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Short Chain Fatty Acids Induce Pro-Inflammatory Cytokine Production Alone And In Combination With Toll-like Receptor Ligands

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Abstract

Problem—Short chain fatty acids (SCFAs), produced at relatively high levels by anaerobic bacteria in bacterial vaginosis (BV), are believed to be anti-inflammatory. BV, a common alteration of the genital microbiota associated with increased susceptibility to HIV infection, is characterized by increased levels of both pro-inflammatory cytokines and SCFAs. We investigated how SCFAs alone or together with TLR-ligands affected pro-inflammatory cytokine secretion.

Method of study—Cytokines were measured by ELISA. Flow was used for phenotyping and reactive oxygen species (ROS) measurement.

Results—SCFAs, at 20mM, induced IL-8, IL-6, and IL-1 β release while lower levels (0.02–2mM) did not induce cytokine secretion. Levels >20mM were toxic to cells. Interestingly, lower levels of SCFAs significantly enhanced TLR2 ligand- and TLR7 ligand-induced production of IL-8 and TNF α in a time- and dose-dependent manner, but had little effect on LPS-induced cytokine release. SCFAs mediated their effects on pro-inflammatory cytokine production at least in part by inducing generation of reactive oxygen species.

Conclusions—Our data suggest that SCFAs, especially when combined with specific TLR ligands, contribute to a pro-inflammatory milieu in the lower genital tract and help further our understanding of how BV affects susceptibility to microbial infections.

Keywords

Short chain fatty acids; inflammation; bacterial vaginosis

Introduction

During innate immune responses at mucosal sites, microbial products, such as lipopolysaccharides (LPS) and peptidoglycans (PGN), are recognized through pattern recognition receptors (PRR) including toll-like receptors (TLRs) and NOD-like receptors (NLRs). The binding of the microbial ligands to their respective receptors results in the production of inflammatory cytokines, chemokines, and anti-microbial products (1, 2). While such responses are beneficial in limiting microbial invasion, chronic inflammation at mucosal sites can also be damaging (3–5) and lead to increased susceptibility to infections such as HIV (6–9). Commensal bacteria influence innate immune responses by inducing or

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suppressing the expression of a broad range of genes involved in inflammatory responses (10).

In addition to extensively-studied PRR ligands such as TLR and NLR ligands, anaerobic bacteria produce short chain fatty acids (SCFA) in the gut, the female genital tract and other mucosal sites (11–14). These microbial products, which include acetic, butyric and propionic acids, have been shown to regulate immune responses, based largely on studies investigating the gut mucosa. Those studies show that SCFA concentrations can reach levels as high as 70–100 mM in the gut lumen; with acetic, propionic and butyric acids comprising on average 60%, 25% and 15%, of the total respectively (15). While healthy microbiota is dominated by *Lactobacillus* species with very low levels of SCFAs, studies by us and others show that SCFAs are found at high concentrations in the lower genital mucosa of women, particularly during a common condition called bacterial vaginosis (BV) (12, 13, 16, 17). At this mucosal site, acetic acid levels may be as high as 20 mM in BV, while butyric and propionic acids' concentrations can range from 5 to 10 fold lower (12, 13, 16, 17).

In many settings, SCFAs exert an anti-inflammatory role by suppressing NF- κ B signaling and inhibiting the production of pro-inflammatory cytokines, by affecting immune cell migration and phagocytosis, and by inducing apoptosis in various cell types including neutrophils (18–23). Given the high prevalence of SCFAs in BV, and the association between BV and susceptibility to HIV (8, 24–28), we investigated the effects of SCFAs on pro-inflammatory cytokine secretion by peripheral blood mononuclear cells (PBMCs) and neutrophils.

Materials and methods

Cell Isolation And Culture

Cells were obtained from at least 5 healthy donors (both men and women) by Ficoll-Hypaque [Lymphocyte Separation Buffer (LSM)] gradient centrifugation of heparinized blood. For neutrophil separation, the cell pellet containing red blood cells and neutrophils was isolated and red cells were removed by hypotonic lysis. For cell cultures, PBMCs and neutrophils were washed and re-suspended in complete medium (RPMI-1640, supplemented with 10% heat inactivated Fetal Bovine Serum, L-glutamine, and 50 μ g/ml gentamicin). All medium reagents were obtained from Lonza (Walkersville, MD). Studies were approved by the Institutional Review Board of Rush University.

