BACTERIAL METABOLITES AND OBESOGENS AS REGULATORS OF

INFLAMMATION AND ADIPOGENESIS IN ADIPOSE TISSUE

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Bacterial Metabolites and Obesogens as Regulators of Inflammation in Adipose Tissue.
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Obesity and the metabolic diseases associated with it, such as diabetes and cardiovascular disease, are areas of major concern in the USA. Obesity is associated with low grade inflammation in adipose tissue; therefore, studying inflammation and better understanding the underlying mechanisms may be useful for developing effective methods to methodically control obesity. This work focuses on investigating the interaction between macrophages and adipocytes within adipose tissue. We shall focus on obtaining the activation profile of key transcription factors found to be associated with adipogenesis upon exposure to xenobiotics such as BPA and TBT. This information will provide us insight onto the metabolic map that occurs in adipocytes that can predispose individuals to obesity. Moreover, we shall observe the expression NF-κB, a key protein involved in immune responses to infection and inflammation. Macrophages are instrumental in the onset, maintenance, and resolution of inflammation. They regulate the immune response through the production of pro- and anti-inflammatory compounds. Bacteria have been shown to influence the development of the immune system, so we would like to assess the inflammatory properties of bacterial metabolites. However, in order to assess these properties, a reporter cell line that allows for measurement of NF-κB must be developed since

NF-κB production cannot be measured directly. The anti-inflammatory capabilities of these metabolites could lead to novel ways of combating obesity and enhance our understanding of how the human microbiota influences immune response.

DEDICATION

"To our cherished families"

ACKNOWLEDGEMENTS

NOMENCLATURE

BPA   bisphenol A

BS   bovine serum

CEBP   CCAAT-enhancer-binding proteins

CREB   cAMP response element-binding

ddH2O   double distilled water

Dex   dexamethasone

DMEM   Dubelco's Modified Eagle Medium

DMF   dimethylformamide

DMSO   dimethyl sulfoxide

FBS   fetal bovine serum

GI   gastro-intestinal

LPS   lipopolysaccharide

NF- κB   nuclear factor kappa B

PPAR-γ   peroxisome proliferator-activated receptors

RFP   red fluorescent protein

TBT   tributyl tin

TNFα   tumor necrosis factor alpha

CHAPTER I

INTRODUCTION

Motivation

Obesity is a growing health and social problem that is reaching epidemic proportions. Moreover, associated metabolic syndrome diseases, such as diabetes, have seen a dramatic increase in incidence over the past two decades[1]. For example, obesity is found to be closely associated with insulin resistance, dyslipidemia, hypertension, cardiovascular disease, and type-2 diabetes [2]. To combat obesity, we must better understand the mechanisms through which obesity-causing agents act. An important fact which must be taken into account is that obesity is accompanied by low grade inflammation of the adipose tissue. This makes obesity an important research topic to push the knowledge boundaries of systems biology and immunology, particularly contributing to work that aims to study inflammation and understand its complicated and intertwined mechanisms. Moreover, such studies will allow us to develop better approaches for combating inflammatory diseases as well as possibly preventing them.

While external physical factors such as decrease in physical activity and increase of caloric intake are significant traditional causes of obesity, there is evidence that environmental agents labeled as obesogens can also "disrupt the normal developmental and homeostatic controls over adipogenesis and energy balance[1,3]." Of particular concern is the dramatic increase in childhood obesity as it is difficult to overcome once the disease has established itself in the young person. In fact, research has shown that even in-utero exposure to organotins predispose a person to

obesity by directing stem cells to pre adipocyte formation rather than bone cell formation[4]. From a different perspective on tackling this problem, recent work has indicated that tryptophan-derived molecules, primarily from bacteria in the GI tract, attenuate indicators of inflammation in multiple cell types. Though there is prior research on the intestinal microbiome and the metabolites produced by it, these metabolites have not been studied as inflammation regulators.

Our aim is to identify how some naturally occurring xenobiotic compounds found in the environment can impact adipocytes. We shall also study how tryptophan-derived bacterial metabolites regulate inflammation in macrophages present in adipose tissue.

Background

TBT, a known organotin, represents a class of widespread organic pollutant that has the potential to disrupt endocrine function in vertebrates and invertebrates[1]. TBT has been shown to be an agonist ligand for nuclear receptors involved in lipid homeostasis and adipogenesis such as peroxisome proliferator activated receptor gamma (PPAR-γ)[1]. Therefore, exposure to certain obesogens in concentrations can lead to a cascade of metabolic changes that will directly lead to adipocyte lipid accumulation and increased risk of being susceptible to obesity. While genetic studies have been performed to study obesity, it was found that genetic variation within a population does indeed affect a person's susceptibility to become obese in the future[5]. However, it is crucially important to note that the observed exponential increase in obesity rates in America cannot be adequately explained by the standard rate of genetic change in the population. As a result, the increased rates of obesity and metabolic disorders can be best explained by environmental factors. To study this hypothesis, it is imperative to identify obesogens that may

be acting in utero or over lifetime exposure to increase obesity through adverse effects on lipid metabolism regulation.

Additionally, the tryptophan-related metabolites are naturally present in the human intestinal tract, so using them to combat disease may be safer than treatments that use synthetic (i.e., non-natural) compounds to do so. Understanding what bacteria-derived molecules have on macrophages will allow us to determine the role microbes play in obesity. Wikoff et al. showed that mammalian metabolism and microbial metabolism are linked through bacterial metabolites that potentially circulate throughout the body[6]. They also found that mice without intestinal bacteria had different tryptophan-derived metabolites than regular mice, indicating that bacteria are necessary for the synthesis of the metabolites that are usually present in the GI tract. A clinical study showed that fecal levels of tryptophan are strongly related to Crohn's disease[7], an inflammatory intestinal disease, suggesting that those with this disease are not utilizing tryptophan or converting it to other compounds and thus may have an imbalance of tryptophan-derived bacterial metabolites. Together these studies imply that the gut microbiome, or the environment created by intestinal bacteria, may be connected to inflammatory disease through the circulation of tryptophan-derived metabolites and their interaction with cells that regulate inflammation; this link is the other key focus of our research. NF-κB, a protein involved in promoting inflammation, cannot be measured directly. Therefore, in order to proceed with our work, the development of a cell line that links NF-κB expression with production of a measureable protein is necessary. Gaussia Luciferase (GLuc) is a secreted protein that glows upon reaction with a particular substrate. Kits for measuring GLuc activity are commonly available, so our lab engineered a plasmid where GLuc production was coupled with NF-κB

expression. Macrophages transduced to contain this plasmid can then be used for further experimentation to determine the effects of bacterial metabolites on NF-κB production. Additionally, constitutive expression of RFP allows cell density to be measured via spectroscopy. GLuc results can then be normalized to RFP values to minimize the impact of well-to-well variations in cell density.

