ADENOSINE 2A RECEPTOR: NUTRITIONAL REGULATION AND ROLE IN DIET-INDUCED INFLAMMATION AND METABOLIC DISEASE

A Dissertation

by

YA PEI

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Chaodong Wu
Committee Members,	Joseph M. Awika
	Susanne Talcott
	Rosemary Walzem
Head of Department,	Boon Chew

December 2017

Major Subject: Nutrition

ABSTRACT

Obesity causes a wide variety of metabolic diseases including fatty liver disease and diabetes. Mechanistically, obesity-associated inflammation has been implicated as a key factor in the development of fat deposition, insulin resistance, and metabolic dysregulation. As a member of the G-protein coupled receptor families, adenosine 2A receptor ($A_{2A}R$) is anti-inflammatory. However, little is known about nutritional regulation of $A_{2A}R$ as it relates to insulin resistance.

In the present study, the expression of $A_{2A}R$ in liver and adipose tissue was examined in wild type (WT) C57BL/6J mice upon feeding a high-fat diet (HFD) or a low-fat diet (LFD). Also, both $A_{2A}R$-deficient mice and WT mice were fed an HFD for 12 weeks to examine the involvement of $A_{2A}R$ in diet-induced inflammation and insulin resistance. Lastly, the effects of major macronutrients, i.e. glucose and palmitate, on the inflammatory responses were examined in adipocytes and macrophages, either from WT or $A_{2A}R$-deficient background.

HFD increased the expression of $A_{2A}R$ in liver and adipose tissue, accompanied with obesity-related inflammation and insulin resistance. It appeared to be a defensive response, which may help protect against inflammatory damage. Nutrients had direct effects on $A_{2A}R$ expression in both adipocytes and macrophages, which indicated $A_{2A}R$ had protection effects on inflammation. When comparing with HFD-fed WT mice, HFD-fed $A_{2A}R$-deficient mice displayed a significant increase in the severity of inflammation and insulin resistance.

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my committee chair, Dr. Wu, and my committee members, Dr. Awika, Dr. Talcott and Dr. Walzem, for their guidance and support throughout the course of my graduate study and this research.

Thanks also go to Wu lab members, including Honggui Li, Yuli Cai, Xin Guo, Shih-lung Woo, Rachel Botchlett, Hang Xu, Ting Guo, Ting Qi, Xiang Hu, Juan Zheng, Lu Chen, Wenya Huang and Xianjun Luo. I am so thankful for the time they took to help with my project.

Thanks also go to the department faculty, staff and students who have made my time at Texas A&M University enjoyable and colorful.

Finally, thanks to my mother and father for their selfless support and love.

CONTRIBUTORS AND FUNDING SOURCES

This work was supported by a dissertation committee consisting of Associate Professor Chaodong Wu [advisor], Associate Professor Susanne Talcott of the Department of Nutrition and Food Science [Home Department], Professor Rosemary Walzem of the Department of Nutrition and Food Science [Home Department] and Associate Professor Joseph M. Awika of the Department of Soil and Crop Science [Outside Department].

The student independently conducted all the research for this dissertation. All the figures depicted in this dissertation will be used for future publication.

This study was supported in part by National Institute of Health (NIH) research project (R01) grants.

NOMENCLATURE

AKT	Protein Kinase B
AC	Adenylyl cyclase
ACC	Acetyl-CoA Carboxylase
ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ADA	Adenosine Deaminase
$A_{2A}R$	Adenosine 2A receptor
$A_{2B}R$	Adenosine 2B receptor
BAT	Brown Adipose Tissue
BMDM	Bone Marrow-Derived Macrophage
cAMP	Cyclic Adenosine Monophosphate
CREB	cAMP responsive element binding protein
ENT	Nucleoside Transporter
FAS	Fatty Acid Synthase
FFAs	Free Fatty Acids
GTT	Glucose Tolerance Test
Gi	G Protein Inhibitory
Gs	G Protein Stimulatory
HFD	High Fat Diet

IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-4	Interleukin-6
IR	Insulin Resistance
ITT	Insulin Tolerance Test
JNK	c-Jun N-terminal Kinase
LPS	Lipopolysacharride
MCP-1	Monocyte Chemotactic Protein-1
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAFLD	Nonalcoholic Fatty Liver Disease
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
PKA	Protein kinase A
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SREBP-1c	Sterol Regulatory Element-Binding Protein 1c
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TRIF	TIR-domain-containing adaptor inducing interferon β
TLR-4	Toll-like Receptor-4
TNF-α	Tumor Necrosis Factor-α
VLDL	Very Low Density Lipoprotein
WAT	White Adipose Tissue
WT	Wild-Type

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Obesity is a serious problem all over the world, and more than 36% of US adults are obese [1]. It involves adipose tissue expansion and dysfunction, which causes dysregulation of adipose tissue derived factors, leading to changed glucose and lipid homeostasis and inflammatory response. If unchecked, imbalanced fatty acids will traffic way from adipose tissue to other tissues such as liver and muscle, which is a main cause for the development of obesity-linked disorders, especially various metabolic diseases. Much of the literature has focused on intracellular sequence of events that resulted in dysfunction of adipose tissue and liver, like key enzymes related to lipolysis and *de novo* lipogenesis. However, there is little literature that covered regulation of adipose tissue and liver function whereby adenosine receptors mediated extracellular signals. Therefore, this dissertation aims at examining the links between adenosine receptors mediation and over-nutrition induced obesity.

$A_{2A}R$ is one of the specific receptor that reported to have regulation on inflammation response. As a key extracellular signaling molecule, adenosine preserves tissue homeostasis through activating adenosine receptors. There are four subtypes of adenosine receptor (A_1, A_{2A}, A_{2B}, A_3), A_{2A} and A_{2B} activation can result in elevated level of cAMP level [2]. Of many cells in immune system, $A_{2A}R$ have effect on suppressing key steps of pro-inflammatory signaling pathways [3-6]. But the nutritional effects in regulating this process and downstream events have not yet been well established. In

virtue of $A_{2A}R$ knock out mice, we would like to focus on exploring the cellular mechanisms of as to how $A_{2A}R$ activation decreases diet-induced inflammation. Therefore, the central hypothesis of this dissertation is that $A_{2A}R$ coordinate metabolic and inflammatory response upon dietary effects in adipose tissue and liver, directly affecting adipocytes and macrophages in an obesity mice model.

CHAPTER II

LITERATURE REVIEW

Obesity-related Inflammation And Metabolic Diseases

The incidence of obesity has been increasing substantially worldwide. In the US, over 1/3 of adults are classified as obese, and similar trends have been observed in countries all over the world [7]. More than 600 million adults were obese in 2014, according to a Global Status Report release by the World Health Organization. The obesity-related metabolic dysfunction includes heart diseases, stroke, type 2 diabetes, nonalcoholic fatty liver disease and certain types of cancer. So finding new pharmacological approaches is crucial in preventing the development or ameliorating they symptoms associated with obesity.

People are living in environments that promote the intake of energy-dense, nutrient-poor food and physical inactivity. Diet packed with added sugar and saturated fatty acid (SFA) will cause excess accumulation of fat stored in adipose tissue. Nowadays, adipose tissue is viewed not only as an inert organ to store food-derived fat, but also as an endocrine organ, which is responsible for releasing several hormones [8]. The excess adipose tissue can trigger increased secretion of FFA and cytokines, which favors infiltration of macrophages and other immune cells into adipose tissue, leading to low-grade chronic inflammation. On the systemic level, altered adipocyte- and macrophage-derived factors secretion can lead to liver fat accumulation and insulin resistance.

SFA and lipotoxicity

Not all fatty acids affect body metabolism and insulin sensitivity the same. SFA, especially palmitic acid, impairs insulin sensitivity and causes inflammation, while unsaturated fatty acids can improve insulin sensitivity [9]. SFA serves as a ligand to bind toll-like receptor 4 (TLR4) and triggers inflammatory responses. TLR4 signaling involves two distinct adaptor proteins, MyD88 and TIR-domain-containing adaptor inducing interferon β (TRIF). MyD88-dependent pathway is associated with the activation of MAP kinase (JNK, p38 MAPK) and NF-κB. MyD99-independent (TRIF) pathway is associated with the phosphorylation of IRF3 and the expression of IFNβ via IKKε/TBK1, which delays MAPK and NF-κB activation [10]. Palmitate also induces mitochondrial dysfunction, which elicits oxidative stress. Meanwhile, accumulated reactive oxygen species (ROS) eventually contribute to the progression of insulin resistance [11].

Another consequence of SFA (particularly palmitate) is the synthesis of ceramide and diacylglycerol (DAG), which causes lipotoxicity. SFAs induce the biosynthesis of ceramide in a TLR-4 mediated fashion. The accumulation of intracellular SFA metabolites also act as "second messengers" that have a potent role in pathogenesis of insulin resistance [12, 13]. Ceramide inhibits the activity of Akt by dephosphorylating it through protein phosphatase 2A (PP2A) and blocking the translocation of Akt via PKCζ to the plasma membrane. The consequence of these two independent mechanisms is reduced translocation of GLUT4 to the plasma membrane and thus reduced glucose uptake [14]. Accumulated DAG in the liver activates PKCε to drive insulin resistance

and hepatosteatosis. PKCε correlates with decreased insulin-stimulated insulin receptor substrate-2 tyrosine phosphorylation and Akt phosphorylation, resulting in the failure of hepatic glycogen synthesis and suppressing gluconeogenesis [15]. PKCδ expression is also higher in the liver of obese mice and humans, which is linked by DAG accumulation. Global or liver-specific knockout of PRKCD gene mice had increased insulin signaling and suppressed glucogenesis and lipogenesis in the liver, which helped protect from hepatosteatosis. In contrast, liver-specific overexpression of the PRKCD gene led to insulin resistance, characterized by increased expression of lipogenetic enzymes, decreased insulin signaling and hepatic steatosis [16].

Added sugar and insulin resistance

Consuming too much sugar causes the pathologies of diabetes and cardiovascular disease in both direct and indirect ways [17]. The direct pathway is altered regulation of hepatic uptake and fructose metabolism that leads to increasing ectopic fat accumulation and uric acid levels. These detrimental effects are related to the consumption of main added sugar, e.g. sucrose, fructose-containing sugars and high fructose corn syrup. While the epidemiological study hold different opinions on the potential impact of added sugar on health, one opinion is that excess sugar causes weight gain and fat accumulation, which leads to diabetes and heart disease in an indirect way [18, 19]. The other opinion is that overconsumption of fructose is just because of the overload of energy [20]. Control the simple sugar intake less than 20% of total energy per day only increases triglycerides but not uniquely related to CVD risk factors [21]. According to

2015-2020 Dietary Guidelines, the maximum limits of added sugars and saturated fats are 10% of calories for a day.

Obese Adipose Tissue, Inflammation And Insulin Resistance

Two kinds of adipose tissue are distributed throughout the body: white adipose tissue (WAT) and brown adipose tissue (BAT). BAT is especially useful in that it has the potential for thermogenesis upon cold and diet, which mediates body temperature and energy expenditure. The prevalence of BAT decreases as humans age [22, 23]. The purpose of WAT is to store extra energy in the form of triglyceride and secret adipose-derived hormones. It protects other tissues and organs from the accumulation of ectopic fat.

WAT contains adipocytes, stromal vascular fraction of cells, endothelial cells, immune cells (i.e., adipose tissue macrophages), lymph nodes, fibroblasts and preadipocytes, some of which are able to secret bioactive products into bloodstream [8, 24]. Adipocytes are able to change size according to the energy requirement of the body. Early onset obesity is characterized as the enlargement of adipocytes (hypertrophy). In humans who are severely obese, hyperplasia is enhanced depending on diet via adipose-derived stem cells differentiation [25].

Adipose tissue-derived factors

On the one hand, WAT secrets pro-hyperglycemic factors during obesity, which is characterized by impaired TAG storage and increased lipolysis. So the excess releasing of FFAs from WAT into other tissues via circulation, accompanied by increased pro-inflammatory cytokines and the altered secretion of adiponectin, leptin,

resistin and retinol binding protein-4 (RBP4) [26]. On the other hand, the level of adipose derived anti-hyperglycemic factors (adiponectin, SFRP5, IL-10) could decrease due to the expansion of adipose tissue. For example, overexpression of adiponectin in enlarged WAT can improve metabolic profiles despite obesity [27]. In all, with up-regulated adipose derived pro-hyperglycemic factors and down-regulated anti-hyperglycemic factors, various metabolic diseases will be induced through desregulation of glucose and lipid homeostasis as well as exacerbation adipose tissue inflammation.

Altered macrophage polarization

In addition to the increased production of pro-inflammatory molecules secreted by adipocytes, recruitment and infiltration by immune cells such as macrophages is also a main cause of inflammation. Both genetically obese mice and diet-induced obese mice showed the increased macrophage cell number in WAT [28, 29]. In lean and obese human WAT, the number of macrophages correlated positively with adipocyte size [30, 31]. Along with a large amount of macrophages recruitment in adipose tissue, there is a switch from anti-inflammatory M2 polarization to pro-inflammatory M1 polarization in phenotype of macrophages. M2 state is characterized by the high level production of anti-inflammatory cytokines, such as interleukin-10 (IL-10). M1 state is characterized by the expression of pro-inflammatory cytokines, such as TNF-α, IL-6. FFAs serve as the ligand to bind TLR4, and activate the TLR4 cascade [32], which stimulate macrophages to secret cytokines including TNF-α, interleukin-1 (IL-1), interleukin-6 (IL-6), etc. [33-35]. WAT inflammation is also characterized by an increased number of adipocytes

surrounded by macrophages in crown-like structures. Increased infiltrated macrophages and cells reside in stromal together, facilitating production of inflammatory cytokines.

Insulin resistance in adipose tissue

Insulin resistance is characterized as decreased insulin sensitivity in tissues and cells (liver, skeletal muscle, adipocytes, etc.), which means these cells and tissues can't respond to insulin properly, leading to hyperglycemia in most cases. As one of the insulin-responsive tissues, adipose tissue stores triglycerides upon insulin stimulation via maturing of adipocytes, increasing glucose uptake, *de novo* lipogenesis and decreased lipolysis [36]. Insulin binds to insulin receptors on the cell membrane, which activates insulin receptor substrate proteins and initiates two insulin-signaling pathways. One is the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, and another one is the Ras-mitogen-activated protein kinase (MAPK) pathway. Phosphorylated insulin receptor substrate-1 binds to and activates PI3K. PI3K produces phosphatidylinositol (3,4,5)-triphsophate (PIP_3), which activates downstream proteins such as AKT. Finally it causes the translocation of glucose transporter 4 to the plasma membrane, acting as a gateway for glucose to enter the cell. The MAPK pathway is not modulating glucose or lipid homeostasis but rather in mitosis and cell growth via insulin.

Fatty Liver Disease And Insulin Resistance

With the expansion of WAT, FFAs released from lipolysis will flow into the portal vein [37]. Thus, FFA reached into liver, increased ectopic fat deposition and induced inflammation. Non-alcoholic fatty liver disease (NAFLD) is a clinic pathologic change, which has been interpreted by the "double-hit" hypothesis. In this model, the

"first hit" includes liver steatosis characterized as TAG accumulation in the hepatocytes. There is an imbalance between lipid input and output in the liver. Since on the one hand, more FFAs from adipose tissue lipolysis and/or chylomicron will be transported to the liver. On the other hand, increased glucose and insulin levels upon carbohydrate intake, leading to increased lipogenesis in liver, decreased FA oxidization and hepatic TAG secretion via packing ApoB to form VLDLs [38]. The following "second hit" is induced by extra fatty acid oxidation, which causes mitochondria dysfunction and reactive oxygen species (ROS) production, leading to oxidative stress. Both saturate fatty acids metabolites, such as ceramide and DAG, together with comprised oxidative status triggers the production of inflammatory mediators by Kupffer cells [39, 40]. Consequently, chronic pro-inflammatory molecules secretion will induce hepatocytes apoptosis and scaring of liver tissue, resulting in NASH.

Insulin resistance in liver

In the fatty liver, hepatocytes become resistant to the effect of insulin because of too much fat deposition. FFAs and ROS block the insulin signaling pathway process, which fails to decrease glucose output. FoxO1 enters the nucleus to upregulate the genes required for gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). But insulin sensitivity remains in the SREBP-1c pathway. An extremely high level of insulin dramatically increases nuclear SREBP-1c levels, which in turn increase lipogenesis and triglyceride biosynthesis, such as acetyl-coenzyme A carboxylase (ACC) and fatty acid synthase (FAS). Excess triglycerides are

packed with VLDL, which are delivered to adipose tissue and muscle. Thus, hyperglycemia and hyperlipidemia together exacerbate systemic insulin resistance [41].

On the other hand, overexpression of IκB in mice hepatocytes activated NFκB and induced insulin resistance in the liver and skeletal muscle. And HFD feeding also activates NFκB in mice' liver, subsequently inducing systemic insulin resistance and inflammation [42]. If the progression of NAFLD can be caught in the early stages, there is a good chance to recover. However, there is limited effective medicine to cure NAFLD and the medication also has some extent of side effects.

Kupffer cells in liver

Liver sinusoids acted as the first line of host defense through acute phase response. Specialized liver resident macrophages, the kupffer cells account for 80-90% of the tissue macrophages present in the body. Under lean conditions, kupffer cells exert a tolerogenic phenotype that is necessary to defend against infected and exhausted cells. They sense invading pathogens such as bacterial products and toxic substances from gut, and produce IL-12 and other monokines (IL-18) to fight against bacterial infections and prevent their circulation. In the meantime, activated liver natural killer (NK) cells and NK1.1 Ag+ T cells produce interferon γ, as a part of first line defense to clear immunoreactive materials [43, 44].

In obesity, the excess fat accumulation results in liver steatosis, which in turn develops into chronic hepatic endoplasmic reticulum stress caused by TLR activation and reactive oxygen species production. Kupffer cells switched from a tolerogenic state to a pathologically activate state. The polarization of kupffer cells changed to M1

phenotype, induced by pro-inflammatory cytokines, adipokines and FFAs secreted from enlarged adipose tissue, or altered gastrointestinal microflora. The adaption of polarization of kupffer cells in turn induce a vicious cycle of cytokines (TNFα, IL-6 and IL-1β), MCP-1, macrophage inflammatory protein (MIP)-1a, MIP1b, oncostatin and prostaglandins [45, 46] that further deteriorates liver functions.

Furthermore, TNFα together with IL-6 limits systemic insulin sensitivity and induces oncostatin M in kupffer cells. Oncostatin M production decreased insulin-dependent Akt activation and glucokinase which contributes to insulin resistance to the development of NASH [47]. Kupffer cells trigger the infiltration of monocyte-derived infiltrating liver macrophages, which amplify the hepatic inflammation and IR via TNFα and IL-6 production [48].

G Protein-coupled Receptors

G protein-coupled receptors are one of the largest gene families studied in eukaryotes. Nowadays, the superfamily of G protein-coupled receptors (GPCRs) has been postulated to contain more than 800 members in the human genome, which is divided into six classes, including Rhodopsin, Secretin, Glutamate, Adhesion, Frizzled/Taste2, and Other [49]. Their typical structure is seven-trans membrane α helix connected by extracellular loops (ELC1-ELC3) and intracellular loops (ICL1-ICL3), carrying an extracellular N terminus and an intracellular C terminus. GPCRs account for 30-50% of best-selling drugs on the market or in clinical trials for cancer, diabetes, pain and neurodegeneration [50]. When a ligand binds to GPCR, it causes the conformation change in the receptor that makes G_{α} subunit leave from $G_{\beta\gamma}$ dimer, with attached

exchanged GTP from GDP. G_α subunit activates other proteins in certain signal transduction pathways. There are four classes of G proteins, G_s, G_i, $G_{q/11}$ and G_{12}. G_s couples adenylate cyclase to produce cAMP, which in turn stimulates protein kinase A (PKA), and then phosphorylates the downstream targets. G_i inhibits making cAMP from ATP. $G_{q/11}$ activates phospholipase C beta, which catalyzes hydrolysis of phosphoinositide 4,5-bisphosphate into two second messengers, inositol 1,4,5-triphosphate and diacylglycerol. $G_{12/13}$ are involved in activation of RhoGTPase nucleotide exchange factors for regulating cells migration.

Adenosine And Its Receptors

Under physiological conditions, adenosine was constantly released from ATP degradation. The concentration of adenosine is around 30-200 nM, which is mainly from dephosphorylating ATP. In order to exhibit immunosuppressive effects under acute or chronic inflammation conditions, adenosine accumulates in extracellular space is up to 1-30 μM [51]. There are two main sources of adenosine. One is from the dephosphorylating extracellular ATP by CD39 and CD73 [52] . These enzymes are abundantly distributed in lymphocytes and endothelial cells, whose activity are intrigued by hypoxia, inflammation and adenosine itself [53, 54]. Another important source of adenosine is from the dephosphorylating intracellular AMP via 5'-nucleotidase. What's more, the conversion of intracellular S-adenosyl homocystein to adenosine and homocysteine also provides a source of adenosine in extracellular space via nucleoside transporter (ENT) [55-57].

Adenosine receptors are a class of G-protein-coupled receptors. There are four subtypes, designated as A_1, A_{2A}, A_{2B} and A_3. $A_{2A}R$ and $A_{2B}R$ positively couple with Gs proteins to activate adenylate cyclase (AC) and increase cyclic AMP (cAMP)'s synthesis. A_1AR and A_3AR couple with Gi/o proteins and can lower cAMP's synthesis. The affinity of adenosine to A_1, A_{2A} and A_3 is high, with 0.2 -0.7μM of EC50 (effective dose). A_{2B} has much less affinity to adenosine with 24 μM of EC50 [58].

$A_{2A}R$ Signaling

There is high abundance of $A_{2A}R$ in nervous system (CNS) and major peripheral tissues, such as liver, heart, lung and the immune system [59, 60]. In the striatum, $A_{2A}R$ couples to G_{olf} proteins. In the peripheral tissues, the major G protein associated with $A_{2A}R$ is G_s [61]. As a sensor of inflammation and tissue damage, the expression of $A_{2A}R$ is enhanced in neutrophils [62], monocytes [63], T cells [64, 65] and platelets [66]. When macrophages and monocytes were treated with LPS, $A_{2A}R$ transcription elevated drastically due to NF-κB activation [67, 68]. Meanwhile, TNFα treatment prevented $A_{2A}R$ desensitization via inhibiting both translocation and association of GPCR kinase-2 to the plasma membrane. Thus, the elevated function of $A_{2A}R$ can outweigh the increment of itself in expression [69].

The binding of adenosine to $A_{2A}R$ triggers the conformation change of this GPCR, also the exchange of GDP to GTP. α subunit of GPCR leaves with attached GTP over to adenylyl cyclase (AC), which increases intracellular cAMP level. One possible mechanism is cAMP activates protein kinase A (PKA). PKA phosphorylates the transcription factor, cAMP responsive element binding protein (CREB), which turns on

downstream genes via binding to cAMP response elements (CRE). Phopho-CREB is response for the inhibition of NF-κB activity, so $A_{2A}R$ stimulation mediates a negative feedback to control the production of cytokines [70, 71]. One downstream target of cAMP is cAMP-regulated guanine nucleotide exchange factor 2 (Epac2), which inhibits TNFα production [72]. Another mechanism is involved in serine/threonine protein phosphatases [73]. Thus, these mechanisms explain why $A_{2A}R$ activation can inhibit inflammation from so many metabolic stresses such as local hypoxia, tissue damage, metabolic stress and inflammation [51, 74, 75].

$A_{2A}R$ Mediates the Anti-Inflammatory Effectors of Adenosine

Wound healing

Wound repair occurs in diabetic disease or long-term corticoid therapy, which is often associated with ulcers, amputations and disability. Wound healing needs the formation of granulation tissue and angiogenesis, including growing microvessels. Compared with WT littermates, $A_{2A}R$ knock out mice made bad performance in wound heal, such as bigger inflammation at the base of the wound. After treating with selective $A_{2A}R$ agonist 2-(p-(carboxyethyl)-phenethylamino)-5′-N-ethyl-carbamido adenosine (CGS21680), $A_{2A}R$ knock out mice had no change of rate in wound repair but WT mice had faster revascularization. Thus, $A_{2A}R$ is required for angiogenesis in wound closure [76]. The mechanism of $A_{2A}R$ in help wound closure is dependent on tissue plasminogen activator, which is product of macrophages and mast cells to activate the enzymes that breakdown fibrin and other matrix protein to migrate at sites of injury. Wound heal is impaired in tissue plasminogen activator-deficient mice compared to WT mice, and

$A_{2A}R$ agonist can increase the rate of wound heal in WT mice significantly but not knock out mice [77].

