Vascular Adhesion Protein 1 Mediates Gut Microbial Flagellin-Induced Inflammation, Leukocyte Infiltration, and Hepatic Steatosis

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Abstract: Toll-like receptor 5 ligand, flagellin, and Vascular Adhesion Protein-1 (VAP-1) are involved in non-alcoholic fatty liver disease (NAFLD). This study aimed to determine whether VAP-1 mediates flagellin-induced hepatic fat accumulation. The effects of flagellin on adipocyte VAP-1 expression were first studied in vitro. Then, flagellin (100 ng/mouse) or saline was intraperitoneally injected to C57BL/6J WT and C57BL/6-Aoc3−/− (VAP-1 KO) mice on high-fat diet twice a week every two weeks for 10-weeks. After that, the effects on inflammation, insulin signaling, and metabolism were studied in liver and adipose tissues. Hepatic fat was quantified histologically and biochemically. Because flagellin challenge increased VAP-1 expression in human adipocytes, we used VAP-1 KO mice to determine whether VAP-1 regulates the inflammatory and metabolic effects of flagellin in vivo. In mice, VAP-1 mediated flagellin-induced inflammation, leukocyte infiltration and lipolysis in visceral adipose tissue. Consequently, increased release of glycerol led to hepatic steatosis in WT but not KO mice. Flagellin-induced hepatic fibrosis was not mediated by VAP-1. VAP-1 KO mice harbored more inflammation-related microbes than WT, while flagellin did not affect the gut microbiota. Our results suggest that by acting on visceral adipose tissue, flagellin increased leukocyte infiltration that induced lipolysis. Further, the released glycerol participated in hepatic fat accumulation. In conclusion, the results describe that gut microbial flagellin through VAP-1 induced hepatic steatosis.

Keywords: gut microbiota; liver; metabolism; inflammation

1. Introduction

Despite the high occurrence of non-alcoholic fatty liver disease (NAFLD), the exact causes of the disease remain largely unknown. However, it is increasingly accepted that the pathogenesis of NAFLD involves multiple simultaneous ‘hits’ that are associated with environmental, host genetics and physiological factors [1] including, for instance, inflammatory mediators [2], increased lipid storage, lipogenesis [3] and mitochondrial dysfunction [4]. Recent studies have also highlighted the
importance of adipose tissue dysfunction and insulin resistance [5], as well as gut-derived signals [6] in the pathogenesis of NAFLD.

During the last decade, the gut microbiota studies have advanced from detecting associations to show some of the underlying mechanisms that link the microbes to NAFLD. Our findings indicated that human subjects with high hepatic fat content over-expressed several Pathogen-associated molecular pattern-recognizing Toll-like receptor (TLR) signaling genes in the subcutaneous adipose tissue [7]. Of the ten TLR family members that are present in humans [8], the adipose tissue TLR5 associated positively with hepatic fat content and reversely with adipose tissue insulin sensitivity [9]. Further, our in vitro experiments suggested that by increasing lipolysis in adipocytes, TLR5 ligand flagellin (FLG) induced fat accumulation in hepatocytes [9]. Whether FLG induces hepatic fat accumulation in vivo is currently unknown.

Nevertheless, the role of TLR5 in hepatic health is not without controversies. In TLR5-deficient mice, gut microbiota-dependent hepatic lipogenesis was reduced [10]. Another study reported that hepatic TLR5 prevented from the onset of gut microbiota-induced liver disease [11]. While these results suggest that TLR5 protects from liver diseases, a prolonged exposure to circulating FLG has been linked to an increased risk for hepatocellular carcinoma [12] and, at high dosage FLG was shown to induce liver injury [13]. Therefore, the physiological effects of FLG need further clarification.

We have shown that also the pro-inflammatory Vascular Adhesion Protein 1 (VAP-1) is involved in NAFLD [14]. VAP-1 is a multifunctional protein with amine oxidase activity that is found in adipocytes and endothelial cells. It supports leukocyte extravasation by enzyme-activity independent and dependent ways and has insulin-like effects on energy metabolism [15]. We have observed that the amine oxidase activity of VAP-1 was involved in bacterial lipopolysaccharide-induced inflammation model [16], but so far, it is unknown whether VAP-1 mediates the inflammatory effects of other bacterial surface molecules including FLG. Therefore, in this study, we first determined whether FLG affects VAP-1 expression in cultured adipocytes that are important mediators of hepatic fat accumulation. Indeed, FLG challenge increased the expression of VAP-1, which is in agreement with our previous findings showing that FLG induced inflammation in adipocytes. Therefore, we further determined whether VAP-1 arbitrates the FLG-induced physiological changes in vivo.