The ultimate goal of our project is to attenuate inflammation. In addition, it is desired to better understand the complex interaction and signaling that occurs between immune system cells such as macrophages with adipocytes within inflamed adipose tissue. Our research is focused specifically on investigating the transcription factors altered by BPA and TBT in the adipogenic differentiation process and enlargement, as well as the activation of NF-κB in macrophages.

The specific results of our project will be of particular importance both in academia and in the healthcare industry. Moreover, our own lab group will benefit greatly from the results of the experiments. In the long term, we aim to fill in the "black box" of metabolic reactions and responses that occur within the cell upon exposure to these obesogenic and bacterial compounds. Therefore, determining the response of each transcription factor to these molecules will bring us one step closer to our goal. Other research groups involved in studying inflammatory disease and factors contributing to obesity will also find application in our results detailing the way in which each transcription factor plays a role in the pathway to adipogenesis and lipid accumulation. We hope that the long term benefits of our work as far as its impact on combatting inflammatory disease will be of use to a much wider range of people. The American Obesity Association, for example, could use information regarding harmful obesogens to advise the public on limiting

exposure to them. The Food and Drug Administration could also use this information to issue

warnings or limits regarding BPA and TBT use in consumer products. Healthcare professionals

and physicians could utilize this knowledge in patient diagnosis, and immunologists could find it

useful in furthering studies on the prevention of inflammatory disease. In summary, our project

will be a key step towards the eventual goal of effectively combating and treating obesity and

better understanding the intricate mechanisms of inflammation.

In vitro studies performed on 3T3-L1 cells suggest that in utero developmental events are

critically and possibly irreversibly affected by exposure to xenobiotic compounds. In fact,

nicotine has experimentally been used to demonstrate this phenomenon by showing that exposed

fetuses are more susceptible to obesity. Environmental estrogenic chemicals like Bisphenol A

were found to promote pre-adipocyte stem cell differentiation into adipocytes[8]. What is not yet

known is whether or not the adipogenesis and lipid accumulation process is transduced

exclusively through an estrogen receptor signaling pathway. Previous studies have indicated that

PPAR-γ agonists are more probable candidates than environmental estrogens such as BPA. This

is due to the finding that in vivo studies of BPA resulted in inhibition of obesity signaling[9]. In

addition, activation of PPAR-γ is vital in the differentiation of preadipocyte cells into adipocytes,

and an upregulation in its activity has been associated with increased fatty acid storage[1].

Tributyltin (TBT), an organotin, is expected to be a PPAR-γ agonist, so the exposure of

preadipocyte cells to TBT is expected to upregulate the activation of this transcription factor. As

a result, adipogenesis is hypothesized to increase in treated cell cultures. Our lab group aims to

measure the direct effect of TBT and BPA on adipogenesis via measurements of lipid

accumulation in adipocyte cells and to map the metabolic pathway taken by these compounds

through the quantitative measurement of transcription factor activity. It is the combination of both methods of quantitative measurements that make the work of our group unique as it not only determines the activation profile of the adipogenesis transcription factors but also directly verifies whether or not the observed profile is consistent with the physiology of the cell.

Our focus is on monitoring the activation of adipogenesis transcription factors such as PPAR-γ and CEBP which play a key role in adipocyte differentiation and energy storage to determine the effect of exposure of preadipocytes to obesogens. 3T3-L1 murine preadipocyte stem cells will be used for experimenting, as this cell line is the standard model for studying adipogenesis. Concentrations of the obesogen in question will be based on the actual levels present in the human body due to everyday exposure. For example, exposure to TBT has been tied to sources such as seafood, fungicides on produce, industrial water systems, and textiles. Levels of TBT in human tissue have been measured and found to range between 3-100 nM[6]. We hypothesize that upregulation of relevant transcription factors will occur in response to the agonization of the TBT ligand to the nuclear receptors. This phenomena is expected to manifest through an increase in number and size of fat droplets present in adipose tissue cells. PPAR-γ activation, for instance, is expected to increase the expression of genes that promote fatty acid storage. The accumulation of lipid will be measured qualitatively through staining procedures.

CHAPTER II

METHODS AND MATERIALS

3T3-L1 cells

Murine 3T3-L1 preadipocyte cell lines are used for this experiment. These cells can be

differentiated into functional adipocytes via exposure to a standard adipogenic hormone cocktail.

The preadipocytes contain a Gaussia Luciferase, or GLuc, reporter gene. The cells were cultured

and differentiated into specific adipocytes over 14 days. The GLuc gene is expressed when a

transcription factor protein binds to the protein site. Therefore, activation of the different

transcription factor can be inferred through monitoring the level of the GLuc production. Two

specific transcription factors investigated in this thesis are CEBP and PPAR.

In addition, the two obesogens studied are Bisphenol-A (BPA) and Tributyltin (TBT).

After the cells were seeded and brought to confluence, they were treated with an obesogen at

specific concentrations on the day of differentiation, or day zero. Three different groups of cell

conditions were used:

- Control group with no treatment
- Control groups treated with 2 μL of the DMF solvent
- BPA treatment groups at 10 μM and 100 μM
- TBT treatment groups at 10 nM and 100 nM

The cells were first unfrozen from a cell bank and then seeded into a T-25 plate (Becton

Dickinson, Franklin Lakes, NJ). Then the cells were passed into six-well plates (Corning, NY)

and another T-25 to continue the use of that cell lineage. The first six-well plate has three wells

for an untreated control and three wells for controls treated with 2 µL of DMF solvent. The second well plate has BPA at 10 µM and 100 µM, and the third well plate was treated with TBT at 10 nM and 100 nM. The procedure for cell culturing is described next.

