**Molecular Mechanisms Regulating Hepatic Fetuin-A Expression**

by

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Abstract

Fetuin-A (Fet-A) is a hepatokine, known to inhibit insulin signaling and is associated with diabetes, cardiovascular outcomes and inflammation. Post-translational modification of fetuin-A at different glycosylation and phosphorylation sites considered to regulate protein expression levels, stability, and biological activity. Previously, it has been suggested that phosphorylation status of Fet-A is critical to inhibit insulin receptor tyrosine kinase activity and downstream insulin signaling. However, certain factors related to its regulation are still unclear. Here we demonstrated that, recombinant Fet-A containing phosphorylation at the site of Ser312 impaired insulin signaling (AKT, MAPK, GSK-3β, and insulin receptor phosphorylation) and action on gluconeogenic enzyme (PEPCK gene expression) and glucose production in HepG2 cells. We have also shown that glucose and free fatty acid (palmitic acid) upregulate expression or secretion of Fet-A and its phosphorylated (pFet-A) forms in human hepatoma HepG2 cells. This upregulation of high glucose-induced Fet-A and pFet-A expression was associated with impairment of insulin signaling. On the other hand, insulin and AMP-activated protein kinase (AMPK) activation downregulate the high glucose induced Fet-A and pFet-A expression.

To further understand the negative regulation by AMPK activation, we examined the effect of AMPK activator, AICAR, on Fet-A expression in HepG2 cells, Hep3B cells and primary rat hepatocytes. We observed that treatment of AICAR, significantly down-

regulated high glucose-induced Fet-A expression without affecting pFet-A expression. Effect of AICAR was associated with activation of AMPK, while inhibition of AMPK activation prevented AICAR-induced downregulation of Fet-A expression. In further exploration of downstream target of AMPK, we observed that AMPK-p38 MAPK axis play a critical role in the regulation of the hepatic Fet-A expression. Further, we demonstrated that short term effect of AICAR on Fet-A expression was mediated by proteosomal degradation. While long term treatment of AICAR is associated with decrease in hepatic expression of C/EBP beta, an important transcription factor involved in Fet-A regulation.

Fet-A is classified as an acute phase protein, divergently regulated during injury and infection. Early inflammatory mediators negatively regulate, while late inflammatory mediators positively regulate Fet-A expression. To understand the differential effect on Fet-A during inflammation, we explore the effect of lipopolysaccharide (LPS) and tumor necrosis factor alpha (TNF-α) on Fet-A and pFet-A expression. Here we propose that Fet-A synthesis and secretion is upregulated with LPS treatment acutely (4hr). While, long term treatment with LPS downregulate Fet-A synthesis as described previously probably due to feedback regulation (24hr). On the other hand, treatment of TNF-α decreases hepatic Fet-A and pFet-A synthesis/secretion. This contradicting effect on Fet-A and pFet-A after 4hr treatment of LPS and TNF-α was observed due to increase and decrease of C/EBP-β expression respectively. Taken together, our study paves the way in understanding of the critical players involved in the regulation Fet-A, a hepatokine associated with insulin resistance, diabetes, and cardiovascular outcomes.

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List of Abbreviations

| | |
|---|---|
| AHSG | Alpha-2-Heremans Schmid glycoprotein |
| AMPK | Adenosine monophosphate-activated protein kinase |
| CVD | Cardiovascular disease |
| Fet-A | Fetuin-A |
| GLUT4 | Glucose transporter 4 |
| IR | Insulin receptor |
| IRS-1 | Insulin receptor substrate-1 |
| MAPK | Mitogen-activated protein kinase |
| GSK | Glycogen synthase kinase |
| pFet-A | Phosphorylated Fetuin-A (Ser312) |
| PI3K | Phosphatidylinositol 3-kinase |
| T2DM | Type 2 diabetes |
| LPS | Lipopolysaccharide |
| TLR-4 | Toll-like receptor-4 |
| TNF-α | Tumor necrosis factor-α |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| AICAR | 5-Aminoimidazole-4-carboxamide ribonucleotide |
| C/EBP-β | CCAAT/enhancer-binding protein beta |
| LPS | Lipopolysaccharide |
| HMGB1 | High mobility group box protein-1 |