In addition Leibovich *et al.* showed LPS and $A_{2A}R$ agonist CGS21680 can together dramatically increase the expression of vascular endothelial growth factor (VEGF) (10 fold), which is much effective than either treatment by itself. But VEGF expression was not up-regulated in TLR4 mutant mice (inactive TLR4 receptors) by macrophages, indicating synergistic interaction of $A_{2A}R$ agonist and LPS signaling through TLR4 pathway [78]. Thus it is synergistic reaction between $A_{2A}R$ and TLR signaling in macrophages, leading the phenotype of macrophages switch from production of pro-inflammatory cytokines to angiogenesis such as elevation the expression of VEGF. Macelo et al. observed the same effect *in vivo,* and its signaling via MyD88, IRAK4 and TRAF6 in virtue of knock-out mice and knockdown cells [79].

Brown fat activation

Gnad *et al.* showed that via $A_{2A}R$, adenosine can activate brown adipocytes and induced white fat into brown-like cells in human and mice [80]. In human and murine adipocytes, $A_{2A}R$ is expressed much higher in brown adipocytes than white adipocytes. And the expression inhibitory effector A_1 is also expressed relatively low in brown adipocytes than white adipocytes. Thus, adenosine concentration is much lower in brown fat than white adipose tissue. However, in the brown fat of the hamsters, the abundance of $A_{2A}R$ and A_1 are similar, so there is no brown fat activation by adenosine in hamsters. $A_{2A}R$ antagonist treatment or $A_{2A}R$-deficient mice caused a decrease of thermogenesis in brown adipose tissue, while $A_{2A}R$ agonist CGS21680 treatment increased lipolysis and

thermogenesis program. In addition, $A_{2A}R$ agonist treatment and white adipocytes infected with lentiviral vectors expressing $A_{2A}R$ can induce white fat into beige cells. This study provides insights into how $A_{2A}R$ expression and activation affect adipocytes turnover and adipogenesis in adipose tissue.

Increase β-cell proliferation

Treatment modalities for individuals suffering from type I or type II diabetes include pancreas transplantation and administration of insulin or insulin sensitizer. Recently, other than insulin or insulin sensitizer, one new strategy was postulated. It stimulates the production of new β-cells from remaining ones, which could benefit diabetes patients and others who suffered from β-cell depletion. Anderson O. *et al.* screened 7186 molecules to identify the enhancers in β-cell proliferation [81]. They found $A_{2A}R$ agonist 5′-N-carboxamido adenosine (NECA) had a stronger effect on increasing β-cell mass and improving glucose homeostasis during β-cell regeneration, rather than under normal conditions. The authors proposed that $A_{2A}R$ activation conserves β-cells to an ideal number, but not acts as promoter for cancer growth. The proliferating and normoglycemia effects also were confirmed in mammals. Mouse islets treated with $A_{2A}R$ agonist had increased multiplication of β-cells. Streptozotocin induced diabetic mice treated with $A_{2A}R$ agonist had a 30% decrease in glycemia and 8-fold increase in β-cell mass. Adenosine signaling is pivotal in the β-cell proliferation since four of five detected enhancers are related to it. Annes et al.' s research also support this point of view. They found adenosine kinase inhibitors also increase β-cell mass in mice, rats and pigs [82]. Since adenosine kinase inhibitors impede the conversion of adenosine

to AMP inside the cells, adenosine level increases, which activates $A_{2A}R$ like what the agonist did.

Schulz N. et al found the endogenous role of adenosine signaling in β-cell regeneration [83]. $A_{2A}R$ agonist increased copying of new β-cells substantially when β-cells were ablated. Under normal conditions, $A_{2A}R$ agonist only increased β-cells modestly. This effect did not depend on the apoptotic microenvironment. The mice with tissue-specific knockout of $A_{2A}R$ in pancreatic β-cells had impaired glucose tolerance and β-cell proliferation during pregnancy, which needs β-cell mass expansion. Under basal state, those mice that lacked $A_{2A}R$ in pancreatic β-cells had no overt phenotype. In addition, islets collected from $A_{2A}R$ conditional knockout mice had decreased level of β-cell proliferation comparing to islets excised from WT mice.

Immunosuppressive effect

Ischemia reperfusion injury (IRI) is characterized as the production of reactive oxygen species, a range of pro-inflammatory cytokines releasing, activated white blood cells adhering to endothelial cells and postischemic tissue [84]. These inflammatory events disrupt vascular reactivity, lipid/ glucose homeostasis and other metabolic processes. Activated white blood cells and endothelial cells in the inner layer of arteries released C-reactive protein, interlukin-1 (IL-1), interlukin-6, tumor necrosis factor α (TNF-α), etc. These cytokines promote glycogenolysis, muscle protein catabolism, which fueling immune response and enabling rapid immune protein synthesis. Inhibition of fatty acid oxidation by inflammatory factors results in hyperlipidemia to neutralize viruses and reduce chemokines infection that induced by IRI. Inflammatory mediators

not only stimulate blood vessels manufacturing vascular cell adhesion molecule-a, causing deposition of fats and other particles in vessel linings, but also contribute the production and infiltration of foam cells, and eventually form plaques. During the reperfusion period, inflammation and micro vascular occlusion initiate tissue damage.

Treatment with $A_{2A}R$ agonist ATL146e can inhibit concanavalin A- induced liver damage by decreasing serum glutamyl pyruvic transaminase and pro-inflammatory cytokines. $A_{2A}R$ agonist also suppressed T cell activation and decreased cytokines that generated by CD4+ T cells [85]. $A_{2A}R$ knock out mice had exacerbated liver injury comparing to WT mice. $A_{2A}R$ agonist treatment had decrease cytokines (IL-1 α, IL-1 β, IL-6, IFN-β, IFN-γ, etc.) and chemokines (monocyte chemotactic protein-1, RANTES (CCL5)) production in WT mice but not to $A_{2A}R$-deficient mice [86]. The anti-inflammatory effect of $A_{2A}R$ exert through cAMP and PKA signaling pathway [62, 87].

Reverse cholesterol transport

Transport cholesterol from periphery back to liver is critical in the prevention of forming foam cells. Foam cells are characterized as monocytes engulfing droplets of cholesterol. Proinflammatory cytokines and immune complex decrease cholesterol 27-hydroxylase, an enzyme plays impartment role in converting cholesterol to oxysterols. Inhibition of cholesterol 27- hydroxylase stops movement of cholesterol from inside to outside of macrophages, results in forming foam cells. Stacked foam cells can form plaques, and over time, plaques narrow blood vessels and make them lose elasticity. Inflammatory mediators also induces immune-mediated disorder and eventually, development of atherosclerotic lesions [88].

$A_{2A}R$ activation reverses foam cells formation via 5'-triphosphate-binding cassette transporter A1 (ABCA1). ABCA1 is involved in an active process dependent of apoA1. ABCA1 transports cholesterol and phospholipid in this manner, assisting the efflux of cholesterol from cells to apoA-1 or HDL in extracellular space [89]. Bingham *et al.* found that $A_{2A}R$ agonist inhibited foam cell formation in THP-1 cells, but has no effect in cells infected with lentiviral vector delivery of siRNA encoding gene of ABCA1. Meanwhile, $A_{2A}R$ agonist CGS21680 increased cholesterol efflux almost two-fold in THP-1 human macrophages but not in ABCA1-deficient cells. As downstream target of cAMP, Epac enhanced ABCA1 and phosphorylated ABCA-1. Thus, $A_{2A}R$ occupancy prevents foam cells formation via reversing cholesterol transport through ABCA1 [90].

Bingham *et al.* also found that $A_{2A}R$ agonist CGS21680 inhibited forming foam cells with lentiviral siRNA infection, which markedly decreased apoE or apoE mRNA in THP-1 cells. But $A_{2A}R$ agonist cannot pause foam cell formation in cholesterol 27-hydroxylase knock down cells. Similarly, $A_{2A}R$ activation decreased cholesterol efflux in apoE knockdown cells but no effect in cholesterol 27-hydroxylase deficient cells. So it is indicated that $A_{2A}R$ occupancy prevents foam cells formation via enhancing the expression of cholesterol 27-hydroxylase [91]. In the liver, the activation of $A_{2A}R$ increased glycogenolysis and gluconeogenesis mediated by cAMP-dependent signaling [92].

Other than anti-inflammatory and altering lipid profile effect, $A_{2A}R$ was studied on its role in the heart and Parkinson's disease, the latter of which was even moved into clinical trails.

A1 Receptor

A_1R is ubiquitous in body, which couples to G_i to inhibit adenyl cyclase, reduce cAMP production and inhibit voltage dependent Ca^{2+} channels that responsible for activating phospholipase C [93]. In adipose tissue, A_1R has been showed to play an important role in adipogenesis and lipid accumulation, including increase lipogenesis and inhibit lipolysis [94]. They also promote leptin secretion and protect against insulin resistance [95, 96]. In addition, stimulation of A_1R favors cardioprotection through modulating adenyl cyclase activity, phospholipiase C- dependent mitochondrial function and nitric oxide-dependent calcium currents [97, 98]. Patients with stable angina treated with A_1R agonist capadenoson had increased total exercise time and time to ischemia [99]. In brain, A_1R has neuroprotection effects such as inhibiting calcium influx and excitatory synaptic transmission via blocking glutamate release. In parallel, A_1R couples to and activate potassium channels via a Gi/o protein, which is sensitive to pertussis toxin [100].

A2B Receptor

In heart, adenosine modulates vascular smooth muscle tone not only via $A_{2A}R$, but $A_{2B}R$. Both $A_{2A}R$ and $A_{2B}R$ are platelet inhibitory mediator, which prevents thrombotic disorders and unstable cardiovascular syndrome [101].

$A_{2B}R$ plays an essential role in protecting from atherosclerosis induced by HFD in apoE-deficient mice. Mice lacking $A_{2B}R$ displayed increased cholesterol and triglyceride level in liver and plasma, and onset of steatosis through activating SREBP-1 and its downstream enzymes for lipognenesis, acetyl- CoA carboxylase and fatty acid synthase [102]. In parallel, ablation of $A_{2B}R$ induced increased M1 and decreased M2 macrophage activation in mice. When BMDM was challenged by LPS or FFA, stimulation of $A_{2B}R$ help decrease TNF-α production [103].

$A_{2B}R$ is overexpressed in various tumor cell types and foster tumor growth [104-106]. By virtue of cancer cell line, $A_{2B}R$ was found to trigger a signaling cascade to decrease the prenylation of Rap1B, which suppress cell-cell adhesion and promote tumor-cell metastasis [107].

A3 Receptor

They are less distributed than other ARs, including lung, aorta, liver, brain and heart. It is overexpressed in cancer and inflammatory cells and less expressed in normal cells, rendering the potential of A_3R agonist as a therapeutic target. A_3R agonist treated mice or rats had suppressed tumor growth and induced apoptosis through dampening NF-κB and Wnt signaling pathways [108, 109]. A_3R agonist exerted anti-inflammatory effects through decreasing PI3K- NF-κB pathway, result in decreased MIP-1α, TNF-α, IL-12 and IL-6 [110, 111]. Thus, A_3R agonist CF101 and CF102 were conducted into clinical trail on its anti-inflammatory effects on rheumatoid arthritis, psoriasis and hepatitis C virus infection [112-114].

Summary

Obesity induced chronic low degree inflammation, is highly associated with altered immune response and insulin sensitivity, and is becoming common cause of metabolic diseases, such as type II diabetes, non-alcoholic fatty liver disease, ischemic stroke, cardio vascular disease. People are bombarded with advertising to buy high fat, poor quality food, and not given enough information of its relation with inflammation during obesity. Since high fat diet (HFD) is energy-dense, high in saturated fatty acid, it increases risk of overeating and excessive accumulation of fat in adipose tissue. Elevated fat deposition in adipose tissue will trigger many positive feedback pathways that overproducing pro-inflammatory proteins (cytokines and chemokines) and hormone. Hyperglycemia and hyperinsulinemia also relate to increased *de novo* lipogenesis and liver fat accumulation. Pro-inflammatory mediators derived from adipose tissue also contribute to the progression of this pathological state. In the context of this slow, subtle and last up to years of inflammation, macrophages paly a critical role in generating cross-talk to the cells in adipose tissue and liver, thereby affecting the immune response of these tissues.

The great challenge is to find potent, endogenous, anti-inflammatory signals, and therapeutically targeting them. Luckily, endogenous adenosine signaling via $A_{2A}R$ was proved to reduce tissue damage and inflammation. With regard to this, $A_{2A}R$ may be one underlying receptor that suppress or compensate for obesity-associated adipose inflammation response upon dietary influences. Therefore, it is of particular interest to

study the effects of $A_{2A}R$ in mediating various cell populations such as the macrophages, adipocytes in improving tissue inflammation and insulin sensitivity.

CHAPTER III

THE PHYSIOLOGICAL RELEVANCE OF $A_{2A}R$ IN ADIPOSE TISSUE UNDER DIFFERENT NUTRITIONAL CONDITIONS

Introduction

Obesity is a worldwide epidemic that is associated with the increase of chronic diseases including type 2 diabetes, cardiovascular disease, stroke and some cancers. During obesity, adipose tissue cannot respond properly to the fluctuations in nutrient and energy supply via altering the size and/or number of adipocytes. The state of obesity can be reversed by increased physical activity and healthy eating pattern.

Actually, not all the adipose tissue expansion is vicious. It has been suggest that 10% to 25 % of people who have metabolically healthy obesity (MHO) [115]. The characteristics of MHO people are that they have normal insulin sensitivity, glucose tolerance, and blood lipid. They seem to resist obesity associated metabolic disease like type II diabetes and cardiovascular disease [116]. MHO people have a lower risk of developing cardiovascular disease when comparing with other obese patients. The main reason is different adipose function between two groups [117]. The problem is how to keep the MHO condition through life span, and not transition to metabolically unhealthy obesity (MUO). Living a healthy life also decreases the gap between energy intake and expenditure, leading to less fat deposition in adipose tissue. Schoeder et al. conducted a survey over nine years, showed that being physically active help maintain a healthy cardio metabolic stage, and inhibit transition to MUO [118]. However, 10% to 27% of

lean people are reported to have higher risk for cardiovascular disease, and they are classified as metabolically unhealthy normal weight (MUNW). Characteristics of these patients are insulin resistance, dyslipidemia and a high percentage of body fat, in particular visceral fat [119].

The underlying mechanism is that under normal conditions, small adipocytes keep the hemostasis of adipose tissue with the coordination and collaboration of secreted adipokines. The relation of visceral fat accumulation but not subcutaneous fat volume to altered lipid profile is independent of body composition and fat distribution [120]. Upon excess energy storage, the hypertrophy and/or hyperplasia of adipocytes is associated with an increase in lypolysis, accompanied by disregulated adipokines production, macrophages infiltration, local hypoxia, fatty acid fluxes and fibrosis [121]. A variety of cell types in adipose tissue contribute in an orchestrated manner to induce adipose tissue remodeling, metabolic stress, and disorders on other organs or on the organism [122]. Increased lipolysis of adipose tissue causes elevated secreted free fatty acids and delivering FFAs to the liver via the portal vain directly, which leads to increased synthesis of VLDL. Increased FFAs secretion inhibits the activity of lipoprotein lipase in adipose tissue and skeletal muscle. In addition, increased synthesis of VLDL in liver inhibits the catabolism of diet fat, which induces hyperglycemia.

From the onset of obesity, many factors drive to the metabolic disorders including increased pro-inflammatory cytokines, decreased adiponectin, and infiltration of immune cells. Visceral adipose tissue is prone to secret inflammatory cytokines and has altered macrophages localization and inflammatory state. With the prevalent

inflammatory factors, adipose tissue endures hypoxia, endoplasmic reticulum stress and adipocytes death. Macrophages in adipose tissue surround the dead adipocytes and form crown-like structures while producing inflammatory cytokines such as TNF-α, macrophage inflammatory protein-1α, etc. [123, 124]. Macrophages presented in crown-like structures link to local and ultimate systemic insulin resistance. Adipocytes and macrophages communicate in a paracrine loop involving FFAs and TNF-α, not only exacerbate inflammatory state of adipose tissue, but also interferes adipocyte insulin signaling and further insulin resistance in the whole body [33].

Adenosine, a well-known purine nucleoside, plays as a key endogenous suppressor in mediating inflammation and immune system response. Adenosine also can be released in the extracellular space to respond to the metabolic disturbance, stress, inflammation or apoptosis. Upon the activation of $A_{2A}R$, the pro-inflammatory cytokines decrease, including TNF-α, IL-12 and nitric oxide produced by M1 macrophages. One proposed mechanism is through cAMP dependent pathway, which activate PKA and phosphorylates CREB. This regulate transcription by binding to the promoter region of CREs [125]. One proposed mechanism of suppression TNF-α production is via an increase in cAMP levels, but PKA and Epac independent way. Instead, okadaic acid can block the inhibitive actions of $A_{2A}R$ agonist by selectively inhibiting protein serine/threonine phosphatases [126]. Another proposed pathway is involving heme oxygnase-1 and its product carbon monoxide, which increasing the level of $A_{2A}R$ and sensitizing the macrophages to the action of adenosine [127, 128].

Materials And Methods

Animal experiments

5-6 weeks old male C57BL/6J mice from the Jackson Laboratory were group housed (3-5 mice/cage), feeding with LFD vs. HFD for 12 weeks. Composition of LFD is 10% fat, 20% protein and 70% carbohydrate of total kcal, while composition of HFD is 60% fat, 20% protein and 20% carbohydrate of total kcal. Both LFD and HFD were gained fro Research Diets, Inc (New Brunswick) and contained same amount of micronutrients per kcal diet, such as vitamin mix and mineral mix, and casein, cellulose, soybean oil as well. LFD is 3.85 kcal/g, including 1260 kcal of cornstarch, 1400 kcal of sucrose, and 180 kcal of lard. While HFD is 5.24 kcal/g, including 275 kcal of sucrose, 2205 kcal of lard and no cornstarch. The mice were maintained in a controlled environment, inverted 12h daylight-cycle (lights on at 06:00). During the 12-week feeding period, body weight and food intake were monitored weekly. After the feeding regimen, mice were anesthetized (0.1 mL/10 g of body weight of 1:1 v/v xylazine 1%/ ketamine 10%, intraperitoneal injection), blood was collected and tissue samples were carefully excised, weighted and stored for further study [129-131]. To determine if the process of over-nutrition affects $A_{2A}R$ expression, adipose tissue were collected from LFD or HFD fed WT mice. The adipose tissue that was dissected for testing involved epididymal, mesenteric and perinephric fat pads. The Institutional Animal Care and Use Committee of Texas A&M University approved the laboratory animal care and handling.

Glucose and insulin tolerance tests

Glucose tolerance test (GTT) measures the body's ability to clear an injected glucose load. Insulin tolerance test (ITT) were used in conjunction with GTT, to determine how well of systemic insulin sensitivity after giving an injection of insulin. Mice were fasted for approximately 4 h and fasted glucose levels are determined before receiving an intra-peritoneal injection of solution of D-glucose (2 g/kg BW) or insulin (1 U/kg BW). For GTT, blood was collected from tail veins at 30, 60, 90 and 120 min after an intraperitoneally injected bolus glucose. For ITT, blood was collected from tail veins at 15, 30, 45 and 60 min after an intraperitoneally injected bolus insulin [132, 133]. The plasma glucose was determined by glucose assay kit (Sigma, St. Louis, MO).

Western blot

Lysates from frozen liver samples applied the lysis buffers (pH 7.4) containing 20 mM HEPEs, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 1% NP-40, 0.1% SDS, 2 mM EDTA and 2 mM navadate. The 5× loading dye contains 1.0 M Tris (pH 6.8), 50% glycerol, 10% SDS and 0.1% bromophenol blue [129, 131]. Cell lysates (50 μg of protein) and protein markers (cell signaling Technology) were subjected to sodium dodecyl sulphate (SDS)- polyacrylamide gel electrophoresis. Proteins were semi-dry transferred to synthetic membranes, and incubated overnight at 4 °C, with primary antibody specific to the protein of interest at a 1: 1 000 dilution. Subsequent immunoblotting of labeled protein were performed for 2h at room temperature, with a 1:10 000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary kit (Immobilon^{TM} Western; EMD Millipore, Billerica, MA USA) [129]. The loading control

is glyceraldehyde 3- phosphate dehydrogenase (GAPDH). The quantitated density of target bands was calculated by using Image Lab ™ software. Ratios of Pp46/p46, Pp65/p65 and AR were normalized to GAPDH and adjusted relative to the average of LFD fed control, which was arbitrarily set as 1 (AU).

RNA extraction and real-time PCR

The detection and quantitative- analysis of pro-inflammatory cytokine genes in WAT were conducted by real-time PCR. Total RNA isolated from frozen adipose tissue was homogenized by using RNA STAT-60™. To extract RNA, 1 vol. of homogenate was added with 0.2 vol. of chloroform. The precipitation of RNA was performed using 0.5 vol. of isopropanol and used 75% ethanol to wash the RNA pellet. Reverse transcription was performed in GoScript™ Reverse Transcription System (Promega). cDNA was prepared from 0.5 μg total RNA by heating for 15 min at 42°C in the presence of AMV reverse transcriptase, recombinant RNasin® ribonuclease inhibitor and oligodeoxythymidilic acid ($oligo(dT)_{15}$). cDNA was subjected to PCR amplification in SYBR Green (LightCycler ® 480 system; Roche) system. The reaction mix contained 1.2 μL cDNA, 0.8 μL primer, 10 μL power Sybr-green PCR master mix and 8 μL nuclease-free water [134, 135]. The mRNA levels were analyzed for $A_{2A}R$, TNF-α, IL-1β, IL-6 and MCP1 in adipose tissue samples. The internal reference gene 18S ribosomal RNA was used to normalize the data.

Statistical analysis

Numeric data are presented as means ± SE (standard error). Two-tailed student's t test was used to assess if the data is statistically significant. The standards of the study is when *P* value <0.05.

Results

HFD induces obesity and insulin resistance

To determine if the process of over-nutrition affects $A_{2A}R$ expression, the adipose tissue were collected from low fat diets (LFD) or HFD fed wild-type (WT) mice. C57BL/6J mice at 5-6 weeks of age were fed ad libitum with a HFD for 12 weeks, which showed profound increases in body weight (diet-induced obesity, DIO). Compared to age- and gender- matched mice that were fed with a LFD, HFD fed mice gained much more body weight after 5 weeks. After 12 weeks feeding, the average body weight in HFD group was 42 g, whereas LFD fed mice was only 30 g (Figure 1 (A)). HFD fed mice displayed decreased glucose tolerance. Comparing to LFD fed mice, HFD fed mice' plasma glucose level increased significantly after ingestion of glucose solution. The plasma glucose level of HFD fed mice kept high after 2 h of ingestion (Figure 1 (B)). ITT showed that HFD fed mice have bad insulin sensitivity. After ingestion of insulin, their plasma glucose level was higher than LFD fed mice group, which indicated that the tissues of HFD fed mice cannot respond to insulin properly (Figure 1 (C)). HFD diet fed mice also gained more visceral fat in terms of epididymal fat, perinephric fat and mesenteric fat than LFD fed mice (Figure 1(D)).

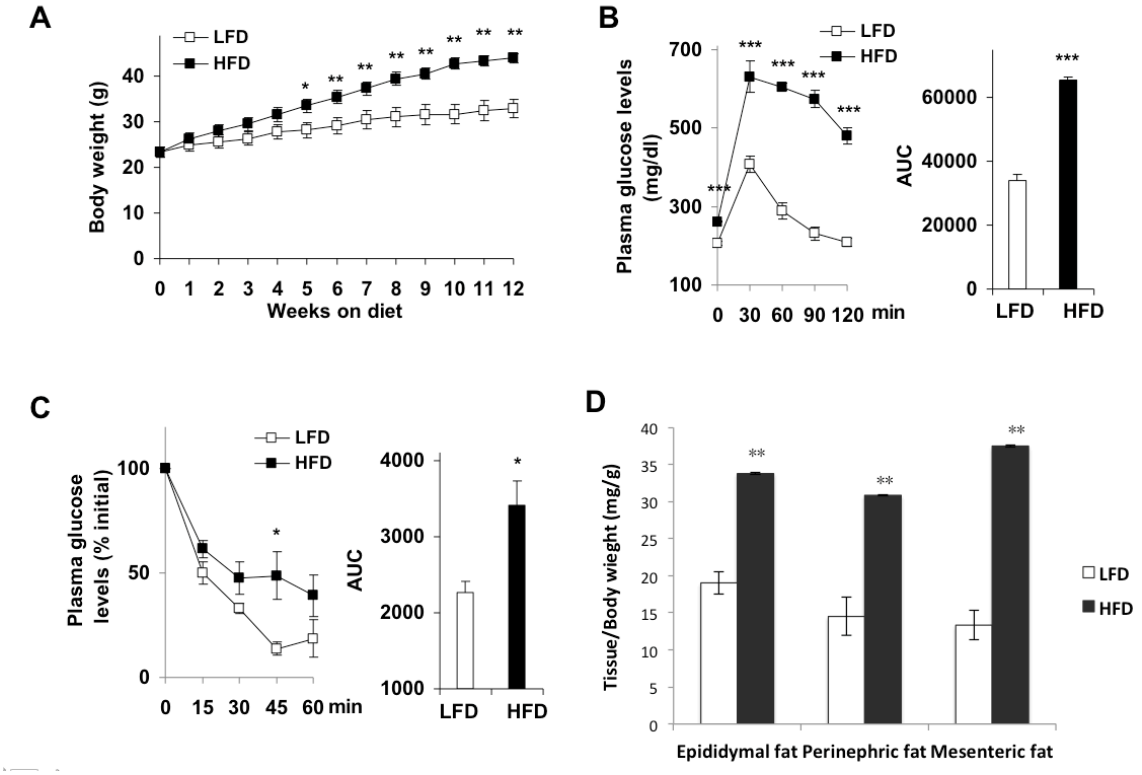

Figure 1. HFD induces obesity and insulin resistance.
Male C57BL/6J mice were fed with a LFD or HFD for 12 weeks. Data are means ± SE, n= 5. (A) Body weight was monitored weekly during the feeding period. (B) GTT. (C) ITT. (D) The ratio of different adipose tissue weight to body weight (mg/g). For B and C, mice were fasted for 4 h and fasted glucose levels are determined before receiving an intra-peritoneal injection of glucose (2 g/kg BW) or insulin (1 U/kg BW). *, P<0.05 and **, P<0.01. LFD fed vs. HFD fed for the same time point (B and C).