2. Materials and Methods

2.1. Animals

The animal experiment was approved by the ethics committee of the Southern Finland Regional State Administrative Agency, Finland and was performed in accordance with the relevant guidelines and regulations. 8-10 weeks old C57BL/6J female mice (WT CTRL) and C57BL/6-Aoc3/- (VAP-1 KO, derived from C5BL/6J) female mice, back-grossed 13 times to their controls, were raised at the University of Turku, Finland. The mice were randomly divided into CTRL and FLG groups (n = 6/group, 3 mice/cage) and were housed in IVC racks. The mice received food and water ad libitum and were maintained on 12/12-hour light/dark cycle. The irradiated high-fat diet (HFD, 58126 DIO Rodent Purified Diet w/60% energy) was purchased from Labdiet/Testdiet, UK. The ultrapure and endotoxin-free Salmonella typhimurium FLG was purchased from Invivogen (San Diego, CA, USA) and was dissolved in endotoxin-free H2O. FLG was injected intraperitoneally twice a week every two weeks for 10-weeks using a dosage of 100ng/mouse dissolved in 100 µl of PBS. The CTRL group received 100 µL of PBS. After that, the mice were euthanized as described below to allow tissue specific analyses.
2.2. Adipocyte Cell Cultures, FLG Exposure and Protein Extraction

The human SGBS adipose cells were a kind gift from Professor Pamela Fischer-Posovszky [17]. SGBS cells were maintained as preadipocytes in DMEM/F12 supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA), 0.17 µM panthotenat plus 0.33 µM biotin (P/B, Sigma Aldrich, St Louis, MO, USA), and penicillin/streptomycin solution (Invitrogen). The preadipocytes were differentiated into the mature adipocytes by incubating them first for four days in DMEM/F12 supplemented with P/B, 0.01 mg/mL transferrin, 20 nM insulin, 100 nM cortisol, 0.2 nM triiodothyronine, 25 nM dexamethasone, 250 µM IBMX, and 2 µM rosiglitazone (all from Sigma-Aldrich). Afterwards, the medium was replaced by DMEM/F12 supplemented with P/B, 0.01 mg/mL transferrin, 20 nM insulin, 100 nM cortisol, and 0.2 nM triiodothyronine for ten days. To challenge the cells, endotoxin-free H2O as a vehicle or 10 ng/mL of FLG was added to the cultures for 4 hours. The total proteins from the challenged adipocytes were extracted at +4 °C using an ice-cold lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, and 1 mM DTT), supplemented with protease and phosphatase inhibitors (Sigma Aldrich). The supernatant was separated by centrifuging the homogenate at +4 °C for 20 min, 12,000 x g.

2.3. Murine Tissue Collection, Multiplex ELISA and Blood Analyses

After the 10-week treatment period, the overnight-fasted mice were anesthetized with CO2 and euthanized by cardiac puncture followed by cervical dislocation. Serum glycerol levels were analysed using KONELAB 20XTi analyser (Diagnostic Products Corporation, Los Angeles, CA, USA). Subcutaneous (SAT) and visceral (VAT) adipose tissue, liver and colon (after emptying the colon contents, see below in Section 2.7) were harvested, weighted, immersed in liquid nitrogen and stored at −80 °C. For the protein and gene expression analyses and functional measurements, the tissues were pulverized in liquid nitrogen to obtain homogeneous mixture of the whole tissues.

Sixteen-plex cytokine ELISA (kit 110349MS) was done from VAT protein homogenates (in 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol and 1 mM DTT) using Quansys and Q-View software (Quansys Biosciences, Logan, UT, USA). The limit of detection for each cytokine was as follows: Interleukin (IL)1α 0.93 pg/mL, IL1β 30 pg/mL, IL2 1.67 pg/mL, IL3 4.78 pg/mL, IL4 24.51 pg/mL, IL5 1.08 pg/mL, IL6 0.83 pg/mL, IL6 0.83 pg/mL, Monocyte chemotactic protein 1 (MCP-1) 5.97 pg/mL, Granulocyte-macrophage colony-stimulating factor (GM-CSF) 0.59 pg/mL, IL17 87.35 pg/mL, IL10 1.16 pg/mL, IL12p70 0.3 pg/mL, Interferon γ (IFN-γ) 0.94 pg/mL, Tumor necrosis factor α (TNFa) 0.49 pg/mL, Macrophage inflammatory protein α (MIP-1a) 0.5 pg/mL and RANTES pg/mL.