Cell Stimulation And Cytokine Measurement

PBMCs or neutrophils were cultured in triplicate in 96-well plates at 250,000 cells/well and stimulated with LPS (Sigma-Aldrich, St. Louis, MO), Pam2CSK4 (InvivoGen, San Diego, CA), iE-DAP, or imiquimod (all from InvivoGen) either alone or in combination with various SCFAs where indicated. The period of culture was 18 hours in most experiments unless otherwise specified. SCFAs [acetic, butyric, and propionic acids (all purchased from Sigma-Aldrich)] were buffered to neutral pH before addition to cultures. For IL-6, IL-8, and TNF α measurements, supernatants were collected from cells after an 18-hour incubation. To measure IL-1 β release, PBMCs were incubated with the indicated stimuli for 6 hours, washed and treated with 1 mM ATP (Sigma-Aldrich) for an additional hour (29). Supernatants were then collected and IL-1 β levels were assessed by ELISA. ELISA kits were purchased either from Invitrogen (IL-8, Carlsbad, CA) or BD Biosciences (TNF α and IL-1 β , San Jose, CA).

Reactive Oxygen Species Measurement

PBMCs were re-suspended at 2×10^6 cells/ml in phosphate buffered saline (PBS, obtained from Lonza) containing 0.1% gelatin (Fisher Scientific, Pittsburgh, PA) and 0.1% glucose (Sigma-Aldrich). They were subsequently loaded with 0.1 mM of 2',7'-dichlorofluorescein diacetate (DCFH-DA, obtained from Sigma-Aldrich) for 15 min at 37°C. After incubation, PBMCs were washed and added to polypropylene tubes containing stimuli in the presence or absence of n-acetylcysteine (NAC, purchased from Sigma-Aldrich) for 45 minutes at 37°C. The oxidative burst was measured in monocytes (gated by light scatter) as a function of the Mean Fluorescence Intensity (MFI) by flow cytometry.

Flow Cytometry

PBMCs were co-stained with anti-human CD14 antibody (BD Biosciences) and anti-human TLR4 antibody (BioLegend, San Diego, CA) to assess the level of expression of these cell surface markers. Events were collected on a FACS Calibur and analyzed using CellQuest software (BD Biosciences).

Statistical Analysis

Data were analyzed using the One-way Analysis of Variance (ANOVA) test. P-values were calculated using the Tukey-Kramer Multiple Comparison post-test.

Results

High Levels of SCFAs Induce The Production of Pro-Inflammatory Cytokines

Despite their documented anti-inflammatory role, there is some evidence that SCFAs may be less inhibitory when used at higher concentrations (22, 30, 31). To learn whether SCFAs by themselves were capable of inducing cytokine secretion at concentrations found at mucosal sites (11–14, 17), we cultured PBMCs with increasing doses of acetic or butyric acid. We found that both acetic and butyric acids, but not propionic acid, induced IL-8, IL-6, and IL-1 β cytokine release when cells were exposed to high levels of SCFAs (20 mM) while lower levels (≤ 2 mM) of these SCFAs did not result in a significant increase in cytokine production (Fig. 1, and data not shown). SCFA concentrations higher than 20 mM were toxic to cells (data not shown).

The Combined Effects of SCFAs and TLR Ligands on Inflammatory Cytokine Production in PBMCs

While lower levels of SCFAs (0.02 to 2 mM) did not by themselves induce pro-inflammatory cytokine production, we hypothesized they could affect responses to other microbial products such as TLR ligands. To test this, we treated PBMCs with Pam2CSK4 (a synthetic TLR2 ligand), imiquimod (a TLR7 ligand), or LPS (a TLR4 ligand) in combination with 0.02 to 2 mM of acetic, butyric, or propionic acids. We found that although treatment with these lower concentrations of SCFAs did not result in an increase in cytokine release (Fig. 1, and data not shown), they significantly enhanced the TLR2 ligand-induced production of IL-8 (Fig. 2A, and Supplementary Fig. 1) and TNF α (Fig. 2C). The lower concentrations of acetic acid and butyric acid also enhanced TLR7 ligand-induced IL-8 production (Fig. 2B). Both TLR2 and TLR7 responses were increased approximately four fold by 2 mM acetic acid while 2 mM butyric acid enhanced TLR2 and TLR7 responses by four and two fold, respectively. As little as 0.02 mM acetic acid significantly increased responses to the TLR2 ligand (Fig. 2A and Supplemental Fig 1). SCFAs enhanced pro-inflammatory cytokine secretion when combined with a range of concentrations of TLR ligands (not shown). Treating cells with a combination of propionic acid and either TLR ligand did not result in a significant increase in IL-8 production (Fig. 2 and data not shown).