# Chapter 1: Introduction

The metabolic syndrome is a cluster of interconnected physiological and biochemical risk factors that increases the probability of heart disease and other health problems, such as diabetes and stroke and thus an approximately 2-fold increase in mortality (1). Metabolic syndrome has been highlighted as a major socioeconomic problem throughout the world as the burden of metabolic syndrome along with its individual risk factors is evident throughout all ethnicities studied. Nearly 35 percent of all U.S. adults and 50 percent of those 60 years of age or older were estimated to have the metabolic syndrome in 2011-2012 (2). The major risk factors for developing metabolic syndrome are physical inactivity and a diet high in fats and carbohydrates, contributing to the central clinical features including insulin resistance, fatty liver, abdominal obesity, atherogenic dyslipidemia, hypertension, elevated plasma glucose, a prothrombotic state, and a proinflammatory state (3).

The present definition of metabolic syndrome does not include fatty liver disease, despite evidence supporting its association. Of note, non-alcoholic fatty liver disease (NAFLD) represents one of the most common liver diseases in the world and has shown a dramatic increase over recent years (4,5). NAFLD affects mainly patients with visceral obesity, dyslipidemia, insulin resistance or impaired glucose tolerance. These features are also included in the definition of the metabolic syndrome. Therefore, NAFLD is considered as a specific manifestation of the metabolic syndrome and has been identified as an independent risk factor for cardiovascular disease (CVD) (6,7). Although the etiology by

which NAFLD increases chronic disease risk remains debatable, insulin resistance appears to be a significant factor (8). Obesity is considered as the most common and prevalent cause of insulin resistance in humans, and the ubiquity of obesity in western populations is the biggest driver of the increasing occurrence of type 2 diabetes (9,10,11). In addition, chronic low-grade inflammation in insulin target tissues is a chief contributor to insulin resistance, and this reflects the interaction of pro-inflammatory immune cells, such as macrophages and lymphocytes, with insulin target cells, predominantly in adipose tissue and liver (12,13,14).

Recently, role of organokines, proteins presenting both paracrine and/or endocrine activities, has been paid more attention and well-studied. These proteins reach other organs where they subsequently exercise their effects. The most known and well-examined of them are adipokines (fat derived): e.g. adiponectin, leptin, resistin, retinol-binding protein 4, visfatin, chemerin, omentine and myokines (skeletal muscle-derived): e.g. irisin, interleukin-6, interleukin-15, myostatin, brain-derived neurotrophic factor, follistatin-related protein 1 (15). Similarly, scientists show how fatty liver, in analogy to the expanded adipose tissue mass, alters its secretion behavior and secretes proteins – termed hepatokines – in varying quantities into the bloodstream. Fetuin-A is considered as one of the most important hepatokines regulating human metabolism. Other important hepatokines include the angiopoietin-related protein 6 (16,17), sex hormone binding globulin (18), fibroblast growth factor 21 (19,20), selenoprotein P (21,22,23), insulin like growth factors, (24) insulin like growth factors binding proteins (24) and leukocyte derived chemotaxin 2 (LECT2) (25).

Fetuin-A, also known as alpha-2-Heremans-Schmid glycoprotein (64 kDa), is produced mainly in the liver (26). This phosphorylated glycoprotein was discovered in 1944 in bovine calves by Pederson and named it “Fetuin”. Later on, second member of the fetuin family was discovered (Fetuin B) and the original fetuin was named as Fetuin-A (27). Structurally fetuin-A belongs to the cystatin super family consist of two amino terminals cystatin-like domains and carboxyl terminal (28, 29). Moreover, fetuin-A consist of binding sites for transforming growth factor-β (TGF-β) super family and calcium featuring its role in bone metabolism and mineralization (28).

