SPHINGOLIPID METABOLISM AND ITS EFFECT ON INTESTINAL HEALTH IN RELATION TO INFLAMMATORY BOWEL DISEASE

By

BRYNN SMITH

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Approved By

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Ashley J. Snider
School of Nutritional Sciences and Wellness
ABSTRACT

Inflammatory bowel disease has been linked with the endoplasmic reticulum stress (ER stress) in response to saturated fatty acids (SFAs). Patients with IBD also have a higher chance of colorectal cancer (CRC). SFA myristate (C:14) and the corresponding ceramide synthases, Cers5 and Cers6, initiate inflammatory pathways within the intestine. To determine the effect of SFAs on IBD, ER stress, and downstream signals of inflammation, we treated colon cells with Cers5, Cers6, and Cers 5 & 6 knockouts with myristate. Once treated, downstream inflammatory signals, such as the protein XBP1 were observed to see if the treatment affected splicing, which would continue the downstream inflammatory signaling, which leads to apoptosis of the inflamed cells. The same pattern can be seen when colon cancer cells are treated with myristate. We can conclude that XBP1 splicing increases when treated with myristate in the healthy intestinal cells and the colon cancer cells. This suggests that myristate plays a larger role in ER stress and intestinal inflammation correlated with IBD and CRC.

INTRODUCTION

Inflammatory bowel disease (IBD) is a broad term used to describe two gastrointestinal diseases, Crohn's Disease (CD) and Ulcerative Colitis (UC). IBD is a chronic affliction that negatively impacts intestinal health, often causing symptoms such as diarrhea, abdominal pain, and weight loss that affects approximately 6.8 million individuals worldwide [1]. Recently, the incidence rate has increased drastically, from a diagnosis rate of roughly 79.5 cases per 100,000 people in 1990 to 84.3 cases per 100,000 people in 2017 [2]. It is estimated that by 2030, the IBD prevalence rate will increase to 325 to 491 per 100,000 people, making this one of the most prevalent intestinal diseases to date [3]. Most IBD cases are diagnosed before 35 years of age, most commonly around 20 to 30 years old but as cases increase, there is a larger variation in diagnosis age [4]. Indeed, 25% of current IBD diagnoses are from patients under 16 years of age.
and 15% are patients over 60 years of age [7]. Individuals with IBD, regardless of status, active or in remission, are at a 2-fold increased risk for colorectal cancer [8].

Recently, IBD has been linked to sphingolipids, which are bioactive lipids within a cell. For many years sphingolipids were only considered to play a role in cell membrane structure but have since been identified as a key factor in inflammation and disease, such as IBD and colorectal cancer [9]. Sphingolipids are made via the de novo pathway, which begins in the endoplasmic reticulum. The de novo pathway starts with the condensation of serine and palmitoyl-CoA, by serine palmitoyl transferase (SPT), which is then rapidly converted to 3-ketodihydrosphingosine and into dihydrosphingosine via an enzyme called 3-ketodihyrdosphingosine reductase. Dihydrosphingosine is converted into dihydroceramide via ceramide synthase(s) (CerS), which is metabolized to ceramide via dihydroceramide desaturase(s). Ceramide, the central lipid in sphingolipid metabolism, can be converted into sphingosine via ceramidase(s). Sphingosine kinases (SK) can then utilize sphingosine to produce sphingosine-1-phosphate (S1P) [9]. S1P can be recycled and converted back into ceramides using sphingosine phosphatase(s) and ceramide synthase(s). Several of these enzymes have been linked to IBD, including SK1 and its lipid product S1P [10].

Ceramides have been linked to endoplasmic reticulum (ER) stress. The ER stress response modifies how protein folding occurs within the ER [9], engaging the unfolded protein response (UPR) which signals specific downstream transcription factors to attenuate protein synthesis. Activation of the inositol-requiring enzyme 1 (IRE1) pathway removes a 26 base pair intron in X box protein 1 (XBP1) and triggers the transcription of downstream inflammatory pathways, which suggests that splicing could play a role in the developments of intestinal inflammation and IBD [11, 12]. Indeed, IRE signaling induced the inflammatory cytokine interleukin 6 (IL6) in human papillomavirus-negative oropharyngeal carcinoma cells and rat intestinal epithelial cells [13, 14]. Our lab has previously documented that when rat intestinal
epithelial cells are treated with myristate, ER stress is induced, causing XBP1 splicing and increased expression of IL6 in a CerS5/6-dependent manner [9, 15-17]. However, the role of ER stress, XBP1 splicing in the IRE1 pathway, and subsequent IL-6 production has not been shown in human cells. The first aim of our study is to define the effects of myristate in human colon epithelial cells. We hypothesize that XBP1 expression will increase in response to ER stress from when intestinal epithelial cells are treated with myristate (C14), therefore signaling the production of IL6 and inflammatory factors that could contribute to IBD. The second aim of our study is to probe the effects of myristate in human colorectal cancer.