Unfreezing cells

A frozen 3T3-L1 reporter cell line was withdrawn from a liquid nitrogen storage tank kept at -72°C. The sample was placed in a water bath (Kendro, Asheville, NC) at 37°C to thaw. The contents of the sample were placed into a 15 mL centrifuge falcon tube (VWR North America, Houston, TX), and the volume was brought to 5 mL by adding Growth Media. The cell sample was centrifuged (Fisher Scientific, Hampton NH) operating at -4°C and 800 RPM for 4 minutes and 30 seconds. The supernatant was decanted and 5 mL of new Growth Media was added to the falcon tube. The tube was shaken and pipetted to ensure that the cells were suspended homogeneously throughout the solution. The entire contents of the falcon tube were then transferred to a T-25 flask. The T-25 flask was then placed in a Hera cell 150 incubator (Heraues) at 37°C at 10% CO_2 for 5 days, with Growth Media being changed every two days to keep the cells nourished.

Passing cells

The Growth Media in the T-25 flask was aspirated out. Five milliliters of PBS was used to wash the cells as it allows trypsin to more easily detach them from the flask. Next, the PBS was aspirated and 1 mL of trypsin (Sigma Aldrich, St. Louis, MO) was added to the T-25 flask. The plate was incubated for 2 minutes to allow the trypsin to suspend the cells. The cells were checked under a light microscope to ensure that suspension had occurred. Four milliliters of

fresh Growth Media were next mixed with the one milliliter of cell suspension in a 15 mL falcon tube. The mixture was centrifuged for 4 minutes and 30 seconds at 800 RPM. The liquid was then decanted and 5 milliliters of fresh Growth Media was added to the falcon tube. The tube was shaken and the cells were pipetted to ensure proper suspension and homogenous mixing. The cells were then seeded into the desired well plate at a 1:10 dilution factor. For a T-25 flask, 500 µL of the cell suspension was added to 4.5 mL of Growth Media. For a six-well plate, 200 µL of cell suspension was added to 1.8 mL of fresh Growth Media.

Cell growth and supernatant collection

The plates were placed into the incubator at 37°C and 10% CO_2 to allow the cells to grow. The media was changed every two days to ensure the cells were adequately nourished. The cells typically became confluent within 3 days; however, they may require longer if they had recently been unfrozen. Once the cells in the six-well plate became 100% confluent, that day was marked as day (-2). Day (0) is marked after the cells become 100% confluent two days later. The media was changed to Differentiation Media 1 on day (0) to begin the process of differentiation into adipocytes. On day (2), the media was changed to Differentiation Media 2. On days (4) onwards, the media was replaced every two days with Maintenance Media. On the last day to be investigated, day (14), no new media was added.

One milliliter of supernatant was collected from each well at each time point and frozen in a 1.5 mL centrifuge tube until the day it is analyzed by a luminometer. Remaining supernatant was removed and two milliliters of fresh media were pipetted into the wells. Wells to be treated with DMF, TBT, or BPA, had the following added to them as well:

16

- 5 µL of 4 mM and 40 mM BPA to make 10 µM and 100 µM BPA well conditions respectively
- 5 µL of 4 µM and 40 µM TBT to make 10 nM and 100 nM TBT well conditions respectively
- 2 µL of DMF to ensure that the solvent does not affect cell viability and the observed response transcription factor.

Culture media

Four types of media were used throughout this experiment; growth media, differentiation media

1 (DM1), differentiation media 2 (DM2), and maintenance media (MM).

Growth media

To prepare 1 L of Growth Media, the following were mixed together in a beaker:

- 880 mL of ddH$_2$O
- 10 g of Dubelco's Modified Eagle Medium (DMEM) (Fisher Scientific)
- 3.7 g of sodium bicarbonate (NaHCO$_3$) (Fisher Scientific)
- 20 mL of penicillin/streptomycin solution at 200 units/mL and 200 µg/mL respectively (Fisher Scientific).
- 110 mg of sodium pyruvate (Fisher Scientific)
- 2M HCl to adjust pH to 7.3

One hundred milliliters of bovine serum (Fisher Scientific) were finally added to make up the

volume of 1 L. The solution was then filtered through a 150 mL, 0.22 µm Steritop Millipore

filter into an autoclaved 1 L bottle.

Differentiation Media 1

To prepare 1 L of Differentiation Media 1, the following were mixed together in a beaker:

- 880 mL of ddH$_2$O
- 10 g of DMEM (Fisher Scientific)
- 3.7 g of NaHCO$_3$ (Fisher Scientific)
- 110 mg of sodium pyruvate (Fisher Scientific)

- 20 mL of penicillin/streptomycin solution at 200 units/mL and 200 ug/mL respectively (Fisher Scientific)
- Dexamethasone (Dex) or 3-isobutyl-1-methylxanthine (IBMX) (Sigma)

Beaker contents were filtered through a 150 mL, 0.22 μm Steritop Millipore filter and stored in an autoclaved container.

Differentiation Media 2

To prepare 1 L of Differentiation Media 1, the following were mixed together in a beaker:

- 880 mL of ddH$_2$O
- 10 g of DMEM (Fisher Scientific)
- 3.7 g of NaHCO$_3$ (Fisher Scientific)
- 110 mg of sodium pyruvate (Fisher Scientific)
- 20 mL of penicillin/streptomycin solution at 200 units/mL and 200 μg/mL respectively (Fisher Scientific)
- 1 μg/mL Insulin
- 2 nM 3-3-5-triiodo-L-thyronine (T3)
- 2 M HCl for pH adjustment to 7.3
- 50 mL of fetal bovine serum (FBS) (Fisher Scientific)

The beaker contents were filtered through a 150 mL, 0.22 μm Steritop Millipore filter and stored in an autoclaved container.

Maintenance media

To prepare 1 L of Maintenance Media, the following were mixed together in a beaker:

- 880 mL of ddH$_2$O
- 10 g of DMEM
- 3.7 g of NaHCO$_3$
- 20 mL of penicillin/streptomycin solution at 200 units/mL and 200 μg/mL respectively.
- 110 mg sodium pyruvate
- 2M HCl to adjust pH to 7.3

One hundred milliliters of FBS was finally added to make up the volume of 1 L. The solution was then filtered through a 150 mL, 0.22 μm Steritop Millipore filter into an autoclaved 1 L bottle.