HFD increases the inflammatory responses in WAT

Inflammatory and metabolic biomarkers were tested through Western Blot and RT-PCR to compare LFD and HFD treatment, which can help to link overnutrition induced inflammation and $A_{2A}R$ expression and activation. In the present study, we confirmed the findings that HFD increased inflammatory responses in the WAT (Sun, 2012 #3009）(Greenberg, 2006 #1733）. Western blot result of WAT samples showed that HFD fed mice had more JNK and NF-κB (p65) phosphorylation than LFD fed mice, which indicated that HFD had effects on the activation of JNK pathway and NF-κB pathway (Figure 2 (A)). In addition, mRNA levels of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and chemokines such as MCP1, were significantly higher in the WAT of HFD fed mice than LFD fed mice (Figure 2 (B)).

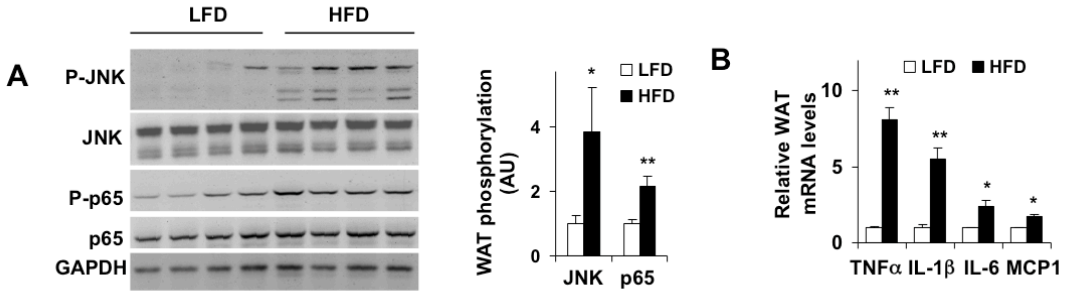

Figure 2. HFD increase the inflammatory responses in WAT.
(A) WAT inflammatory signaling. Protein extracts from WAT were subjected to western blots. The degree of phosphorylation was determined from the measured ratio of phosphorylated JNK1 to total JNK1 (Pp46/p46) and phosphorylated NF-κB p65 to total p65 (Pp65/p65), via GAPDH normalization (AU, arbitrary unit). (B) The detection and quantitative- analysis of pro-inflammatory cytokine genes in WAT were conducted by real-time PCR. For bar graphs (A and B), data are means ± SE, n= 5. *, $P<0.05$ and **, $P<0.01$.

$A_{2A}R$ level increases significantly in WAT

Tissue distribution of adenosine receptor has been examined in the mice by using of Western blots. High levels of adenosine receptors are found in brain, spleen and liver of WT mice (Figure 3 (A)). Western blots result showed HFD feeding increased the level of adenosine receptors (Figure 3 (B)). The mRNA level of $A_{2A}R$ in adipose tissue from HFD-fed mice were significantly higher than that from LFD fed mice (Figure 3 (C)).

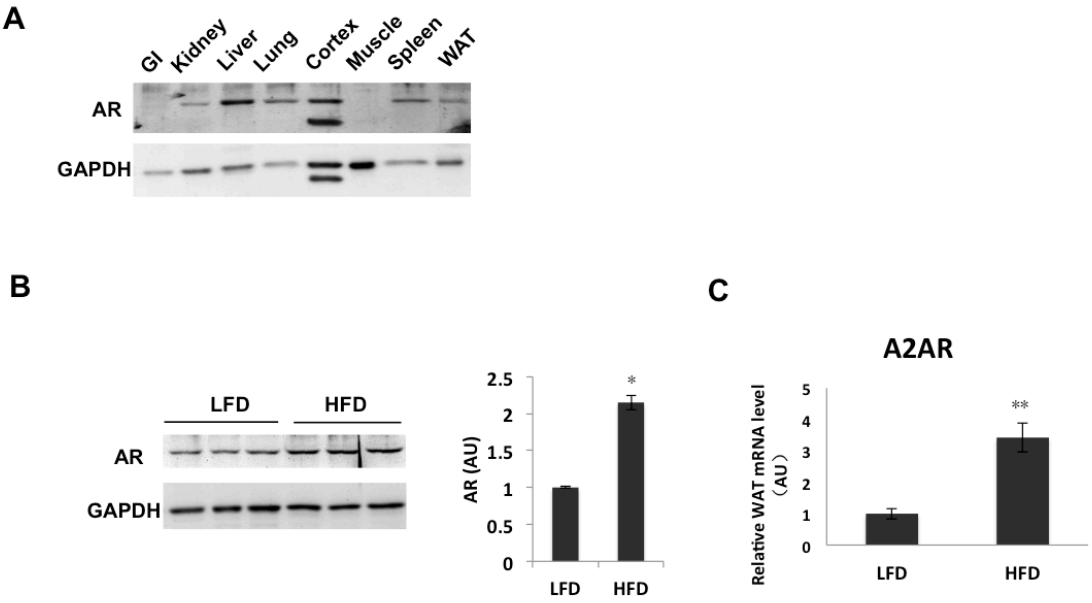

Figure 3. The level of $A_{2A}R$ increases significantly in WAT.
(A) Tissue distribution of adenosine receptor in mice. (B) WAT adenosine receptor signaling. Protein extracts from WAT were subjected to western blots. The level of adenosine receptor was determined from the measured ratio of adenosine receptor to GAPDH (AU, arbitrary unit). (C) The detection and quantitative- analysis of $A_{2A}R$ gene in WAT were conducted by real-time PCR. For bar graphs (B and C), data are means ± SE, n= 5. *, $P<0.05$ and **, $P<0.01$.

Discussions

C57BL/6J mice had profound increase in body weight and visceral fat on a HFD, compared with LFD fed WT mice. Adipose tissue of HFD fed WT mice had increased JNK and NF-κB phosphorylation and increased pro-inflammatory gene expression. Concurrently, HFD-fed WT mice had decreased glucose tolerance and insulin sensitivity. These are consistent with the results gained from the mouse model [132, 133, 136, 137]. Obesity activates IKKβ/ NF-κB signaling pathway, by which IKK (NF-κB activator) phosphorylates IkBα (NF-κB inhibitor) and releases NF-κB that was sequestered in cytoplasm. NFκB-p65 is phosphorylated and translocate to the nucleus to mediate the transcription of target genes such as TNF-α, IFN-γ and iNOS, etc. IKKβ protects adipocytes from death in diet-induced obesity and is required for the expansion or contraction of adipose tissue according to different nutrient status [138]. HFD-fed mice displayed 2-fold enhanced NF-κB luciferase illumination and translocation to the nucleus in adipose tissue macrophages compared to their lean counterparts. Furthermore, HFD-fed mice had also increased activation of IKKε and NF-κB in adipose tissue and liver. IKKε- deficient mice maintained normal glucose tolerance, insulin sensitivity and insulin signaling, which also protected against hepatic steatosis [139].

The pro-inflammatory cytokines and FFAs activate JNK1 (p46) and JNK2 (p54) in adipose tissue. JNK activation promotes pro-inflammatory cytokines production (IL-6, TNF-α etc.), lipolysis, and insulin resistance in adipose tissue of diet-induced obesity animal models. Global knockout of JNK maintained insulin sensitivity and insulin signaling [140-142]. Because JNK causes serine/threonine phosphorylation of IRS1 and

IRS2 and impairs tyrosine phosphorylation in response to insulin, it decreases PI3K/AKT signaling [143]. A double knockout of JNK1 and JNK 2 specific in macrophages retained similar obesity in HFD-fed mice compared to their WT littermates, but knock mice had reduced inflammation and insulin resistance owing to decreased macrophages infiltration into adipose tissue. It was proposed that JNK1 and JNK2 have redundant function, and omission of both genes in myeloid cells would induce insulin resistance and inflammation [144].

The excess adiposity causes insulin resistance, which is characterized as impaired suppression of lipolysis and diminished glucose uptake. Interestingly, insulin resistance is incomplete in adipose tissue, by which GLUT trafficking is impaired, yet nuclear exclusion of Forkhead box O-1 (FoxO1) is preserved [145]. Limited expansion of subcutaneous adipose tissue leads to the enlargement of visceral adipose tissue. The mechanisms link visceral fat accumulation and insulin resistance so that the secreted pro-inflammatory cytokine from visceral adipose tissue impairs insulin sensitivity. Also, the accumulation of visceral fat happens in parallel with ectopic fat accumulation, such as in the liver and muscles, which causes the lipotoxicity and insulin resistance in these tissues.

The present study suggests that the increased $A_{2A}R$ level in adipose tissue of HFD-fed mice is a defensive response, which may help protect against inflammatory damage. The underlying mechanism could be inhibiting immune cell trafficking and proliferation in adipose tissue, decreasing pro-inflammatory cytokines production, or

cytotoxicity. These hypotheses are proved and discussed further in the following chapters.

CHAPTER IV

THE PHYSIOLOGICAL RELEVANCE OF $A_{2A}R$ IN LIVER UNDER DIFFERENT NUTRITIONAL CONDITIONS

Introduction

Nonalcoholic fatty liver disease (NAFLD) is highly prevalent in the obese population, which is characterized by swelling of the liver with fat. It is particularly astonishing since it can develop into the stage of cirrhosis, hepatocellular carcinoma and ultimately liver failure. There are 3-12% of adults in the U.S. are affected by this kind of severe NAFLD, termed as nonalcoholic steatohepatitis (NASH). The main causations of NAFLD are insulin resistance and compensatory hyperinsulinaemia by which excess accumulation of triglyceride is produced in the liver. In adipose tissue, diminished insulin action causes impaired suppression of lipolysis and increased release of non-esterified fatty acids [146]. Thus, increased blood sugar caused by inhibited glucose uptake in skeletal muscle, together with elevated secreted fatty acids from adipose tissue promote hepatic fat uptake, *de novo* lipogenesis and impede β- oxidation. Consequently, the liver will enlarge with triglyceride accumulation, which results in hepatic steatosis. Steatosis means more than 5% of liver weight is lipid content. It is the most frequent liver lesion striking up to 70% of overweight adults [147].

A variety of fatty acids are cytotoxic, such as SFA and arachidonic acid, via producing pro-inflammatory cytokines like IL-1, IL-6 and TNF-α. Thus, a mechanism for fatty acid trapping is needed to avoid cell toxicity, which involves insulin and

acylation stimulating protein/C3adesArg pathway [148]. Insulin metabolism contains a complex insulin-signaling network. Binding of insulin activates its receptor Tyr kinase, leading to phosphorylation and recruitment of insulin receptor substrate (IRS) that related two main signaling pathway activation, PI3K-AKT/PKB pathway and MAPK pathway. PI3K is one target of IRS, which produces PIP3 to activate PDK kinase to phosphorylate and activate Akt kinase. Consequently, this signaling results in translocation of GLUT4 to plasma membrane, and increasing glucose uptake. MAPK activates mitogenic and growth effects of insulin.

In the liver, the insulin resistance is incomplete, which is displayed as impaired suppression of gluconeogenesis but conserved lipogenesis [145]. The underlying mechanism is that downstream effectors of insulin signaling pathways are differently affected, prominently the isoforms PI3K and Akt kinases. For instance, p110α isoform of PI3K and Akt2 isoform of Akt are essential in both insulin-regulated hepatic glucose and lipid metabolism [146, 149-151].

Adenosine acts as $A_{2A}R$ to stimulate short interruption of blood flow, which protects the liver from damage followed by a subsequent ischemia/reperfusion. It is a mechanism of liver tolerance to avoid the damage from irreversible tissues by starving them. Pretreatment of $A_{2A}R$ agonists displayed hepatoprotection against ischemia/reperfusion injury or hypoxia. Upon $A_{2A}R$ activation, those genes for intracellular survival were expressed [152]. Besides, an indirect mechanism is inhibiting the suppressant effect of ischemia/ reperfusion on nitric oxide synthase in the liver endothelial cells [153].

People with steatosis significantly decrease tissue tolerance after ischemia/reperfusion injury from liver surgery [154]. Moreover, these people had estimated twice the risk of postoperative complications, and triple on risk of liver failure to those with excess steatosis [155]. The infiltration of FFAs and their metabolites relates to hepatotoxic effects such as increase of oxidative stress, mitochondrial dysfunction and decrease of ATP, which leads to liver intolerance and to ischemia/ reperfusion injury. $A_{2A}R$ activation inhibited apotosis in rats primary hepatocytes challenged with FFAs and ameliorated the progression of NAFLD in rat fed with methionine choline-deficient diet [156]. $A_{2A}R$ agonist inhibited JNK1/2 activation via impeding MKK4 (mitogen activated protein kinase-4)/ SEK1 (stress-activated protein kinase/extracellular-signal regulated kinase 1). These studies underscore the need to better understand the mechanisms of protective effects of $A_{2A}R$ against steatosis. Thus, using the liver collected from diet-induced obesity model in the previous chapter, we hypothesized that $A_{2A}R$ activation could decrease the inflammatory status and protect hepatocytes lipotoxicity.

Materials And Methods

Animal experiments

5-6 weeks old male C57BL/6J mice from the Jackson Laboratory were group housed (3-5 mice/cage), feeding with LFD vs. HFD for 12 weeks. Composition of LFD is 10% fat, 20% protein and 70% carbohydrate of total kcal, while composition of HFD is 60% fat, 20% protein and 20% carbohydrate of total kcal. Both LFD and HFD were gained fro Research Diets, Inc (New Brunswick) and contained same amount of

micronutrients per kcal diet, such as vitamin mix and mineral mix, and casein, cellulose, soybean oil as well. LFD is 3.85 kcal/g, including 1260 kcal of cornstarch, 1400 kcal of sucrose, and 180 kcal of lard. While HFD is 5.24 kcal/g, including 275 kcal of sucrose, 2205 kcal of lard and no cornstarch. The mice were maintained in a controlled environment, inverted 12h daylight-cycle (lights on at 06:00). During the 12-week feeding period, body weight and food intake were monitored weekly. After the feeding regimen, mice were anesthetized (0.1 mL/10 g of body weight of 1:1 v/v xylazine 1%/ ketamine 10%, intraperitoneal injection), blood was collected and tissue samples were carefully excised, weighted and stored for further study [129-131]. To determine if the process of over-nutrition affects $A_{2A}R$ expression, liver will be collected from LFD or HFD fed WT mice. The Institutional Animal Care and Use Committee of Texas A&M University approved the laboratory animal care and handling.

Lysates from frozen liver samples applied the lysis buffers (pH 7.4) containing 20 mM HEPEs, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 1% NP-40, 0.1% SDS, 2 mM EDTA and 2 mM navadate. The 5× loading dye contains 1.0 M Tris (pH 6.8), 50% glycerol, 10% SDS and 0.1% bromophenol blue [129, 131]. Cell lysates (50 μg of protein) and protein markers (cell signaling Technology) were subjected to sodium dodecyl sulphate (SDS)- polyacrylamide gel electrophoresis. Proteins were semi-dry transferred to synthetic membranes, and incubated overnight at 4 °C, with primary antibody specific to the protein of interest at a 1: 1 000 dilution. Subsequent immunoblotting of labeled protein were performed for 2h at room temperature, with a 1:10 000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary kit

(Immobilon™ Western; EMD Millipore, Billerica, MA USA) [129]. The loading control is glyceraldehyde 3- phosphate dehydrogenase (GAPDH). The quantitated density of target bands was calculated by using Image Lab ™ software. Ratios of Pp46/p46, Pp65/p65 and AR were normalized to GAPDH and adjusted relative to the average of LFD fed control, which was arbitrarily set as 1 (AU).

RNA extraction and real-time PCR

The detection and quantitative- analysis of pro-inflammatory cytokine genes in liver were conducted by real-time PCR. Total RNA isolated from frozen adipose tissue was homogenized by using RNA STAT-60™. To extract RNA, 1 vol. of homogenate was added with 0.2 vol. of chloroform. The precipitation of RNA was performed using 0.5 vol. of isopropanol and used 75% ethanol to wash the RNA pellet. Reverse transcription was performed in GoScript™ Reverse Transcription System (Promega). cDNA was prepared from 0.5 μg total RNA by heating for 15 min at 42°C in the presence of AMV reverse transcriptase, recombinant RNasin® ribonuclease inhibitor and oligodeoxythymidilic acid (oligo(dT)$_{15}$). cDNA was subjected to PCR amplification in SYBR Green (LightCycler ® 480 system; Roche) system. The reaction mix contained 1.2 μL cDNA, 0.8 μL primer, 10 μL power Sybr-green PCR master mix and 8 μL nuclease-free water [134, 135]. The mRNA levels were analyzed for $A_{2A}R$, TNF-α, IL-1β, IL-6 and MCP1 in liver samples. The internal reference gene 18S ribosomal RNA was used to normalize the data.

Statistical analysis

Numeric data are presented as means ± SE (standard error). Two-tailed student's t test was used to assess if the data is statistically significant. The standards of the study is when *P* value <0.05.

Results

HFD induces the inflammatory responses in liver

To determine if excess SFAs affect $A_{2A}R$ expression, the liver were collected from LFD or HFD fed WT mice. In addition, inflammatory and metabolic biomarkers were tested through Western Blot and RT-PCR to compare LFD and HFD treatment, which can help to link overnutrition induced inflammation and $A_{2A}R$ activation and modulation. In the present study, we also confirmed that HFD increased inflammatory responses in the liver. Western blot result of liver samples showed that HFD fed mice have more JNK and NF-κB (p65) phosphorylation than LFD fed mice, which indicated HFD had effects on activation of the JNK pathway and NF-κB pathway (Figure 4 (A)). In addition, mRNA levels of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 were significantly higher in WAT of HFD fed mice than LFD fed mice (Figure 4 (B)). Also, HFD mice had much higher liver tissue weight relative to body weight (Figure 4 (C)).

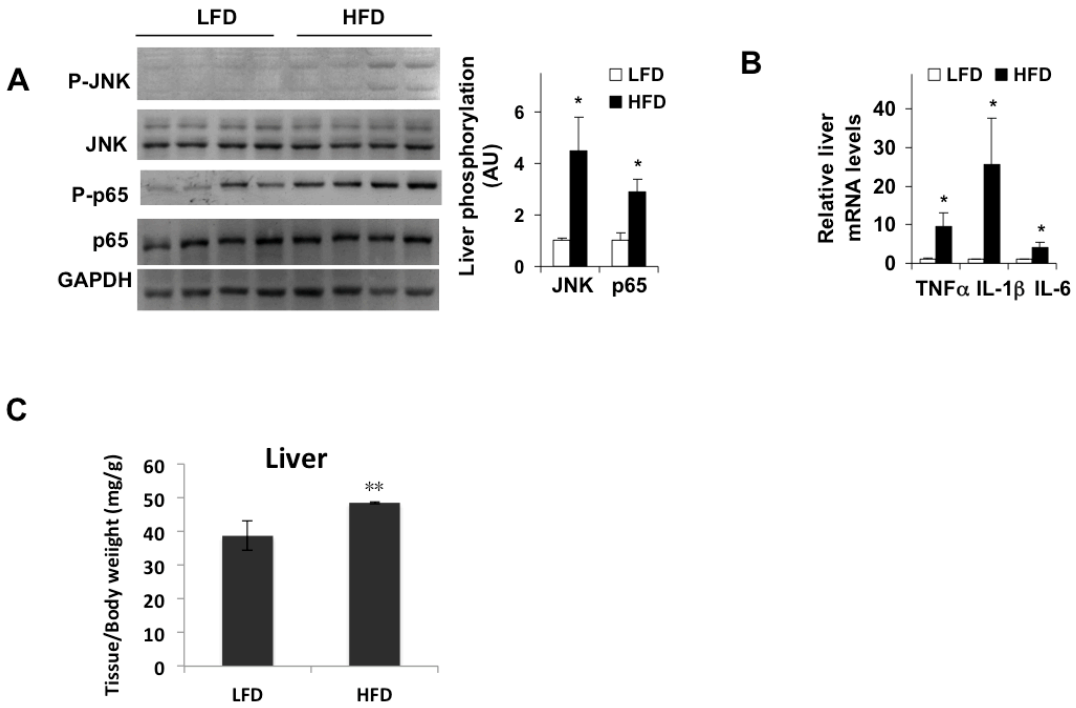

Figure 4. HFD increase the inflammatory responses in liver.
(A) Liver inflammatory signaling. Protein extracts from liver were subjected to western blots. The degree of phosphorylation was determined from the measured ratio of phosphorylated JNK1 to total JNK1 (Pp46/p46) and phosphorylated NF-κB p65 to total p65 (Pp65/p65), via GAPDH normalization (AU, arbitrary unit). (B) The detection and quantitative- analysis of pro-inflammatory cytokine genes in liver were conducted by real-time PCR. For bar graphs (A and B), data are means ± SE, n= 5. *, $P<0.05$ and **, $P<0.01$. (C) The ratio of liver weight to body weight (mg/g).

$A_{2A}R$ level increases significantly in liver

Western blot of liver samples showed that $A_{2A}R$ level was much higher in HFD fed mice than LFD fed mice (Figure 5).

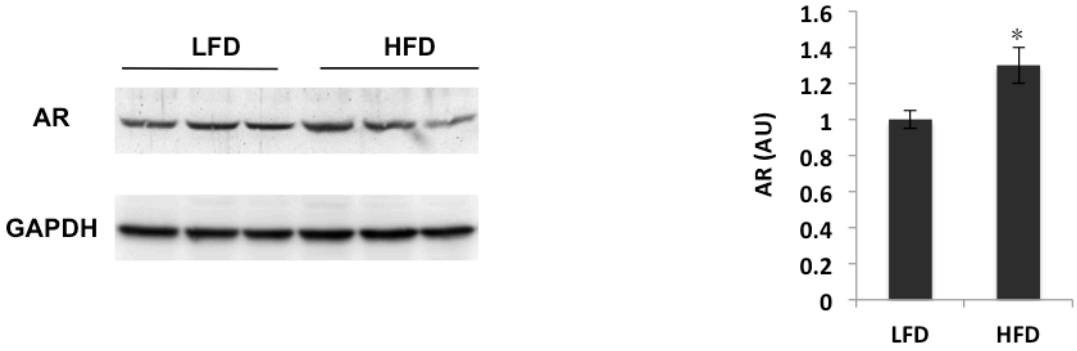

Figure 5. The level of $A_{2A}R$ increases significantly in liver.
(A) Liver adenosine receptor signaling. Protein extracts from WAT were subjected to western blots. The level of adenosine receptor was determined from the measured ratio of adenosine receptor to GAPDH (AU, arbitrary unit). For bar graphs, data are means ± SE, n= 5. *, P<0.05 and **, P<0.01.

Discussions

In the present study, 12-week HFD feeding caused swelling of the liver compared to LFD fed mice. Impaired glucose tolerance and insulin tolerance occurred with altered lipid metabolism in liver. This is consistent with widely accepted concept that insulin resistance in adipose tissue results in increased lipolysis and subsequent FFAs secretion. FFAs, especially from visceral fat, is taken up by the liver directly from the portal vein, which results in decreased insulin clearance in liver and further hyperinsulinemia. Hyperinsulinemia compensated for insulin resistance and led to steatosis through increased *de novo* lipogenesis, decreased β- oxidation, decreased hepatic lipid output and increased flux of FFAs. FFAs promote impaired suppression of

insulin stimulated gluconeogenesis, glucose output, and conserved lipogenesis lead to a combination of hyperglycemia and hyperlipidemia [145]. In addition, FFAs ligate to toll-like receptor 4 (TLR-4) complex, activating MyD 88-NF-κB-denpendent pathway and increase TNF-α release [157].