2.4. Liver Fat Content Measurement

The total hepatic lipids were extracted by homogenizing ~20 mg of pulverized liver in chloroform-methanol. The homogenates were then agitated, and the solvents washed with 0.9% NaCl. After separating the phases by centrifugation, the organic phase was evaporated under vacuum (Speedvac Savant, Thermo Scientific, Waltham, MA, USA). The dried extract was dissolved in ethanol and the triglyceride and cholesterol content was determined with KONELAB 20XTi analyser.

2.5. Real-Time Quantitative mRNA, Protein, Citrate Synthase, Xanthine Oxidase, 3-hydroxyacyl-CoA Dehydrogenase 8, and AST and ALT Analyses

The total RNA was extracted from ~100 mg of pulverized SAT and VAT, and ~20 mg of pulverized liver and colon by homogenizing with TissueLyser (Qiagen, Valencia, CA, USA) in Trizol reagent (Invitrogen) according to the supplier’s protocol. Total RNA was reversely transcribed according to the manufacturer’s instructions using High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA).

Real-time quantitative PCR (qPCR) analysis was done according to MIQE guidelines using in-house designed primers (from Invitrogen), iQ SYBR Supermix and CFX96™ Real-time PCR Detection
System (Bio-Rad Laboratories, Richmond, CA, USA). The sequences of the primers used are presented in Table 1. The expression levels of mRNA were normalized to mRNA levels of the housekeeping gene, β-actin (Actb), and the relative expression levels were calculated using a standard curve.

Table 1. The sequences of the primers used in real-time quantitative PCR.

| Primer name  | Primer sequence 5’ > 3’                  |
|--------------|-----------------------------------------|
| mACTB fwd    | GGCTGTATTTCCCTCCATCG                    |
| mACTB rev    | CCAGTGTTAACAATGCCATGT                   |
| mIL1B fwd    | TGTGAAATGCCACCTTTTA                   |
| mIL1B rev    | GGTCAAAAGITTGAAGGCA                    |
| mMMP9 fwd    | AGACGACATAGACGGCATCC                    |
| mMMP9 rev    | CTGTCGGCCTGTTTCAGT                     |
| mPLIN fwd    | TGAAGCAGGCCACCTCTC                     |
| mPLIN rev    | GACACCACCTGATCCGCT                     |
| mSOD1 fwd    | CAGGAATCTATTTAATCCTC                   |
| mSOD1 rev    | TGCCAGGTCTCCACAT                      |
| mTLR5 fwd    | AAGTTCCCCGGGAATCTGT                   |
| mTLR rev     | GCATAGCTGAGCGCTTTC                     |
| mTjp1 fwd    | GAGCGGGCTACCTTTACAA                    |
| mTjp1 rev    | GTCACTCTTTTCGGAGGCA                    |

Notes: fwd, forward; rev, reverse.

The total proteins from ~100 mg of pulverized SAT and VAT were extracted in ice-cold lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol and 1 mM DTT). The proteins from ~20 mg of pulverized liver were extracted at +4 °C using 10 x weight of ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, and 0.5% Sodium deoxycholate, 0.1% SDS and 1 mM DTT). Each buffer was supplemented with protease and phosphatase inhibitors (Sigma Aldrich, St. Louis, MO, USA). The tissues were homogenized with TissueLyser (Qiagen), kept on ice for 30 min and centrifuged for 10 min at 12,000 x g. To remove excess lipids, adipose tissue protein extracts were centrifuged twice.

A total of 20–40 micrograms of the protein extracts were separated by SDS-Page using 4%–20% Criterion gradient gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes. After blocking, the membranes were probed overnight at +4 °C with primary antibodies purchased from Cell Signaling Technology (Danvers, MA, USA) and Sigma-Aldrich (anti-GAPDH). Odyssey antirabbit IRDye 800CW or anti-rat IRDye 680RD (LI-COR Biosciences, Lincoln, NE, USA) was used as a secondary antibody. Finally, the blots were scanned and quantified using Odyssey CLX Infrared Imager of LI-COR and manufacturer’s software. All samples and results were normalized to two Ponceau S-stained bands due to differences in the expression levels of housekeeping GAPDH between the groups, except VAT and adipocyte VAP-1 that could be normalized to GAPDH.

