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Introduction

Gut microbiota refers specifically to the various microorganisms that live in mammalian gastrointestinal tracts [10]. Gut microbiota is diverse and host-specific, and can change over time as a result of internal and external factors. Due to its proposed influence on health and disease, the microbiome has become an increasingly researched area [3]. Specifically, intestinal microbiota has been linked to behavior, stress response, autism, mental illnesses such as depression and anxiety, and neurological diseases including multiple sclerosis, Parkinsons disease, and Alzheimer disease [3,10,11]. Further, it has been linked to type two diabetes and obesity, two of the most prevalent diseases in the United States affecting over 30 million and 100 million Americans, respectively [3,14,15]. This relationship between the microbiome and disease has led to the investigation of factors that communicate between gut microbiota and other parts of the body.

Several studies of mice have linked intestine colonization by microbiota to metabolic function. One proposed mechanism is through regulation of angiopoeitin-like 4 (ANGPTL4) expression [3,7]. ANGPTL4 is a protein secreted by a variety of tissues including liver, muscle, and colon tissue. It has multiple functions such as regulation of lipid and glucose homeostasis, as well as inhibition of cell migration and angiogenesis. Specifically, ANGPTL4 blocks lipoprotein lipase activity, an enzyme that catalyzes the uptake of circulating lipids into tissues. This effectively increases blood triglyceride
levels, a symptom of metabolic syndrome which can lead to weight gain and obesity [3,16]. Therefore, an increase in ANGPTL4 expression is believed to influence metabolic activity and lead to weight gain.

ANGPTL4 expression is regulated by peroxisome proliferator-activated receptors (PPAR), a class of nuclear receptor proteins. PPARs act as transcription factors and regulate cell differentiation, development, and metabolism [7,12]. ANGPTL4 mRNA levels—an indicator of ANGPTL4 levels—are regulated in the liver and small intestine by PPARα, in skeletal muscle and macrophages by PPARδ, and in adipocytes by PPARγ [3]. ANGPTL4 mRNA expression in the human colon is less researched, where PPARγ is believed to be the primary regulator. Ultimately, PPARγ regulates ANGPTL4 expression in the colon, part of the small intestine, and ANGPTL4 expression is predicted to influence metabolic activity and cause weight gain. Therefore, regulation of ANGPTL4 expression by PPARγ is proposed to play a role in the relationship between intestine microbiota and metabolic activity.

PPARγ is widely studied and is known to be involved in regulation of fatty acid metabolism via β-oxidation, cell proliferation, and gut homeostasis [3]. Several synthetic and natural ligands, such as free fatty acids, act as PPARγ activators [7]. It is hypothesized that short-chain fatty acids (SCFAs) also activate PPARγ. SCFAs are the products of bacterial fermentation of dietary fiber, and the main source of energy for colonocytes, epithelial cells in the colon [7]. Essentially, gut bacteria in the microbiome process fiber into SCFAs, which are crucial for colonocyte metabolism [7]. Further, through recapitulation of gene expression and chromatin states, SCFAs have been shown to effectively counteract the negative effects of a Western diet, which causes poor gut health and decreases microbiome diversity [9,13]. Since SCFA are a potential activator of PPARγ, they are believed to affect ANGPTL4 regulation by activating PPARγ, a potential mechanism for the microbiome influencing metabolic syndrome and weight gain. Butyrate is a SCFA and a known inhibitor of histone deacetylase, and thus a proposed activator of PPARγ [13].
Butyrate can induce ANGPTL4 expression through both independent and PPARγ mediated mechanisms, suggesting the possibility of two differing activation functions.

SCFAs, a product of the microbiome, are essential in colonocyte metabolism, and since SCFAs are a potential activator of PPARγ, investigating the relationship between SCFAs, ANGPTL4 expression and PPARγ will provide insight into the relationship between gut microbiota and metabolic activity. Ultimately, the microbiome plays a large role in health and disease development, specifically metabolic syndrome which leads to obesity and diabetes. Therefore, researching the factors that connect gut microbiota to metabolism and weight gain can aid in improving our understanding of obesity and diabetes development, and thus prevention.