By virtue of C57B6/J mice, we observe that after the onset of steatosis, the liver is more vulnerable to the factors and interactions induced by HFD feeding, which delineated as increased JNK and NF-κB phosphorylation, increased pro-inflammatory genes expression. A number of papers also demonstrated that other than pro-inflammatory cytokines, adipokines, endoplasmic reticulum stress, mitochondrial stress etc. promote the progress of liver fibrosis [26, 124, 158]. Saturated fatty acids in HFD serve as a ligand to TLR4, triggering both MyD88-dependent and MyD88-independent (TRIF) pathways. The downstream of MyD88-dependent pathway is IκB kinase (IKK) complex activation, which subsequently phosphorylates IKKα, and ubiquination of IKK thereafter by the proteasome and free NF-κB to nucleus. As a transcription factor, phosphorylation and the activation of NF-κB lead to the activation of vicarious members of MAPK such as p38 and JNK. The TRIF pathway is due to IRF3 phosphorylation and induced expression of IFNβ that interacts with RIP1 and activates NF-κB [10]. As a result of NF-κB, SFAs (mainly palmitic acids) induce the pro-inflammatory gene products such as IL1 and TNF-α.

In addition, JNK plays a central role in causing cellular inflammation and insulin resistance, leading to NAFLD. JNK causes both hepatic and systemic insulin resistance through serine/threonine phosphorylation of IRS1 and IRS2, resulting in impaired

tyrosine phosphorylation of these molecules, which is required for insulin signaling [143]. Mice with genetic deletion of JNK in the liver not only reversed insulin resistance in the liver but also improved systemic insulin sensitivity. Activation of JNK1 (p46) induced phosphorylation of SIRT1 with its subsequent degradation. The inhibition of SIRT1 catalytic activity increased the level of genes related to *de novo* lipogenesis such as LXRα, SREBP-1 and SCD1. In addition, together with SIRT1 inhibition, decreased LKLB1 and AMPK activity lead to pathogenesis of steatosis [159]. In the mesenteric fat, ablation of SIRT1 contributed to the elevation of FFAs secretion, which was further taken up by the hepatocytes [160]. JNK1 (p46) and 2 (p54) ablation specific in myeloid cells had attenuated hepatic steatosis, liver inflammation, immune cells infiltration and better insulin sensitivity [144]. JNK also promotes the development of inflammation through AP-1 dependent transcription.

Intriguingly, increased $A_{2A}R$ level in the liver of HFD-fed mice is a defensive response, which may help protect against inflammatory damage. This result is consistent with the previous study done by the Italian group who conducted the research on pharmacological activation of $A_{2A}R$ in hepatocytes and in rats [161]. When the hepatocytes were challenged with stearic acid, treatment of $A_{2A}R$ agonist (CGS21680) ameliorated JNK1/2 activation and decreased apoptosis. $A_{2A}R$ agonist inhibited induction of apoptosis from blocking stress-signaling kinase MKK4/SEK1, mediated by PI3K/Akt. Administration of $A_{2A}R$ agonist to MCD-fed rats prevented MKK4/SEK1-mediated JNK signaling, improved liver inflammation and fibrosis, and thus ameliorated NASH.

CHAPTER V

DIRECT ROLE OF $A_{2A}R$ ON ADIPOCYTES AND MACROPAHGES

Introduction

When there is an excessive amount of energy consumption that is not immediately oxidized for cellular energy, will be stored as fat. To deposit excessive energy from diet for compensation, preadipocytes differentiate into mature adipocytes. Increased inflammation and insulin resistance in an obese body induce dramatically decreased number of preadipocytes to undergo differentiate. The mechanism may be the increased expression of mitogen-activated protein 4 kinase 4 (MAP4K4), which inhibit peroxisome proliferator-activated receptor-gamma (PPARγ), one of the key transcription factors that governing the adipogenesis process. TNF-α promoted Wnt10b expression to inhibit differentiation of the preadipocytes and switch preadipocytes into pro-inflammatory phenotype, involving increased expression of cytokines and chemokines [162, 163].

SFAs, such as steric acid and palmititc acid, activate TLR4 cascade, and subsequently induce inflammation attributes to JNK and NF-κB pathways. In addition, SFAs cause ER stress involving increased phosphorylation of signal transduction proteins such as PPKR-like endoplasmic reticulum kinase (PERK) and inositol-requiring enzyme 1α (IRE-1α) [164]. Thus, TLR4 activated by SFAs may serve as the molecular link between metabolic and immunologic effects in adipose tissue [165].

Adipose tissue is infiltrated by macrophages upon obesity, and these macrophages surround the dead adipocytes to form crown-like structures, leading to pro-inflammatory cytokines production such as IL-1, TNF-α and IL-1β. These cytokines and SFAs contribute to a paracrine loop between adipocytes and macrophages. The crosstalk between these two kinds of cells leads to inflammation transformation and insulin resistance of adipose tissue via activation of JNK1/2 [166, 167]. Co-culture of adipocytes and macrophages with LPS in a transwell system markedly up-regulated IL-6, IL-8, MCP-1 and RANTES [168]. Macrophages attacked and phagocytosed adipocytes, which led to synthesis and release of IL-6 and MCP-1 in a NF-κB dependent fashion [169].

Kupffer cells are resident hepatic macrophages in the linings of the liver sinusoids. They exert a tolerogenic state upon immunoreactive materials into the sinusoids including gut-derived bacteria and microbial products that to be transported via the portal vein, and also dead or damaged cells during the circulation of the liver [170]. While kupffer cells, together with monocyte-derived macrophages, switch to immunogenic state in the setting of chronic liver injuries and inflammasome activation caused by such as NAFLD/ NASH [171]. Palmitate stimulates death receptor 5 (DR5/ TNFSF10B), leading to the release of extracellular vesicles bearing TNF-related apoptosis-inducing ligand (TRAIL/ TNFSF10) from hepatocytes and subsequent activation of macrophages to shift to inflammatory state. THP-1 macrophages challenged with extracellular vesicles or TRAIL can activate NF-κB pathways in presence of elevated p65 translocation [172]. Another danger signal related to

macrophage activation and polarization is liver- derived histidine-rich glycoprotein (HRG). Genetic deletion of HRG had ameliorated liver fibrosis, reduced macrophages number and shifted macrophages toward M2 polarization in livers of mice fed with CCI4 or MCD diet. Patients with NAFLD dramatically increased HPG expression in liver, which was related to the shifting of M1 phenotype of macrophages [173]. In addition, the decrease of the glucocorticoid- induced leucine zipper (GILZ) in the liver with nonalcoholic steatosis results in macrophage populations skews toward M1 polarization. Unlike monocyte-derived macrophages, kupffer cells are the scavenger for antigens to induce systemic tolerance and antigen-specific CD4 and CD8 T cell activation. In chronic liver disease, monocyte-derived macrophages infiltration is correlated to the failing of kupffer cells to induce T cell responses, which leads to liver tolerance abrogation [171].

Adenosine, an extracellular signaling molecule via the activation of four GPCRs, is incessantly released from adipocytes. Among adenosine receptors, $A_{2A}R$ and $A_{2B}R$ are dominant in preadipocytes, while A_1R is predominantly expressed on mature adipocytes. A_1R activation induces adipocytes differentiation, inhibits lipolysis and leptin secretion. Lipolysis itself can induce vicious cycle of inflammatory alteration in adipose tissue, including elevated FFAs secretion and impaired insulin sensitivity. $A_{2A}R$ expression promotes differentiation and proliferation of adipocyte progenitors via elevating the expression of transcription factors in adipogenesis process, PPARγ and C/EBPα [174]. $A_{2B}R$ expression inhibits adipogenesis via continued expression of Krüppel-like factor 4 (KLF4), which is involved in regulation of stem cells [175]. Concomitant with the

inhibited adipogenesis is $A_{2B}R$- stimulated osteoblast including increased osteocalcin and alkaine phosphatase (ALP) [176].

The anti-inflammatory effects of adenosine on M1 macrophages are mediated predominantly by $A_{2A}R$ through inhibition of TNF-α, IL-6, nitric oxide and increase of IL-10 production. Adenosine released upon phagocytosis of adipocytes by macrophages activated $A_{2A}R$, which inhibited chemokines production such as MIP-2 and KC [177].

From the result of Chapter III and IV, we demonstrated that increased $A_{2A}R$ level in diet-induced obesity mice serve as defensive response. Therefore, in this chapter, we are interested in investigating the direct effect of $A_{2A}R$ on a cellular level.

Materials And Methods

Cell culture and treatment

3T3-L1 adipocytes were cultured in low glucose (5.5 mM) vs. high glucose (27.5 mM) DMEM medium. DMEM was supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. To differentiate 3T3-L1 cells into adipocyte-like cells, the 1 d post-confluent cells were incubated in induction medium including 1μM dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine and 10 μg/mL insulin (Day 0). On day 3, changed the induction medium into DMEM supplemented with 10 μg/mL insulin for an another 6-8 days. Differentiated into adipocyte-like cells were treated with or without palmitate (250 μM) for 24h. To test the effects of each macronutrient on $A_{2A}R$ expression, RT-PCR results among groups were determined. Inflammatory and metabolic biomarkers like TNFα, IL-6 and adiponectin were examined by RT-PCR, which address the relationship between nutritional factors and

inflammatory responses. Whole cell lysates were prepared and conducted Western Blot, the phosphorylation of JNK and NF-κB p65 were measured.

Bone marrow was isolated from the tibias and femurs of WT mice and was cultured in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal bovine serum. To initiate the outgrowth of macrophages, IMDM was added with 15% (v/v) L929 culture supernatant for 8 days. After differentiation, BMDM were treated with either LG (5.5 mM) or HG (27.5 mM) medium, with or with palmitate (250 μM) for 24 h. After harvesting the cells, total RNA of each treatment cells was extracted to measure the expression level of $A_{2A}R$, TNF-α, IL-1β and IL-6 via RT-PCR. Whole cell lysates were collected to measure the phosphorylation of JNK and NF-κB p65 via Western Blot.

Bond marrow was isolated from $A_{2A}R^{-/-}$ and $A_{2A}R^{+/+}$ mice. BMDM were cultured in either LG (5.5 mM) or HG (27.5 mM) medium, with or without palmitate (250 μM) treatment for 24 h. After harvesting the cells, total RNA of each treatment cells was used to measure the expression level of $A_{2A}R$, TNF-α, IL-1β and IL-6 via RT-PCR. Whole cell lysates were used to measure the phosphorylation of JNK and NFκB p65 via Western Blot.

Western blot

Lysates from cells applied the lysis buffers (pH 7.4) containing 20 mM HEPEs, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 1% NP-40, 0.1% SDS, 2 mM EDTA and 2 mM navadate. The 5× loading dye contains 1.0 M Tris (pH 6.8), 50% glycerol, 10% SDS and 0.1% bromophenol blue [129, 131]. Cell lysates (50 μg of protein) and protein markers (cell signaling Technology) were subjected to sodium

dodecyl sulphate (SDS)- polyacrylamide gel electrophoresis. Proteins were semi-dry transferred to synthetic membranes, and incubated overnight at 4 °C, with primary antibody specific to the protein of interest at a 1: 1 000 dilution. Subsequent immunoblotting of labeled protein were performed for 2h at room temperature, with a 1:10 000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary kit (Immobilon™ Western; EMD Millipore, Billerica, MA USA) [129]. The loading control is glyceraldehyde 3- phosphate dehydrogenase (GAPDH). The quantitated density of target bands was calculated by using Image Lab ™ software. Ratios of Pp46/p46, Pp65/p65 and AR were normalized to GAPDH and adjusted relative to the average of WT control with BSA treatment, which was arbitrarily set as 1 (AU).

RNA extraction and real-time PCR

The detection and quantitative- analysis of pro-inflammatory cytokine genes in cells were conducted by real-time PCR. Total RNA isolated from frozen adipose tissue was homogenized by using RNA STAT-60™. To extract RNA, 1 vol. of homogenate was added with 0.2 vol. of chloroform. The precipitation of RNA was performed using 0.5 vol. of isopropanol and used 75% ethanol to wash the RNA pellet. Reverse transcription was performed in GoScript™ Reverse Transcription System (Promega). cDNA was prepared from 0.5 μg total RNA by heating for 15 min at 42°C in the presence of AMV reverse transcriptase, recombinant RNasin® ribonuclease inhibitor and oligodeoxythymidilic acid (oligo$(dT)_{15}$). cDNA was subjected to PCR amplification in SYBR Green (LightCycler ® 480 system; Roche) system. The reaction mix contained 1.2 μL cDNA, 0.8 μL primer, 10 μL power Sybr-green PCR master mix and 8 μL

nuclease-free water [134, 135]. The mRNA levels were analyzed for $A_{2A}R$, TNF-α, IL-1β, IL-6 and MCP1 in adipose tissue samples. The internal reference gene 18S ribosomal RNA was used to normalize the data.

Statistical analysis

Numeric data are presented as means ± SE (standard error). Two-tailed student's t test was used to assess if the data is statistically significant. The standards of the study is when *P* value <0.05.

Results

Palmitate enhanced the inflammatory responses in 3T3-L1 cells

To investigate the effects of carbohydrate and SFAs on $A_{2A}R$, differentiated 3T3-L1 adipocytes were cultured in low glucose or high glucose DMEM medium, with or without palmitate treatment for 24 hours. In differentiated 3T3-L1 adipocytes, high glucose and palmitate treatment groups had more JNK (p46) phosphorylation comparing to PBS control group, which indicated both high glucose and palmitate had effects on the activation of JNK pathway. Palmitate treatment also elevated adenosine receptors level, in both low glucose and high glucose groups (Figure 6 (A)). In addition, mRNA levels of pro-inflammatory cytokines, such as TNF-α and IL-6 were significantly higher in palmitate treatment groups than in control groups. Thus, palmitate treatment induced inflammatory responses in 3T3-L1 differentiated adipocytes, which was consistent with previous findings. The higher $A_{2A}R$ expression was also induced by palmitate treatment, either in low glucose or high glucose condition (Figure 6 (B)).

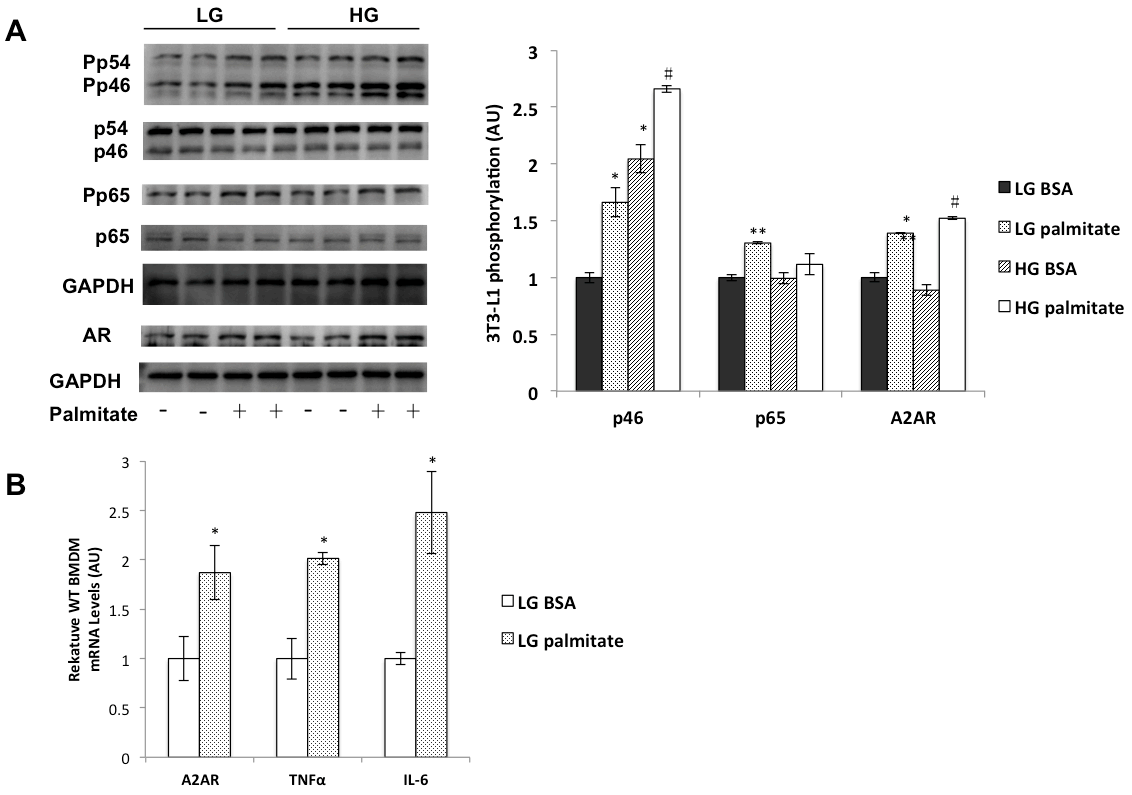

Figure 6. Palmitate enhanced the inflammatory responses in 3T3-L1 cells.
(A) 3T3-L1 adipoctyes inflammatory signaling. Protein extracts from whole cells were subjected to western blots. The degree of phosphorylation was determined from the measured ratio of phosphorylated JNK1 to total JNK1 (Pp46/p46) and phosphorylated NF-κB p65 to total p65 (Pp65/p65), via GAPDH normalization (AU, arbitrary unit). (B) The detection and quantitative- analysis of pro-inflammatory cytokine genes and $A_{2A}R$ in cells were conducted by real-time PCR. For bar graphs (A and B), data are means ± SE, n= 5. *, P<0.05 and **, P<0.01.

Palmitate enhanced the inflammatory responses in WT BMDM

To examine the effects of carbohydrate and SFAs on WT mice macrophages, bone marrow-derived macrophages (BMDM) were cultured in low glucose or high glucose DMEM medium, with or without palmitate treatment for 24 h. In BMDM,

palmitate treatment groups had more NF-κB p65 (Ser 536) phosphorylation in both low glucose and high glucose treatment groups, which indicated palmitate had effects on the activation of NF-κB pathway (Figure 7 (A)). The mRNA levels of pro-inflammatory cytokine TNF-α was significantly higher in palmitate treatment group than in control group. Also, palmitate treatment increased $A_{2A}R$ gene expression in WT macrophages (Figure 7 (B)).

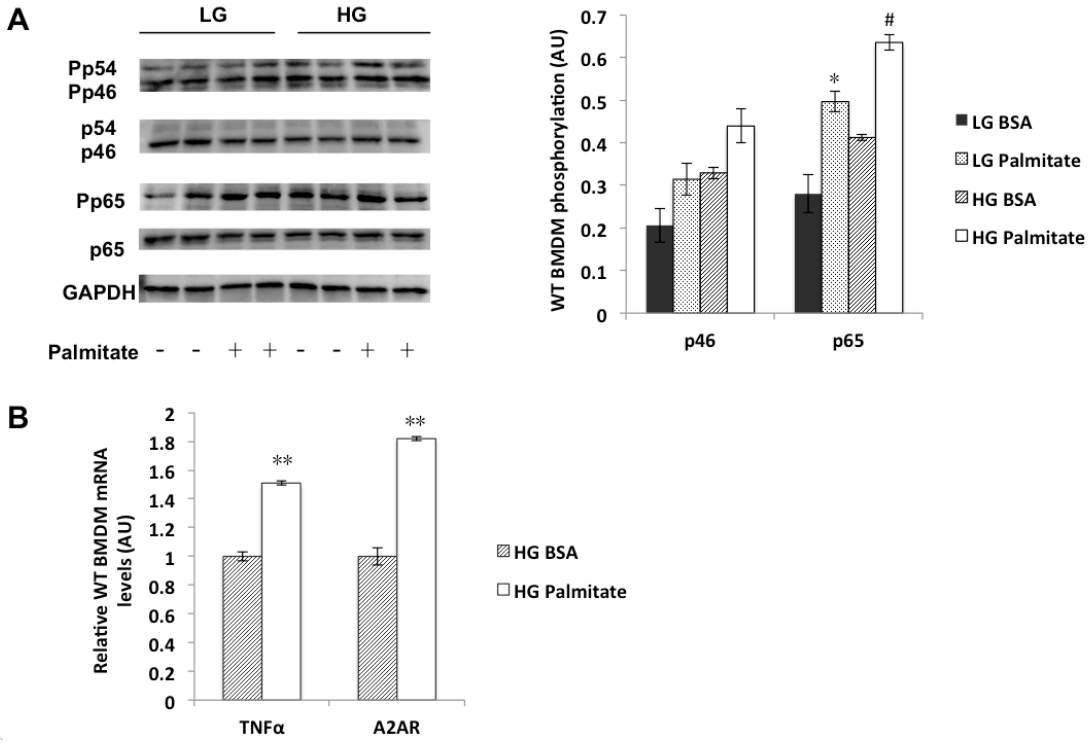

Figure 7. Palmitate enhanced the inflammatory responses in BMDM.
(A) BMDM inflammatory signaling. Protein extracts from whole cells were subjected to western blots. The degree of phosphorylation was determined from the measured ratio of phosphorylated JNK1 to total JNK1 (Pp46/p46) and phosphorylated NF-κB p65 to total p65 (Pp65/p65), via GAPDH normalization (AU, arbitrary unit). (B) The detection and quantitative- analysis of pro-inflammatory cytokine genes and $A_{2A}R$ in cells were conducted by real-time PCR. For bar graphs (A and B), data are means ± SE, n= 5. *, $P<0.05$ and **, $P<0.01$.

$A_{2A}R$ KO BMDM had enhanced pro-inflammatory responses

To examine if $A_{2A}R$ have effects on macrophages upon high carbohydrate and SFAs treatment, bond marrow were isolated from $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice. BMDM were cultured in low glucose or high glucose DMEM medium, with or without palmitate treatment for 24 h. In BMDM with genetic deletion of $A_{2A}R$, high glucose and palmitate treatment groups had significantly higher phosphorylation of JNK (p46) and NF-κB p65 (Ser 536), meaning both high glucose and palmitate had effects on the activation of JNK and NF-κB pathways (Figure 8(A)). Comparing to WT macrophages, $A_{2A}R$ deficient macrophages had significantly higher JNK (p46) phosphorylation, in both BSA and palmitate treatment groups (Figure 8 (B)). The mRNA levels of pro-inflammatory cytokines were significantly higher in genetic deletion of $A_{2A}R$ group than in WT control group (Figure 8 (C)). Together, these results suggested that $A_{2A}R$ had a direct effect upon palmitate-induced inflammation.

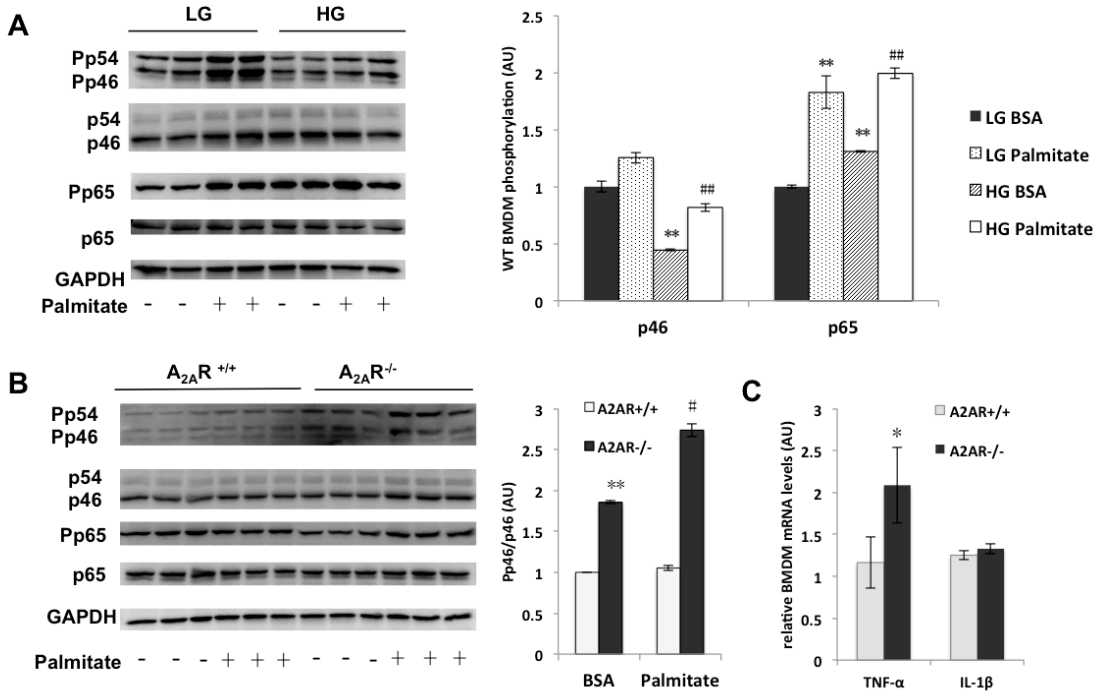

Figure 8. $A_{2A}R$ KO BMDM had enhanced pro-inflammatory responses.
(A) $A_{2A}R$ KO BMDM inflammatory signaling. Protein extracts from whole cells were subjected to western blots. The degree of phosphorylation was determined from the measured ratio of phosphorylated JNK1 to total JNK1 (Pp46/p46) and phosphorylated NF-κB p65 to total p65 (Pp65/p65), via GAPDH normalization (AU, arbitrary unit). (B) Inflammatory signaling in WT and $A_{2A}R$ KO BMDM. Protein extracts from whole cells were subjected to western blots. The degree of phosphorylation was determined from the measured ratio of phosphorylated JNK1 to total JNK1 (Pp46/p46) and phosphorylated NF-κB p65 to total p65 (Pp65/p65), via GAPDH normalization (AU, arbitrary unit). (C) The detection and quantitative- analysis of pro-inflammatory cytokine genes and $A_{2A}R$ in cells were conducted by real-time PCR. For bar graphs (A and B), data are means ± SE, n= 5. *, $P<0.05$ and **, $P<0.01$.