Interestingly, none of the SCFAs significantly enhanced LPS-mediated IL-8 production by PBMCs (Fig. 2D). It should be noted that in some experiments, the combination of acetic acid plus LPS was significantly lower than LPS alone although this reduction was not significant when averaged across all experiments (Fig. 2D). Similarly, butyric or acetic acids did not increase IL-8 production by PBMCs in response to iE-DAP, a peptidoglycan-derived molecule which binds to NOD1 (data not shown).

The Combined Effects of SCFAs and TLR Ligands on Inflammatory Cytokine Production in Neutrophils

We also tested the ability of SCFAs to enhance TLR-mediated cytokine production in neutrophils. Similar to PBMCs, 0.02 to 2 mM doses of SCFAs alone did not result in IL-8 production by neutrophils (not shown), and both acetic and butyric acids enhanced TLR2-mediated IL-8 production in neutrophils in some donors. However, the overall impact of the SCFAs on neutrophils was less pronounced than in PBMCs as only acetic acid at 2 mM modestly, but significantly, enhanced the levels of TLR2-ligand-induced IL-8 (Fig. 3). Also, similar to what was observed with PBMCs, SCFAs did not increase LPS-induced IL-8 production (Fig. 3). We did not detect TNF α production in neutrophil cultures (data not shown).

SCFA Effects on CD14 And TLR4

The above experiments showed that pro-inflammatory cytokine secretion due to Pam2CSK4 (TLR2) and imiquimod (TLR7), but not LPS (TLR4), was increased by SCFAs. To determine why LPS-induced cytokine secretion was not enhanced by SCFAs, we examined whether 2 mM acetic acid or butyric acid reduced expression of CD14 or TLR4, two receptors known to play important roles in LPS responses (32). A 4-hour treatment with LPS alone or LPS plus acetic or butyric acids, reduced the frequency of monocytes expressing both TLR4 and of CD14 by up to 25% (Fig. 4). Acetic acid, either alone or in combination with LPS, had little effect on the frequency of cells co-expressing CD14 and TLR4 when compared to the no-stimulation or LPS-only controls. Moreover, acetic acid did not reduce the level of expression of TLR4 or CD14 on monocytes (Supplementary Table I).

Although we observed some donor-to-donor variability, butyric acid alone did not significantly reduce CD14 expression at 4 hours post stimulation when measured across experiments (Supplementary Table I), nor did it reduce the frequency of monocytes expressing both CD14 and TLR4 (Fig. 4). Interestingly, however, butyric acid greatly reduced CD14 levels at 18-hours and 48-hours post stimulation when compared to the no-stimulation or LPS-only controls, (Fig. 4 and Supplementary Table I). This reduction was even greater when cells were treated with butyric acid and LPS combined. Butyric acid did not markedly affect TLR4 expression when compared with the controls (Supplementary Table I).

The Simultaneous Presence of SCFAs and TLR7 Ligand is Necessary For The Increase in IL-8 Production By PBMCs

To determine how SCFAs increased the IL-8 response to TLR ligands, we treated PBMCs with both 2 mM acetic acid and imiquimod simultaneously. Alternatively, cells were treated with acetic acid for 1 or 4 hours before stimulation with imiquimod. As shown in Fig. 5, IL-8 levels were highest when PBMCs were exposed to acetic acid and imiquimod simultaneously. The sequential treatment of cells with acetic acid first and imiquimod next diminished the enhancing effects of acetic acid on imiquimod-induced IL-8 production in a time-dependent manner. In fact, addition of imiquimod 4 hours after acetic acid, completely abrogated the acetic acid-induced enhancement.

The Induction of Pro-Inflammatory Cytokine Release Is Dependent Upon The Generation of Reactive Oxygen Species

Reactive oxygen species (ROS) have been reported to be involved in some pro-inflammatory cytokine responses (33, 34). Notably, we and others have found that SCFAs, including butyric and acetic acids, can induce ROS production in cells (22). The ROS scavenger *N*-acetylcysteine (NAC) was added to cultures to assess whether ROS were involved in the increases in levels of pro-inflammatory cytokine production induced by TLR ligands. As expected, SCFAs induced ROS in PBMCs and NAC greatly reduced the levels of ROS measured in these cells (Supplementary Fig. 2). NAC also diminished the ability of acetic acid to enhance imiquimod-mediated IL-8 production by 50% (Fig. 6). However, NAC did not significantly reduce the butyric acid-enhancing effects (data not shown).