Experimental Techniques

Enzyme-linked immunosorbent assay (ELISA) is a quick and reliable immunochemical technique that is used to detect antigens and antibodies in molecular constituents of blood and other bodily fluids [1,2]. It involves an immune reaction, an enzymatic chemical reaction, and signal detection and quantification [4]. Enzymes frequently used include alkaline phosphatase, horseradish peroxidase, and B-galactosidase [4]. ELISA is relatively cheap in comparison to florescence detection systems, and it is safe as it does not use radiochemicals [2]. The general procedure involves four steps--coating, blocking, detection, and readout--with a wash completed between each step, as shown in Figure 1. First, the antigen or antibody is adsorbed into a well in the ELISA plate in coating buffer. Several washes are done to remove buffer and unbound materials. Next, free well sites are blocked using a buffer containing unrelated proteins. The wash is repeated. Then the enzyme-conjugated detection antibody binds the antigen. The wash is repeated. Lastly, the substrate is catalyzed by the enzyme to generate a colored readout.
Quantitative reverse transcription polymerase chain reaction (RT-qPCR) is a technique that combines reverse transcription of RNA into DNA with amplification of specific DNA targets using PCR to measure the amount of desired RNA in a sample [5]. Complimentary DNA (cDNA) is synthesized from RNA by reverse transcriptase, and the cDNA is then used as a template qPCR [6]. This technique can be performed in one or two steps as shown in Figure 2. One step assays combine reverse transcription and qPCR amplification in a single tube with two enzymes (reverse transcriptase and DNA polymerase) and sequence-specific primers. Reverse transcription generates cDNA and then the reverse transcriptase and cDNA are denatured and the DNA polymerase amplifies the cDNA. A gene-specific primer is used so only the desired region is reverse-transcribed and amplified. Two-step assays use two separate tubes, one for reverse transcription and one for PCR, with differing buffers, reaction conditions, and priming strategies [5].
Figure 2. Diagram of one-step versus two-step RT-qPCR basic procedure. [5]

Current Research

Evidence 1: (Alex et al. 2013): Induction of ANGPTL4 by butyrate is mediated by PPARγ [3]

Since ANGPTL4 is a well-known target of PPARγ in colonocytes, Alex et al. hypothesized that butyrate, a SCFA, increases ANGPTL4 expression in colonocytes by activating PPARγ. To test this hypothesis, T84 cells were cultured in Dulbecco’s modified Eagle medium, grown until 80 to 90% confluence, and treated with synthetic PPARγ agonist rosiglitazone (1 uM) and/or antagonist GW9662 (5 uM) in dimethylsulfoxide (DMSO). Cells were preincubated with alpha-amanitin for one hour, and then 24 hours with or without sodium butyrate (1 mM). Cell medium was collected and ANGPTL4 was analyzed via enzyme-linked immunosorbent assay (ELISA).

ELISA was carried out as follows. Ninety-six well plates were coated with anti-human ANGPTL4 polyclonal goat IgG antibody and incubated overnight. Plates were washed extensively, blocked, 100 uL of cell medium was applied, and the plates were incubated. The antibody was added, followed by streptavidin-conjugated horseradish peroxidase for 20 minutes and then tetramethyl benzidine substrate reagent for six minutes. The reaction was stopped and absorbance was recorded at 450 nm.

ANGPTL4 concentration in the cell, quantified by ELISA, was increased in the
presence of either butyrate or synthetic PPARγ agonist rosiglitazone (Figure 3). As shown on the first bar of Figure 3, the ANGPTL4 concentration with just control is less than 0.1 mg/mL. The addition of solely butyrate, as seen in the third bar, increases the concentration to almost 0.7 mg/mL, and the addition of solely rosiglitazone, as shown on bar five, increases the concentration to over 0.4 mg/mL. The addition of synthetic PPARγ antagonist GW9662 in the presence of rosiglitazone significantly decreased ANGPTL4 levels to less than 0.2 mg/mL, as seen on the sixth bar. Similarly, the fourth bar indicates induction of ANGPTL4 by butyrate was significantly decreased in the presence of antagonist GW9662, resulting in a concentration of roughly 3 mg/mL.