To analyze the activities of citrate synthase (CS), xanthine oxidase (XO), 3-hydroxyacyl-CoA dehydrogenase 8 (β-HAD), aspartate aminotransferase (AST) and alanine aminotransferase (ALT), ~20 mg of pulverized livers were homogenized in ice-cold lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, and 1 mM DTT), supplemented with protease and phosphatase inhibitors (Sigma Aldrich, St Louis, MO, USA) using TissueLyser (Qiagen, Valencia, CA, USA). After centrifugation at 12,000 x g, the activity of CS, XO, AST and ALT was measured from liver protein extracts with a KONELAB 20XTi analyzer using commercial kits. β-HAD activity was measured with KONELAB 20XTi in a solution that contained 50 mM Triethanolamine-HCl (pH 7.0), 4 mM EDTA, 0.04 mM NADH, and 0.015 mM S-Acetoacyl CoA.

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2.6. Histological and Immunohistochemical Analyses

The adipose tissues were fixed in Tris-buffered zinc fixative (2.8 mM calcium acetate, 22.8 mM zinc acetate, 36.7 mM Zinc chloride in 0.1 M Tris-buffer, pH 7.4). After paraffin and endogenous peroxidase removal, the sections were stained using Vectastain Elite ABC kit (PK-6104 Vector Laboratories) according to the manufacturer’s instructions. The first stage antibody was anti-mouse CD45 (clone 30F11, BD 553076) 1 µg/mL (overnight at +4 °C) or negative control antibody. Diaminobenzedine (DAKO) was used as a chromogen, and the sections were counterstained using Mayer’s hematoxylin. CD45 cells were counted from 10 to 15 randomly selected fields and the results are shown as the number of cells divided by the number of fields. The adipocyte sizes from 500 randomly selected cells in each sample were determined with CellProfiler 2.2.0 from the H&E stained SAT sections.

The frozen liver sections were stained with Oil Red O as previously described [18]. The fat content was scored according to the droplet sizes as 0 (smallest), 1, 2, and 3 (largest). Fibrosis was studied with Van Gieson staining that was semi-quantitatively scored as follows: 0–0.5 no damage, 1–2 damage, and 3 severe damage.

2.7. 16S rRNA Gene Amplicon Sequencing and the Analysis of the Gut Microbiota Composition

The colon contents were collected and snap-frozen immediately in liquid nitrogen. The samples were stored at −75 °C. DNA was extracted with semi-automated GenoXtract and Stool Extraction Kit (Hain Lifesciences, Nehren, Germany) accompanied with an additional homogenization by bead-beating in 0.5 mm Glass PowerBead Tubes (Qiagen, Hilden, Germany) with MO BIO PowerLyzer 24 Homogenizer (MO BIO Laboratories, Inc., CA, USA). The microbiota composition was analyzed with next-generation sequencing as previously described [19]. Briefly, the V4 region of the bacterial 16S rRNA gene was amplified with barcoded primers and the PCR products were purified with Agencourt AMPure XP Magnetic beads (Beckman Coulter, Inc., Brea, CA, USA) utilizing the DynaMag™-96 mageneric plate (Invitrogen). The product length and DNA integrity were checked with TapeStation (Agilent Technologies Inc., CA, USA), and the final DNA concentrations were measured with Qubit 2.0 fluorometer (Invitrogen). The sequencing was performed with Illumina Miseq system. The quality of the sequence data was checked with fastQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and the dataset and statistics were analyzed with QIIME 1.9 pipeline [20].

2.8. Statistical Analyses

All statistical analyses were done using IBM SPSS Statistics version 24 for Windows (SPSS, Chicago, IL, USA). The group comparisons were done by analyzing the data with either ANOVA or Mann-Whitney U Test depending on whether the data was normally distributed according to Shapiro Wilks test in SPSS. The group differences in the gut microbiota composition were analyzed by Kruskal-Wallis test using QIIME 1.9 pipeline followed by Benjamini-Hochberg false discovery rate (FDR) correction [20]. The statistical significance was set at \( p < 0.05 \) unless otherwise stated.

