IKK/NF-κB-regulated Inflammatory Pathway in Human Adipocytes: Implication in Subsequent Contribution to Insulin Resistance and Atherosclerosis

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IKK/NF-κB–regulated Inflammatory Pathway in Human Adipocytes: Implication in Subsequent Contribution to Insulin Resistance and Atherosclerosis

Bimjhana Bishwokarma

A Thesis in the Field of Biotechnology
for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

Obesity is associated with a state of chronic inflammation that is thought to be major contributor to disease and atherosclerosis. Many inflammatory pathways that contribute to the development of insulin resistance and atherosclerosis are regulated by IKK- NF-κB signaling. Several studies during the past two decades have highlighted the key role of the IKK/NF-κB pathway in the induction and maintenance of the state of inflammation that underlies metabolic diseases. We addressed the stipulated role of IKKβ to produce proinflammatory cytokines in cultured human adipocytes using small molecule inhibitor. We hypothesized that IKK/NF-κB signaling plays a critical role in inflammatory pathway in human adipocytes which may subsequently contribute to insulin resistance and atherosclerosis. We have developed an effective protocol for deriving adipocytes from in vitro culture of pre-adipocytes and demonstrated upregulation of proinflammatory cytokines in this system. Our results show that IKK inhibition abrogates inflammatory cytokine secretion during adipogenesis and mature adipocytes in dose dependent manner. In conclusion, this study provides the critical role of IKK/NF-kB-mediated inflammatory pathways in human adipocytes; and represents proof of concept for use of appropriate IKK inhibitor as an innovative therapeutic strategy to treat obesity and cardiometabolic diseases.
Importance of this work: Obesity is a rapidly growing epidemic representing a serious threat to the health of the population in almost every country around the world. There is an imperative need to understand the mechanisms underlying the obesity and associated cardiometabolic disorders. In this thesis, we have shown that inhibition with specific small molecular inhibitor of IKK abrogated the proinflammatory cytokine release. This study provides a foundation for targeting the IKK NF-kB signaling as therapeutic strategy for metabolic diseases.
IKK/NF-kB Target Validation in Obesity-Associated Metabolic Disorders

Figure 1: The Vicious Cycle of Obesity, Inflammations and Associated Metabolic Disorders
Author’s Biographical Sketch

Hailed from Nepal, the author of this thesis currently resides in Brookline, Massachusetts, USA with her husband and three children. Ms. Bishwokarma works as an Immuno-Oncology Scientist in the prestigious Pharmaceutical Company Merck & Co., Inc.

What drives Ms. Bishwokarma is an earnest desire to contribute to the betterment of society at every walk of life. Besides her scientific career, the author is a poet and loves to travel with her family. Her other hobbies include cooking, writing, and photography. Ms. Bishwokarma believes in making as many good memories as possible for her children, and her children keep her motivated and grounded.
Dedication

This Thesis is Dedicated to The Ones Who Perpetually Give Wings to
My Dreams:

My Mother, Mrs. Mithai Devi Bishwokarma

&

My Father, Mr. T.R. Bishwokarma
Acknowledgments

This work would not have come to fruition had it not been for all the supports I received from people from different walks of my life. First and foremost, I would like to thank immensely to my Thesis Director Dr. Vilas Wagh, without whom this thesis would not have been possible. I appreciate all your expertise, time, support, and your patience! My heartfelt gratitude is also extended to my Thesis Advisor, Dr. Steven Denkin, for his immense support, encouragement, and inspirational contributions towards this thesis work. I thank you for providing me with the opportunity to obtain an ALM degree from Harvard Extension School. I would like to thank Ms. Maura McGlame, my Research Advisor, for the encouragements and support throughout my tenure at Harvard. Without you, navigating all the routes towards my degree would have been very difficult! I would also like to thank Dr. Min Lu for his expertise, valuable inputs, and advices for this thesis work. All the staffs and members of Harvard that I have crossed paths with, who helped me with myriads of things throughout my journey and who made my experience at Harvard memorable, are also very much appreciated!

Last, but not the least, my heartfelt gratitude is extended to my family: to my husband for his immense patience, encouragements, support, and unwavering confidence in me, and for loving me unconditionally since the day I met him; to my children Zaid, Isra, and Sophie for being the source of inspiration for me and for providing me with the much needed bliss and panacea during difficult times; to my father whose blessings protect me from even the afterworld; to my mother and sister for their unwavering support and faith in me, and for always being there; to my brother and
brother-in-law for always encouraging me towards getting this degree and not letting me lose sight when I wanted to quit; and finally to my sister-in-law, my nephew, nieces; my extended family; and family-in-law for their support and understandings! And last, but not least, to all my friends who always had been there for me. You all are my rock!