Figure 3. Induction of ANGPTL4 by butyrate is mediated by PPARγ. (A) Inhibitory effect of PPARγ antagonist GW9662 (5 uM) on induction of ANGPTL4 secretion in medium by rosiglitazone (10 nM) and butyrate (1 mM) in T84 cells using ELISA. Bars represent the mean recovery plus ranges from two independent experiments. Error bars represent SD except when indicated otherwise. An asterisk indicates a significantly different result from that of the control according to Student’s t test (P < 0.05)

The authors conclude that butyrate increases ANGPTL4 expression through activating PPARγ. The ELISA results support the claim that the effect of butyrate on ANGPTL4 is mediated by PPARγ because the addition of synthetic PPARγ antagonist GW9662 caused butyrate-induced ANGPTL4 expression to decrease. This evidence demonstrates that PPARγ antagonist GW9662 can decrease butyrate-induced ANGPTL4 expression, supporting my thesis by suggesting that butyrate-induced ANGPTL4 expression is PPARγ mediated.

The authors used an effective control (DMSO), and conducted a student’s t test to show that the difference between the control and antagonist GW9662 was significant,
allowing the reader to be confident in the data presented. However, more data is needed to determine whether butyrate increases ANGPTL4 expression by direct activation of PPARγ. These results do not show a direct measurement of binding, therefore it is possible that PPARγ activates other proteins that indirectly induce ANGPTL4. It cannot be concluded from this experiment that butyrate increases ANGPTL4 expression through activating PPARγ.

**Evidence 2 (Korecka, et al. 2013): Butyrate induced ANGPTL4 expression is PPARγ independent**

Korecka et al. hypothesizes that butyrate induces ANGPTL4 expression in a PPARγ-independent manner. To test this hypothesis, HT-29 cells were pretreated with selective PPARγ inhibitor GW9662 (10 uM) or DMSO control (0.1%) for 1 h, after which cells were treated with butyrate (2 mM), rosiglitazone (5 uM), or a combination of both for 6 h. The control was treated with 0.025% DMSO. ANGPTL4 expression was normalized to beta-actin. RNA was isolated using Qiagen Rneasy Mini-kit and cDNA was synthesized with Super-Script II (Invitrogen) then used for qPCR reaction performed using gene-specific primers. Relative expression was calculated. Each sample was measured in duplicates and the experiment performed in triplicate.

Pretreatment with antagonist GW9662 significantly decreased rosiglitazone-induced ANGPTL4 expression quantified by RT-qPCR (Figure 4). The treatment with rosiglitazone and control DMSO, shown in white, expressed mRNA levels (fold change) of around 750. The ANGPTL4 mRNA levels are used as an indicator of overall ANGPTL4 levels. The addition of antagonist GW9662 to rosiglitazone, shown in black, resulted in mRNA levels of less than 50. However, butyrate-induced ANGPTL4 expression was not decreased, but instead increased after pretreatment with the PPARγ antagonist. Treatment with butyrate and control, as shown in white, resulted in mRNA levels of roughly 200. The addition of the antagonist increased ANGPTL4 mRNA levels to just under 500, as seen in black. The treatment of
rosiglitazone, butyrate, and control, as seen in white, resulted in mRNA levels of just under 2000. The addition of PPARγ antagonist, as shown in black, resulted in a decrease of ANGPTL4 mRNA, with a fold change of around 500.