In humans, fetuin-A gene is located on chromosome 3q27, which was identified as susceptibility locus for type 2 diabetes and metabolic syndrome (28). Due to which very recently its role in metabolic and cardiovascular disorders has been explored and established. Fetuin-A directly bind with the beta chain of insulin receptor and act as a natural inhibitor of the insulin receptor tyrosine kinase, leading to insulin resistance in rodents (30). Apart from its direct effect, fetuin-A promote insulin resistant by propagating proinflammatory state. Fetuin-A act as an endogenous ligand of TLR-4 to promote lipid-induced insulin resistant (31). Also, fetuin-A reduced adiponectin expression and increases inflammatory cytokine expression in adipocytes and monocyte (32). Additionally, fetuin-A act as an efficient chemokine involved in macrophage migration and polarization from anti-inflammatory M2 to proinflammatory M1 subtype significantly contributes to develop a link between inflammation and insulin resistance (33).

Expression, stability and biological activity of fetuin-A considered to be regulated by posttranslational modification at different glycosylation and phosphorylation sites.

Phosphorylation of fetuin-A suggested as critical for inhibition insulin receptor auto-phosphorylation (34). Though, regulation of fetuin-A and its phosphorylated forms in relation to insulin resistance is not completely understood. Fetuin-A is considered as acute phase protein, divergently regulated in injury versus infection, and acute versus chronic low-grade chronic inflammation. Fet-A gene expression is down regulated by tumor necrosis factor-α (TNF- α), IL-6, and IL-1β in rat or human hepatocytes (35, 36). In contrast, other late inflammatory mediators including high-mobility group protein-1 (HMGB1) increases Fet-A expression, suggesting that cytokines differently regulate hepatic Fet-A expression (37). However, there is a significant gap in our knowledge of the mechanisms underlying this differential regulation of Fet-A. In addition, regulation of phosphorylated forms of fetuin-A during inflammation is not known.

In contrast, glucose and free fatty acid driving the upregulation of Fet-A by ERK1/2 and NF-kB pathway respectively (38,39). While, glucocorticoid including dexamethasone increases fetuin-A expression by C/EBP beta, a transcription factors responsible for basal level of fetuin-A (40). In addition, estrogen and endoplasmic reticulum stress also found to increases fetuin-A expression (38,41). However, status of pFet-A expression by these factors is not known. Also, there are very limited study regarding negative regulation of fetuin-A. Previously, it has been shown that pioglitazone (antidiabetic compound) decreases fetuin-A level, while metformin treatment or exercise for six month have no impact on it in patients with type 2 diabetes (42). Same group also observed that pioglitazone but not metformin inhibits fetuin-A expression in FAO cells and its effect is mediated through direct activation of PPAR-γ (43). On contrary, Haukeland *et al* have shown decrease in fetuin-A secretion in NAFLD patients and in HepG2 cell line after

treatment with metformin (44). Fetuin-A levels also decreased significantly with short term exercise in patients with NAFLD and is associated with improvement of glucose tolerance and insulin sensitivity (45). In addition, salsalate, adiponectin and exendin-4 have been shown to suppress palmitate-induced fetuin-A expression through AMPK (46,47). However, mechanisms of fetuin-A regulation by AMPK is not known. So, our main purpose of study was to,

1. **Characterize effects of metabolic mediators on regulation of hepatic fetuin-A and its phosphorylated forms in relation to insulin resistance.**
2. **Determine regulatory pathways involved in AMPK activator, AICAR-induced downregulation of hepatic Fetuin-A expression.**
3. **Analyze effects of AICAR treatment on lipopolysaccharide-induced hepatic Fetuin-A expression**

# Chapter 2: Review of Literature

## 2.1 Metabolic Syndrome

### 2.1.1 Definition and epidemiology

Metabolic syndrome become a major public health concern worldwide, in that it is considered to be the etiology of the current epidemic of diabetes and cardiovascular disease. Initially, highland *et al* first described the cluster of hypertension, hyperglycemia and gout (48). Thereafter, in 1979 association of obesity, diabetes and cardiovascular disease has been reported (48). Later, in 1988, Reaven *et al* described insulin resistant as a central pathophysiological feature in the cluster of metabolic syndrome (49). Afterward, over a period of time, different definitions of the metabolic syndrome have been proposed but all center around the metabolic abnormalities of central obesity, hypertension, decreased high-density lipoproteins and elevated triglycerides with insulin resistance as the uniting physiologic factor (50).