3. Results

3.1. VAP-1 Mediated the FLG-Induced Inflammation and Lipolysis in VAT and Consequently Hepatic Fat Accumulation

FLG challenge increased the expression of VAP-1 monomer in cultured human adipocytes \( (p = 0.021) \) and in VAT of the WT mice \( (p = 0.036, \text{Figure 1a}) \). As expected, no VAP-1 expression was detected in VAT of the KO mice.
Figure 1. FLG increased VAP-1 expression in VAT (V) that mediated the FLG-induced increase in inflammation and lipolysis in VAT (V, visceral). (a) FLG increased the expression of VAP-1 monomer in cultured human adipocytes and VAT (V) of the WT mice. No VAP-1 expression was detected in KO mouse; (b) FLG increased the concentrations of IL1β, MCP-1 and GM-CSF in VAT that were impeded by VAP-1 KO; (c) FLG-injected WT mice had more CD45-positive inflammatory cells (in photographs indicated with arrows, scale bar 100 µm) in VAT compared to the FLG-injected VAP-1 KO. One entire section was scored for each mouse. Approximately, 5 fields/section were scored (range 4–9). The results are shown as the number of cells divided by the number of high power fields. (d) FLG increased the lipolysis-activating phosphorylation of HSL that was impeded by VAP-1 KO in VAT. FLG treatment decreased the expression of lipid droplet membrane protein, Plin1 mRNA that was hampered by VAP-1 KO in VAT. The increased VAT lipolysis was reflected in higher serum glycerol levels in FLG-injected WT mice compared to controls. The FLG-injected KO mice had lower levels of serum glycerol than the WT. The data are presented as mean ± SD, n = 4–6/group. The mRNA levels were calculated against a standard curve and are presented relative to Actb housekeeping mRNA. The expression and phosphorylation levels of the proteins are presented as arbitrary units (AU) normalized to GAPDH housekeeping protein. The samples run in different gels were further normalized to calibrator sample. The membranes were cut horizontally to analyze several proteins of the samples in the same run. The blots were scanned with infrared imager to avoid exposure differences between the blots. Abbreviations: PBS-injected wild type control, WT CTRL; Flagellin (FLG)-injected wild type control, WT FLG; PBS-injected VAP-1 knockout (KO) control, VAP-1 KO CTRL; Flagellin (FLG)-injected VAP-1 knockout (KO) control, VAP-1 KO FLG.

Because VAP-1 mediates leukocyte infiltration into the adipose tissue [21], we next determined the effects of FLG on VAT inflammation and lipolysis. Sixteen cytokines were analyzed from VAT protein homogenates. We found that in WT mice, FLG induced an increase in the concentrations of Interleukin 1 beta (IL1β, p = 0.048), MCP-1 (CCL2, p = 0.01), and GM-CSF, (CSF2, p = 0.016) (Figure 1b). These increases were not detected in KO mice and, FLG-injected groups did not differ from each other. The most remarkable increase was in MCP-1, which was undetected in WT CTRL (<5.97 pg/mL) and over 60 pg/mL in FLG-treated mice. VAP-1 also mediated the tendency of FLG to increase IL6 (p = 0.063, Figure 1c). No differences were observed in IL1a, IL3, IL4, IL5, IL12, IL17 or RANTES (data not shown). Under the detection limit remained IL2 (<1.67 pg/mL), IL10 (<1.16 pg/mL), IFN-γ (<0.97 pg/mL), and MIP-1α (CCL3 < 0.5 pg/mL). However, VAP-1 KO mice had higher levels of MCP-1 (p = 0.038) and GM-CSF (p = 0.01) than the WT CTRL, suggesting thus that only the FLG-induced
increase in IL1β was truly VAP-1 mediated. Therefore, the increased expression of IL1b mRNA in FLG-treated WT mice was further confirmed by qPCR ($p = 0.043$, Figure 1b).

In agreement with the increased leukocyte-attracting cytokines in VAT, the FLG-treated WT mice had more CD45-positive inflammatory cells ($p = 0.003$, Figure 1c) in VAT that was impeded by VAP-1 KO. VAP-1 also regulated FLG-induced phosphorylation of the lipolysis-activating Hormone sensitive lipase (HSL) ($p = 0.029$, Figure 1d) and FLG-suppressed expression of the lipid droplet membrane protein, Perilipin (Plin1) mRNA ($p = 0.01$, Figure 1d). Consequently, the increased lipolysis was reflected in higher serum levels of the lipolysis end product, glycerol in the FLG-injected WT mice compared to CTRL ($p = 0.009$, Figure 1d). Because lipolysis was not increased in VAP-1 KO mice, they had lower levels of serum glycerol than the WT ($p = 0.006$, Figure 1d). The higher serum glycerol that serves for triglyceride synthesis in the liver, led to a higher hepatic fat content in FLG-treated WT mice ($p = 0.005$, Figure 2a) indicating steatosis that was regulated by VAP-1. The histological results were confirmed by extracting the total lipids from liver. The FLG-injected WT but not KO mice had higher hepatic triglyceride content than the WT CTRL ($p = 0.002$) and FLG-injected KO mice ($p < 0.001$) (Figure 2b). We further determined whether the hepatic fat accumulation was accompanied with fibrosis. Van Gieson staining showed more fibrotic livers in the FLG-injected WT ($p = 0.024$) and KO ($p = 0.002$) mice (Figure 2c). VAP-1 also mediated FLG-induced expression of Mmp9 mRNA ($p = 0.004$, Figure 2c) that has been previously linked to hepatic fibrosis [22].