Fluorescence measurements

Red fluorescence intensity was measured in the 3T3-L1 cells as a measure of cell density to account for variation in the cell density between experiments. Measurements were made using a Spectra MAX Gemini FM (Molecular Devices). The excitation and emission wavelengths were 550 nm and 600 nm, respectively.

Luminescence measurements

Gaussia Luciferase (GLuc) activity in the samples determined using a BioLux® Gaussia Luciferase Assay Kit (New England Biolabs Inc) and measured using a luminometer.

Dilution procedure

Samples were diluted 1:200 (1:10 followed by a 1:20 dilution) for measuring luciferase activity. The original supernatant sample and intermediate diluents were vortexed to ensure proper mixing of the tube contents.

Operating the luminometer

20 μL of each sample were used in each well of a 96-well plate (Whatman Incorporated, Piscataway, NJ) in duplicate.

Luminometer data processing

The output of the luminometer was processed with Microsoft Office Excel. The Relative

Luminescence Unit (RLU) of each well was normalized by the RFP reading. The data was also

normalized with respect to the time between the supernatant collections.

The data normalized from each well was then further normalized to the day (0) reading. The

luminometer data ($\frac{\text{RLU}}{\text{RFP·time}}$) was then plotted against time to observe how each well condition

responded compared to the negative control well.

Errors in measurement were taken into account and plotted as error bars by statistical analysis of

the data to obtain standard deviation.

Adipored stain

In addition to luciferase activity, lipid accumulation in 3T3-L1 cells was quantitatively measured

using adipored staining of lipid droplets. Cells were rinsed with 1 mL of PBS, and 5 mL of PBS

was then pipetted into each well with 140 μL AdipoRed™ stain (Lonza). The well plates were

then incubated for 15 minutes, after which they were analyzed through fluorescence readings at

excitation and emission wavelengths of 485 nm and 572 nm, respectively. The results were be

normalized with RFP measurements to account for differences in cell density between wells.

Bacterial metabolites

Cell culture

The J774A.1 cell line (Sigma Aldrich's, St. Louis, MO), consisting of murine macrophages, was utilized for the bacterial metabolite portion of our research. These cells were cultured in Dulbeco's Modified Eagle Medium supplemented with 2 mM glutamine and 10% fetal bovine serum and grown at 37°C and 5% CO_2.

Reporter cell line

Our lab used lentiviral transduction to introduce a reporter element for NF-κB into macrophages, which allowed NF-κB levels to be monitored by measuring the reporter GLuc activity.

Dilutions

LPS was stored in a stock form of 5 mg/mL. Twelve microliters of stock was added to 12 mL of media to create a 5 µg/mL solution. One microliter of the stock was added to media-filled wells desired to have a concentration of 10 µg/mL. For wells to have a concentration of 1 µg/mL, 100 µL of the refreshed media was removed and replaced with the 5 µg/mL solution. For wells to have a concentration of 0.1 µg/mL, 10 µL of media was replaced with the 5 µg/mL LPS solution.

Data collection

After incubating the exposed cells for 8 h, 20 µL of the supernatant from each well was collected every 4 h. Twenty-four hours after the cells were exposed to LPS, all of the supernatant was collected. A spectrometer reading at 550 nm excitation and 600 nm emission was taken to determine the relative number of cells in each well.

The GLuc assay was performed using the luminometer, as described in the methodology for

3T3-L1 cells, on all collected samples.

CHAPTER III

RESULTS

3T3-L1 cells

Experiment 1

The first experiment investigated the effect of obesogens on the activation of two transcription factors PPAR-γ and CEBP. The control plate consisted of three untreated control wells along with three wells treated with the solvent only, DMF to ensure that it does not alter the expression of the transcription factors, or compromise cell viability. The BPA and the TBT well plates included both low and high concentration treatments. BPA treatment included 10 μM and 100 μM while TBT included 10 nM and 100 nM treatment, similar to values found in the literature. For this experiment, cell viability became an issue due to the toxicity of the DMF solvent utilized (0.5% vol/vol). Therefore, the adipocytes died at the higher concentrations (100 μM and 100 nM) for both BPA and TBT treatment respectively. This phenomenon, was observed for both transcription cell-lines. Therefore, the dilutions of the stocks was manipulated such that the DMF in each well does not exceed 0.2% (vol/vol). Because of this complication, the data obtained from the first experiment includes treatment at only the lower concentrations (10 mM for BPA and 10 nM for TBT). The data was normalized to the initial time-point of each respective condition. Plots were generated that depict transcription activation through RLU readings versus time from the onset of differentiation. Each well condition was then compared to the control wells as shown in Figures 1 and 2.