In 1998, WHO first define the metabolic syndrome suggesting insulin resistance as an absolute requirement in the pathophysiology of metabolic syndrome indicated by impaired fasting glucose or impaired glucose tolerance, high HOMA-IR value or glucose infusion rate during euglycemic hyperinsulinemic clamp (51). In 1999, the European Group for the Study of Insulin Resistance (EGIR) simplify the measure of insulin resistance by fasting plasma insulin value (52). They also simplified obesity criteria to waist circumference from waist to hip ratio or body mass index (52). Later, in 2001 National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III)

devised the metabolic syndrome as the presence of any of the three criteria from waist circumference over 40 inches (men) or 35 inches (women), blood pressure over 130/85 mmHg, fasting triglyceride (TG) level over 150 mg/dl, fasting high density lipoprotein (HDL) cholesterol level less than 40 mg/dl (men) or 50 mg/dl (women) and fasting blood sugar over 100 mg/dl (53). In 2005, the International Diabetes Foundation (IDF) published new criteria proposes that obesity but not insulin resistance necessarily be present (54).

Data from National Health and Nutrition Examination Survey (NHANES) observed reduction in age adjusted prevalence from 25.5% to 22.9% from 1999/2000 to 2009/10 in United States (55). While, wong et al observed increased in overall prevalence of the metabolic syndrome increased from 32.9% (95% CI, 31.6%- 34.2%) in 2003-2004 to 34.7% (95% CI, 33.5%-36.0%) in 2011-2012 according to NHANES data (2). Trends from 2007-2008 to 2011-2012, overall prevalence of the metabolic syndrome remained stable from 36.1% in 2007-2008 to 34.7% in 2011-2012 (2).

### 2.1.2 Genetics and environmental factors

Appreciation of genetic component in the pathophysiology of metabolic syndrome (MetS) was first evidence with family and twin studies. For example, heritability estimates from the Northern Manhattan Family Study were 46 % for waist circumference, 24 % for fasting glucose levels, 47 % for triglyceride levels, 60 % for HDL cholesterol, and 16 and 21 % for systolic and diastolic BP (56). These evidences prompted the search of gene variants for the MetS. Till now, many approaches have been used including multiple candidate gene association studies, linkage studies, and more recently by genome-wide association (GWA) studies to search genetic determinant (57). Linkage study

demonstrated a quantitative trait locus on chromosome 3q27 strongly associated with six metabolic syndrome-related traits (58, 59). Although several candidate genes regulating primarily lipid metabolism, adiposity, or insulin resistance have been found to be associated with metabolic syndrome, they provide so far only a limited evidence for common genetic background explaining the clustering of metabolic traits.