Discussions

In virtue of *in vitro* study, we were able to separately illustrate that $A_{2A}R$ activation not only exerts a protective effect on adipocytes, but also directly mediates

macrophage inflammation. Upon palmitate treatment, 3T3-L1 adipocytes had increased JNK, NF-κB phosphorylation, TNF-α, IL-6 expression and increased $A_{2A}R$ levels. According to the literature, it is agreeable that accumulated SFAs contribute to the inflammatory responses in adipocytes and macrophages [158]. Obesity-induced low-grade inflammatory responses are characterized by the secretion of adipokines from adipocytes, such as TNF-α, IL-6 and MCP1. These adipokines induced the infiltration and polarization of macrophages in adipose tissue. There is direct connection of adipocytes and macrophages due to crown-like structure formation.

Adipocytes hypertrophy and hyperplasia impair adipose tissue function including glucose intolerance, insulin resistance and inflammation [178]. The turnover of the adipocytes may be one mediator that is responsible for the different adipose morphologies upon obese-induced inflammation. The annual turnover rate of adipocytes is approximately 10% of the total fat cell pool. To clear the dead cells, macrophages form a crown-like structure to scavenge the cellular debris that endured apoptosis, to keep the balance of cell number. In lean adipose tissue, resident macrophages exert the alternatively activated phenotype, which is critical in maintaining adipose tissue homeostasis. Onset of obesity increases the ratio of macrophages in adipose tissue from 5% up to 50%. The prevalence of macrophages predominantly switch to the classically activated phenotype that promotes inflammatory responses. Those macrophage pro-inflammatory genes highly express on stromal vascular fraction of obese adipose tissue comparing to adipocytes [179].

FFAs have been implicated in activating the TLR4 cascades [32], which stimulate M1 polarization and secret cytokines including TNF-α, IL-1, IL-6, etc. [33-35]. Adipocytes and macrophages act in a paracrine loop involving FFAs and TNF-α and contribute to inflammation and insulin resistance in adipose tissue. It is a vicious cycle that pro-inflammatory cytokines released from macrophages cause insulin resistance in adipose tissue, and subsequently increase lipolysis and elevated FFAs production from adipocytes. The uncontrolled FFAs secretion further activates macrophages for the transcription of inflammatory cytokines upon TLR4 cascade activation.

Kupffer cells are heterogeneous population and the microenvironment in the liver determines the prevalence and phenotype of these liver-resident macrophages. LPS acts like a ligand to attach to TLR-4 on kupffer cells, and triggers the acute inflammation via inducing the release of IL-6. During chronic liver injury such as NASH, the monocyte-derived macrophages infiltrate into liver and cause the ablation of hepatic tolerance in presence of TNF production by activated kupffer cells [180]. Secreted TNF in turn promotes the additional infiltration of neutrophil, and stimulates ROS production in hepatocytes, which contributes to apoptosis of hepatocytes and inflammation in the liver. When mice were fed with MCD diet to induce steatosis and fibrosis, it was showed that kupffer cells activated hepatic stellate cells induced tissue inhibitor of metalloproteinase 1 and suppressed metalloproteinase synthesis through the release of TNF. Genetic deletion of TNF receptor 1 and 2 showed amelioration in liver fibrosis. Furthermore, hepatic macrophages activate the progression of fibrosis by maintaining hepatic stellate cell number in a NF-κB dependent manner. By applying CCl_4 treatment and bile duct

ligation to induce fibrosis in mice, it showed activation of NF-κB pathway increased the transcription of IL-1 and TNF, which did not activate hepatic stellate cells but sustained the myofibroblast survival [181].

This chapter showed that exposure of BMDM to palmitate induced inflammatory signaling and pro-inflammatory cytokines' secretion, a typical M1-like phenotype. We therefore tested the hypothesis that genetic deletion of $A_{2A}R$ in BMDM would accentuate the inflammatory potential. We crossed the $A_{2A}R^{+/-}$ with $A_{2A}R^{+/-}$ to develop a global knockout model ($A_{2A}R^{-/-}$) with WT littermates ($A_{2A}R^{+/+}$). Upon high glucose and palmitate treatment, $A_{2A}R$-deficient macrophages had increased JNK and NF-κB phosphorylation. Comparing to WT macrophages, $A_{2A}R$-deficient macrophages had increased JNK phosphorylation, and increased pro-inflammatory cytokines. Given that ablation of $A_{2A}R$ exacerbates inflammatory responses upon palmitate treatment in BMDM, it is postulated that $A_{2A}R$ has direct effect on macrophages to mediate immunologic effects.

CHAPTER VI

$A_{2A}R$ PROTECTS AGAINST OVERNUTRTION-INDUCED CHRONIC INFLAMMATION AND SYSMETIC INSULIN RESISTANCE

Introduction

During cellular stress or hypoxia, the purine nucleoside, adenosine, is produced from dephosphorylation of ATP, ADP or AMP. Adenosine attaches to adenosine receptors and mediates tissue protection mainly achieved by suppressing immune system responses. However, it is still unappreciated that $A_{2A}R$ expressed on liver and adipose tissue can control metabolic and immunologic events such as glucose/ lipid metabolism and inflammation. The aim of this chapter is to evaluate the effects of $A_{2A}R$ ablation, focusing on liver and adipose tissue inflammation induced by obesity in mice.

The *de novo* lipogenesis exists in the liver to generate fat from glucose, which contributes to increased fat mass and subsequent obesity-induced tissue dysfunction. Transcription factors such as SREBP-1c exert significant activating effects on *de novo* synthesis of fatty acids since SREBP-1c regulates many genes that code for the rate-limiting enzymes in fatty acids biosynthesis pathways such as ACC, FAS, ATP-citrate lyase (ACL) and stearoyl-CoA desaturase (SCD). SREBPs reside in the endoplasmic reticulum (ER) membranes as inactive precursors (~125k Da). SREBPs translocate from ER to the Golgi to undergo a proteolytic process upon demand signals for fatty acids, escorted by a SREBP cleavage activating protein (SCAP). The SREBPs are cleaved by the Site-1 and Site-2 protease, and a N-terminal fragment of SREBPs then transfers into

the nucleus and acts as transcription factor. In the nucleus, SREBPs bind to the promoters of the sterol response elements (SREs), resulting in the "turning on" of transcription. The activity of SREBP is regulated by its phosphorylation and acetylation.

The activity of SREBP-1c is correlated with SIRT1. During fasting, SIRT1 induces the deacetylation of SREBP-1c. Under feeding conditions, increased insulin and glucose levels caused SIRT1 to lose interaction with SREBP-1c, thus increasing the acetylation SREBP-1c. In diet-induced obese mice, acetylation of SREBP-1c increased abnormally, which worsen the state of obesity by enhancing fatty acid *de novo* synthesis. It is supposed that inflammatory signaling (JNK pathway) caused the degradation of SIRT1 and the lose of interaction with SREBP-1c, so the level of *de novo* lipogenesis increased and so did liver steatosis. Overexpression of SIRT1 in the liver for 1 week could decrease the acetylation of SREBP-1c and exerted beneficial effects [159, 182].

Acetyl-CoA is the acyl group donor in the process of *de novo* fatty acid synthesis, which is generated from citrate using the enzyme ACL. The fatty acid chain that transformed from Acetyl-CoA carboxylate to malonyl-CoA, which is a committed step of fatty acids biosynthesis pathway, catalyzed by ACC. Malonyl-CoA is indispensable in fatty synthesis since on the one hand, it provides 2-carbon units in the synthesis of palmitate. On the other hand, it inhibits β-oxidation by binding and blocking carnitine palmitoyltransferase (CPT1), which is the requisite transporter of fatty acid across mitochondrial membrane to undergo mitochondrial oxidation.

Malonyl-CoA donates 2-carbon units every time to the fatty acid chain and finally generates palmitate, using the enzyme FAS. Recent research revealed that

macrophage FAS was essential in determining phospholipid composition on membrane, thus altering lipid raft domain upon diet-induced inflammation [183]. Genetic deletion of *Fasn* specific in myeloid cells attenuated HFD-induced inflammation and resistance in mice. In FAS deficient macrophages, the membrane order was changed, retention of lipid raft was impaired and Rho GTPase activity was reduced. Rho GTPase mediated macrophage cell motility, including migration, adhesion and activation. These findings suggested that FAS was needed in endogenous fatty acids synthesis, which resulted in membranes composition perturbation, and macrophages recruiting upon inflammatory signaling.

The anti-inflammatory effects of adenosine on macrophages are characterized as inhibition of M1 macrophages via $A_{2A}R$ regulation and activation of M2 macrophages via $A_{2B}R$ mediation, which subsequently decreases pro-inflammatory cytokine, chemokine secretion and nitric oxide produced by M1 macrophages and increasing IL-10 release from M2 macrophages [184]. HFD fed Swiss mice treated with the $A_{2A}R$ agonist (CGS-21680) showed improved glucose and insulin tolerance with decreased inflammation. TNF-α and plasminogen activator inhibitor-1 (PAI-1) were reduced in Swiss mice' blood. TNF-α and MCP-1 were also reduced in visceral adipose tissue. Adipose tissue macrophages secrete these cytokines and chemokines, which lead to the activation JNK1/2 pathway. JNKs phosphorylate insulin receptor at serine/threonine, and inhibit insulin signaling, which results in increased lipolysis in adipose tissue and generate a vicious cycle of inflammation. Thus, $A_{2A}R$ activates upon agonist ameliorated adipose tissue inflammation and thus systemic low-grade inflammation and insulin

resistance. In adipose tissue, $A_{2A}R$ agonist treatment increased p38 mitogen activated protein kinase (MAPK) signaling [185]. From the result of Chapter V, we demonstrated that an increased $A_{2A}R$ level serves as a defensive response at the cellular level. Therefore, in this chapter, we are interested in investigating HFD induced inflammation using $A_{2A}R$ knockout mice.

Materials And Methods

Animal experiments

5-6 weeks-old $A_{2A}R^{-/-}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{+/+}$ mice litter mates were group housed (3-5 mice/cage), feeding with HFD for 12 weeks. Composition of LFD is 10% fat, 20% protein and 70% carbohydrate of total kcal, while composition of HFD is 60% fat, 20% protein and 20% carbohydrate of total kcal. HFD were gained fro Research Diets, Inc (New Brunswick) and composition was 5.24 kcal/g, including 275 kcal of sucrose, 2205 kcal of lard and no cornstarch. The mice were maintained in a controlled environment, inverted 12h daylight-cycle (lights on at 06:00). During the 12-week feeding period, body weight and food intake were monitored weekly. After the feeding regimen, mice were anesthetized (0.1 mL/10 g of body weight of 1:1 v/v xylazine 1%/ ketamine 10%, intraperitoneal injection), blood was collected and tissue samples were carefully excised, weighted and stored for further study [129-131]. To determine if the process of over-nutrition affects $A_{2A}R$ expression, tissues such as liver and adipose tissue were collected from LFD or HFD fed WT mice. The adipose tissue that was dissected for testing involved epididymal, mesenteric and perinephric fat pads. The Institutional Animal Care

and Use Committee of Texas A&M University approved the laboratory animal care and handling.

Glucose and insulin tolerance tests

Glucose tolerance test (GTT) measures the body's ability to clear an injected glucose load. Insulin tolerance test (ITT) were used in conjunction with GTT, to determine how well of systemic insulin sensitivity after giving an injection of insulin. Mice were fasted for approximately 4 h and fasted glucose levels are determined before receiving an intra-peritoneal injection of solution of D-glucose (2 g/kg BW) or insulin (1 U/kg BW). For GTT, blood was collected from tail veins at 30, 60, 90 and 120 min after an intraperitoneally injected bolus glucose. For ITT, blood was collected from tail veins at 15, 30, 45 and 60 min after an intraperitoneally injected bolus insulin [132, 133]. The plasma glucose was determined by glucose assay kit (Sigma, St. Louis, MO).

Histology

Liver and epididymal fat were fixed in formalin (10%, Sigma). Tissue blocks were cut into 5 μm sections for F4/80 with rabbit anti-F4/80 (1:100) (AbD Serotec, Raleigh, NC) [186]. For lipid detection, frozen liver sections were fixed with formalin followed by Oil-Red-O in isopropanol. Images of Oil-Red-O staining were taken with a Canon Powershot SD300 digital camera. Optical density was measured at 510 nm.

Western blot

Lysates from frozen liver samples applied the lysis buffers (pH 7.4) containing 20 mM HEPEs, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 1% NP-40, 0.1% SDS, 2 mM EDTA and 2 mM navadate. The 5× loading dye contains 1.0 M Tris (pH

6.8), 50% glycerol, 10% SDS and 0.1% bromophenol blue [129, 131]. Cell lysates (50 μg of protein) and protein markers (cell signaling Technology) were subjected to sodium dodecyl sulphate (SDS)- polyacrylamide gel electrophoresis. Proteins were semi-dry transferred to synthetic membranes, and incubated overnight at 4 °C, with primary antibody specific to the protein of interest at a 1: 1 000 dilution. Subsequent immunoblotting of labeled protein were performed for 2h at room temperature, with a 1:10 000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary kit (Immobilon™ Western; EMD Millipore, Billerica, MA USA) [129]. The loading control is glyceraldehyde 3- phosphate dehydrogenase (GAPDH). The quantitated density of target bands was calculated by using Image Lab ™ software. Ratios of Pp46/p46, Pp65/p65 and AR were normalized to GAPDH and adjusted relative to the average of LFD fed control, which was arbitrarily set as 1 (AU).

RNA extraction and real-time PCR

The detection and quantitative- analysis of pro-inflammatory cytokine genes in liver were conducted by real-time PCR. Total RNA isolated from frozen adipose tissue was homogenized by using RNA STAT-60™. To extract RNA, 1 vol. of homogenate was added with 0.2 vol. of chloroform. The precipitation of RNA was performed using 0.5 vol. of isopropanol and used 75% ethanol to wash the RNA pellet. Reverse transcription was performed in GoScript™ Reverse Transcription System (Promega). cDNA was prepared from 0.5 μg total RNA by heating for 15 min at 42°C in the presence of AMV reverse transcriptase, recombinant RNasin® ribonuclease inhibitor and oligodeoxythymidilic acid ($oligo(dT)_{15}$). cDNA was subjected to PCR amplification

in SYBR Green (LightCycler ® 480 system; Roche) system. The reaction mix contained 1.2 μL cDNA, 0.8 μL primer, 10 μL power Sybr-green PCR master mix and 8 μL nuclease-free water [134, 135]. The mRNA levels were analyzed for $A_{2A}R$, TNF-α, IL-1β, IL-6 and MCP1 in liver samples. The internal reference gene 18S ribosomal RNA was used to normalize the data.

Statistical analysis

Numeric data are presented as means ± SE (standard error). Two-tailed student's t test was used to assess if the data is statistically significant. The standards of the study is when *P* value <0.05.

Results

$A_{2A}R$-deficient male mice had increased body weight and insulin resistance

To study the extent to which $A_{2A}R$ disruption exacerbates inflammation, adipose tissue and liver were obtained from HFD-fed $A_{2A}R^{-/-}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{+/+}$ mice litter mates. Systemic glucose tolerance, insulin sensitivity and inflammatory biomarkers were analyzed and compared among groups. Male $A_{2A}R^{+/-}$ mice gained more body weight than $A_{2A}R^{+/+}$ mice, and male $A_{2A}R^{-/-}$ mice showed significant increased bodyweight comparing to $A_{2A}R^{+/-}$ mice (Figure 9 (A)). There is no difference among $A_{2A}R^{-/-}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{+/+}$ mice in daily food intake during 12-week HFD feeding (Figure 9 (B)). An intraperitoneal glucose challenge showed that male $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice had decreased glucose tolerance. Comparing to $A_{2A}R^{+/+}$ mice, plasma glucose level of $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice significantly increased after ingestion of glucose solution. The plasma glucose kept high after 2h of ingestion (Figure 9 (C)). ITT showed that male

$A_{2A}R$ knockout mice had a dysfunctional insulin signaling. After ingestion of insulin, the plasma glucose levels of $A_{2A}R^{+/-}$ and $A_{2A}R^{+/+}$ mice were higher than $A_{2A}R^{+/+}$ mice, which indicated that tissues of $A_{2A}R$ knockout mice cannot respond to insulin properly (Figure 9 (D)). HFD-fed $A_{2A}R^{-/-}$ mice had much higher liver tissue weight relative to body weight than $A_{2A}R^{+/+}$ mice. In addition, $A_{2A}R^{+/-}$ and $A_{2A}R^{+/+}$ mice had significant higher adiposity, which indicated they accumulated more visceral fat than $A_{2A}R^{+/+}$ mice (Figure 9 (E)).

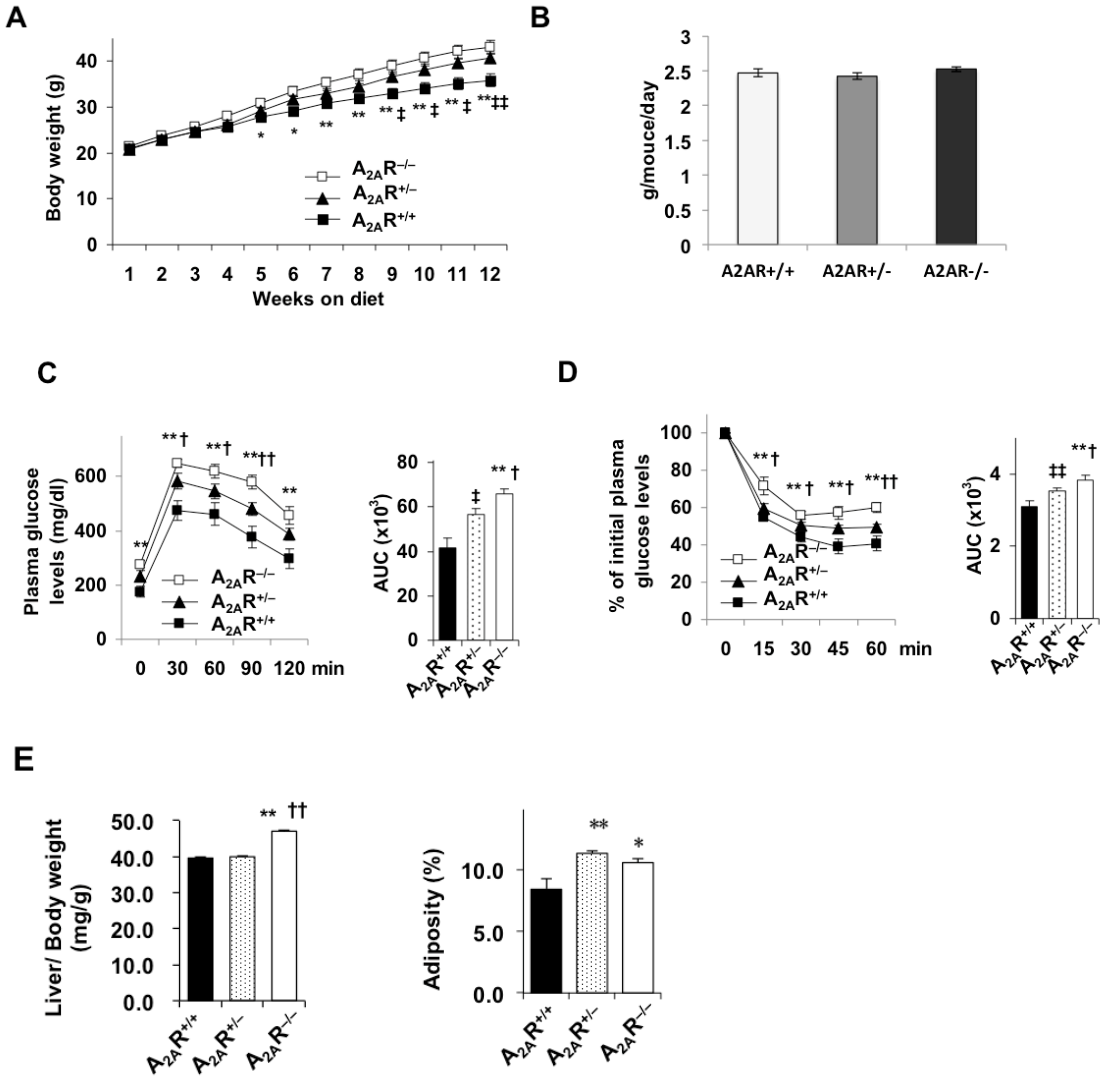

Figure 9. $A_{2A}R$-deficient male mice had increased body weight and insulin resistance.

Male mice ($A_{2A}R^{-/-}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{+/+}$) were fed with HFD for 12 weeks. Data are means ± SE, n= 10. (A) Body weight was monitored weekly during the feeding period. (B) Food intake. (C) GTT. (D) ITT. (E) The ratio of liver weight to body weight (mg/g), and the rate of visceral fat to body weight (%). For C and D, mice were fasted for 4 h and fasted glucose levels are determined before receiving an intra-peritoneal injection of glucose (2 g/kg BW) or insulin (1 U/kg BW). *, $P<0.05$ and **, $P<0.01$. $A_{2A}R$ WT vs. $A_{2A}R$ knockout for the same time point (C and D).

$A_{2A}R$-deficient female mice had insulin resistance and increased adiposity

There were no significant differences of body weight among the $A_{2A}R^{+/+}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ female mice (Figure 10 (A)). There was no difference between $A_{2A}R^{+/-}$ and $A_{2A}R^{+/+}$ mice in daily food intake during 12-week HFD feeding, but $A_{2A}R^{-/-}$ mice had significant decreased daily food intake (Figure 10 (B)). GTT and ITT were conducted to assess systemic glucose level and insulin sensitivity. GTT showed that female $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice had decreased glucose tolerance. Comparing to $A_{2A}R^{+/+}$ mice, the plasma glucose level of $A_{2A}R^{-/-}$ and $A_{2A}R^{+/-}$ mice increased significantly after ingestion of glucose solution. The plasma glucose kept high after 2h of ingestion. (Figure 10 (C)). ITT showed that female $A_{2A}R^{+/-}$ mice had worse insulin sensitivity than $A_{2A}R^{+/+}$ mice, and $A_{2A}R^{-/-}$ mice had the worst insulin sensitivity. After ingestion of insulin, the plasma glucose levels of $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice were higher than $A_{2A}R^{+/+}$ mice (Figure 10 (D)). We did not observe the liver weight difference between female $A_{2A}R^{-/-}$ mice and $A_{2A}R^{+/+}$ mice. $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice had significant higher adiposity, which showed that they accumulated more visceral fat than $A_{2A}R^{+/+}$ mice (Figure 10 (E)).