METHODS

Reagents and materials

Dulbecco’s modified Eagle’s medium (DMEM) and PureLink RNA Mini Kit were purchased from Thermo Fischer Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was from GE Healthcare Life Sciences (Logan, UT, USA). qScript cDNA SuperMix purchased from Quantabio (Beverly, MA, USA). iTAQ Master Mix obtained from Bio-Rad (Hercules, CA, USA). Fatty acid free, low-endotoxin bovine serum albumin (BSA) and myristate purchased from MilliporeSigma (Burlington, MA, USA). Tunicamycin obtained from Tocris Biosciences (Bristol, United Kingdom, UK).

Cell Culture

DLD1 human colorectal adenocarcinoma cells were obtained from the University of Arizona Cancer Center, where they were originally purchased from American Type Culture Collection (Manassas, VA, USA). As described in Choi et al. [9], DMEM was supplemented with
and 10% (v/v) fetal bovine serum. In brief, the cells were kept in a humidified incubator at 37°C with 5% CO₂. Cells were seeded and treated as needed 24 hours after plating. Cells were grown to a final confluence of approximately 70%.

**Treatment**

Saturated fatty acids (SFAs) were prepared as described in Ross et al. [18] and Choi et al. [9]. In brief, SFAs were dissolved in 100% ethanol to a concentration of 100µM, and subsequently distributed and dried by nitrogen. The SFAs were altered to designated concentrations using DMEM that was supplemented with 2% fatty acid free, low-endotoxin BSA. SFAs were conjoined to BSA via brief sonication, with incubation at 55°C for 15 min, and were cooled to 37°C. Before SFA treatment, cells were serum starved for 8 hours, and the media was changed to a media containing SFAs conjoined to BSA for the indicated treatments. [9, 18, 19].

**RNA extraction and real time qPCR**

RNA extraction occurred following cell collection. As previously noted, RNA extraction was performed according to the manufacturer’s protocol using the PureLink RNA Mini Kit [9]. 0.5µg of RNA was used for cDNA synthesis using the qScript cDNA SuperMix per the manufacturer’s protocol.

As documented in Choi et al. [9], real-time RT PCR was performed using the QuantStudio 3 Real-Time PCR System (Thermo Fischer Scientific). The following TaqMan probes (Thermo Fischer Scientific) were used: human C/EBP homologous protein (CHOP), human ER localized DnaJ 4 (ERdj4), and human ribosomal protein lateral stalk subunit P0 (RPLP0) as a housekeeping gene. In brief, cycle threshold (Ct) values were obtained for each gene of interest. ΔCt values were calculated, and then we subsequently calculated the relative gene expression normalized to control samples from ΔΔCt values.

**XBP1 Splicing**
ER stress–induced processing of XBP1 mRNA was evaluated by RT-PCR [9, 20]. The 601 base pair product of XBP1 was amplified using the following XBP1 primers: human sXBP1 forward (5’ CTGAGTCCGCAGCAGGTG 3’), human uXBP1 forward (5’ TCCGCAGCACTCAGACTACG 3’), and human s/uXBP1 (5’ AGTTGTCCAGAATGCCCAACA 3’). The thermal cycling profile consisted of 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Samples were then separated on a 3% agarose gel. Optical density of bands representing XBP1s and XBP1u were measured by ImageJ program (NIH).

**Statistical Analysis**

Statistical analyses were carried out using GraphPad Prism (GraphPad Software, LaJolla, CA, USA). Data are presented as means ± SEM and analyzed by a one-way ANOVA. P Values of <0.05 were considered statistically significant.

**RESULTS**

![Figure 1: siRNA XBP1 splicing](image-url)
It has been previously documented that myristate treatment increased XBP1 splicing in a Cers5/6 dependent manner in rat intestinal epithelial cells [9], but the effects of myristate in human colon epithelial cells is unknown. To determine if myristate induced ER stress in a similar manner in human colon epithelial cells, we transfected HCEC 1CT cells treated with siRNA for CerS5, CerS6 or CerS5/6 and treated with 600µM myristate. Following mRNA extraction and cDNA synthesis, spliced XBP1 was amplified using PCR and visualized on a 3% agarose gel. XBP1 splicing occurred basally with CerS knockdown in these cells and was increased with myristate treatment in all siRNA transfected cells (Figure 1).

**Figure 2: Quantification of siRNA XBP1 splicing**

Visually in Figure 1, we determined that XBP1 splicing increased in all siRNA transfected cells but were unable to determine the amplitude of the increased splicing. XBP1 splicing was quantified using ImageJ analysis. The light density of Figure 1 for spliced XBP1 were divided by the unspliced light intensity to determine the OD ratio. (Figure 2). It is clear to see that siCers6
has the highest spliced/ unspliced ratio, therefore indicating that ceramide synthase 5 expression induces the most XBP1 splicing in human colon epithelium.