Figure 4. Butyrate-induced ANGPTL4 expression is peroxisome proliferator-activated receptor PPARγ-independent. C: inhibition of PPAR-γ does not block butyrate-induced ANGPTL4 expression. Quantitative RT-PCR results for effects of PPARγ inhibition on ANGPTL4 mRNA expression in HT-29. Cells were pretreated with GW9662 (10 uM) or DMSO (0.1%) for 1 h, after which cells were treated with butyrate (2 mM), rosiglitazone (5 uM), or a combination of both for 6 h. Control was treated with 0.025% DMSO. ANGPTL4 expression was normalized to beta-actin. 2-way ANOVA **P < 0.01; ***P < 0.001 between indicated bars. Bars and error bars show means +/- SD. [7]

The decrease in rosiglitazone-induced ANGPTL4 expression confirms that rosiglitazone is PPARγ mediated. Contrastingly, the increase in ANGPTL4 mRNA levels after pretreatment with GW9662 support the author’s hypothesis that butyrate-driven ANGPTL4 expression is PPARγ independent. Ultimately, the authors conclude that butyrate-induced ANGPTL4 expression is PPARγ independent. A two-way ANOVA test was performed and a P value of less than 0.001 was observed for the experiment with rosiglitazone, and a P value of less than 0.01 was observed for the experiment with butyrate (Figure 4). The differences between the treatments with and without GW9662 are very significant, providing confidence in the data. They used duplicates of each
sample and did the experiment in triplets leading to six trials of each experiment, which is sufficient, but more trials would be ideal. The data set using both rosiglitazone and butyrate with and without GW9662 pretreatment provides minimal information regarding ANGPTL4 expression by specifically butyrate, but confirms a successful assay and shows that one of the two ligands induces ANGPTL4 via PPARγ. However, the data set using butyrate with and without GW9662 provides strong evidence for my thesis by demonstrating that PPARγ inhibitor GW9662 can increase ANGPTL4 expression by butyrate, suggesting that butyrate-induced ANGPTL4 expression is PPARγ independent, or alternatively, has two different activation functions.

**Significance and Future Directions**

Further investigation is required to make a conclusion regarding the exact mechanism of butyrate-induced ANGPTL4 expression, and the role of PPARγ. Evidence 1 demonstrated that PPARγ antagonist GW9662 decreased butyrate-induced ANGPTL4 expression, supporting PPARγ mediated expression of ANGPTL4 by butyrate. This experiment effectively supports the portion of my thesis arguing that PPARγ antagonist GW9662 can decrease butyrate-induced ANGPTL4 expression suggesting that butyrate-induced ANGPTL4 expression is PPARγ mediated. However, the authors make the claim that butyrate increases ANGPTL4 expression through direct activation of PPARγ, and this cannot be concluded from evidence 1. More data is needed. Evidence 2 showed the presence of GW9662 increases butyrate-driven ANGPTL4 expression, providing support for PPARγ independent expression of ANGPTL4 by butyrate. This effectively supports the portion of my thesis arguing that PPARγ inhibitor GW9662 can increase ANGPTL4 expression by butyrate which suggests that butyrate-induced ANGPTL4 expression is PPARγ independent. These are conflicting arguments surrounding the role of PPARγ in butyrate-induced expression of ANGPTL4, suggesting the possibility of multiple activation pathways. More research is needed to better understand the relationship between PPARγ activation and butyrate in the expression of ANGPTL4.

**Future Direction**
In order to further investigate the role of PPARγ in butyrate-induced expression of ANGPTL4, it is suggested to perform siRNA knockdown experiments. Further, in order to ensure sufficient knockdown, the HT-29 cells can be treated with rosiglitazone and the ANGPTL4 mRNA levels can be quantified using qPCR. siRNA is a type of gene knockdown, an experimental technique where the expression of one or more genes are reduced. They are reduced either through genetic modification or treatment with a reagent such as a short DNA or RNA oligonucleotide containing a complementary sequence to either gene or an mRNA transcript [8]. HT-29 cells will be plated and PPARγ will be downregulated using Silencer Select siRNA products directed against PPARγ. Cells will be treated with rosiglitazone or butyrate 24 hours after transfection, and a control with DMSO. ANGPTL4 and PPARγ expression will then be measured using qPCR. If silencing of PPARγ in cells treated with butyrate results in blocking of ANGPTL4 expression, this will further support the argument that expression of ANGPTL4 by butyrate is independent of PPARγ. [7] Ultimately, understanding how butyrate, a SCFA, affects PPARγ, a known regulator of ANGPTL4, will provide mechanistic insight into how microbiota communicates with the rest of the body.
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