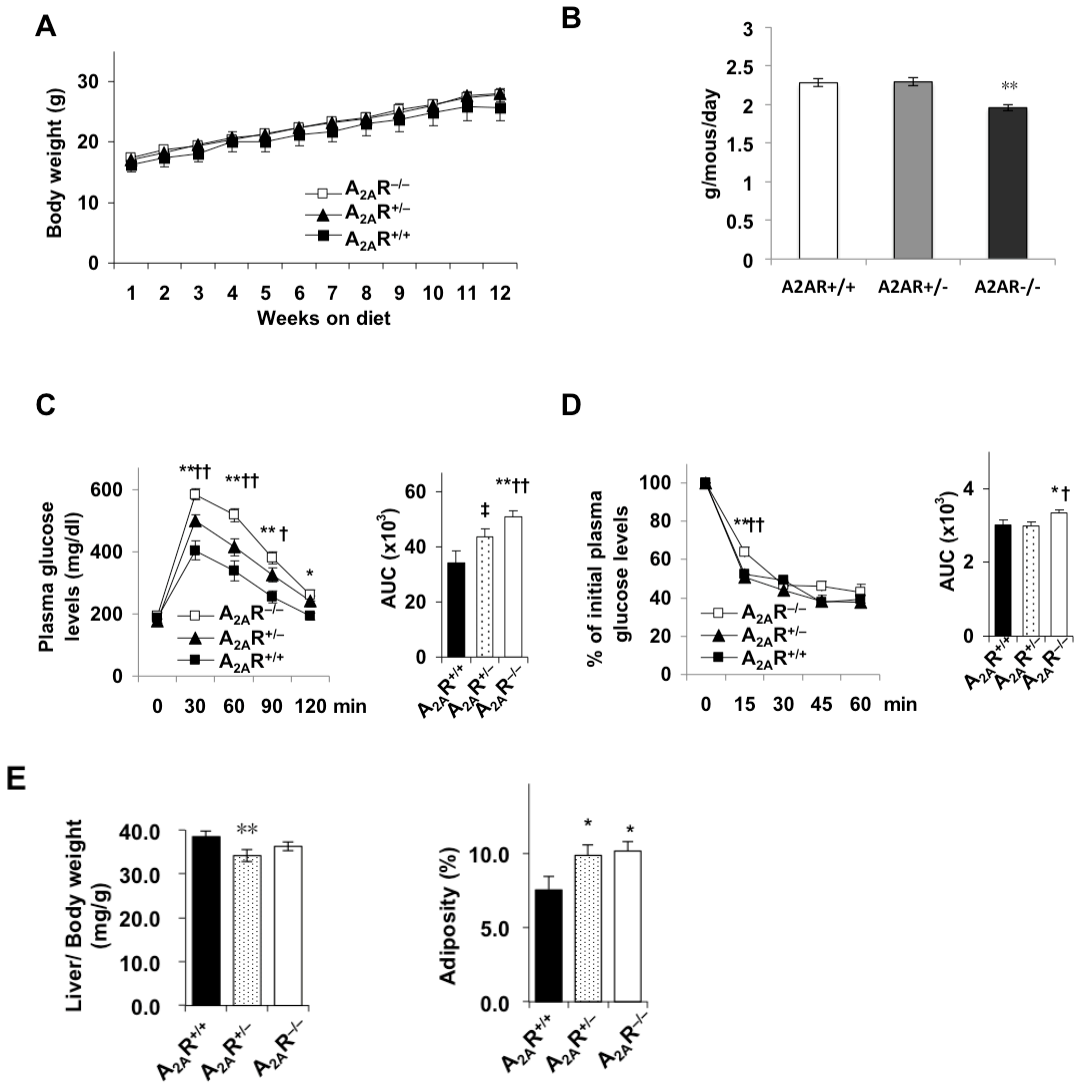

Figure 10. $A_{2A}R$-deficient female mice had insulin resistance and increased adiposity.

Female mice ($A_{2A}R^{-/-}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{+/+}$) were fed with HFD for 12 weeks. Data are means ± SE, n= 10. (A) Body weight was monitored weekly during the feeding period. (B) Food intake. (C) GTT. (D) ITT. (E) The ratio of liver weight to body weight (mg/g), and the rate of visceral fat to body weight (%). For C and D, mice were fasted for 4 h and fasted glucose levels are determined before receiving an intra-peritoneal injection of glucose (2 g/kg BW) or insulin (1 U/kg BW). *, $P<0.05$ and **, $P<0.01$. $A_{2A}R$ WT vs. $A_{2A}R$ knockout for the same time point (C and D).

A_{2A}R-deficient mice displayed severe inflammation and insulin resistance in liver

$A_{2A}R^{+/-}$ mice had much more fat deposition than $A_{2A}R^{+/+}$ mice. In addition, $A_{2A}R^{-/-}$ mice demonstrated increased fat accumulation in the liver than $A_{2A}R^{+/-}$ mice. F4/80 staining showed $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice accumulated much more hepatic macrophages (Figure 11 (A)). Gene expression of pro-inflammatory cytokines, such as TNF-α and IL-6 were significantly higher in $A_{2A}R$ deficient mice. The genes expression that involved in lipogenesis, such as ACC, FAS, CPT1a and SREBP1c were also significantly higher in $A_{2A}R$ deficient mice (Figure 11 (B)). Comparing to $A_{2A}R^{+/+}$ mice, $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice had more NF-κB (p65) phosphorylation in livers, which indicated the deficiency of $A_{2A}R$ augmented the activation of JNK pathway and NF-κB pathway (Figure 11 (C)). To examine the connection between $A_{2A}R$ and insulin sensitivity, liver samples were collected from $A_{2A}R^{+/+}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice that injected with insulin before harvesting. Genetic deletion of $A_{2A}R$ in mice had decreased phosphorylation of AKT in liver (Figure 11 (D)).

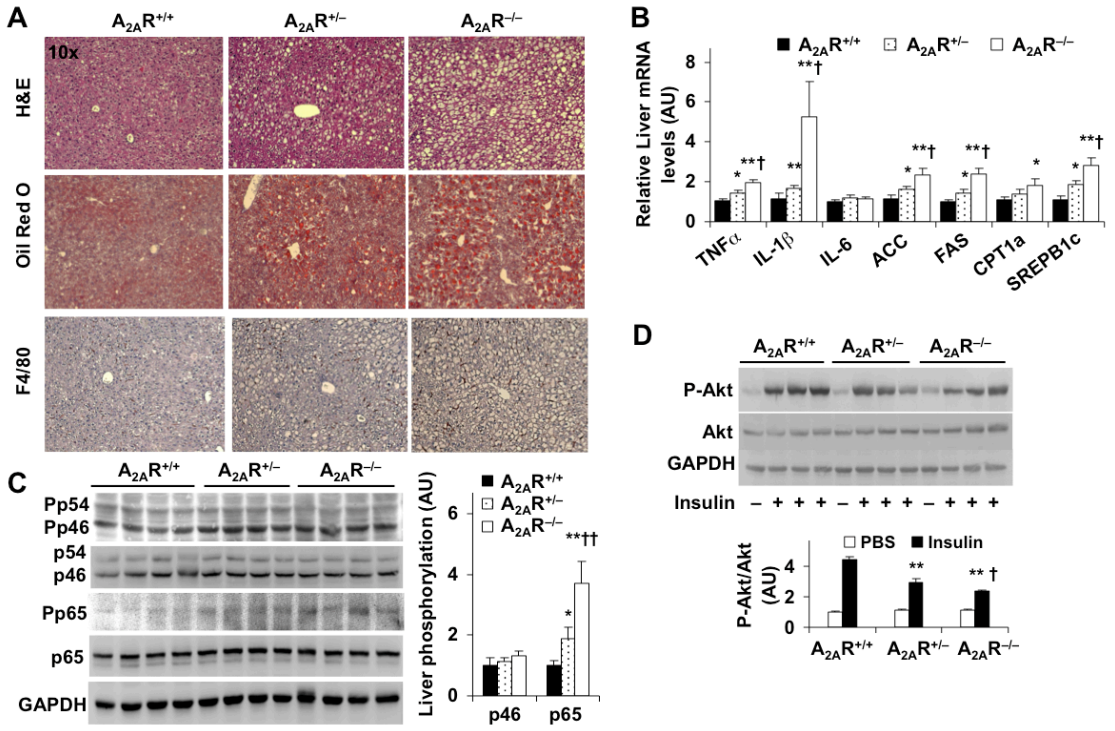

Figure 11. $A_{2A}R$-deficient mice displayed severe inflammation and insulin resistance in liver.

(A) Liver histology. Top panels, H&E staining; middle panels, Oil-Red-O staining; Bottom panels, F4/80 staining. (B) The detection and quantitative- analysis of pro-inflammatory cytokine genes and fatty acids biosynthesis genes in liver were conducted by real-time PCR. (C) Liver inflammatory signaling. Protein extracts from liver were subjected to western blots. The degree of phosphorylation was determined from the measured ratio of phosphorylated JNK1 to total JNK1 (Pp46/p46) and phosphorylated NF-κB p65 to total p65 (Pp65/p65), via GAPDH normalization (AU, arbitrary unit). (D) Liver insulin signaling. $A_{2A}R^{-/-}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{+/+}$ mice were injected with insulin before harvesting. Protein extracts from liver were subjected to western blots. The degree of phosphorylation was determined from the measured ratio of phosphorylated Akt to total Akt, via GAPDH normalization (AU, arbitrary unit). For bar graphs (B, C and D), data are means ± SE, n= 10. *, $P<0.05$ and **, $P<0.01$.

A_{2A}R-deficient mice displayed sever inflammation and insulin resistance in adipose tissue

$A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice had bigger adipocytes and accumulated much more adipose tissue macrophages than $A_{2A}R^{+/+}$ mice (Figure 12 (A)). There was also a switch of phenotype from anti-inflammatory M2 to pro-inflammatory M1 macrophages (Figure 12 (B)). Comparing to $A_{2A}R^{+/+}$ mice, $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice had more JNK and NF-κB (p65) phosphorylation in WAT, which indicated the deficiency of $A_{2A}R$ augmented the activation of JNK pathway and NF-κB pathway in the adipose tissue (Figure 12 (C)). Genetic deletion of $A_{2A}R$ in mice had decreased phosphorylation of AKT in the adipose tissue (Figure 12 (D)). $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice expressed higher level of pro-inflammatory cytokines than $A_{2A}R^{+/+}$ mice. Also, $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice expressed lower levels of anti-inflammatory gene (adiponetkin) and lipolysis enzyme genes (Figure 12 (E)).

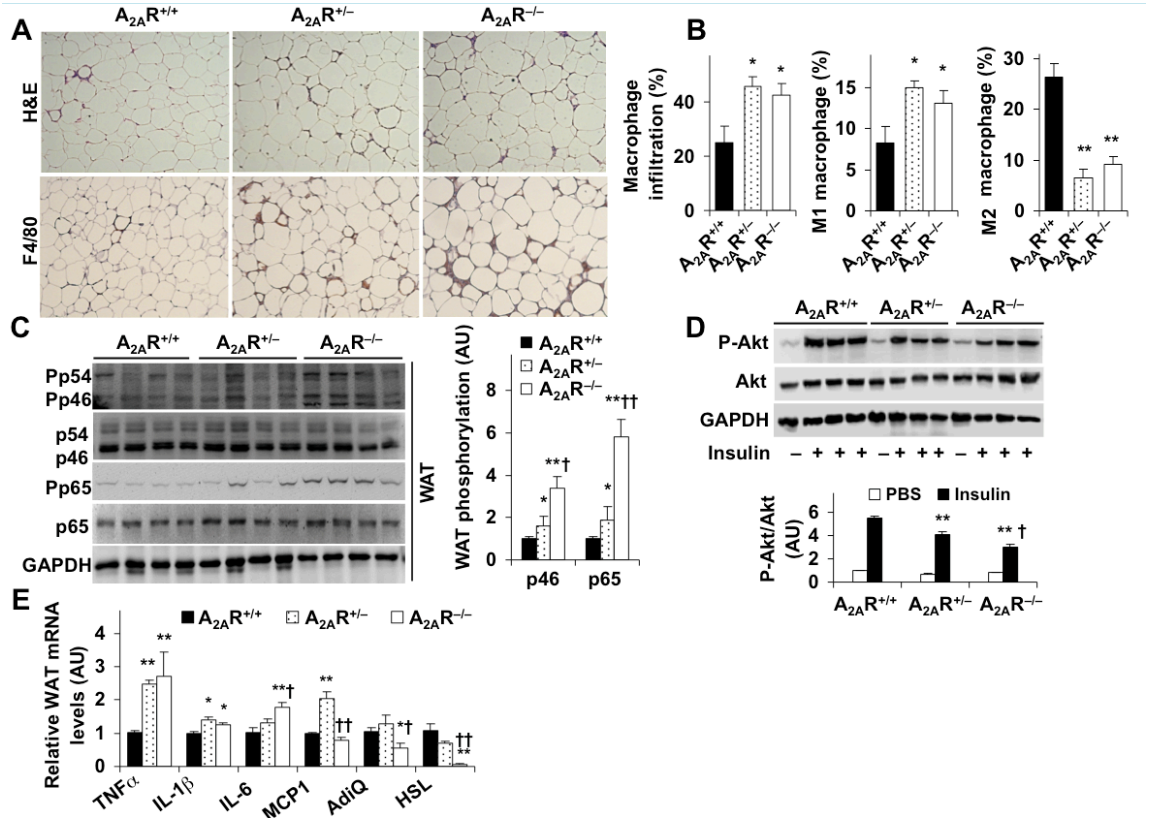

Figure 12. $A_{2A}R$-deficient mice displayed severe inflammation and insulin resistance in adipose tissue.

(A) Adipose tissue histology. Top panels, H&E staining; Bottom panels, F4/80 staining. (B) Polarization of macrophages in adipose tissue by using flow cytometry. (C) Adipose tissue inflammatory signaling. Protein extracts from liver were subjected to western blots. The degree of phosphorylation was determined from the measured ratio of phosphorylated JNK1 to total JNK1 (Pp46/p46) and phosphorylated NF-κB p65 to total p65 (Pp65/p65), via GAPDH normalization (AU, arbitrary unit). (D) Adipose tissue insulin signaling. $A_{2A}R^{-/-}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{+/+}$ mice were injected with insulin before harvesting. Protein extracts from adipose tissue were subjected to western blots. The degree of phosphorylation was determined from the measured ratio of phosphorylated Akt to total Akt, via GAPDH normalization (AU, arbitrary unit). (E) The detection and quantitative- analysis of pro-inflammatory cytokine genes and lipolysis genes in adipose tissue were conducted by real-time PCR. For bar graphs (B, C and D), data are means ± SE, n= 10. *, P<0.05 and **, P<0.01.

Discussions

HFD feeding caused inflammation and insulin resistance in the liver and adipose tissue, which involved liver steatosis and infiltration of macrophages to adipose tissue. This study showed that $A_{2A}R$ is requisite in quenching diet-induced inflammation. $A_{2A}R$-deficient mice had higher body weight, decreased glucose tolerance and insulin sensitivity, compared to WT mice. The adipose tissues and the livers of $A_{2A}R$-deficient mice had increased JNK and NF-κB (p65) phosphorylation, pro-inflammatory gene expression and lipogenesis gene expression. Furthermore, our data showed increased macrophage recruitment and the dominant classically activated phenotype in the adipose tissue of $A_{2A}R$ knockout mice. Aside from affecting inflammation and glucose homeostasis, ablation of $A_{2A}R$ also induced a worsening phenotype of ectopic fat accumulation with elevated *de novo* lipogenesis genes expression.

The anti-inflammatory effect of $A_{2A}R$ has also been studied in diabetic retinopathy. WT mice treated with $A_{2A}R$ agonist (CGS21680) showed dramatic decreases in retinal cell death (especially neuronal cells) stimulated by hyperglycemia, and TNF-α secretion. Genetic deletion of $A_{2A}R$ in diabetic mice resulted in enhanced TNF-α secretion, and expression of genes that correlated to retinal vascular inflammation in diabetic patients such as intercellular adhesion molecule-1 (ICAM-1) expression and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-(TUNEL). The $A_{2A}R$ signaling was investigated by treating primary retinal microglia with amadori-glycated albumin to induce diabetic disorders. Activation of $A_{2A}R$ ameliorated TNF-α secretion mediated in a cAMP-dependent manner and suppressed C-

Raf/ERK cascade. Thus, $A_{2A}R$ possessed a retinal protective effect by reducing inflammatory signaling and rescuing cell death in diabetic retinopathy [187].

Human epithelium, in specific urothelium, $A_{2A}R$ has been implicated in mediating inflammation. Human urothelium cells challenged with *Escherichia coli* or pro-inflammatory cytokines displayed increased $A_{2A}R$ expression. $A_{2A}R$ agonist (CGS21680) treatment did not increase cAMP generation or CREB phosphorylation profoundly. $A_{2A}R$ agonist (CPCA) activated cAMP/CREB pathway and inhibited MAPK-family (ERK, JNK, p38 and STAT3) signaling. However, despite the inhibition of these inflammatory signaling, neither CGS21680 nor CPCA could functionally suppress *Escherichia coli* stimulated IL-8 production [188].

The immunomodulatory effects of $A_{2A}R$ on immune cells were studied back to ohta and sitkovsky's study [189]. Adenosine receptors, particular $A_{2A}R$ has been shown to prevent excessive M1 macrophage activation in terms of the decreasing expression of reactive nitrogen and oxygen species, macrophage inflammation protein-1 α, IL-12, and especially TNF-α secretion [184]. The cAMP/ CREB pathway involved the activation of PKA by cAMP, which in turn phosphorylates the transcription factor, CREB. CREB binds to CRE to turn on the transcription of the downstream genes. Phopho-CREB is the response for the inhibition of NF-κB activity. So $A_{2A}R$ stimulation mediates negative feedback to control the production of cytokines [70, 71]. Murine alveolar macrophages were challenged with broken down fragments of extracellular matrix hyaluronan had increased pro-inflammatory cytokines and chemokines production. $A_{2A}R$ stimulation suppressed TNF-α generation in a PKA independent manner, which via cAMP (Epac-1)

[190]. In addition, $A_{2A}R$ activation also augmented anti-inflammatory cytokine IL-10 secretion. $A_{2A}R$ ablation mice failed to increase IL-10 secretion in peritoneal macrophages from mice, while WT mice were able to stimulate IL-10 production by *E. coli* stimulation [191, 192].

$A_{2A}R$ also supported macrophage metabolism through the induction of glycolysis, which may facilitate in switching their phenotype to M2 polarization to help tissue restoration. Adenosine activated glycolysis via the synergic effects of LPS and $A_{2A}R$ agonist in elevating the expression of PFKFB3 isozyme of 6PFK1 in a Specific protein 1 dependent manner [193]. $A_{2A}R$ exerted an immunosuppression effect not only through the inhibition of inflammatory responses but also the clearance of apoptotic cells by macrophages. The level of adenosine increased significantly to activate $A_{2A}R$. The neutrophil migration factors such as macrophage inflammatory protein-2 were inhibited in the cAMP/PKA pathway. $A_{2A}R$ null mice failed to inhibit neutrophil-attracting chemokines production [194].

Intriguingly, our study demonstrated ablation of $A_{2A}R$ displayed switched phenotype of macrophages in liver, which also contributed to the vicious cycle of FFAs and pro-inflammatory cytokines production. Altogether, our data indicate that $A_{2A}R$ is one of mediators in suppressing inflammation and *de novo* lipogenesis.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Summary

In the present study, WT mice were fed with HFD to induce inflammation and insulin resistance as an animal model to investigate the immunomodulatory and metabolic effects of $A_{2A}R$ *in vivo*. As expected, HFD fed mice had increased body weight and tissue weight such as visceral fat and liver, compared to LFD fed mice. Other than unregulated glucose homeostasis, HFD fed mice also exhibited inflammation characterized as increased inflammatory signaling (JNK and NF-κB pathway), and pro-inflammatory cytokines and chemokines (TNF, IL-1β, IL-6 and MCP-1). Intriguingly, the level of $A_{2A}R$ increased in the liver and adipose tissue of HFD fed mice, which may help to protect tissue from this high content of saturated fatty acids diet.

The direct effects of nutrients on $A_{2A}R$ and inflammatory responses were also examined *in vitro* study. Adipocytes and BMDM showed increased inflammation upon high glucose and palmitate treatment, in terms of increased phosphorylation of JNK (p46) and NF-κB (p65) and pro-inflammatory cytokines production. Consistent with the result from mice, 3T3-L1 adipocytes and BMDM showed increased $A_{2A}R$ levels upon saturated fatty acid stimulation. In addition, $A_{2A}R$-deficient macrophages provided evidence for the direct effect of $A_{2A}R$ in modulating inflammatory responses as shown by an increase in phosphorylation of JNK (p46) and NF-κB (p65) upon high glucose and palmitate treatment, and an increase in mRNA expression of TNF, IL-1β and IL-6. $A_{2A}R$

deletion augments of inflammation in macrophages with increased JNK (p46) phosphorylation upon both BSA and palmitate treatment. Given this, the protective effect of $A_{2A}R$ was proven by using $A_{2A}R$ knockout BMDM.

In virtue of $A_{2A}R$-deficient mice, we were able to illustrate the effect of $A_{2A}R$ on the diet-induced obesity animal model. Ablation of $A_{2A}R$ caused mice significant increased body weight, compared to WT mice. Global knockout mice fed with HFD displayed impaired glucose clearance and insulin sensitivity. In the liver, genetic deletion of $A_{2A}R$ showed a significant increase in fat accumulation upon HFD feeding, shown by H&E and Oil-Red-O staining, comparing to WT mice. A significant increase in mRNA expression of *de novo* lipogenesis such as ACC, FAS, CPT1a and SREBP1c indicated abnormal lipid homeostasis in liver. In addition, F4/80 staining showed elevated macrophage population in liver, which also indicated the elevated production of pro-inflammatory molecules from liver macrophages. Moreover, an increase in phosphorylation of JNK (p46) and NF-κB (p65), expression of pro-inflammatory cytokines (TNF, IL-1β and IL-6) in liver suggested $A_{2A}R$ possess a beneficial effect on diet-induced chronic inflammation. $A_{2A}R$- deficient mice injected insulin before harvest showed decreased phosphorylation of AKT comparing to WT mice, which indicated impaired insulin signaling. In adipose tissue, F4/80 staining showed increased macrophages infiltration in $A_{2A}R$-deficient mice, and prolonged classical activated macrophages polarization indicated pro-inflammatory cytokine generation, which was confirmed by increased gene expression of MCP1. Genetic deletion of $A_{2A}R$ also exacerbated adipose tissue inflammation as shown by an increase in phosphorylation of

JNK (p46) and NF-κB (p65) and increased mRNA level of TNF, IL-1β and IL-6. $A_{2A}R$ heterozygous knockout mice showed decreased Akt phosphorylation in adipose tissue after insulin injection, compared to WT mice. And $A_{2A}R$ homozygous mice had significant reduced phosphorylated Akt, comparing to both heterozygous knockout and WT mice.

Conclusions

The presented study provided evidence that $A_{2A}R$ had both metabolic and immunologic effects in adipose tissue and liver. This was supported by the results of C57BL/6J mice fed with HFD, which showed an increase in the expression of $A_{2A}R$ in liver and adipose tissue, accompanied with obesity-related inflammation and insulin resistance. It appeared to be a defensive response, which may help protect against inflammatory damage. Further in vitro studies confirmed nutrients had direct effects on $A_{2A}R$ expression in both adipocytes and macrophages, which indicated that $A_{2A}R$ had protective effects on inflammation. Lastly, when comparing with HFD fed WT mice, HFD fed $A_{2A}R$ deficient mice displayed a significant increase in the severity of inflammation and insulin resistance. And the ablation of $A_{2A}R$ exacerbated the liver steatosis and adipose tissue macrophages infiltration and polarization. Together, these results suggested that $A_{2A}R$ is requisite in improving diet-induced obesity, characterized by regulating glucose/ lipid homeostasis, insulin signaling and most prominently, the anti-inflammatory effects via mediating the population and phenotype of macrophages that resided in both liver and adipose tissue.

REFERENCES

1. Ogden, C. L., Carroll, M. D., Fryar, C. D. and Flegal, K. M., *Prevalence of obesity among adults and youth: United States, 2011–2014.* NCHS data brief, 2015. **219**.
2. Haskó, G., Linden, J., Cronstein, B. and Pacher, P., *Adenosine receptors: therapeutic aspects for inflammatory and immune diseases.* Nat Rev Drug Discov, 2008. **7**(9): p. 759-770.
3. Day, Y.-J., Li, Y., Rieger, J. M., Ramos, S. I., Okusa, M. D. and Linden, J., *A_{2A} adenosine receptors on bone marrow-derived cells protect liver from ischemia-reperfusion injury.* J Immunol, 2005. **174**(8): p. 5040-5046.
4. Day, Y.-J., Marshall, M. A., Huang, L., McDuffie, M. J., Okusa, M. D. and Linden, J., *Protection from ischemic liver injury by activation of A_{2A} adenosine receptors during reperfusion: inhibition of chemokine induction.* Am J Physiol Gastrointest Liver Physiol, 2004. **286**(2): p. G285-293.
5. Day, Y. J., Huang, L., McDuffie, M. J., Rosin, D. L., Ye, H., Chen, J. F., Schwarzschild, M. A., Fink, J. S., et al., *Renal protection from ischemia mediated by A2A adenosine receptors on bone marrow-derived cells.* J Clin Invest, 2003. **112**(6): p. 883-891.
6. Okusa, M. D., Linden, J., Macdonald, T. and Huang, L., *Selective A2A adenosine receptor activation reduces ischemia-reperfusion injury in rat kidney.* Am J Physiol Renal Physiol, 1999. **277**(3): p. F404-412.

7. WHO Consultation on Obesity (1999: Geneva, S. W. H. O., *Obesity : preventing and managing the global epidemic : report of a WHO consultation.* Geneva : World Health Organization, 2000.

8. Kershaw, E. E. and Flier, J. S., *Adipose tissue as an endocrine organ.* J Clin Endocrinol Metab, 2004. **89**(6): p. 2548-2556.

9. Gadgil M.D., A. L. J., Yeung E., Adnerson C.A., Sacks F.M. and Miller E.R.3rd, *The effects of carbohydrate, unsaturated fat, and protein intake on measures of insulin sensitivity: results from the OmniHeart trial.* Diabetes Care, 2013. **36**(5): p. 1132-7.