The hepatic TCA cycle activity was assessed by determining the activities of CS, AST and ALT from liver protein homogenates, and beta-oxidation rate by measuring β-HAD activity. While no differences in the activity of CS or β-HAD were found (data not shown), both AST ($p = 0.041$) and ALT activity ($p = 0.004$, Figure 2d) that end up supplying substrates to TCA cycle were decreased in the FLG-injected WT mice, but in the absence of VAP-1, FLG increased AST ($p = 0.015$, Figure 2d). We also determined whether the hepatic fat accumulation was accompanied with increased redox state. The activity of XO that produces superoxides did not differ between the groups (Figure 2e), but the FLG-induced expression of the mRNA of superoxide dismutase (Sod1) that eliminates the superoxides ($p = 0.046$, Figure 2e) was mediated by VAP-1.
Figure 2. FLG increases liver (L) fat content that is mediated by VAP-1. (a) The FLG-treated WT mice had higher hepatic fat content according to Oil Red O staining that was not observed in the liver of the KO mice (scale bar 100 µm). The hepatic fat content was scored according to the droplet sizes as 0 (smallest), 1, 2 and 3 (largest); (b) FLG increased hepatic triglyceride content in WT but not KO mice; (c) FLG increased hepatic fibrosis that was assessed with Van Gieson staining and by determining Mmp9 mRNA. Fibrosis seemed not be mediated by VAP-1. Van Gieson staining was semi quantitatively scored: 0–0.5 no damage, 1–2 damage and 3 severe damage; (d) FLG decreased the activity of hepatic AST and ALT in WT mice, but in the absence of VAP-1 in KO mice FLG increased both activities; (e) FLG did not affect xanthine oxidase activity but induced an increase in Sod1 mRNA that was absent in the liver of the VAP-1 KO mice. The data are presented as mean ± SD, n = 4–6/group. The mRNA levels were calculated against a standard curve and are presented relative to Actb housekeeping mRNA. Abbreviations: PBS-injected wild type control, WT CTRL; Flagellin (FLG)-injected wild type control, WT FLG; PBS-injected VAP-1 knockout (KO) control, VAP-1 KO CTRL; Flagellin (FLG)-injected VAP-1 knockout (KO) control, VAP-1 KO FLG.

3.2. VAP-1 Mediated the FLG-Induced Decrease in Insulin-Sensitive ERK1/2 Phosphorylation in SAT

FLG did not increase SAT mass (data not shown) and neither did the groups differ from each other in adipocyte sizes (Figure 3a). VAP-1 mediated the decrease in the phosphorylation of ERK1/2 (p = 0.032, Figure 3b) caused by the FLG treatment. While FLG did not increase the number of CD45-positive cells in SAT, a reduced number of cells was detected in the KO CTRL compared to the WT CTRL (p = 0.042, Figure 3c).
Figure 3. VAP-1 mediated the FLG-induced decrease in insulin-sensitive ERK1/2 phosphorylation in SAT (SC, subcutaneous). (a) No differences in the sizes of SAT adipocytes were found between the groups; (b) The phosphorylation of insulin-sensitive ERK1/2 phosphorylation decreased in SAT of FLG-treated WT mice but not VAP-1 KO mice; (c) FLG did not affect the number of CD45-positive cells in SAT. A reduced number of cells were detected in the KO controls compared to the WT CTRL. The arrows are pointing out some CD45-positive cells. Scale bar 100 µm. One entire section was scored for each mouse. Approximately 7.7 fields were scored (range 4–11). The results are shown as the number of cells divided by the number of high power fields. The data are presented as mean ± SD, n = 4–6/group. The phosphorylation levels are presented as arbitrary units (AU) normalized to Ponceau staining. The samples run in different gels were further normalized to calibrator sample. The membranes were cut horizontally to analyze several proteins of the samples in the same run. The blots were scanned with infrared imager to avoid exposure differences between the blots.