This work is as much all of yours as mine!
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Chapter I
Introduction and Background

Public Health Relevance

Chronic positive energy balance, where calorie intake is higher than energy expenditure, leads to obesity; which gives rise to the multitude of metabolic pathologies in its wake. The fast-growing epidemic obesity and associated metabolic consequences like diabetes, high cholesterol/triglyceride, hypertension, and cardiovascular disease, collectively pose a global health threat; placing high financial burden on the global economy (www.cdc.org ). The failure and/or limitation of current therapies in managing obesity and associated metabolic disorders warrant the development of novel therapeutic interventions.

Obesity is associated with both increased adipocyte size (hypertrophy) and adipocyte number (hyperplasia). Adipocyte number is a major determinant of fat mass in adults and about 10% of the body’s adipocytes are annually regenerated in adults (Rodeheffer et al., 2008; Spalding et al., 2008). This annual increase in adipocytes directly correlates with obesity; hence deeming the regulation of new adipocyte production as a potential therapeutic target to treat obesity (Rodeheffer et al., 2008). Type 2 Diabetes Mellitus (T2DM) is one of the consequences of obesity. Owing to its relevance in obesity, the ideal model to test the translatable modalities of T2DM would be human adipocytes. Insulin resistance is a hallmark of obesity and a risk factor for T2DM (Aronne and Segal, 2002; Wilcox, 2005). Inflammation is a mutual trait
attributed to both obesity and insulin resistance. Extensive research implicated directly proportional relationship between obesity-associated chronic low-grade inflammation and T2DM and Atherosclerosis (Gregor and Hotamisligi, 2011). Thus, delineation of inflammatory pathway is a crucial step in understanding the mechanisms underlying this pathology. Many of these inflammatory pathways are regulated by the transcriptional factor NF-kB, which regulates innate and adaptive immune responses (Hayden and Ghosh, 2008). NF-kB activation requires IkB kinase (IKK) β, which is a major catalytic subunit of IKK complex (Hayden and Ghosh, 2008; Sui et al., 2014). Thus, we seek to delineate IKK/NF-κB – regulated Inflammatory Pathway in human adipocytes.

IKKβ has been defined as a crucial molecular link between obesity, inflammation, and associated metabolic disorders (Baker et al., 2011; Solinas et al., 2010). Cai et al. have shown that constitutive activation of IKKβ in the liver causes insulin resistance (Cai et al., 2005). IKKβ activation in hypothalamus has also been interconnected with obesity and associated metabolic syndrome (Zhang et al., 2008). Taken together, these data stipulate a role of IKKβ in obesity, diabetes, and insulin resistance. Thus, our research study would lead a way to elucidate the role of IKK/NF-kB in contributing to Insulin Resistance and Atherosclerosis.

We hypothesize that IKK/NF-kB plays a critical role in inflammatory pathway in human adipocytes and it may subsequently contribute to Insulin Resistance and Atherosclerosis. We have proposed to test this hypothesis via two specific aims; by evaluating the role of IKK inhibition on adipogenic differentiation and further assess the role of IKK inhibition on inflammatory response. This work will pave the way to further evaluate whether IKK confers insulin resistance in the human adipocytes and whether
IKK inhibition can reverse insulin resistance and hence, render the adipocytes sensitive to insulin.

The limited efficacy of current therapies for obesity and associated metabolic disorders necessitates the search for new therapies. Data generated from this study may define the role of IKK/NF-κB in adipogenesis and atherosclerosis and further disclose a critical link between inflammation and cardiometabolic disease. This study will also help fortify establishment of the human adipocytes as relevant cellular model for subsequent research studies. Our data may demonstrate the IKK/NF-κB-mediated inflammatory pathways in that cell line; further establishing IKK as a therapeutic target for obesity and associated metabolic diseases, such as, diabetes and atherosclerosis.

**Obesity and Associated Metabolic Disorder**

The global prevalence of obesity has rendered it an epidemic. According to the statistics from Centers for Disease Control & Prevention, approximately 35.7% of adults and 17% of children are struggling with obesity in the United States alone, burdening American finances with high annual medical cost (www.cdc.org). Thus, the research studies that elucidate the molecular mechanism underlying obesity have evoked interests worldwide. The grave consequences of obesity are metabolic diseases. Extensive research studies have shown that obesity contributes significantly to widespread epidemics like Type-2 Diabetes Mellitus (T2DM) and other metabolic syndromes, such as increase in bad cholesterol, hypertension, atherosclerosis, and some cancers (Guh et al., 2009).
Obesity, Diabetes, and Insulin Resistance

The National Diabetes Educational Program (NDEP) by the National Institute of Health (NIH) has reported that about 13 million men and 12.6 million women have diabetes, 90 to 95% of which is accountable to T2DM; contributing to be the 7th leading cause of death in the U.S. (www.ndep.nih.gov). Insulin resistance is a hallmark of obesity and a risk factor for T2DM (Aronne and Segal, 2002; Wilcox, 2005). Insulin is a peptide hormone secreted by the pancreatic β cells and plays an important role in the metabolic activity of the body, such as maintaining normal blood glucose levels (Fu et al., 2013). Insulin resistance is defined as a metabolic disorder in which the body cannot use the insulin it produces, resulting in glucose being built up in the blood instead of being absorbed and eventually leading to T2DM (Zeyda and Stulnig, 2009; Malik et al., 2013). The intricate relationship between obesity, insulin resistance, and T2DM constitutes a vicious cycle, playing causal/effect role in each other’s etiology. Despite substantial efforts, the management of obesity and the associated metabolic conditions remains the area of significant unmet medical need.