![Figure 3: XBP1 splicing following a dose response of Myristate. Spliced XBP1 band is at 120 bp and the unspliced XBP1 is at 141 bp.](image)

It has been well documented that myristate treatment induces ER stress in multiple tissues, such as the liver and adipose [21-23] and our lab previously determined that myristate induces ER stress in rat intestinal epithelial cells [9]. After determining that myristate induced ER stress in human intestinal epithelial cells we next set out to determine if human cancer cells exhibited a similar response. Human colorectal cancer cells (DLD1) were treated with increasing doses of myristate (100 µM, 300 µM, 600 µM, and 900 µM) and XBP1 splicing examined, using tunicamycin as a control (Figure 3). In Figure 1, XBP1 splicing increased in a dose dependent manner, suggesting that increased doses of C14, elicit increased ER stress response in colon cancer cells.
In order to quantify XBP1 splicing in response to myristate, band intensities were quantified using ImageJ. There is a statistically significant increase in XBP1 splicing between the untreated control and 300 µM, 600µM, 900µM C14 doses. Upon closer evaluation, XBP1 splicing was the highest in the 600µM dose, increasing from the control group by an approximate 1.5-fold change (Figure 4). These data indicate that treatment with myristate resulted in ER stress and IRE1 signaling in human colorectal cancer cells, with 600 µM eliciting the greatest response.
To determine if myristate induced transcription of downstream target genes real time RT-PCR was utilize to measure ERDJ4, a known downstream target of the IRE1 pathway. Realtime data were analyzed and normalized to the housekeeping gene RPLPO and significance assessed using a one-way ANOVA test. ERDJ4 expression increased with increasing doses of myristate, except for the 900µM dose which suggests that maximal response for myristate is at 6000µM (Figure 5). ERDJ4 expression was significantly increased at 300 µM and 600µM, which indicated that ER stress and the IRE1 pathway are upregulated with 600µM treatment of myristate.
CHOP is a known downstream target of ER stress that is involved in the apoptotic ER stress pathway [24]. To determine if CHOP expression was induced in DLD1 cells when treated with myristate, RT-qPCR was used. CHOP expression was normalized to RPLPO and the control and subsequently analyzed using a one-way ANOVA test (Figure 6). CHOP expression remains relatively identical, despite the increasing dose of C14.
DISCUSSION

In this study we aimed to define the effects of myristate on human colon epithelium. Our lab has previously determined that 600µM of myristate induced XBP1 splicing in a CerS 5/6 dependent manner in IEC6 rat intestinal epithelial cells. Therefore, we treated the HCEC 1CT human colon epithelial cells with 600µM of myristate. To extend our previous findings from rat intestinal cells to a human colon epithelial cells. HCEC 1CT cells were transfected with small interfering RNA for CerS 5, CerS 6, and CerS 5 and 6. In HCEC cells XBP1 splicing in response to myristate did not require CerS5 or 6. The results indicated that while each set of knockdown cells showed increased XBP1 splicing, the greatest increase and most statistically significant change occurred in the siRNA CerS 6 knockdown. These data suggest that XBP1 splicing and myristate incorporation has a greater correlation with the CerS 5 enzyme, which is different from the results our lab found in the rat intestinal epithelium [9]. More studies should be performed to verify that CerS 5 is the ceramide synthase that is directly involved in myristate incorporation into sphingolipids, therefore causing ER stress, the UPR, and IRE signaling.

The second aim of our study was to probe for the effects of myristate in human colorectal cancer. Based on the results shown from the HCEC 1CT cells, we hypothesized that myristate would act similarly in DLD1 colorectal cancer cells. Since 600µM of myristate induced the highest XBP1 splicing, we hypothesized that myristate in the DLD1 cells would exert a similar response. As XBP1 splicing is an indicator that the IRE1 pathway is being activated, we examined at known downstream proteins and transcription factors, ERDJ4 and CHOP, and expected that they would have the greatest expression when treated with 600µM myristate as well. While this is true for the ERDJ4 gene, the change in CHOP expression from the untreated control group and the myristate doses are not statistically significant. This could be due to the fact that CHOP expression is also dependent on other ER stress pathways such as PKR-like ER kinase (PERK) and it's downstream activation transcription factor 4 (ATF4) pathway [25], and the ATF6 pathway. IRE1 can also
activate an additional pathway besides the XBP1 splicing, namely the apoptotic signaling kinase 1 (ASK1) to Jun-N-terminal kinase (JNK) and/or p38 mitogen-activated protein kinase (p38MAPK), which all increase CHOP expression [26]. Since so many other pathways are involved in CHOP expression, it is likely that one of them has a greater downstream impact on expression than XBP1 splicing.

While 600µM of myristate did exhibit the most XBP1 splicing, future studies should perform a dose response with smaller increments to determine whether the activation of the XBP1 transcription factor occurs with less myristate in human colorectal cancer cells as the fold change in XBP1 splicing and ERDJ4 gene expression from the 100µM dose to the 300µM dose is so high. It is possible that since IBD correlates with a higher incidence of CRC, CRC cells begin to incorporate myristate into the cell at smaller concentrations due to their disease state. Studies of this nature would not only verify mechanism but indicate disease progression from an IBD to CRC state.

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References