Abbreviations: PBS-injected wild type control, WT CTRL; Flagellin (FLG)-injected wild type control, WT FLG; PBS-injected VAP-1 knockout (KO) control, VAP-1 KO CTRL; Flagellin (FLG)-injected VAP-1 knockout (KO) control, VAP-1 KO FLG.

3.3. VAP-1 Knockout Modified the Gut Microbiota Composition and Intestinal Gene Expression

Because the gut microbiota has been shown to associate with liver fat content [7], we determined whether the gut microbiota had contributed to the FLG-induced steatosis. The 16S rRNA gene amplicon sequencing did not reveal any differences in alpha or beta diversity between the groups (Figure 4a), or any taxonomic changes in response to FLG. However, interestingly, VAP-1 KO mice differed significantly from the WT mice. The PBS-injected KO mice had lower relative abundance of bacteria of an unidentified genus of Bacteroidales order and Desulfovibrionaceae family ($p = 0.049$ for
both, Figure 4b), and more *Flexispira* and of an unidentified genus of Helicobacteriaceae (*p* = 0.049 for both, Figure 4b). The FLG-injected KO mice differed from the WT counterparts on the higher relative abundance of *Lactobacillus* and *Oscillospira* (*p* = 0.027 for both, Figure 4b) and lower of an unidentified genus of Bacteroidales and Desulfovibrionaceae (*p* = 0.023 for both, Figure 4b). Due to the differences in inflammation-associated microbes, we determined whether VAP-1 affected intestinal inflammation or integrity. While no differences in the mRNA of tight junction protein 1 (*Tjp1*) were found between the groups, VAP-1 KO decreased the expression of *Il1b* mRNA (*p* = 0.006) that was independent of *Tlr5* expression (Figure 4c).

**Figure 4.** VAP-1 KO mice differed from the WT mice in the gut microbiota composition and intestinal Il1b expression. (a) The Shannon indices shown as sequences per sample (upper graph) did not reveal any differences in the alpha-diversity of the gut microbiota between the groups, neither did the groups differ from each other in beta-diversity as observed in the PCoA plot of all groups (lower graph); (b) The CTRL and FLG-injected KO mice had lower abundance of an unidentified (ud) genus of Bacteroidales order and Desulfovibrionaceae family than the WT mice. In addition, the CTRL KO mice had more *Flexispira* and of unidentified (ud) genus of Helicobacteriaceae, and the FLG-injected KO mice had higher abundance of *Lactobacillus* and *Oscillospira*. The bars are representative of the average abundances for each group. * indicates a significant difference in the given taxon (FDR < 0.05); (c) The KO CTRL mice expressed less intestinal *Il1b* than the WT CTRL mice. FLG did not affect the expression of *Tjp1*, *Il1b* or *Tlr5* mRNA. The gene expression data are presented as mean ± SD, n = 4–6/group. The mRNA levels were calculated against a standard curve and are presented relative to *Actb* housekeeping mRNA. Abbreviations: PBS-injected wild type control, WT CTRL; Flagellin (FLG)-injected wild type control, WT FLG; PBS-injected VAP-1 knockout (KO) control, VAP-1 KO CTRL; Flagellin (FLG)-injected VAP-1 knockout (KO) control, VAP-1 KO FLG.

### 4. Discussion

Our recent studies have suggested that FLG increases hepatocyte fat content in vitro by acting on adipocytes [9] and that the amine oxidase activity of VAP-1 is involved in NAFLD [14]. In this study, we extend our findings by demonstrating that VAP-1 mediated FLG-induced hepatic steatosis in vivo.
It is increasingly accepted that VAT and SAT inflammation and insulin resistance play an important role in the pathogenesis of NAFLD [23]. In this study, we found that in SAT, VAP-1 mediated the FLG-induced decrease in insulin-sensitive ERK1/2 phosphorylation. No other major effects were detected in SAT. By contrast, VAT that is more detrimental for metabolic health than SAT, was more inflamed and showed metabolic changes in the FLG-injected mice. VAP-1 regulated the FLG-induced elevations of IL1β, GM-CSF and MCP-1 cytokines. However, VAP-1 KO mice had higher levels of MCP-1 and GM-CSF than the WT controls that partly hamper our findings on them. Thus, it may be that only the FLG-induced increase in IL1β is truly VAP-1 mediated. Nevertheless, in WT mice the stimulation of GM-CSF and MCP-1 by FLG is in agreement with previous studies [24,25]. In addition, the increase in MCP-1 and circulating neutrophils, monocytes and lymphocytes-recruiting GM-CSF can explain the increased infiltration of CD45-positive leukocytes into the VAT of FLG-treated mice that seemed to be VAP-1-mediated.

