemokine production and neutrophil recruitment. The results suggest that blocking of GPR55 might have different effects on neutrophil pathophysiology depending on the stage of atherogenesis. Confirming previous findings (36, 37), our results demonstrated that neutrophils might exert different proatherosclerotic activities when exposed to early and advanced atherogenesis as well as different levels of hypercholesterolaemia.

**In vitro study**

In vivo results were supported by in vitro experiments using neutrophils from ApoE<sup>-/-</sup> mice, in which serum from mice with high chemokine levels potentiate the cell migration to both medium and CXCL2. In addition, neutrophil degranulation was dose-dependently induced by CID16020046. Although our pharmacological approach might imply some concerns on the selectivity of GPR55 inhibition, the effective role of CID16020046 on GPR55 was confirmed by the use of GPR55 agonists (i.e. AM251 and the more selective Abn-CBD) that abrogated degranulation induced by the antagonist. In addition, we measured the potential release of endocannabinoids and related ligands) in the neutrophil supernatants. An increase in the levels of AEA was found with both PMA and CID16020046, suggesting this endocannabinoid is produced as an adaptive response to degranulation induced either by PKC activation by PMA, or the blockade of the degranulation-inhibitory action of unidentified GPR55 ligands. Since the GPR55 antagonist, unlike PMA, also elevated the levels of 2-AG and OEA, it is possible that GPR55 tonically inhibits the biosynthesis/release of these three mediators, which are otherwise likely to inhibit degranulation via other mechanisms (i.e. CB<sub>2</sub> activation). This interpretation is highly speculative and might be controversial, since previous studies, but not others (17) suggested for AEA and PEA a role as GPR55 ligands. These discrepancies might be explained by the finding that GPR55 may influence other transmembrane receptors (i.e. CB2) (16) at a post-receptorial levels (intracellular GTPases) (10) or by other molecular mechanisms, such as co-capping or heteromerisation (38, 39). If it were to be confirmed that compounds like PEA and AEA can activate GPR55, and that this receptor negatively controls neutrophil degranulation, the neutrophil activating effect of...
CID16020046 might be due to the counteraction of the tonic action of these compounds, which were in fact found in measurable amounts also in non-stimulated cells.

Conclusion

We showed that treatment with CID16020046 was not able to reduce atherosclerotic plaque size in two mouse models of atherogenesis. Within atherosclerotic plaques, GPR55 antagonism did not affect macrophage or Th cell-mediated inflammation, but impacted on neutrophil activation and recruitment in early atherogenesis and on degranulation in the more advanced phases of the disease. GPR55 antagonism in neutrophils might block beneficial effects of endocannabinoids in atherogenesis and the modulation/activation of this receptor might be targeted for more effective and anti-atherosclerotic treatments.

Conflict of interest

None declared.

References