Atherosclerosis

Atherosclerosis is a disease of arteries, in which plaque buildups in the arterial walls clog the arteries and make them narrow, contributing to disruptions in the blood flow and fatal strokes, heart attacks, etc. (www.mayoclinic.org). These plaques or fatty deposits constitute cholesterol, triglycerides, fatty substances, cellular debris, calcium, and fibrin (a clotting material in the blood) (Wattanakit et al., 2005).
Obesity, Inflammation, oxidative stress, T2DM, and Atherosclerosis

Inflammation and oxidative stress are the hallmarks of obesity and associated metabolic syndrome (Grattagliano et al., 2008; Pennathur et al., 2007). Extensive research implicated directly proportional relationship between inflammation and T2DM and Atherosclerosis (Gregor and Hotamisligi, 2011). C-reactive protein (CRP), a biochemical marker for low grade inflammation, is elevated in T2DM (Wu et al., 2002). Many studies have linked oxidative stress with insulin resistance. Oxidative stress has been attributed as an initial causal event in high fat diet-induced insulin resistance and obesity (Matsuzawa-Nagata et al., 2008). The Obesity-associated oxidative stress can start the process of lipid peroxidation, via attacking of free radicals, such as hydroxyl radicals and peroxynitrite, on the unsaturated bonds of the lipids linoleic and arachidonic acids; subsequently generating 4-hydroxynonenal (4-HNE) (Mark et al., 1997; Kruman et al., 1997). 4-HNE has shown to be elevated in obese patients – in muscle (Russell et al., 2003) and in adipocytes (Grimsrud et al., 2007); and is thought to play an important role in obesity-associated metabolic syndrome. Downregulation of 4-HNE is associated with enhanced insulin sensitivity (Morris et al., 2008; Vincent et al., 2001). Other lipid peroxidation product like prostaglandin is also implicated in obesity and metabolic syndrome (Reginato et al., 1998). Thus, delineation of inflammatory pathway is a crucial step in understanding the mechanisms underlying this pathology.

Obesity and Adipocytes
The Adipose tissue plays a crucial role in maintaining energy homeostasis through various mechanisms (Frayn, 2001). Adipocyte number is a major determinant of fat mass in adults (Rodeheffer et al., 2008). This annual increase in adipocytes directly correlates with obesity. Obesity is associated with both increased adipocyte size (hypertrophy) and adipocyte number (hyperplasia) (Rodeheffer et al., 2008). Thus, adipocytes would be an ideal model to test the translatable modalities of obesity-associated metabolic diseases. Most of the experiments in this field have been carried out in mouse adipocytes and hence, not very translatable to human studies. Establishing human adipocytes as a relevant cellular model to be utilized for downstream in vitro modalities to study T2DM would be a great milestone in the translational research in T2DM therapeutic space. Our study would establish adipocytes as translatable cellular model and hence, provide this monumental step in the obesity and diabetes research.

Inflammation, NF-κB, and IKKβ

Many of the inflammatory pathways are regulated by the transcriptional factor NF-κB, which regulates innate and adaptive immune responses (Hayden and Ghosh, 2008). NF-κB activation require IkB kinase (IKK) β, which is a major catalytic subunit of IKK complex (Hayden and Ghosh, 2008; Sui et al., 2014). IKKβ has been defined as a crucial molecular link between obesity, inflammation, and associated metabolic disorders (Baker et al., 2011; Solinas et al., 2010). IKKβ activation in hypothalamus has also been interconnected with obesity and associated metabolic syndrome (Zhang et al., 2008). Constitutive activation of IKKβ in the liver has shown to have caused insulin resistance (Cai et al., 2005). Taken together, these data stipulate a role of IKKβ in obesity, diabetes,
and insulin resistance (figure 1). Thus, elucidation of the role of IKK/NF-kB in contributing to Insulin Resistance and Atherosclerosis is imperative. Data generated from this proposal may define the role of IKK/NF-kB in adipogenesis and atherosclerosis and further disclose a critical link between inflammation and cardiometabolic disease.