A substantial error was observed in some of the data points of Figures 1 and 2. We identified mixing and dilution limitations in our experimental procedure that could contribute to the observed errors. We improved upon the procedure by incorporating vortexing and utilizing larger tubes to ensure better mixing in our dilution procedure prior to obtaining luminescence readings from the luminometer.
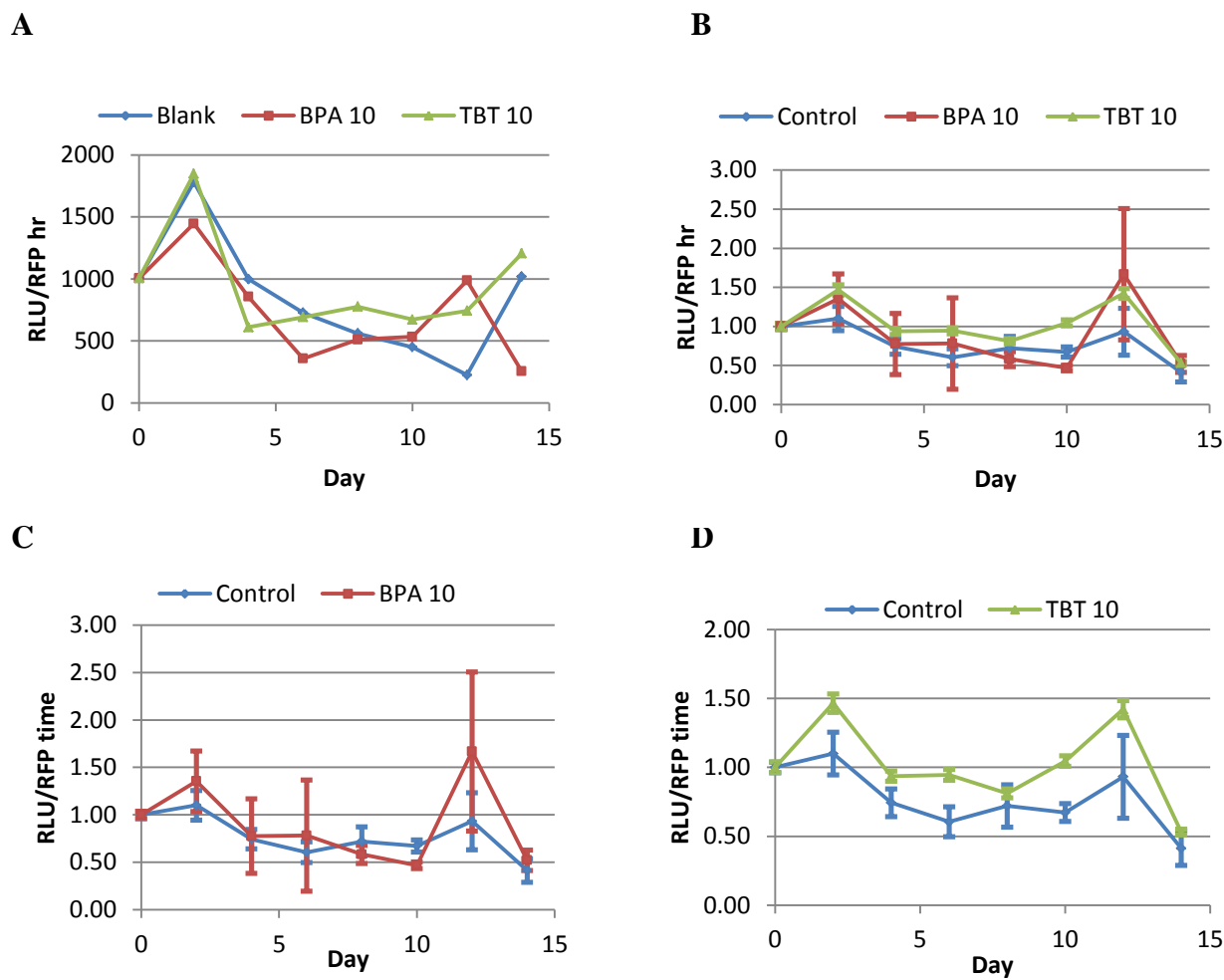
A

B

C

D

Figure 1 (A-D): (A) PPAR-γ Reporter profile without relative normalization. (B) PPAR Reporter profile normalized with respect to day 0. (C) PPAR-γ Reporter profile of control vs. BPA at low concentration normalized with respect to day 0. (D) PPAR-γ Reporter profile of control vs. TBT at low concentration normalized with respect to day 0.
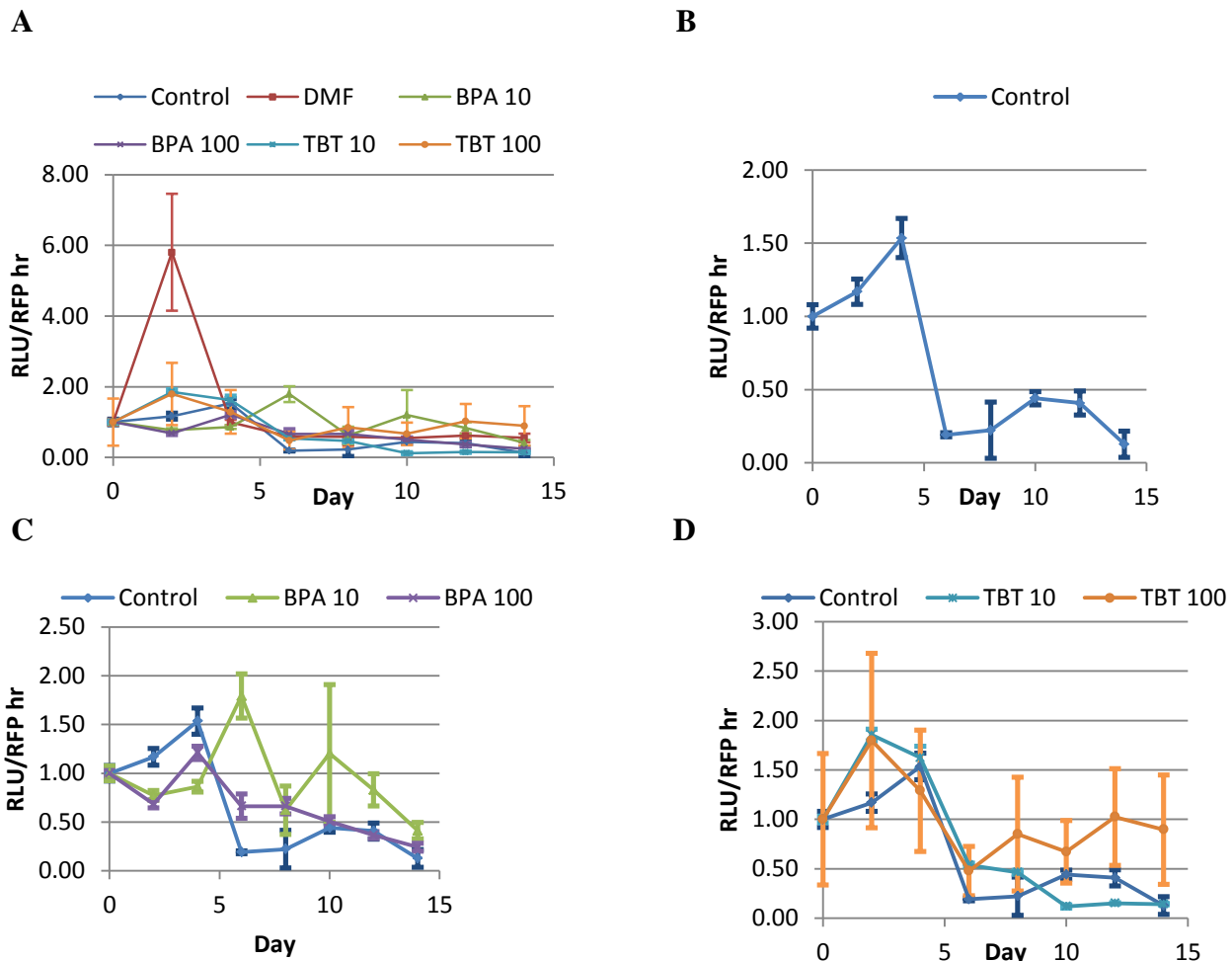
A

B

C

D

Figure 2 (A-D): (A) Overall response profile of CEBP with respect to data from day 0. (B) Response profile of untreated CEBP cells. (C) Response profile of CEBP to 10 μM and 100 μM BPA compared to control. (D) Response profile of CEBP to 10 nM and 100 nM TBT compared to control.