IL1β has been reported to accelerate lipolysis in VAT during an acute phase of inflammation [26]. While one evident lipolysis-increasing factor in the FLG-injected mice of this study was an enhanced phosphorylation of HSL that is the rate-limiting enzyme of the catabolism of diacylglycerols [27], the other underlying cause for the increased release of glycerol was the decreased expression of PLIN1. Perilipins are membrane phosphoproteins of the triacylglycerol droplets, which act to prevent lipases from hydrolyzing triacylglycerol and thus, prevent the release of glycerol and fatty acids from the droplets [26]. Our present results from mice are supported by our own findings showing that FLG increased inflammation [28], decreased Plin1 expression in cultured SGBS adipocytes and consequently caused degradation of lipid droplets and release of glycerol [9].

Due to that VAT drains into the portal vein [29], the released glycerol can reach the liver to be used for triglyceride synthesis [30], which may be further exacerbated by circulating IL1β [31] and MCP-1 [32] even in the absence of hepatic inflammation [35]. The VAT-secreted cytokines have been recently implicated in initiating and maintaining the systemic inflammatory state that is observed in NAFLD [33]. We lately showed that in vitro exposure of SGBS adipocytes to FLG increased fat accumulation in hepatocytes by increasing inflammation in adipocytes and releasing glycerol [9]. In this study, we confirm in vivo that FLG increased lipolysis, glycerol release and hepatic fat accumulation and further identified VAP-1 as a novel regulatory protein in this process. In addition to glycerol, triglyceride synthesis requires free fatty acids. Unfortunately, due to the scarcity of serum samples, we could not faithfully determine the levels of fatty acids, and therefore the exact underlying mechanisms of hepatic fat accumulation need to be fully determined in the future. In addition, data showing where glycerol is entering hepatic metabolism should be produced. However, the hepatic fat accumulation in this study may be partly explained by decreased oxidation because FLG decreased the activity of AST and ALT that supply oxaloacetate and pyruvate, respectively, to the TCA cycle. We cannot fully explain why FLG increased their activity in VAP-1 KO mice, and why the KO controls had lower activities than the WT controls. The latter may be a consequence of decreased glucose transport [34] due to the absence of VAP-1, which would result in lower gluconeogenesis decreasing subsequently AST and ALT activities [35].

Upon liver fat accumulation, hepatic mitochondria produce more reactive oxygen species that can cause oxidative damage. The damage can be protected by SOD1 that scavenges oxygen radicals [36]. Therefore, the FLG-induced decrease in Sod1 mRNA may have increased redox state in liver, but unfortunately, due to the instability of lipid peroxidation products, such as malondialdehyde [37] we were unable to measure peroxidation. However, we have earlier shown that FLG induced the production of reactive oxygen species in cultured hepatocytes [9] and that overexpression of VAP-1 increased redox state [38]. In addition to steatosis, FLG increased hepatic fibrosis in mice according to Van Gieson staining. The state of fibrosis was supported also by the increased expression of Mmp9 in the liver of the FLG-treated mice. It has been shown that in steatotic and fibrotic liver, MMP-9 increased extracellular matrix remodeling to promote leukocyte infiltration and angiogenesis [22]. In this study, the FLG-induced fibrosis was not mediated by VAP-1.

Interestingly, the absence of VAP-1 also modulated the composition of the gut microbiota. This may be due to a decreased inflammation because the KO mice expressed less intestinal Il1b that is
normally produced during an active infection and is critical for pathogen eradication [39]. Thus, the decreased Il1b may have allowed an increased growth of inflammation-associated Helicobacteriaceae and Oscillospira [40]. On the other hand, the WT mice had higher relative abundance of Desulfovibrionaceae than the KO mice. This might be a result of H2O2 produced by VAP-1 that could enhance the growth of catalase-positive sulfate reducing Desulfovibrionaceae [41]. In the future, the mechanisms through which VAP-1 modifies the gut microbiota would be an interesting area of research.

5. Conclusions

Based on the present findings, we propose that by acting on visceral adipose tissue FLG increased inflammation and leukocyte infiltration that subsequently increased lipolysis. Lipolysis released glycerol that can reach the liver to contribute to hepatic steatosis due to a defective TCA cycle activity. Importantly, all these effects of FLG were mediated by VAP-1. In addition, FLG induced hepatic fibrosis that may ultimately lead to severe steatohepatitis.

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