Discussion

In this study, we found that SCFAs alone can cause the production of pro-inflammatory cytokines IL-8, IL-6 and IL-1 β . This effect was dose-dependent as SCFAs at ≤ 2 mM, were unable to induce PBMCs to produce the aforementioned cytokines. Interestingly, however, even low doses of SCFAs significantly increased TLR2- and TLR7-mediated IL-8 and TNF α release. Taken together, our results indicate that SCFAs have pro-inflammatory effects at concentrations found at mucosal sites including the lower genital tract (12, 13, 16, 17).

SCFAs have long been known to modulate a wide range of immune responses such as cytokine production, immune cell migration and phagocytosis (18–23). Many of these studies show that SCFAs, particularly butyric acid, can have anti-inflammatory effects on immune cell function (18–23). However, some investigators found that depending on the type and the dose of the SCFAs used, in addition to the particular cell type, these compounds may not hamper immune responses and could in fact even be stimulatory (35). Indeed, Bailon *et al.* recently reported that butyric acid inhibited cytokine production only in proliferating cells (36). Moreover, Cavaglieri *et al.* showed that whereas butyric acid inhibited IFN γ production by activated rat lymphocytes, acetic and propionic acids increased the release of this cytokine (37). Furthermore, acetic, propionic, and butyric acids have been shown to promote neutrophil chemotactic responses in a dose-dependent manner (30). In agreement with those previous studies, our data indicate that induction of cytokine release is dependent on the concentration of the SCFA used (Fig. 1).

SCFAs may also affect immune responses by virtue of the fact that they are weak acids with a *pK* of 4.8 (11, 14, 15, 38). To our knowledge, how changes in pH shape SCFA-mediated immune responses remains to be carefully examined. In the current study, to more closely model the BV vaginal environment (which has a relatively high pH), we tested the ability of SCFAs to affect innate immune responses at the neutral pH of 7. However, we found that un-buffered SCFAs at a concentration of 2–20 mM induced apoptosis in PBMCs and neutrophils (data not shown). We also used doses similar to those found in vaginal fluids from women with BV [0.2–20 mM, reviewed in (17)]. Our dose response studies showed that SCFAs at doses higher than 20 mM had toxic effects on cells *in vitro*, even when buffered to a pH of 7 (data not shown).

Similar to other studies (18), we found that SCFAs did not significantly enhance IL-8 production when combined with LPS. Our data suggest this lack of responsiveness is not due to decreased signal transduction due to reduced CD14 or TLR4, as neither acetic nor butyric acid greatly reduced the level of expression of these 2 molecules at 4 hours (Fig. 4, and Supplementary Table I). Although our data indicate that the lack of IL-8 production in response to SCFA plus LPS is not caused by changes in CD14 levels, we did observe 3

patterns of CD14 expression depending on the type and duration of the stimulus used. As shown in Fig. 4 and Supplementary Table I, we found that cells treated with 2 mM butyric acid expressed substantially lower levels of CD14 on their surface at 18 and 48 hours post-stimulation when compared to the no-stimulation or LPS-only cultures. A decrease in CD14 expression was more pronounced when cells were exposed to a combination of butyric acid and LPS, where CD14 levels were almost completely obliterated after an 18-hour stimulation. This suggests that there might exist multiple ways in which CD14 expression is controlled in the face of a sustained exposure to these microbial products. The exact mechanism(s) by which such regulation might occur remains to be determined.

Given the effects of some SCFAs on gene expression (19–21, 39–42), we predicted that the addition of SCFAs before TLR ligands in cultures would enhance IL-8 secretion just as effectively as simultaneous addition of SCFA and TLR ligand by increasing expression of genes involved in the response; for example, SCFA pre-treatment could increase expression of TLR2 or TLR7 so that when the TLR ligands are added an enhanced response would occur. However, we found this not to be the case as IL-8 levels were highest only when PBMCS were exposed simultaneously to both acetic acid and imiquimod (Fig. 5) or acetic acid and Pam2CSK4 (data not shown). That simultaneous exposure to SCFAs and TLRs were required for the largest cytokine response suggested a mechanism that could involve molecules with a relatively short half-life, such as ROS.

As ROS have been reported to be involved in a wide array of inflammatory responses (33, 34), and because we and others find that SCFAs can induce the generation of these products in cells [(22) and Supplementary Fig. 2], we hypothesized that the presence of ROS may lead to the induction of pro-inflammatory cytokine production. Our data suggest that the production of ROS may only partly be responsible for combined effects of SCFA + TLR-ligands on cytokine production. It will be important to discern what other pathways may play a role in SCFA-mediated immune activation.