For experiment trial 1, Adipored readings were obtained on day (14) to measure lipid formation for each of the treatment conditions. These readings were normalized to RFP measurements for each well. The Adipored readings provide us with an additional independent data dimension to compare the effects of the obesogens on the production of lipid, which is hypothesized to increase, as shown in Figure 3.
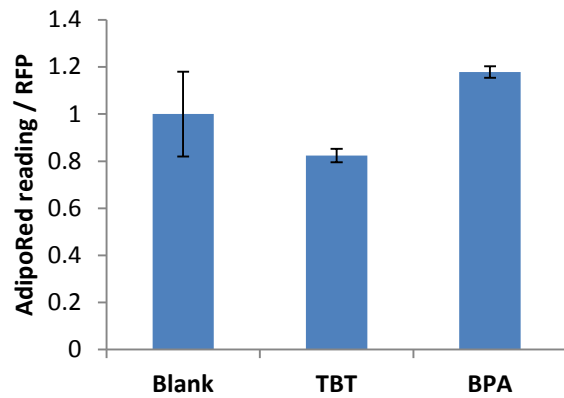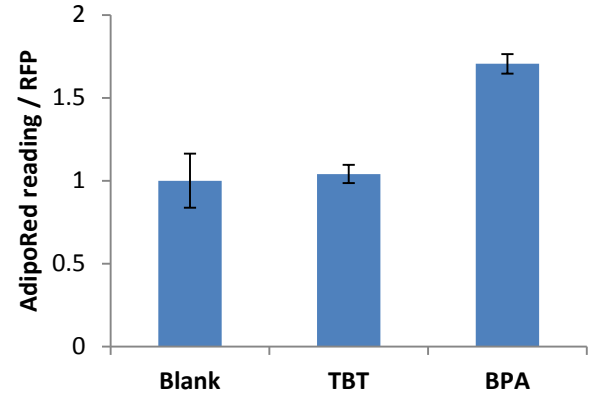
A

B

Figure 3 (A-B): (A) Adipored reading comparisons for CEBP reporter cell line at day 14. (B) Adipored reading comparisons for PPAR-γ reporter cell line at day 14.

The PPAR-γ reporter cell line was imaged on days (10) and (16) for visual observation of lipid formation in response to exposure to BPA and TBT as compared to lipid formation in the control wells, as can be seen in Figure 4 A-F.
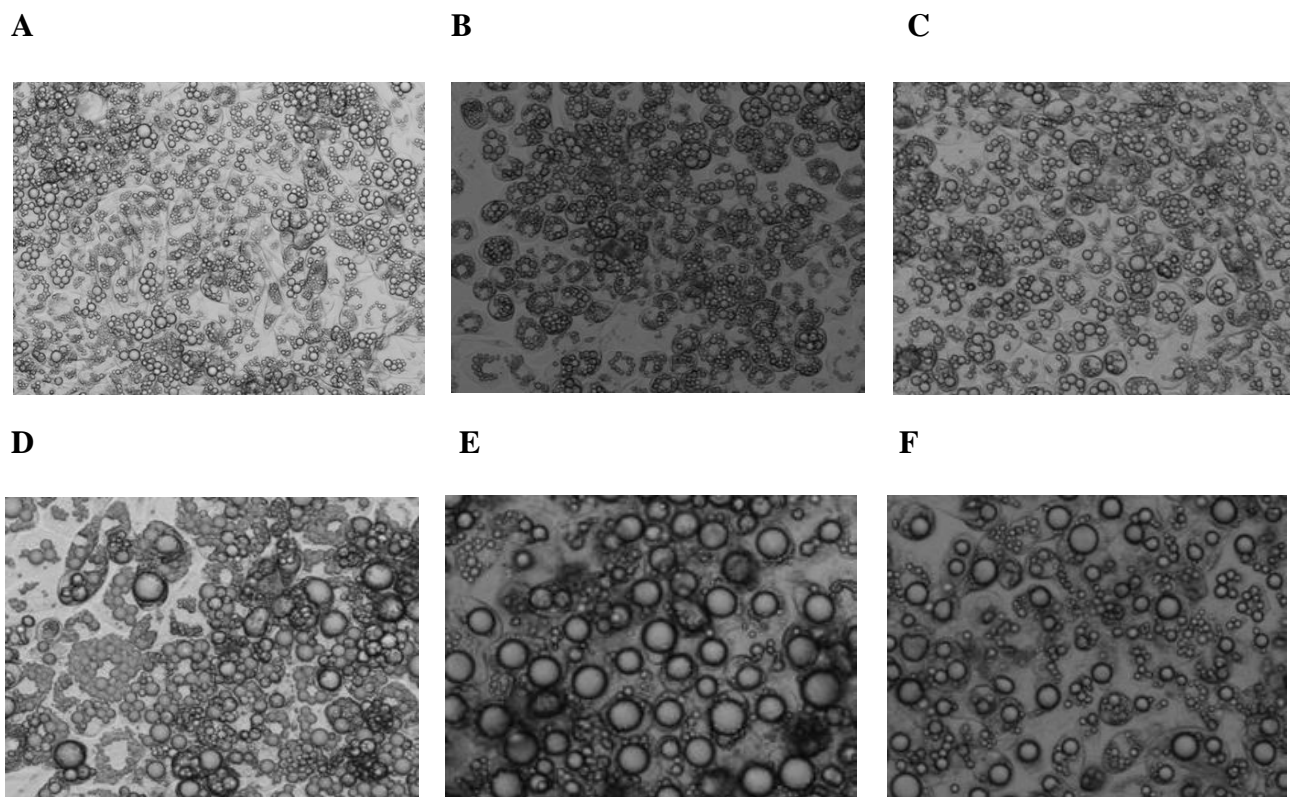
A   **B**   **C**

D   **E**   **F**

Figure 4 (A-F): (A) Untreated cells at day 10, 10x magnification. (B) Cells treated with 10 µM BPA at day 10, 10x magnification. (C) Cells treated with 10 nM TBT at day 10, 10x magnification. (D) Untreated cells at day 16, 10x magnification. (E) Cells treated with 10 µM BPA at day 16, 10x magnification. (F) Cells treated with 10 nM TBT at day 16, 10x magnification.