10. Takeda, K. and Akira, S., *TLR signaling pathways.* Semin Immunol., 2004. **16**(1): p. 3-9.

11. Kraegen, E. W. and Cooney, G. J., *Free fatty acids and skeletal muscle insulin resistance.* Curr Opin Lipidol., 2008. **19**(3): p. 235-41.

12. Evans, W., *Oxygen-carrying proteins in meat and risk of diabetes mellitus.* JAMA Intern Med., 2013. **173**(14): p. 1335-1336.

13. Samuel, V. T. and Shulman, G. I., *Mechanisms for insulin resistance: common threads and missing links.* Cell, 2012. **148**(5): p. 852-871.

14. Stratford, S., Hoehn, K. L., Liu, F. and Summers, S. A., *Regulation of insulin action by ceramide: dual mechanisms linking ceramide accumulation to the inhibition of Akt/protein kinase B.* J Biol Chem, 2004. **279**(35): p. 36608-36615.

15. Jornayvaz, F. R. and Shulman, G. I., *Diacylglycerol Activation of Protein Kinase Cε and Hepatic Insulin Resistance.* Cell Metabolism, 2012. **15**(5): p. 574-584.

16. Bezy, O., Tran, T. T., Pihlajamäki, J., Suzuki, R., Emanuelli, B., Winnay, J., … Kahn, C. R. , *PKCδ regulates hepatic insulin sensitivity and hepatosteatosis in mice and humans.* The Journal of Clinical Investigation, 2011. **121**(6): p. 2504-2517.

17. Macdonald, I. A., *A review of recent evidence relating to sugars, insulin resistance and diabetes.* Eur J Nutr. , 2016. **55**: p. 17-23.

18. Stanhope, K. L., *Sugar consumption, metabolic disease and obesity: The state of the controversy.* Crit Rev Clin Lab Sci. , 2016. **53**(1): p. 52-67.

19. Stanhope, K. L., Medici, V., Bremer, A. A., Lee, V., Lam, H. D., Nunez, M. V., Chen, G. X., Keim, N. L., et al., *A dose-response study of consuming high-fructose corn syrup-sweetened beverages on lipid/lipoprotein risk factors for cardiovascular disease in young adults.* Am J Clin Nutr. , 2015. **101** (6): p. 1144-1154.

20. van Buul, V. J., Tappy, L. and Brouns, F. J., *Misconceptions about fructose-containing sugars and their role in the obesity epidemic.* Nutr Res Rev. , 2014. **27**(1): p. 119-130.

21. Rippe, J. M. and Angelopoulos, T. J., *Fructose-containing sugars and cardiovascular disease.* Adv Nutr. , 2015. **6**(5): p. 430-439.

22. Saito, M., Okamatsu-Ogura, Y., Matsushita, M., Watanabe, K., Yoneshiro, T. and Nio-Kobayashi, J., *High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity.* Diabetes 2009. **58**(7): p. 1526-1531.

23. Yach, D., Stuckler, D. and Brownell, K. D., *Epidemiologic and economic consequences of the global epidemics of obesity and diabetes.* Nat. Med., 2006. **12**(1): p. 62-66.

24. Fain, J. N., Madan, A. K., Hiler, M. L., Cheema, P. and Bahouth, S. W., *Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans.* Endocrinology, 2004. **145**(5): p. 2273-2282.

25. Hausman, D. B., DiGirolamo, M., Bartness, T. J., Hausman, G. J. and Martin, R. J., *The biology of white adipocyte proliferation.* . Obes. Rev., 2001. **2**: p. 239-254.

26. Jung, U. J. and Choi, M. S., *Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease.* . Int. J. Mol. Sci. , 2014. **15**: p. 6184-6223.

27. Kim, J. Y., van de Wall, E., Laplante, M., Azzara, A., Trujillo, M. E., Hofmann, S. M., Schraw, T., Durand, J. L., et al., *Obesity-associated improvements in metabolic profile through expansion of adipose tissue.* J Clin Invest, 2007. **117**(9): p. 2621-2637.

28. Davis, J. E., Gabler, N. K., Walker-Daniels, J. and Spurlock, M. E., *Tlr-4 deficiency selectively protects against obesity induced by diets high in saturated fat.* Obesity (Silver Spring), 2008. **16**(6): p. 1248-1255.

29. Murano, I., Barbatelli, G., Parisani, V., Latini, C., Muzzonigro, G., Castellucci, M. and Cinti, S., *Dead adipocytes, detected as crown-like structures, are*

prevalent in visceral fat depots of genetically obese mice. J Lipid Res, 2008. **49**(7): p. 1562-1568.

30. Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L. and Ferrante, A. W. J., *Obesity is associated with macrophage accumulation in adipose tissue.* J Clin Invest, 2003. **112**(12): p. 1796-1808.

31. Cinti, S., Mitchell, G., Barbatelli, G., Murano, I., Ceresi, E., Faloia, E., Wang, S., Fortier, M., et al., *Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans.* J Lipid Res, 2005. **46**: p. 2347–2355.

32. Shi, H., Kokoeva, M. V., Inouye, K., Tzameli, I., Yin, H. and Flier, J. S., *TLR4 links innate immunity and fatty acid-induced insulin resistance.* J Clin Invest, 2006. **116**(11): p. 3015-3025.

33. Suganami, T., Nishida, J. and Ogawa, Y., *A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor a.* Arterioscler Thromb Vasc Biol, 2005. **25**(10): p. 2062-2068.

34. Rotter, V., Nagaev, I. and Smith, U., *Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects.* . J. Biol. Chem. , 2003. **25**: p. 2062-2068.

35. Amrani, A., Jafarian-Tehrani, M., Mormède, P., Durant, S., Pleau, J. M., Haour, F., Dardenne, M. and Homo-Delarche, F., *Interleukin-1 effect on glycemia in the*

non-obese diabetic mouse at the pre-diabetic stage. J. Endocrinol., 1996. **148**: p. 139-148.

36. Kahn, B. B. and Filer, J. S., *Obesity and insulin resistance.* J. Clin. Invest. , 2000. **106**(3): p. 473-481.

37. Nielsen, S., Guo, Z., Johnson, C. M., Hensrud, D. D. and Jensen, M. D., *Splanchnic lipolysis in human obesity.* J Clin Invest, 2004. **113**: p. 1582-1588.

38. Berlanga, A., Guiu-Jurado, E., Porras, J. A. and Auguet, T., *Molecular pathways in non-alcoholic fatty liver disease.* Clin. Exp. Gastroenterol. , 2014. **7**: p. 221-239.

39. Serviddio, G., Bellanti, F. and Vendemiale, G., *Free radicalbiology for medicine: learning from nonalcoholicfatty liver disease.* Free. Radic. Biol. Med., 2013. **65**: p. 952-968.

40. Schuppan, D., Gorrell, M. D., Klein, T., Mark, M. and Afdhal, N. H., *The challenge of developing novel pharmacological therapies for non-alcoholic steatohepatitis.* Liver Int., 2012. **30**(6): p. 795-808.

41. Brown, M. S. and Goldstein, J. L., *Selective versus Total Insulin Resistance: A Pathogenic Paradox.* Cell Metab., 2008. **7**(2): p. 125-134.

42. Cai, D., Yuan, M., Frantz, D. F., Melendez, P. A., Hansen, L., Lee, J. and Shoelson, S. E., *Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB.* Nat Med, 2005. **11**: p. 183-190.

43. Liaskou, E., Wilson, D. V. and Oo, Y. H., *Innate Immune Cells in Liver Inflammation.* Mediators of Inflammation, 2012: p. 21.

44. Dixon, L. J., Barnes, M., Tang, H., Pritchard, M. T. and Nagy, L. E., *Kupffer Cells in the Liver.* . Comprehensive Physiology, 2013. **3**(2): p. 785-797.

45. Jager, J., Aparicio-Vergara, M. and Aouadi, M., *iver innate immune cells and insulin resistance: the multiple facets of Kupffer cells.* . J Intern Med 2016. **280**(2): p. 209-220.

46. Ray, I., Mahata, S. K. and De, R. K., *Obesity: An Immunometabolic Perspective.* Front. Endocrinol., 2016.

47. Henkel, J., Gärtner, D., Dorn, C., Hellerbrand, C., Schanze, N., Elz, S. R. and Püschel, G. P., *Oncostatin M produced in Kupffer cells in response to PGE2: possible contributor to hepatic insulin resistance and steatosis.* Lab Invest. , 2011. **91**(7): p. 1107-1117.

48. Morinaga, H., Mayoral, R., Heinrichsdorff, J., Osborn, O., Franck, N., Hah, N., Walenta, E., Bandyopadhyay, G., et al., *Characterization of distinct subpopulations of hepatic macrophages in HFD/obese mice.* Diabetes, 2015. **64**(4): p. 1120-1130.

49. Bjarnadóttir, T. K., Gloriam, D. E., Hellstrand, S. H., Kristiansson, H., Fredriksson, R. and Schiöth, H. B., *Comprehensive repertoire and phylogenetic analysis of the G protein-coupled receptors in human and mouse.* Genomics, 2006. **88**(3): p. 263-273.

50. Klabunde, T. and Hessler, G., *Drug design strategies for targeting G-protein-coupled receptors.* Chembiochem, 2002. **3**: p. 928-944.

51. Fredholm, B. B., *Adenosine- a physiological or pathophysiological agent?* J. Mol. Med., 2014. **92**(3): p. 201-206.

52. Yegutkin, G. G., *Nucleotide- and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade.* Biophys. Acta, 2008. **1783**: p. 673-694.

53. Narravula, S., Lennon, P.F., Mueller, B.U., and Colgan, S.P. , *Regulation of endothelial CD73 by adenosine: paracrine pathway for enhanced endothelial barrier function.* J. Immunol. , 2000. **165**: p. 5262-5268.

54. Niemela, J., Henttinen, T., Yegutkin, G.G., Airas, L., Kujari, A.M., Rajala, P., and Jalkanen, S. , *IFN- induced adenosine production on the endothelium: a mechanism mediated by CD73 (ecto-5'-nucleotidase) up-regulation.* J. Immunol., 2004. **172**: p. 1646-1653.

55. Eltzschig, H. K., Thompson, L. F., Karhausen, J., Cotta, R. J., Ibla, J. C., Robson, S. C., Colgan, S. P. , *Endogenous adenosine produced during hypoxia attenuates neutrophil accumulation: coordination by extracellular nucleotide metabolism.* Blood 2004. **104**: p. 3986 –3992.

56. Linden, J., *Molecular approach to adenosine receptors: receptor- mediated mechanisms of tissue protection.* Annu. Rev. Pharmacol. Toxi- col., 2001. **41**: p. 775–787.

57. Zimmermann, H., *Extracellular metabolism of ATP and other nucleotides.* . Naunyn Schmiedebergs Arch. Pharmacol. , 2000. **362**: p. 299–309.

58. Fredholm, B. B., Ijzerman, A. P., Jacobson, K. A., Klotz, K.-N. and Linden, J., *International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors.* Pharmacol Rev, 2001. **53**: p. 527–552.

59. Ledent, C., Vaugeois, J. M., Schiffmann, S. N., Pedrazzini, T., El Yacoubi, M., Vanderhaeghen, J. J., Costentin, J., Heath, J. K., et al., *Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor.* Nature, 1997. **388**: p. 674-678.

60. Lee, Y. C., Chien, C. L., Sun, C. N., Huang, C. L., Huang, N. K., Chiang, M. C., Lai, H. L., Lin, Y. S., et al., *Characterization of the rat A2A adenosine receptor gene: a 4,8-kb promoter-proximal DNA fragment confers selec- tive expression in the central nervous system.* Eur. J. Neurosci., 2003. **18**: p. 1786-1796.

61. Kull, B., Svenningsson, P. and Fredholm, B. B., *Adenosine A2A receptors are colocalized with and activate Golf in rat striatum.* Mol. Pharmacol., 2000. **58**: p. 771-777.

62. Sullivan -G., W., Rieger, J. M., Scheld, W. M., MacDonald, T. L. and Linden, J., *Cyclic AMP-dependent inhibition of human neutrophil oxidative activity by substituted 2-propynylcyclohexyl adenosine A2A receptor agonists.* Br. J. Pharmacol., 2001. **132**: p. 1017-1026.

63. Link, A. A., Kino, T., Worth, J. A., McGuire, J. L., Crane, M. L., Chrousos, G. P., Wilder, R. L. and Elenkov, I. J., *Ligand-activation of the adenosine A2a receptors inhibits IL-12 production by human monocytes.* J Immunol, 2000. **164**(1): p. 436-442.

64. Huang, S., Apasov, S., Koshiba, M. and Sitkovsky, M., *Role of A2a extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion.* Blood, 1997. **90**: p. 1600-1610.

65. Koshiba, M., Kojima, H., Huang, S., Apasov, S. and Sitkovsky, M. V., *Memory of extracellular adenosine A2A purinergic receptor-mediated signaling in murine T cells.* J. Biol. Chem. , 1997. **272**: p. 25881–25889.

66. Cooper, J. A., Hill, S. J., Alexander , S. P., Rubin, P. C. and Horn, E. H., *Adenosine receptor-induced cyclic AMP generation and inhibition of 5-hydroxytryptamine release in human platelets.* Br. J. Clin. Pharmacol. , 1995. **40**: p. 43-50.

67. Murphree, L. J., Sullivan, G. W., Marshall, M. A. and Linden, J., *Lipopolysaccharide rapidly modifies adenosine receptor transcripts in murine and human macrophages: role of NF-kB in A_{2A} adenosine receptor induction.* Biochem J, 2005. **391**(3): p. 575-580.

68. Khoa, N. D., Montesinos, M. C., Reiss, A. B., Delano, D., Awadallah, N. and Cronstein, B. N., *Inflammatory cytokines regulate function and expression of adenosine A2A receptors in human monocytic THP-1 cells.* J. Immunol. , 2001. **167**: p. 4026-4032.

69. Khoa, N. D., Postow, M., Danielsson, J. and Cronstein, B. N., *Tumor necrosis factor- prevents desensitization of G s-coupled receptors by regulating GRK2 association with the plasma membrane.* Mol. Pharma- col., 2006. **69**: p. 1311-1319.

70. Bshesh, K., Zhao, B., Spight, D., Biaggioni, I., Feokistov, I., Denenberg, A., Wong, H. R. and Shanley, T. P., *The A2A receptor mediates an endogenous regulatory pathway of cytokine expression in THP-1 cells.* J. Leukoc. Biol. , 2004. **72**: p. 1027-1036.

71. Lukashev, D., Ohta, A., Apasov, S., Chen, J.-F. and Sitkovsky, M., *Cutting edge: Physiologic attenuation of proinflammatory transcription by the G_s protein-coupled A2A adenosine receptor in vivo.* J Immunol, 2004. **173**(1): p. 21-24.

72. Scheibner, K. A., Boodoo, S., Collins, S., Black, K. E., Chan-Li, Y., Zarek, P., Powell, J. D. and Horton, M. R., *The adenosine A2A receptor inhibits matrix-induced inflammation in a novel fashion.* Am. J. Respir. Cell Mol. Biol., 2009. **40**: p. 251-259.

73. Kreckler, L. M., Gizewski, E., Wan, T. C. and Auchampach, J. A., *Adenosine suppresses lipopolysaccharide- induced tumor necrosis factor-α production by murine macrophages through a protein kinase A- and exchange protein activated by cAMP-independent signaling pathway.* . J. Pharmacol. Exp. Ther. , 2009. **331**: p. 1051-1061.

74. Antonioli, L., Blandizzi, C., Pacher, P. and Hasko, G., *Immunity inflammation and cancer: a leading role for adenosine.* Nat. Rev. Cancer, 2013. **13**(12): p. 842-857.

75. Cekic, C. and Linden, J., *Purinergic regulation of the immune system.* Nature reviews. Immunology, 2016. **16**(3): p. 177-192.

76. Montesinos, M. C., Desai, A., Chen, J.-F., Yee, H., Schwarzschild, M. A., Fink, J. S., Cronstein, B. N., *Adenosine Promotes Wound Healing and Mediates Angiogenesis in Response to Tissue Injury Via Occupancy of A2A Receptors.* The American Journal of Pathology, 2002. **160**(6): p. 2009-2018.

77. Montesinos, M. C., Desai-Merchant, A. and Cronstein, B. N., *Promotion of Wound Healing by an Agonist of Adenosine A2A Receptor Is Dependent on Tissue Plasminogen Activator.* Inflammation, 2015. **38**(6): p. 2036-41.

78. Leibovich, S. J., Chen, J. F., Pinhal-Enfield, G., Belem, P. C., Elson, G., Rosania, A., Ramanathan, M., Montesinos, C., et al., *Synergistic up-regulation of vascular endothelial growth factor expression in murine macrophages by adenosine A2A receptor agonists and endotoxin.* Am J Pathol, 2002. **160**: p. 2231-2244.

79. Macedo, L., Pinhal-Enfield, G., Alshits, V., Elson, G., Cronstein, B. N. and Leibovich, S. J., *Wound Healing Is Impaired in MyD88-Deficient Mice: A Role for MyD88 in the Regulation of Wound Healing by Adenosine A2A Receptors.* The American Journal of Pathology, 2007. **171**(6): p. 1774-1788.

80. Gnad T., S. S., von Kügelgen I., Scheele C., Kilić A., Glöde A., Hoffmann LS., Reverte-Salisa L., Horn P., Mutlu S., El-Tayeb A., Kranz M., Deuther-Conrad W., Brust P., Lidell ME., Betz MJ., Enerbäck S., Schrader J., Yegutkin GG., Müller CE., Pfeifer A., *Adenosine activates brown adipose tissue and recruits beige adipocytes via A2A receptors.* Nature, 2014. **516**: p. 395-399.

81. Andersson, O., Adams, B. A., Yoo, D., Ellis, G. C., Gut, P., Anderson, R. M., German, M. S. and Stainier, D. Y., *Adenosine signaling promotes regeneration of pancreatic β cells in vivo.* Cell Metab., 2012. **15**(6): p. 885-94.

82. Annes, J. P., Ryu, J. H., Lam, K., Carolan, P. J., Utz, K., Hollister-Lock, J., Arvanites, A. C., Rubin, L. L., Weir, G., Melton, D. A. , *Adenosine kinase inhibition selectively promotes rodent and porcine islet β-cell replication.* . Proc. Natl. Acad. Sci. USA, 2012. **109**: p. 3915-3920.

83. Schulz, N., Liu, K.-C., Charbord, J., Mattsson, C. L., Tao, L., Tworus, D., Andersson, O. , *Critical role for adenosine receptor A2a in β-cell proliferation.* Molecular Metabolism, 2016. **5**(11): p. 1138-1146.

84. Lappas, C. M., Day, Y. J., A., M. M., H., E. V. and Linden, J., *Adenosine A2A receptor activation reduces hepatic ischemia reperfusion injury by inhibiting CD1d-dependent NKT cell activation.* J Exp Med., 2006. **203**: p. 2639-2648.

85. Odashima, M., Otaka, M., Jin, M., Horikawa, Y., Matsuhashi, T., Ohba, R., Linden, J. and Watanabe, S., *A selective adenosine A2A receptor agonist, ATL-146e, prevents concanavalin A-induced acute liver injury in mice.* Biochem Biophys Res Commun., 2006. **347**(4): p. 949-954.

86. Day, Y. J., Marshall, M. A., Huang, L., McDuffie, M. J., Okusa, M. D. and Linden, J., *Protection from ischemic liver injury by activation of A2A adenosine receptors during reperfusion: inhibition of chemokine induction.* Am J Physiol Gastrointest Liver Physiol. , 2004. **2004**(286): p. G285-293.

87. Raskovalova, T., Lokshin, A., Huang, X., Jackson, E. K. and Gorelik, E., *Adenosine-mediated inhibition of cytotoxic activity and cytokine production by IL-2/NKp46-activated NK cells: involvement of protein kinase A isozyme I (PKA I).* . Immunol Res., 2006. **36**(36): p. 91-99.

88. Hansson, G. K., *Inflammation, atherosclerosis, and coronary artery disease.* N. Engl. J. Med., 2005. **352**: p. 1685-1695.

89. Oram, J. F., *HDL apolipoproteins and ABCA1: partners in the removal of excess cellular cholesterol Arterioscler.* Thromb. Vasc. Biol., 2003. **23**: p. 720-727.

90. Bingham, T. C., Fisher, E. A., Parathath, S., Reiss, A. B., Chan, E. S. and Cronstein, B., *A2A adenosine receptor stimulation decreases foam cell formation by enhancing ABCA1-dependent cholesterol efflux.* Journal of Leukocyte Biology, 2010. **87**: p. 683-690.

91. Bingham, T. C., Parathath, S., Tian, H., Reiss, A. B., Chan, E. S., Fisher, E. A. and Cronstein, B. N., *Cholesterol 27-hydroxylase but not apolipoprotein apoE contributes to A2A adenosine receptor stimulated reverse cholesterol transport.* Inflammation, 2012. **35** (1): p. 49-57.

92. González-Benítez, E., Guinzberg, R., Díaz-Cruz, A. and Piña, E., *Regulation of glycogen metabolism in hepatocytes through adenosine receptors. Role of Ca2+ and cAMP.* Eur J Pharmacol, 2002. **437**(3): p. 105-111.

93. Sachdeva, S. and Gupta, M., *Adenosine and its receptors as therapeutic targets: An overview.* Saudi Pharmaceutical Journal, 2013. **21**(3): p. 245-253.

94. Gharibi, B., Abraham, A. A., Ham, J. and Evans, B. A., *Adenosine receptor subtype expression and activation influence the differentiation of mesenchymal stem cells to osteoblasts and adipocytes.* J Bone Miner Res, 2011. **26**(9): p. 2112-2124.

95. Johansson, S. M., Salehi, A., Sandstrom, M. E., Westerblad, H., Lundquist, I., Carlsson, P. O., Fredholm, B. B. and Katz, A., *A1 receptor deficiency causes increased insulin and glucagon secretion in mice.* Biochem Pharmacol., 2007. **74**: p. 1628-1635.

96. Szkudelska, K., Nogowski, L. and Szkudelski, T., *The inhibitory effect of resveratrol on leptin secretion from rat adipocytes.* . Eur J Clin Invest., 2009. **39**: p. 899-905.

97. Greene, S. J., Sabbah, H. N., Butler, J., Voors, A. A., Albrecht-Küpper, B. E., Düngen, H. D., Dinh, W. and Gheorghiade, M., *Partial adenosine A1 receptor agonism: a potential new therapeutic strategy for heart failure.* Heart Fail Rev., 2016. **21**(1): p. 95-102.

98. Xiang, F., Huang, Y. S., Zhang, D. X., Chu, Z. G., Zhang, J. P. and Zhang, Q., *Adenosine A1 receptor activation reduces opening of mitochondrial permeability transition pores in hypoxic cardiomyocytes.* Clin Exp Pharmacol Physiol, 2010. **37**: p. 343-349.

99. Tendera, M., Gaszewska-Zurek, E., Parma, Z., Ponikowski, P., Jan- kowska, E., Kawecka-Jaszcz, K., Czarnecka, D., Krzeminska-Pakula, M., et al., *The new oral*

adenosine A1 receptor agonist capade- noson in male patients with stable angina. Clin Res Cardiol, 2012. **101**: p. 585-591.

100. Mangmool, S. and Kurose, H., *Gi/o Protein-Dependent and -Independent Actions of Pertussis Toxin (PTX).* Toxins, 2011. **3**: p. 884-899.

101. Yang, D., Chen, H., Koupenova, M., Carroll, S. H., Eliades, A., Freedman, J. E., Toselli, P. and Ravid, K., *A new role for the A2b adenosine receptor in regulating platelet function.* J Thromb Haemost, 2010. **8**(4): p. 817-827.

102. Koupenova, M., Johnston-Cox, H., Vezeridis, A., Gavras, H., Yang, D., Zannis, V. and Ravid, K., *A2b adenosine receptor regulates hyperlipidemia and atherosclerosis.* Circulation, 2012. **125**(2): p. 354-363.

103. Csóka, B., Koscsó, B., Törő, G., Kókai, E., Virág, L., Németh, Z. H., … Haskó, G. , *A2B Adenosine Receptors Prevent Insulin Resistance by Inhibiting Adipose Tissue Inflammation via Maintaining Alternative Macrophage Activation.* Diabetes, 2014. **63**(3): p. 850-866.

104. Cekic, C., Sag, D., Li, Y., Theodorescu, D., Strieter, R. M., and Linden, J., *Adenosine A2B receptor blockade slows growth of bladder and breast tumors.* . J. Immunol. , 2012. **188**: p. 198-205.

105. Vecchio, E. A., Tan, C. Y., Gregory, K. J., Christopoulos, A., White, P. J., and May, L. , *Ligand-independent adenosine A2B receptor constitutive activity as a promoter of prostate cancer cell proliferation.* . J. Pharmacol. Exp. Ther., 2016. **357**: p. 36-44.