Inflammation and Proinflammatory Cytokines

The cytokines have overlapping biologic activity and are secreted by many different cell types in response to multiple types of stimuli. The proinflammatory cytokines are produced specifically in response to inflammatory stimuli (Medzhitov R, 2008). The major members of proinflammatory cytokines are tumor necrosis factor (TNF-α), interferon-γ (IFN-γ), and the interleukins (ILs) such as IL-1b, IL-2, IL-6, IL-8, IL-12, etc. TNF and IL-1 are known to be the primary mediators of the inflammatory response and contribute in eliciting the local response through cell activation and subsequently triggering a cytokine cascade (Tisoncik et al., 2012).

Adipose tissue and adipokines

The Adipose tissue also contribute to the systemic low-grade inflammation and progression. Many cytokines and chemokines play important roles in different regulatory pathways in the hypertrophic adipose tissue (Greenberg and Obin, 2006). In addition to storing excess energy as triacylglycerol, adipose tissue also secretes the adipokines. These adipokines (cytokines and chemokines associated with adipose tissue) can directly regulate the insulin sensitivity; and adipokine secretion from the adipose tissue of obese
individuals was shown to contribute to the development of systemic insulin resistance and associated metabolic diseases (Zhang et al., 1994). Thus, unearthing the link to chronic low-grade inflammation in adipose tissue can lead to development of much-needed prospective therapeutics.

Research Significance

Obesity is an epidemic that is engulfing the entire world. Obesity is the root cause of several widespread diseases like diabetes, hypertension, high cholesterol/triglyceride, and cardiovascular disease. The limited efficacy of current therapies for obesity and associated metabolic disorders necessitates the search for new and novel therapeutic interventions. Data generated from this proposal may define the role of IKK/NF-kB in adipogenesis and atherosclerosis and further disclose a critical link between inflammation and cardiometabolic disease. Establishing human adipocytes as a relevant cellular model to be utilized for downstream in vitro modalities to study T2DM would be a great milestone in the translational research in T2DM therapeutic space. Our data may demonstrate the IKK/NF-kB-mediated inflammatory pathways in that cell line; further establishing IKK as a therapeutic target for obesity and associated metabolic disorders; which may help the mankind in combating the devastating consequences of obesity.
Chapter II.
Materials and Methods

Reagents

Growth medium DMEM-Ham’s F-12 medium (DMEM-F12), Phosphate Buffers (PBS), and fetal bovine calf serum (FBS), were supplied by GIBCO (Burlington, ON, Canada). Isobutylmethylxanthine (IBMX), Insulin, and dexamethasone were obtained from Life Technologies (Carlsbad, CA). TrypLE was supplied by Gibco. Oil Red O solution was purchased from Sigma. Probes for relevant genes were obtained from ABI Biosystems. The selective IKK inhibitors VII (IKK-16) and BMS-345541 (4(2′-Aminoethyl) amino-1,8-dimethylimidazo(1,2-a) quinoxaline) were purchased from Abcam (Abcam, Cambridge, UK). The catalogue numbers of common reagents used are as listed in table 2.

Cell Culture

Human preadipocytes (hPADs) were obtained from Cell Applications Inc. (San Diego, CA). The human preadipocytes were routinely cultured in “0FC” culture media (DMEM-F12 supplemented with 3.3mM Biotin, 1.7mM Panthotenat, and 10% fetal bovine calf serum) as described in figure 2. The hPADS were maintained in culture at 37°C humidified incubator with 5% CO2 and were subdivided at ~70% confluence.
Preadipocytes were differentiated post-confluence to adipocytes using our lab-modified protocol, as described in detail in the results section, as this is part of our specific aim. The hPADs were differentiated in “QD” induction media (DMEM/F12, 3 mM Biotin, 1.7 mM Panthotenat, 10% FBS, 0.01 mg/ml Trasferrin, 20 nM Insulin, 100 nM Cortisol, 0.2 nM T3, 25 nM Dexamethasone, 250 µM IBMX, and 2uM rosiglitazone) for the first 4 days of differentiation and subsequently maintained in “3FC” maintenance media (DMEM/F12, 3.3 mM Biotin, 1.7 mM Panthotenat, 10% FBS, 0.01 mg/ml Trasferrin, 20 nM Insulin, 100 nM Cortisol, 0.2 nM T3). All media are free of antibiotics.

Cell Proliferation Assays

Cell confluency and viability was determined by automated ViCell cell counter, using trypan blue dye exclusion method and confluency was expressed as number of cells in millions. Additionally, cells were monitored in real time via Live Imaging system IncuCyte (Essen BioScience, Ann Arbor, Michigan, USA). To this end, cells were seeded and treated as described in the respective experiments as outlined in the result sections, incubated within the IncuCyte system inside the incubator, and followed for the entire incubation period. The system acquired an image every 2 hours and the confluency was measured using the algorithm in the IncuCyte software (Essen BioScience). Percent confluency was expressed as area occupied in the IncuCyte.