Experiment 2

The second experiment tested the separate conditions on each transcription factor. CEBP and

PPAR-γ cells were subjected to treatment of 10 µM and 100 µM of BPA; 10 nM and 100 nM of

TBT as well. Two control wells, one untreated and one containing the DMF solvent, were

included for each reporter cell line. Due to the toxicity of DMF at higher concentrations observed

in the previous experiment, new stock solutions of BPA and TBT were prepared so that a lower

volume of DMF was added in each well. This implementation resolved the cell viability

complication and cells under all treatment conditions survived the entire course of the

experiment. Another difficulty encountered during trial 2 was that the PPAR-γ cell line did not fully differentiate. This phenomena is due to the high pass-number of the utilized cell-line, which greatly decreases the viability of the cells and introduces substantial lift off. Once again, luminometer readings were obtained and normalized with respect to time and the RFP reading of each well at day 7. This data is compared to the control to obtain an overall response profile as shown in Figure 5 (A-D).
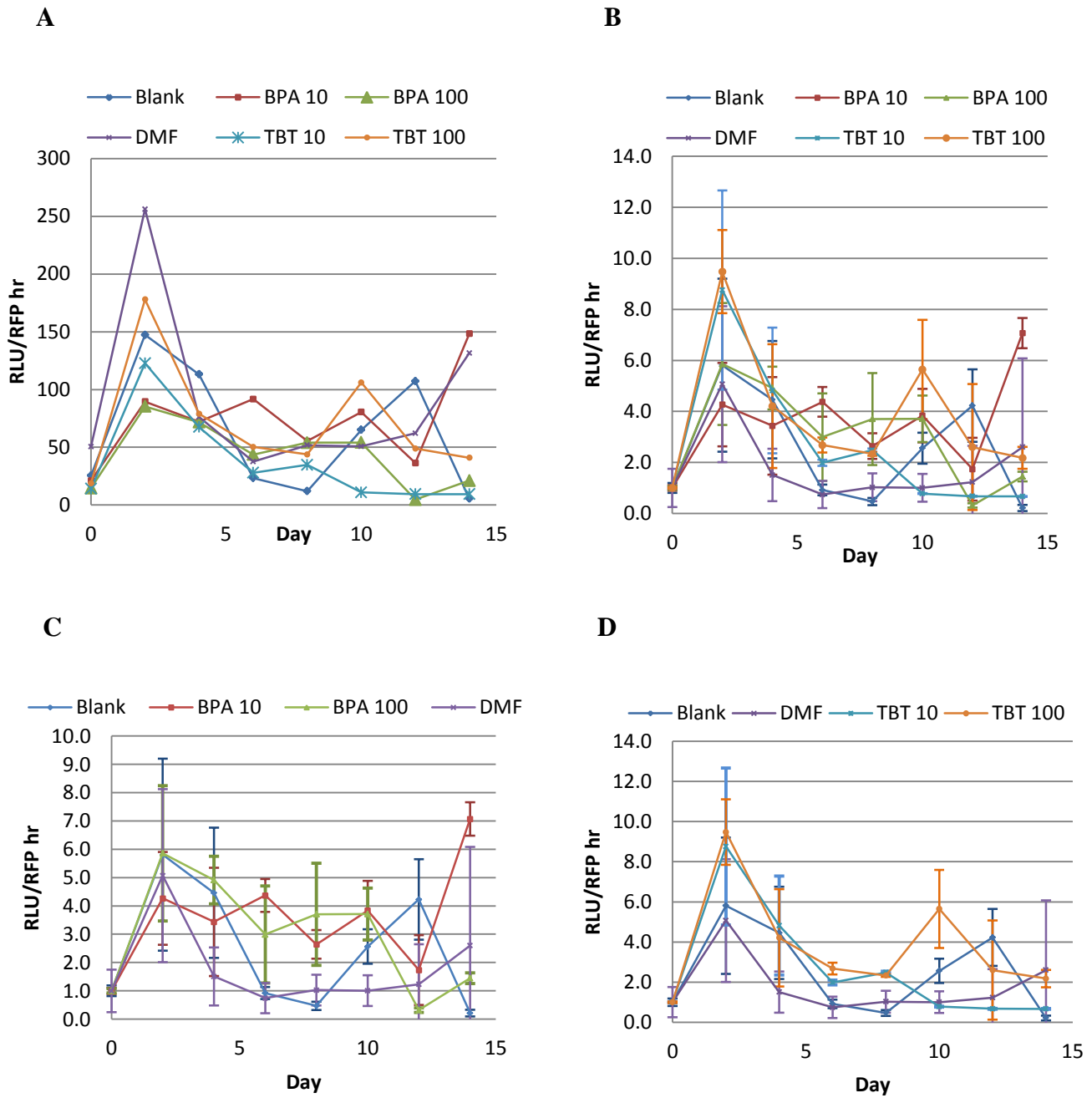
A

B

C

D

Figure 5: (A) CEBP response profile without relative normalization. (B) Response profile normalized with respect to day 0. (C) Response profile of BPA with respect to day 0. (D) Response profile of TBT with respect to day 0.