Using SCFAs at doses and pHs that are found in the vaginal mucosa, the data presented here show that these microbial products can activate immune responses, alone or when combined with specific TLR ligands. A limitation of the current study is that it was done *in vitro*. Moreover, this study does not directly examine the types of cells that are present in the vaginal environment. However, our findings suggest that the multimicrobial milieu in BV can alter innate immune responses, and set the platform where such effects can further be assessed *in vivo*. These studies will enhance our understanding of how such interactions may render the lower genital tract more susceptible to pathogenic infections, such as HIV, during BV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. High levels of SCFAs induce the production of pro-inflammatory cytokines 250,000 PBMCs from healthy donors were cultured in the presence of increasing concentrations of acetic (AA) or butyric acid (BA). To measure the level of IL-8 (A) or IL-6 (B) production, supernatants were collected from cells after an 18-hour incubation. To measure IL-1 β release (C), cells were incubated with the indicated stimuli for 6 hours, washed and treated with 1 mM ATP for an additional hour at which time supernatants were collected. Cytokine release was assessed by ELISA. Values shown are means $\pm$ SD of triplicate cultures. Data shown are representative of at least 3 independent experiments from multiple donors. *** P-value < 0.001, and * P-value < 0.05, were determined by Tukey-Kramer Multiple Comparison post-test, comparing PBMCs treated with 20 mM SCFAs to those that were untreated.

Figure 2. The combined effects of SCFAs and TLR ligands on inflammatory cytokine production in PBMCs

PBMCs were collected as described and cultured in the presence of Pam2CSK4 (0.1 μ g/ml, designated as Pam in A and C), imiquimod (10 μ g/ml, B), or LPS (1 μ g/ml, D). Supernatants were collected after 18 hours of culture, and IL-8 (A, B, and D,) or TNF α (C) levels were assessed by ELISA. In A, B, and D, data were pooled from 3–5 independent experiments using various donors, and levels of cytokine production were normalized to the levels seen in TLR-ligand-only-stimulated groups. Values shown are means $\pm$ SD of multiple experiments from multiple donors (A, B and D), or triplicate cultures (C). *** P-value < 0.001, ** P-value < 0.01, and * P-value < 0.05, were determined by Tukey-Kramer Multiple

Comparison post-test, comparing cells treated with the TLR ligand to those treated with a combination of TLR ligand plus SCFA.

Figure 3. The combined effects of SCFAs and TLR ligands on inflammatory cytokine production in neutrophils

Neutrophils were cultured in the presence of Pam2CSK4 (0.1 $\mu\text{g/ml}$, designated as Pam), or LPS (1 $\mu\text{g/ml}$). Supernatants were collected after 18 hours of culture, and IL-8 levels were assessed by ELISA. Data were pooled from at least 3 independent experiments using various donors, and levels of IL-8 production were normalized to the levels seen in TLR-ligand-only-stimulated groups. Values shown are means $\pm$ SD of multiple experiments. * P-values <0.05 were determined by Tukey-Kramer Multiple Comparison post-test, comparing cells treated with the TLR ligand to those treated with a combination of TLR ligand plus SCFA.

Figure 4. SCFA effects on CD14 and TLR4

PBMCs were cultured in the presence of no addition, LPS (1 μ g/ml), or 2 mM SCFAs $\pm$ LPS, as indicated. Cells were then harvested at indicated time points, co-stained with antibodies against CD14 and TLR4 and analyzed by flow cytometry gating by light scatter on monocytes. Data shown are representative of 3 independent experiments, using 5 different donors.

Figure 5. The simultaneous presence of SCFAs and TLR7 ligand is necessary for the increase in IL-8 production by PBMCs

PBMCs were cultured either alone or with imiquimod (Imiq at 10 μ g/ml) in the absence or in presence of 2 mM acetic acid (AA). Stimuli were added either simultaneously, or addition of imiquimod was delayed as depicted in the Figure. Supernatants were collected 18 hours after addition of imiquimod and IL-8 levels were measured by ELISA as described in Fig. 1. Data shown are representative of 4 independent experiments, using multiple donors.

Figure 6. The induction of pro-inflammatory cytokine release is dependent upon the generation of reactive oxygen species

PBMCs were cultured as described in Fig. 1 and stimulated as indicated in the presence or absence of 10 mM NAC. IL-8 levels were measured by ELISA. Values shown are means ($\pm$ SD) of triplicate cultures. Results are representative of at least 3 independent experiments, using multiple donors. *** P-values <0.001 were determined by Tukey-Kramer Multiple Comparison post-test, comparing cells treated with imiquimod (10 μ g/ml) to those treated with a combination of imiquimod plus acetic acid (AA at 2 mM).