Oil Red O Staining
hPADs and differentiated adipocytes were rinsed with phosphate-buffered saline twice and washed with 60% isopropanol, followed by staining of cells for 10 minutes in a diluted Oil Red O solution. Stained cells were washed four times with double-distilled water, and the stained lipids in the adipocytes were visualized using light microscopy (Olympus) and photographed. Images were quantified using ImageJ software.

Cell Viability/Cytotoxicity Assay via CellTiter Glo

Cytotoxic effects of VII and BMS-345541 on the hPADs and adipocytes were evaluated by assessing the long-term cell viability via CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI), a reagent with a luminescent readout that reflects cell viability via the measurement of ATP metabolism (Crouch et al., 1993). Respective cells were seeded at an appropriate density (after optimized density gradient) in opaque-walled 96-well plates and treated with the titration of the compound and incubated for 3 days and 5 days. These time points were selected based on pilot time course experiments that had depicted unambiguous treatment effects at these points with control untreated cells still growing in the exponential growth phase. Three independent experiments were performed with 2-3 biological replicates each time. On the end of the experiment, viability was measured by using CellTiter Glo. The assay was performed according to the manufacturer’s instructions. Luminescence intensity was recorded using the SpectraMax luminometer (Molecular Devices, San Jose, CA). Results from the CellTiter Glo were expressed as Luminiscence (RLU); and error bars indicate standard deviation. EC\textsubscript{50} were calculated from the dose response curve and graphs were plotted in GraphPad Prism Software (GraphPad Software Inc., San Diego, CA).
Multiplex Mesoscale Assay

The proprietary MSD® MULTI-Spot® v-plex Human Proinflammatory Panel 1 kit (Cat # K15049D) measured cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, 12p70, IL-13, and TNF-α) that are important in inflammation response and immune system regulation, in addition to various other biological processes. Lyophilized cytokine standards were provided as a cocktail mix.

Supernatants from the experimental groups were collected at appropriate time points as listed within the experimental parameters and 10-plex panel of proinflammatory cytokines levels were measured via v-plex mesoscale assay using the Vendor-recommended protocol was followed. At the end of the assay, the MSD plates were read on the MSD Sector Imager 2400 plate reader. The raw data was measured as electrochemiluminescence signal (light) detected by photodetectors and further analyzed using the MSD software Discovery Workbench 3.0. A 4-parameter logistic fit curve was generated for each analyte using the standards and the concentration (pg/mL) of each sample calculated. Graphs were plotted in GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Real-time PCR

Gene signature of hPADs were initially characterized in this lab as a preliminary validation (figure 8C). Cells were lysed, homogenized, & total RNA was extracted using RNeasy Mini Kit (Qiagen, Gaithersburg, MD) and RNase-Free DNase Set treatment, following vendor’s protocol. RNA concentration was determined via the NanoDrop. First strand cDNA was synthesized using Superscript cDNA kit (Life Technologies).
Subsequently real time PCR was performed in ABI 7900 thermocycler using Taqman assay probes (Life Technologies, Carlsbad, CA). Relative mRNA levels were calculated with the PCR product for each primer set normalized to PPIB mRNA using $2^{\Delta\Delta C_t}$ method.

Statistical Analyses

Each experiment had at least 2 Biological replicates, with 3-5 independent repeats. All quantitative values are expressed as the average of the group ± standard error of the mean (SEM). P values < 0.05 are defined as statistically significant. Statistical significance was derived by t-test analyses and one-way or two-way ANOVA. All graphs were plotted in GraphPad Prism Software (GraphPad Software Inc., San Diego, CA).
Chapter III

Results

Setting up the human adipocyte model *in vitro* from hPADs differentiation

Our first aim was to evaluate the role of IKK/NF-κB in adipogenesis, and to do so, it is crucial to set up the cell platform. So here we aspired to successfully differentiate human preadipocytes (hPADs) into adipocytes. We cultured human preadipocytes line (hPADs) per the recommended protocol. Please see *figure 2* for the snapshot of differentiation method and media formulations. We cultured hPADs through 2-3 passages in “0FC” media before differentiating them into adipocytes. We needed to make sure that the cells did not get confluent beyond 55-65% in each passage. Optimal cell seeding density and optimal plate formats were established as a part of optimization steps. Cell confluency and viability was determined by automated ViCell cell counter, and confluency was expressed as number of cells in millions. Additionally, cells were monitored in real time via Live Imaging system IncuCyte (*figure 3*). Percent confluency was expressed as area occupied in the IncuCyte. Once, optimal confluency of optimal passage number is achieved for differentiation, the hPADs were then subjected to adipogenic differentiation. The “0FC” culture media was swapped for the differentiation media or induction media (“QD”). After 4 days, the differentiation media was swapped for maintenance media (“3FC”) and cells were maintained in this media for 14 days or more, at the end of which they become adipocytes. Thenceforth, preadipocytes and mature adipocytes were characterized via morphological assessment.
Characterization of differentiated adipocytes