106. Wei, Q., Costanzi, S., Balasubramanian, R., Gao, Z. G., and Jacobson, K. A. , *A2B adenosine receptor blockade inhibits growth of prostate cancer cells.* . Purinergic Signal. , 2013. **9**: p. 271-280.

107. Ntantie, E., Gonyo, P., Lorimer, E. L., Hauser, A. D., Schuld, N., McAllister, D., Kalyanaraman, B., Dwinell, M. B., et al., *An adenosine-mediated signaling pathway suppresses prenylation of the GTPase Rap1B and promotes cell scattering.* Sci Signal., 2013. **28**(6): p. 277.

108. Bar-Yehuda, S., Stemmer, S. M., Madi, L., Castel, D., Ochaion, A., Cohen, S., Barer, F., Zabutti, A., et al., *The A3 adenosine receptor agonist CF102 induces apoptosis of hepatocellular carcinoma via de-regulation of the Wnt and NF-kappaB signal transduction pathways.* Int J Oncol., 2008. **33**(2): p. 287-295.

109. Fishman, P., Bar-Yehuda, S., Ardon, E., Rath-Wolfson, L., Barrer, F., Ochaion, A. and Madi, L., *Targeting the A3 adenosine receptor for cancer therapy: inhibition of prostate carcinoma cell growth by A3AR agonist.* Anticancer Res. , 2003. **23**(3A): p. 2077-2083.

110. Fishman, P., Bar-Yehuda, S., Madi, L., Rath-Wolfson, L., Ochaion, A., Cohen, S. and Baharav, E., *The PI3K-NF-kappaB signal transduction pathway is involved in mediating the anti-inflammatory effect of IB-MECA in adjuvant-induced arthritis.* Arthritis Res Ther., 2006. **8**(1): p. R33.

111. Szabó, C., Scott, G. S., Virág, L., Egnaczyk, G., Salzman, A. L., Shanley, T. P. and Haskó, G., *Suppression of macrophage inflammatory protein (MIP)-1alpha*

production and collagen-induced arthritis by adenosine receptor agonists. Br J Pharmacol. , 1998. **125**(2): p. 379-387.

112. Asarch, A., Barak, O., Loo, D. S. and Gottlieb, A. B., *Th17 cells: a new paradigm for cutaneous inflammation.* J Dermatolog Treat., 2008. **19**(5): p. 259-266.

113. Silverman, M. H., Strand, V., Markovits, D., Nahir, M., Reitblat, T., Molad, Y., Rosner, I. and Rozenbaum, M., *Clinical evidence for utilization of the A3 adenosine receptor as a target to treat rheumatoid arthritis: data from a phase II clinical trial.* J Rheumatol. , 2008. **35**(1): p. 41-48.

114. Fishman, P., Bar-Yehuda, S., Liang, B. T., Jacobson, K. A., *Pharmacological and Therapeutic Effects of A3 Adenosine Receptor (A3AR) Agonists.* Drug Discovery Today, , 2012. **17**(7-8): p. 359-366.

115. Blüher, M., *The distinction of metabolically 'healthy' from 'unhealthy' obese individuals.* Current Opinion in Lipidology, 2012. **21**(1): p. 38-43.

116. Denis, G. V. and Obin, M. S., *'Metabolically healthy obesity': Origins and implications.* . Molecular Aspects of Medicine, 2013. **34**(1): p. 59-70.

117. Badoud, F., Perreault, M., Zulyniak, MA., Mutch, D. M., *Molecular insights into the role of white adipose tissue in metabolically unhealthy normal weight and metabolically healthy obese individuals.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2014. **29**(3): p. 748-758.

118. Schro¨der, H., Ramos, R., Baena-D´ıez, J., Mendez, M., Canal, D., F´ıto, M., Sala, J., Elosua, R。, *Determinants of the transition from a cardiometabolic normal to abnormal overweight/obese phenotype in a Spanish population.* Eur. J. Nutr. , 2013. **53**: p. 1345-1353.

119. Lee, S. H., Ha, H. S., Park, Y. J., *Identifying metabolically obese but normal-weight (MONW) individuals in a nondiabetic Korean population: the Chungju Metabolic disease Cohort (CMC) study.* Clin. Endo- crinol. (Oxf.), 2011. **75**: p. 475-481.

120. Sam, S., Haffner, S., Davidson, M.H., D'Agostino, R.B., Sr., Feinstein, S., Kondos, G., Perez, A., Mazzone, T., *Relationship of abdominal visceral and subcutaneous adipose tissue with lipoprotein particle number and size in type 2 diabetes.* . Diabetes. , 2008. **57**: p. 2022-2027.

121. Rodríguez, A., Ezquerro, S., Méndez-Giménez, L., Becerril, S. and Frühbeck, G., *Revisiting the adipocyte: a model for integration of cytokine signaling in the regulation of energy metabolism.* Am J Physiol Endocrinol Metab. , 2015. **309**(8): p. E691-714.

122. Choe, S. S., Huh, J. Y., Hwang, I. J., Kim, J. I., Kim, J. B., *Adipose Tissue Remodeling: Its Role in Energy Metabolism and Metabolic Disorders.* Frontiers in Endocrinology, 2016. **7**(30).

123. Gericke, M., Weyer, U., Braune, J., Bechmann, I. and Eilers, J., *A method for long-term live imaging of tissue macrophages in adipose tissue explants.* Am J Physiol Endocrinol Metab. , 2015. **308**(11): p. E1023-1033.

124. Boutens, L., Stienstra, R. , *Adipose tissue macrophages: going off track during obesity.* Diabetologia., 2016. **59**: p. 879-894.

125. Yang, Y., Wang, H., Lv, X., Wang, Q., Zhao, H., Yang, F., Yang, Y. and Li, J., *Involvement of cAMP-PKA pathway in adenosine A1 and A2A receptor-mediated regulation of acetaldehyde-induced activation of HSCs.* Biochimie. , 2015. **115**: p. 59-70.

126. Kreckler, L. M., Gizewski, E., Wan, T. C. and Auchampach, J. A., *Adenosine suppresses lipopolysaccharide-induced tumor necrosis factor-alpha production by murine macrophages through a protein kinase A- and exchange protein activated by cAMP-independent signaling pathway.* J Pharmacol Exp Ther., 2009. **331**(3): p. 1051-1061.

127. Haschemi, A., Wagner, O., Marculescu, R., Wegiel, B., Robson, S. C., Gagliani, N., Gallo, D., Chen, J. F., Bach, F. H., Otterbein, L. E. , *Cross-regulation of carbon monoxide and the adenosine A2A receptor in macrophages.* . Journal of Immunology., 2007. **178**: p. 5921-5929.

128. Weis, N., Weigert, A., von Knethen, A., Brune, B. , *Heme oxygenase-1 contributes to an alternative macrophage activation profile induced by apoptotic cell supernatants.* Molecular Biology of the Cell. , 2009. **20**: p. 1280-1288.

129. Wu, C., Kang, J. E., Peng, L., Li, H., Khan, S. A., Hillard, C. J., Okar, D. A. and Lange, A. J., *Enhancing hepatic glycolysis reduces obesity: Differential effects on lipogenesis depend on site of glycolytic modulation.* Cell Metab, 2005. **2**(2): p. 131-140.

130. Wu, C., Okar, D. A., Newgard, C. B. and Lange, A. J., *Overexpression of 6-phosphofructo-2- kinase/fructose-2,6-bisphosphatase in mouse liver lowers blood glucose by suppression of hepatic glucose production.* J Clin Invest, 2001. **107**(1): p. 91-98.

131. Wu, C., Khan, S. A., Peng, L. J., Li, H., Camela, S. and Lange, A. J., *Perturbation of glucose flux in the liver by decreasing fructose-2,6-bisphosphate levels causes hepatic insulin resistance and hyperglycemia.* Am J Physiol Endocrinol Metab, 2006. **291**(3): p. E536-543.

132. Huo, Y., Guo, X., Li, H., Wang, H., Zhang, W., Wang, Y., Zhou, H., Gao, Z., et al., *Disruption of inducible 6-phosphofructo-2-kinase ameliorates diet-induced adiposity but exacerbates systemic insulin resistance and adipose tissue inflammatory response.* J Biol Chem, 2010. **285**: p. 3713-3721.

133. Guo, X., Xu, K., Zhang, J., Li, H., Zhang, W., Wang, H., Lange, A. J., Chen, Y. E., et al., *Involvement of inducible 6-phosphofructo-2-kinase in the anti-diabetic effect of PPARg activation in mice.* J Biol Chem, 2010. **285**(31): p. 23711–23720.

134. Guo, X., Li, H., Xu, H., Halim, V., Zhang, W., Wang, H., Ong, K. T., Woo, S. L., Walzem, R. L., Mashek, D. G., Dong, H., Lu, F., Wei, L., Huo, Y., Wu,C., *Palmitoleate induces hepatic steatosis but suppresses liver inflammatory response in mice.* PLoS One 2012. **7**: p. e39286.

135. Guo, X., Li, H., Xu, H., Halim, V., Thomas, L. N., Woo, S.-L., Huo, Y., Chen, Y. E., Sturino, J. M., Wu, C. , *Disruption of inducible 6- phosphofructo-2-kinase*

impairs the suppressive effect of PPARg activation on diet-induced intestine inflammatory response. J. Nutr. Biochem. , 2013. **24**: p. 770-775.

136. Wang, C.-Y. and Liao, J. K., *A Mouse Model of Diet-Induced Obesity and Insulin Resistance.* . Methods in Molecular Biology (Clifton, N.J.), 2012. **821**: p. 421-433.

137. van der Heijden, R. A., Sheedfar, F., Morrison, M. C., Hommelberg, P. P., Kor, D., Kloosterhuis, N. J., Gruben, N., Youssef, S. A., et al., *High-fat diet induced obesity primes inflammation in adipose tissue prior to liver in C57BL/6j mice.* Aging (Albany NY). , 2015. **7**(4): p. 256-268.

138. Park, S. H., Liu, Z., Sui, Y., Helsley, R. N., Zhu, B., Powell, D. K., Kern, P. A. and Zhou, C., *IKKβ Is Essential for Adipocyte Survival and Adaptive Adipose Remodeling in Obesity.* Diabetes. , 2016. **65**(6): p. 1616-1629.

139. Chiang, S.-H., Bazuine, M., Lumeng, C. N., Geletka, L. M., Mowers, J., White, N. M., Saltiel, A. R., *The protein kinase IKKε regulates energy expenditure, insulin sensitivity and chronic inflammation in obese mice.* Cell, 2009. **138**(5): p. 961-975.

140. Vallerie, S. N., Furuhashi, M., Fucho, R. and Hotamisligil, G. S., *A predominant role for parenchymal c-Jun amino terminal kinase (JNK) in the regulation of systemic insulin sensitivity.* PLoS One, 2008. **3**(9): p. e3151.

141. Li, H. and Yu, X., *Emerging role of JNK in insulin resistance.* Curr Diabetes Rev, 2013. **9**(5): p. 422-428.

142. Perry R.J., C. J. P., Kursawe R., Titchenell P.M., Zhang D., Perry C.J., *Hepatic acetyl CoA links adipose tissue inflammation to hepatic insulin resistance and type 2 diabetes.* Cell, 2015. **160**: p. 745-748.

143. Solinas, G. and Becattini, B., *NK at the crossroad of obesity, insulin resistance, and cell stress response.* Mol Metab, 2017. **6**(2): p. 174-184.

144. Han, M. S., Jung, D. Y., Morel, C., Lakhani, S. A., Kim, J. K., Flavell, R. A. and Davis, R. J., *JNK expression by macrophages promotes obesity-induced insulin resistance and inflammation.* Science, 2013. **339**(6116): p. 218-222.

145. Gonzalez, E., Flier, E., Molle, D., Accili, D. and McGraw, T. E., *Hyperinsulinemia leads to uncoupled insulin regulation of the GLUT4 glucose transporter and the FoxO1 transcription factor.* Proc Natl Acad Sci U S A. , 2011. **108**(25): p. 10162-10167.

146. Liu, Q., Bengmark, S., Qu, S., *The role of hepatic fat accumulation in pathogenesis of non-alcoholic fatty liver disease (NAFLD).* Lipids Health Dis. , 2010. **9**: p. 42.

147. Farrell, G. C. and Larter, C. Z., *Nonalcoholic fatty liver disease: From steatosis to cirrhosis.* Hepatology, 2006. **43**(S1): p. S99-S112.

148. Klop, B., Elte, J. W. F., Castro Cabezas, M. , *Dyslipidemia in Obesity: Mechanisms and Potential Targets.* Nutrients, 2013. **5**(4): p. 1218-1240.

149. Knight, Z. A., Gonzalez, B., Feldman, M. E., Zunder, E. R., Goldenberg, D. D., Williams, O., Loewith, R., Stokoe, D., et al., *A pharmacological map of the PI3-*

K family defines a role for p110alpha in insulin signaling. Cell, 2006. **125**(4): p. 733-747.

150. Foukas, L. C., Claret, M., Pearce, W., Okkenhaug, K., Meek, S., Peskett, E., Sancho, S., Smith, A. J., et al., *Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta).* Nature, 2006. **441**(7091): p. 366-370.

151. Leavens, K. F., Easton, R. M., Shulman, G. I., Previs, S. F. and Birnbaum, M. J., *Akt2 is required for hepatic lipid accumulation in models of insulin resistance.* Cell Metab., 2009. **10**(5): p. 405-418.

152. Alchera, E., Imarisio, C., Mandili, G., Merlin, S., Chandrashekar, B. R., Novelli, F.,, *Pharmacological Preconditioning by Adenosine A2a Receptor Stimulation: Features of the Protected Liver Cell Phenotype.* . Biomed Res Int. , 2015: p. 286746.

153. Serracino-Inglott, F., Virlos, I. T., Habib, N. A., Williamson, R. C. N. and Mathie, R. T., *Adenosine preconditioning attenuates hepatic reperfusion injury in the rat by preventing the down-regulation of endothelial nitric oxide synthase.* BMC Gastroenterology, 2002. **2**(1): p. 22.

154. Chu, M. J. J., Hickey, A. J. R., Phillips, A. R. J. and Bartlett, A. S. J. R., *The impact of hepatic steatosis on hepatic ischemia-reperfusion injury in experimental studies: a systematic review,.* Seminars in Liver Disease, 2013. **21**(1).

155. De Meijer, V. E., Kalish, B. T., Puder, M., & IJzermans, J. N. M., *Systematic review and meta‐analysis of steatosis as a risk factor in major hepatic resection.* British Journal of Surgery, 2010. **97**(9): p. 1331-1339.

156. Imarisio, C., Alchera, E., Sutti, S., Valente, G., Boccafoschi, F., Albano, E., Carini, R. , *Adenosine A2a receptor stimulation prevents hepatocyte lipotoxicity and non-alcoholic steatohepatitis (NASH) in rats.* . Clinical Science, 2012. **123**(4): p. 323-332.

157. Suganami. T., N. J., Ogawa. Y, *Role of free fatty acids and tumor necrosis factor alpha.* Arterioscler. Thromb. Vasc. Biol. , 2005. **25**: p. 2062-2068.

158. Johnson, A. R., Milner, J. J., Makowski, L. , *The inflammation highway: metabolism accelerates inflammatory traffic in obesity.* Immunological Reviews, 2012. **249**(1).

159. Gao, Z., Zhang, J., Kheterpal, I., Kennedy, N., Davis, R. J. and Ye, J., *Sirtuin 1 (SIRT1) Protein Degradation in Response to Persistent c-Jun N-terminal Kinase 1 (JNK1) Activation Contributes to Hepatic Steatosis in Obesity.* J Biol Chem. , 2011. **286**(25): p. 22227-22234.

160. Cheng, J., Liu, C., Hu, K., Greenberg, A., Wu, D., Ausman, L. M., McBurney, M. W. and Wang, X., *Ablation of systemic SIRT1 activity promotes nonalcoholic fatty liver disease by affecting liver-mesenteric adipose tissue fatty acid mobilization.* FASEB Journal, 2017. **31**.

161. Imarisio, C., Alchera, E., Sutti, S., Valente, G., Boccafoschi, F., Albano, E. and Carini, R., *Adenosine A(2a) receptor stimulation prevents hepatocyte lipotoxicity*

and non-alcoholic steatohepatitis (NASH) in rats. Clin Sci (Lond). , 2012. **123**(5): p. 323-332.

162. Chon, S.-H. and Pappas, A., *Differentiation and characterization of human facial subcutaneous adipocytes.* adipocytes, 2015. **4**(1): p. 13-21.

163. Isakson, P., Hammarstedt, A., Gustafson, B. and Smith, U., *Impaired preadipocyte differentiation in human abdominal obesity: role of Wnt, tumor necrosis factor-alpha, and inflammation.* Diabetes., 2009. **58**(7): p. 1550-1557.

164. Boß, M., Newbatt, Y., Gupta, S., Collins, I., Brüne, B., Namgaladze, D. , *AMPK-independent inhibition of human macrophage ER stress response by AICAR.* Sci Rep. , 2016. **6**: p. 32111.

165. Fresno, M., Alvarez, R. and Cuesta, N., *Toll-like receptors, inflammation, metabolism and obesity.* Arch Physiol Biochem. , 2011. **117**(3): p. 151-164.

166. Schäffler, A. and Schölmerich, J., *Innate immunity and adipose tissue biology.* Trends Immunol. , 2010. **31**(6): p. 228-235.

167. Yamashita, A., Soga, Y., Iwamoto, Y., Asano, T., Li, Y., Abiko, Y. and Nishimura, F., *DNA microarray analyses of genes expressed differentially in 3T3-L1 adipocytes co-cultured with murine macrophage cell line RAW264.7 in the presence of the toll-like receptor 4 ligand bacterial endotoxin.* Int J Obes (Lond). , 2008. **32**(11): p. 1725-1729.

168. Bassols, J., Ortega, F. J., Moreno-Navarrete, J. M., Peral, B., Ricart, W. and Fernández-Real, J. M., *Study of the proinflammatory role of human differentiated omental adipocytes.* J Cell Biochem. , 2009. **107**(6): p. 1107-1117.

169. Sárvári, A. K., Doan-Xuan, Q.-M., Bacsó, Z., Csomós, I., Balajthy, Z., Fésüs, L. , *Interaction of differentiated human adipocytes with macrophages leads to trogocytosis and selective IL-6 secretion.* Cell Death Dis, 2015. **6**(1): p. e1613.

170. Krenkel, O. and Tacke, F., *Liver macrophages in tissue homeostasis and disease.* Nat Rev Immunol., 2017. **17**(5): p. 306-321.

171. Heymann, F., Peusquens, J., Ludwig-Portugall, I., Kohlhepp, M., Ergen, C., Niemietz, P., Martin, C., van Rooijen, N., et al., *Liver inflammation abrogates immunological tolerance induced by Kupffer cells.* Hepatology, 2015. **62**(1): p. 279-291.

172. Hirsova, P., Ibrahim, S. H., Krishnan, A., Verma, V. K., Bronk, S. F., Werneburg, N. W., Charlton, M. R., Shah, V. H., et al., *Lipid-Induced Signaling Causes Release of Inflammatory Extracellular Vesicles From Hepatocytes.* Gastroenterology., 2016. **150**(4): p. 956-967.

173. Bartneck, M., Fech, V., Ehling, J., Govaere, O., Warzecha, K. T., Hittatiya, K., Vucur, M., Gautheron, J., et al., *Histidine-rich glycoprotein promotes macrophage activation and inflammation in chronic liver disease.* Hepatology, 2016. **63**(4): p. 1310-1324.

174. Eisenstein, A. and Ravid, K., *G protein-coupled receptors and adipogenesis: a focus on adenosine receptors.* J Cell Physiol., 2014. **229**(4): p. 414-421.

175. Eisenstein, A., Carroll, S. H., Johnston-Cox, H., Farb, M., Gokce, N. and Ravid, K., *An adenosine receptor-Krüppel-like factor 4 protein axis inhibits adipogenesis.* J Biol Chem., 2014. **289**(30): p. 21071-21081.

176. Gharibi, B., Abraham, A. A., Ham, J. and Evans, B. A., *Contrasting effects of A1 and A2b adenosine receptors on adipogenesis.* Int J Obes (Lond). , 2012. **36**(3): p. 397-406.

177. Haskó, G., Cronstein, B. , *Regulation of Inflammation by Adenosine.* Front Immunol, 2013. **4**: p. 85.

178. Arner, E., Westermark, P. O., Spalding, K. L., Britton, T., Rydén, M., Frisén, J., Arner, P., *Adipocyte Turnover: Relevance to Human Adipose Tissue Morphology.* Diabetes, 2010. **59**(1): p. 105-109.

179. Hill, A. A., Bolus, W. R., Hasty, A. H. , *A Decade of Progress in Adipose Tissue Macrophage Biology.* Immunol Rev. , 2014. **262**(1): p. 134-152.

180. Abdullah, Z. and Knolle, P. A., *Liver macrophages in healthy and diseased liver.* Pflugers Arch., 2017. **469**(3-4): p. 553-560.

181. Pradere, J. P., Kluwe, J., Minicis, S., Jiao, J. J., Gwak, G. Y., Dapito, D. H., Dragomir, A. C., *Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice.* Hepatology, 2013. **58**(4): p. 1461-1473.

182. Ponugoti, B., Kim, D. H., Xiao, Z., Smith, Z., Miao, J., Zang, M., Wu, S. Y., Chiang, C. M., et al., *SIRT1 deacetylates and inhibits SREBP-1C activity in regulation of hepatic lipid metabolism.* J. Biol. Chem. , 2010. **285**(44): p. 33959-33970.

183. Wei, X., Song, H., Yin, L., Rizzo, M. G., Sidhu, R., Covey, D. F., Ory, D. S. and Semenkovich, C. F., *Fatty acid synthesis configures the plasma membrane for inflammation in diabetes.* Nature, 2016. **539**(7628): p. 294-298.

184. Haskó, G. and P., P., *Regulation of macrophage function by adenosine.* Arterioscler Thromb Vasc Biol., 2012. **32**(4): p. 865-869.

185. DeOliveira, C. C., Paiva Caria, C. R., Ferreira Gotardo, E. M., Ribeiro, M. L. and Gambero, A., *Role of A1 and A2A adenosine receptor agonists in adipose tissue inflammation induced by obesity in mice.* Eur J Pharmacol. , 2017. **799**: p. 154-159.

186. Zhang, J., Fu, M., Cui, T., Xiong, C., Xu, K., Zhong, W., Xiao, Y., Floyd, D., et al., *Selective disruption of PPARg2 impairs the development of adipose tissue and insulin sensitivity.* Proc Natl Acad Sci USA, 2004. **101**(29): p. 10703-10708.

187. Ibrahim, A. S., El-shishtawy, M. M., Zhang, W., Caldwell, R. B., Liou, G. I. , *A2A Adenosine Receptor (A2AAR) as a Therapeutic Target in Diabetic Retinopathy. .* Am J Pathol. , 2011. **178**(5): p. 2136-2145.

188. Säve, S. and Persson, K., *Effects of Adenosine A(2A) and A(2B) Receptor Activation on Signaling Pathways and Cytokine Production in Human Uroepithelial Cells.* Pharmacology, 2010. **86**(3): p. 129-137.

189. Ohta, A. and Sitkovsky, M., *Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage.* Nature, 2001. **414**(6866): p. 916-920.

190. Scheibner, K. A., Boodoo, S., Collins, S., Black, K. E., Chan-Li, Y., Zarek, P., Powell, J. D. and Horton, M. R., *The adenosine a2a receptor inhibits matrix-induced inflammation in a novel fashion.* Am J Respir Cell Mol Biol, 2009. **40**(3): p. 251-259.

191. Csoka, B., Nemeth, Z. H., Selmeczy, Z., Koscso, B., Pacher, P., Vizi, E. S., Deitch, E. A. and Hasko, G., *Role of a(2a) adenosine receptors in regulation of opsonized e. Coli-induced macrophage function.* Purinergic Signal., 2007. **3**: p. 447-452.

192. Nemeth, Z. H., Lutz, C. S., Csoka, B., Deitch, E. A., Leibovich, S. J., Gause, W. C., Tone, M., Pacher, P., et al., *Adenosine augments il-10 production by macrophages through an a2b receptor-mediated posttranscriptional mechanism.* J Immunol, 2005. **175**: p. 8260-8270.

193. Ruiz-García, A., Monsalve, E., Novellasdemunt, L., Navarro-Sabaté, A., Manzano, A., Rivero, S., Castrillo, A., Casado, M., et al., *Cooperation of adenosine with macrophage Toll-4 receptor agonists leads to increased glycolytic flux through the enhanced expression of PFKFB3 gene.* J Biol Chem, 2011. **286**(22): p. 19247-19258.

194. Köröskényi, K., Duró, E., Pallai, A., Sarang, Z., Kloor, D., Ucker, D. S., Beceiro, S., Castrillo, A., et al., *Involvement of adenosine A2A receptors in engulfment-dependent apoptotic cell suppression of inflammation.* J Immunol, 2011. **186**(12): p. 7144-7155.