Bacterial metabolites

Experiments are currently in progress to determine the response of J774 A.1 cells to tryptophan-derived metabolites. Lentiviral transduction to produce J774 A.1 cells containing a reporter plasmid for a GLuc and RFP was successful. Figure 6 shows that the transduced cells exhibit a time-dependent response to LPS stimulation.
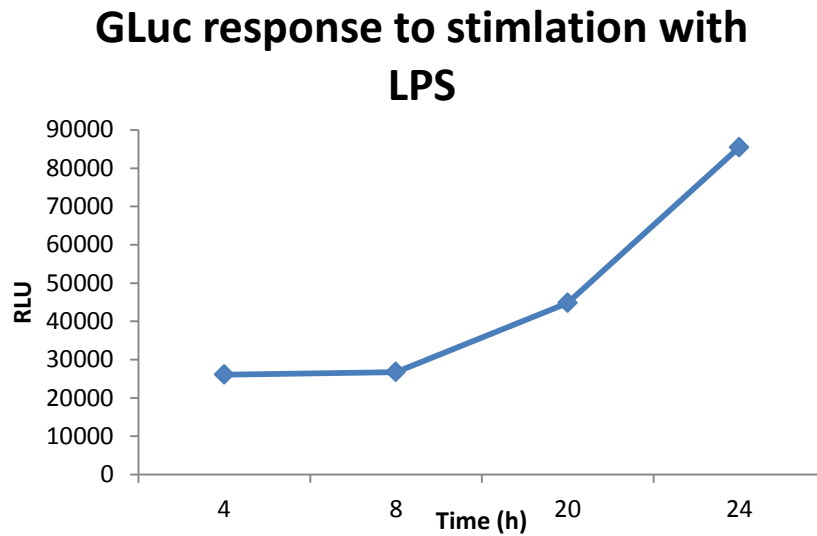
GLuc response to stimlation with LPS

Figure 6: GLuc response of transduced J774A.1 cells to stimulation with 250 ng/mL LPS.

Another objective of the reporter cell line is the cells should constantly express RFP so red fluorescence can be used as an indicator of cell density so GLuc readings can be normalized to RFP expression and thus not reflect well-to-well variations in cell density. Table 1 shows RFP readings for blank wells as well as non-stimulated J774A.1 reporter cells.

Table 1: RFP measurements without stimulation	
	RFP fluorescence
Blank well	0.440
Transduced J774 A.1 cells	1.355

CHAPTER IV

CONCLUSION

Transcription factor profiling experiments

From the graphs in Figure 2, we draw conclusions on the effect of obesogens on the PPAR- γ reporter. These results are tested for statistical significance with the Q test at a 90% confidence interval. Except for the data-point of day 12, it can be concluded that BPA at 10 µM does not have a statistically significant effect on PPAR activation. In comparison to the control wells, the large error observed in Figure 2 (C) for the BPA treated cells makes the output of PPAR- γ activation of 10 µM BPA treated cells to be statistically indiscernible from that of the control cells.

On the other hand, Figure 2 (D) indicates that cells treated with TBT at 10 nM increase activation of PPAR- γ throughout the course of the experimental period. Nevertheless, the activation profile of PPAR- γ is observed to be the same between the control and TBT treated cells.

The graph in Figure 3 (C) suggests that the cells treated with 10 µM of BPA have an increased activation of the CEBP reporter. However, at 100 µM BPA, the increased effect is not observed. Instead, a decrease in activation of CEBP is noted. This decrease in activation of CEBP can be explained by inhibitory or even toxic effects of BPA at 100 µM on the cells.

TBT, on the other hand, does not seem to have a significant effect on the activation of the CEBP reporter, as can be seen in Figure 3 (D). At 10 nM TBT, the activation appears to increase only initially up to day 2. However, this activation of quickly decreases afterwards and reaches the same level as the control wells. TBT at 100 nM does not give statistically significant results. The error associated with the obtained data overlaps with the control wells. Therefore, no conclusions can be withdrawn about the effect of TBT at 100 nM on the CEBP reporter.

Lipid accumulation analysis

Comparison to the control well in Figure 4 (A and B) shows that cells treated with 10 nM TBT had approximately equivalent amounts of lipid as the control wells. On the other hand, cells treated with 10 μM BPA displayed significant increase in lipid accumulation, as can be seen from the column labeled BPA in Figure 4.

The microscope images shown in Figure 5 (D-F) provide visual support for the Adipored stain results. Indeed, Figure 5 (E) shows a larger number and size of lipid droplets on the adipocytes that were treated with 10 μM BPA on day 14 in comparison with Figure 5 (D) which resembles the control cells on day 14.

Metabolic pathway conclusions

We observe from our research that TBT at 10 nM causes an increased activation in PPAR- γ. This is consistent with what has been observed and reported by other studies [1]. Furthermore, an initial increase in CEBP activation is observed for cells treated with TBT. This is an interesting observation which provokes the question of how the rest of the transcription factors associated

with adipogenesis respond to stimulation by TBT. This needs to be further investigated by knocking out the reporter genes one at a time and observing how the profiles of the rest of the reporters continue to behave upon similar stimulation.

As for BPA stimulation, we observed that BPA does not affect the PPAR- γ profile. Therefore, we can conclude that BPA does not act through the PPAR- γ pathway. However, BPA did increase the activation of CEBP, and the Adipored results as well as the microscope images show that BPA increases lipid accumulation. As a result, the logical conclusion for those observations is that BPA causes metabolic changes independent of the PPAR- γ. Similar to the recommendation given for TBT stimulation, the profiles of other transcription factors must also be investigated to correctly map out the metabolic pathway for the mechanism of BPA on adipogenesis.

Another recommendation is to utilize low concentrations of the stimulants when testing out the rest of the reporters. The concentrations to utilize are 10 μM and 10 nM for BPA and TBT respectively. This recommendation stems from the viability issues encountered during experimentation when the cells were exposed to toxic levels of DMF solvent. In addition, the toxicity threshold of BPA and TBT is undetermined, but is sure to exist. Therefore, it is best to avoid cell viability issues by minimizing the effect of the solvent, and preventing the stimulant from killing the cells.

Bacterial metabolites - reporter cell line development

Figure 6 shows that GLuc levels increase with stimulation, indicating the increased production of NF-κB in response to pro-inflammatory molecules corresponds to an increase in GLuc production; which is one of the key objectives of the reporter cell line. The second objective is constant production of RFP. Table 1 shows that the cells express RFP even when unstimulated. More rigorous analysis of RFP production could be executed by comparing the transduced reporter cells to regular J774A.1 cells as well as comparing stimulated and unstimulated cells. This analysis was only carried out in a qualitative fashion; quantitative analysis will be performed in future work. However, current analysis indicates that the reporter line has successfully been developed as intended with Gluc expression corresponding to NF-κB activation and constitutive expression of RFP. These cells can now be used in experiments to determine the effects of various bacterial metabolites on NF-κB expression in macrophages. Activating the inflammatory response allows us to measure the efficacy of the metabolites as anti-inflammatory agents by observing how much they reduce the inflammatory response compared to the control.

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