The differentiation was tracked via automated live cell imaging, using IncuCyte; and phase contrast images were collected at appropriate time points. Phase contrast images of HPADs differentiation from preadipocytes to adipocytes shows gradual intracellular fat accumulation (figure 4). Adipocyte differentiation was further quantified after 14 days via Oil-Red O, using light microscopy; and via morphological assessments, using phase contrast images (figure 5). Adipocyte differentiation is a complex and multi-step process. Once differentiated, the cells function as mature adipocytes and manifest a gene expression profile comparable to that of mature human fat cells, as characterized previously in this lab (figure 6). Oil Red O staining intensity was stronger in differentiated cells, which directly deciphers into lipid intensity, hence substantiates the accumulation of lipid (figure 5). RT-qPCR analysis showed upregulated gene expression of adipocyte markers, such as LEPR, ADIPOQ, and FABP (figure 6).

Expression of inflammatory markers in the adipocyte model

The gene expression of hPADs was performed previously using RNAseq. hPADs were differentiated in the similar manner as stated above and gene expression of NF-kB signaling pathway family was assessed during different day of adipogenic differentiation. The heatmap in figure 7 shows that the expression levels of inflammatory genes intensify as the adipogenic differentiation advances, with highest expression of the key inflammatory genes being attributed to the mature adipocyte. The result achieved here
corroborates with the preestablished facts that the adipose tissue itself contributes to the systemic low-grade inflammation and progression (Wabitsch M et al., 2001). We further assessed the cytokine profile, as secreted by the hPADs and adipocytes during differentiation process. hPADs were subjected to differentiation as described and supernatants were collected from day 1, 7 and 12, followed by measurement of the secreted cytokines, using MSD multiplex assay (figure 8), as described in the methods section. A panel of multiple proinflammatory cytokines were evaluated (IFN-γ, IL-1b, IL-4, IL-6, IL-8, IL-12p70, IL-13, and TNF-α) during adipogenesis. Figure 8 depicts that there is a gradual increase in proinflammatory cytokines as the adipogenic differentiation progresses and hPADs become mature adipocytes.

Toxicity assessment of IKK inhibitors

Next, we wanted to define the role of IKK inhibition on adipogenic differentiation. We used a small molecule BMS-345541 (figure 9A), which is a highly selective inhibitor of the catalytic subunits of IKK-2 and IKK-1 (Burke et al., 2003). An additional IKK inhibitor IKK-16 or VII was used as a confirmatory tool. IKK 16 (figure 9B) is a selective IκB kinase (IKK) inhibitor for IKK2, IKK complex and IKK1 (Waelchli et al., 2006). We performed a dose response of IKK inhibitors on hPADs using CellTiter Glo Luminescent Cell Viability Assay. The time-course assays (table 1) were performed to determine the mean efficacious dose (MED) of the IKK inhibitors BMS-345541 (figure 10) and VII (data not shown). It was shown that 10 μM was toxic to the cells (figure 10B) and EC₅₀ of ~ 3 μM was calculated for BMS-345541 from the dose response curve (figure 10A). Once the
optimal conditions for IKK inhibitors were established, a titration of BMS-345541 spanning the MED was utilized in the downstream applications.

Inhibition of IKK abrogates inflammatory cytokine secretion during adipogenesis

Here we evaluated the effect of the inhibition of IKK/NF-kB pathway on adipogenic differentiation. Figure 11 depicts the representative Assay Flow Chart of one of several experimental set-ups used in the assay to address the role of IKK in adipogenesis. So, three sets each with of undifferentiated hPADs were cultured. One set was used as control hPADs, another set of hPADs were subjected to routine adipogenic differentiation to differentiate into human adipocytes normally, and last set had differentiating condition but with IKK-Inhibitor BMS-345541. As the differentiation advanced, the cells were assessed morphologically, and live cell analysis was performed via IncuCyte. Phase contrast images were obtained and thence, comparisons were made (data not shown). Mesoscale Assay was performed, as described earlier herein, to measure the cytokines secretion during adipogenesis in presence of IKK inhibitor (figure 12). The results here show that inhibition of IKK during adipogenic differentiation abrogates the proinflammatory cytokine secretion.

Mature adipocytes significantly inhibit cytokine secretion in presence of IKK inhibitor

Since, human body does not go through adipogenesis after certain point; we needed to depict the role of IKK in the mature adipocytes, for rendering our study more clinically relatable. So, here we have evaluated the role of IKK/NF-kB on inflammatory
pathway and determined the inflammatory response of mature human adipocytes upon IKK inhibition (figures 13 and 14). First, hPADs were subjected to routine adipogenic differentiation. Differentiated adipocytes were validated via morphological assessments and oil red staining. Once we confirmed that differentiated cells are adipocytes, RNA was isolated from the mature adipocytes and a baseline gene signature was ascertained prior to looking at the alteration in that gene signature upon IKK inhibition, to be assessed via qPCR (as previously established in this lab). Biological replicates of adipocytes were incubated with or without BMS-345541 &/or vehicle for a range of time points; such as, 4 hrs., 8 hrs., and 24 hrs. 3 independent experiments were performed here for respective downstream applications. Subsequently, supernatants were retrieved to further quantitate the cytokines secretion by mature adipocytes, in presence or absence of IKK inhibitor, via MSD Mesoscale Assay (figure 13). It was shown that mature adipocytes significantly inhibit cytokine secretion in presence of IKK inhibitor (figure 13). Furthermore, Oil red O staining was utilized to quantify lipid intensity (figures 14A and 14B); and the similar corroborative results were achieved.
Chapter IV.
Discussion

It is generally accepted that obesity is associated with a state of chronic low-grade inflammation that is a major contributor to insulin resistance and type 2 diabetes, yet the detailed molecular mechanisms remain elusive (Gaal et al., 2006). IκB kinase β (IKKβ), a central coordinator of inflammatory responses through activation of nuclear factor-κB (NF-κB) (Sui et al., 2014) has been implicated as a key molecular link between obesity and inflammation (Baker et al., 2011). As inflammatory responses, IKKβ signaling in multiple tissues including liver, pancreas, and brain have been associated with obesity and obesity-related inflammatory processes (Yuan et al., 2009; Arkan et al., 2005; and Zhang et al., 2011). Deletion of IKKβ in the liver improved diet-induced insulin resistance, and deficiency of IKKβ in myeloid cells rendered global insulin sensitivity upon HF feeding (Arkan et al., 2005). By contrast, constitutive activation of IKKβ in the liver caused systemic insulin resistance (Cai et al., 2005).

Compared with other tissues and cell types involved in obesity, IKKβ signaling in context to adipose tissue remains incompletely understood. Obesity is associated with elevated activity of IKKβ and another key stress/inflammatory kinase, c-Jun amino-terminal kinase (JNK), (Hotamisligil et al.) in adipose tissue (Jiao et al., 2011). Although the role of IKK in inflammatory factor–mediated adipocyte insulin resistance has been confirmed by reported (Jiao et al., 2009), most of these studies using transgenic mouse model have generated inconsistent results. Hence there is a pressing need to develop a model that recapitulate the molecular events based on human origin cells or tissues. Here we have developed an in vitro model for adipocyte differentiation from primary
preadipocytes. We show that hPADs cells show consistent adipogenic potential upon induction and exhibits the makers, gene expression and most importantly intracellular fat accumulation in two weeks of culture. The cells also show robust increase in proinflammatory markers upon differentiation and can be maintained in culture for several weeks.

The constitutive active form of IKKβ in adipose tissue has been reported to increased systemic and tissue inflammation in transgenic mice (Tang et al., 2009). A recent study demonstrated the adipocyte specific IKKβ signaling plays an important role in inflammation resolution by inducing interleukin 13 (IL-13) expression in adipose tissue (Kwon et al., 2014). We tested several proinflammatory cytokine in the human adipocyte model. Upon selective IKK inhibition we observed a significant reduction in all the cytokine, including IL-13. Deficiency of IKKβ in adipocyte precursor cells inhibited adipocyte differentiation and protected mice from diet-induced obesity and metabolic disorders (Sui et al., 2014). However, in our \textit{in vitro} adipocyte model, we have not observed inhibition of adipogenicity upon IKK inhibition, this could be due to the species differences, which argues to need to utilize human based models for mechanistic understanding of metabolic disorders. These findings suggest that the functions of IKKβ signaling in adipose tissue are complex and are species intrinsic and that further studies should focus on human cell physiological model.

In summary, we have revealed a role of IKK mediated NF-κB signaling in human adipocytes as central mediator of inflammation and subsequent immune responses. Our results show adipocytic differentiation is associated with increase in proinflammatory mediator that can be significantly inhibited upon IKK antagonism. Our data suggest that
targeting IKKβ in adipocyte lineage cells may represent a novel therapeutic approach to reduce visceral adipose tissue mass in obesity and inflammation.

Conclusion

The data attained from this thesis work are expected to define the role of IKK in adipogenesis and reveal a critical link between inflammation and cardiometabolic disease. Additionally, by establishing human adipocytes as a relevant cellular model to be utilized for downstream in vitro modalities to study T2DM, we have achieved a great milestone in the translational research in T2DM and metabolic syndromes therapeutic space. Our study has paved a pathway to further study the implications of IKK/NF-kB as a therapeutic target in insulin resistance; which may help the mankind in combating the devastating consequences of obesity and associated diabetes and metabolic syndromes.
Appendix 1.

Figures

Figure 1. The Vicious Cycle of Obesity, Inflammation, and Associated Metabolic Disorder

IKK/NF-κB Target validation in obesity-associated metabolic disorders.
Figure 2. Differentiation of Human preadipocytes into the mature adipocytes

Timeline and media formulation for differentiation of human preadipocytes (hPAdS) to mature adipocytes.
Figure 3. hPAD Viability

*Growth characteristics of HPAD measured by A) cell counting on ViCell Counter and B) Percent confluence as shown via incuCyte.*
Figure 4. Phase contrast images during adipogenesis

*Phase contrast images of HPADs differentiation from preadipocytes to adipocytes shows gradual intracellular fat accumulation. White arrow head shows fat accumulation.*
Figure 5. Phase contrast and Oil red O stained images of differentiated adipocytes.

*HPAD differentiated into adipocytes shows accumulation of intracellular lipid A) phase contrast images and B) Oil Red O stained images.*
Figure 6. hPADs differentiation confirmed by adipocyte specific markers.

A) Oil Red Staining intensity stronger in differentiated cells, B) Phase contrast image of Day12 adipocytes stained with Oil Red O, (zoomed in images), C) RTq-PCR analysis shows increase of gene expression of adipocyte markers.
Figure 7. NF-kB signaling pathway family in hPAD Gene expression of in HPADs

*NF-kB signaling pathway genes as assessed in the RNAseq data generated for hPAD differentiations.*
Figure 8. Inflammatory cytokine secreted by HPAD during differentiation

Cytokine secretion was measured via multiple Mesoscale Assay.
Figure 9. Chemical structure of IKK inhibitors

A) Chemical structure of BMS-34554 (N-(1,8-Dimethylimidazo[1,2-a]quinoxalin-4-yl)-1,2-ethanediamine hydrochloride (CAS Number 547757-23-3, Molecular Weight 291.78). B) Chemical structure of IKK-16 (VII) (CAS Number 873225-46-8, Molecular Weight 483.63).
Figure 10. HPAD cellular toxicity of BMS-345541

A) Dose-response curve measured by cell titer glow assay, B) relative luminescence measured at various concentrations.
Figure 11. The Assay Flow Chart

The role of IKK inhibition on adipogenic differentiation.
Figure 12. Cytokine secretion during adipogenesis in presence of IKK inhibitor.
Fig. 12 continued. Cytokine secretion during adipogenesis in presence of IKK inhibitor

*Mesoscale assay of secreted cytokines*
Figure 13. Proinflammatory cytokine panel.

Mesoscale Assay of secreted cytokines
Figure 14. Lipid intensity quantitation.

*Oil red O* staining (A) and *Normalized pixel intensity* (B)
Table 1. Toxicity Assessment of IKK/NF-κB Inhibitors: Tentative experimental plan

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Replicates</th>
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<tbody>
<tr>
<td>Vehicle</td>
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</tr>
<tr>
<td>BMS-345541</td>
<td>3</td>
</tr>
<tr>
<td>(Titration)</td>
<td></td>
</tr>
<tr>
<td>IKK -16 (VII)</td>
<td>3</td>
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<td>(Titration)</td>
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*HPAD cellular toxicity of IKK inhibitors BMS-345541 and IKK-16 (VII)*
### Table 2. Reagents and Suppliers’ catalogue numbers

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<tr>
<td>3-Isobutyl-1-methylxanthine (IBMX)</td>
<td>Sigma I5879</td>
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<td>Dexamethasone</td>
<td>Sigma D1756</td>
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<td>bovine serum Albumin (fatty acid-free BSA)</td>
<td>Invitrogen 235000-054</td>
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<tr>
<td>Insulin</td>
<td>Life Technologies 12585014</td>
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<tr>
<td>Oil Red O solution</td>
<td>Sigma 01391</td>
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<tr>
<td>TrypLE</td>
<td>Gibco™ 12563029</td>
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</table>

The list of common reagents used.
Appendix 3.

Definition of terms

- 4-HNE: 4-hydroxynonenal
- CDC: Centers for Disease Control and Prevention
- CRP: C-reactive protein (CRP), a biochemical marker for low grade inflammation.
- Drug Target: a biological target that can be modified by external small molecules to change its modulatory activities within the cell.
- GLP: Good Lab Practice
- hPADs: human preadipocytes
- IKK-16: IKK Inhibitor VII, an ATP-competitive inhibitor of IKK
- IKKβ: IκB kinase (IKK) - an enzyme complex which propagates the cellular response to inflammation.
- IncuCyte: Live-Cell Analysis System.
- Lipopolysaccharide (LPS): Component of gram-negative bacteria that induces cytokine release.
- MED: Mean Effective Dose
- NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells
- NDEP: National Diabetes Educational Program
• NIH: National Institute of Health

• qPCR: see RT-PCR below.

• RT-PCR: reverse transcription polymerase chain reaction, a method of detecting mRNA transcripts for assessing gene signatures.

• T2DM: Type 2 Diabetes Mellitus


diseases. Nature Reviews Immunology, 8(12), 923-934. doi:10.1038/nri2449