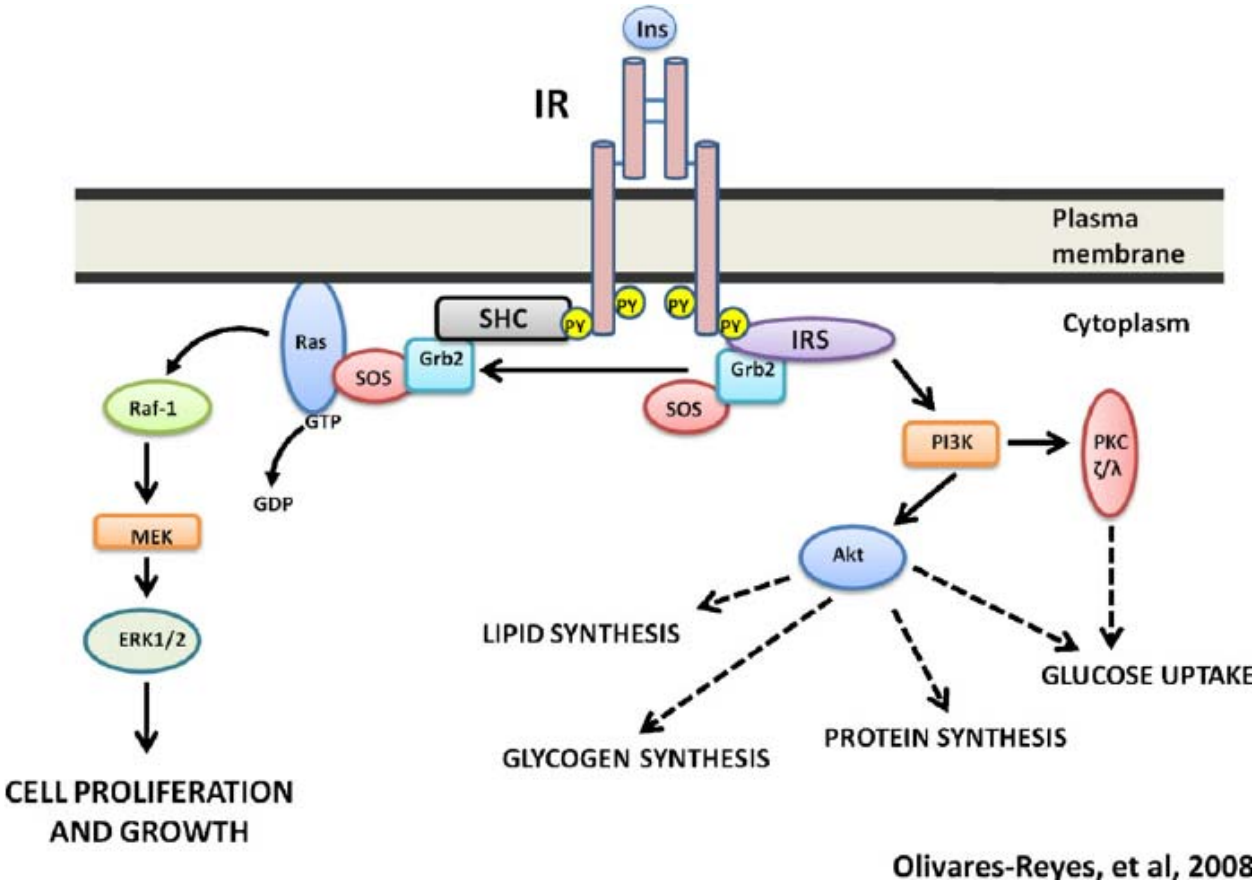
Recently, apart from genetic factors contribution of environmental factors is also much appreciated to the susceptibility and causation of multifactorial diseases including MetS (60). Environmental factors notably via diet modulate multiple gene responsible for obesity, lipid levels, and markers of inflammation and increases the risk for developing MetS, diabetes, and CVD (60). There are many susceptible gene interactions with environment have been identified, but major challenge is to translation of this knowledge into practical public health applications.

### 2.1.3 Insulin signaling

As insulin resistant play a significant role in the pathophysiology of metabolic syndrome, insulin signaling cascade have been elucidated to a good extent. The action of insulin initiated with binding to plasma membrane receptor, and activation of its intrinsic protein tyrosine kinase activity, resulting in phosphorylation of tyrosine residues. This promotes phosphorylation and recruitment of different substrate adaptors, including the IRS family of proteins. Among these, IRS1 and IRS2 appear to be the adapter molecules playing a major role in the coupling to the PI3K-AKT and MAPK downstream kinases (61). Tyrosine phosphorylated IRS1/2 recruit the heterodimeric p85/p110 PI3K at the plasma membrane, where it produces the lipid second messenger PIP3. PIP3 activates a serine/threonine phosphorylation cascade of PH-domain containing proteins which

include PDK1, PKB and aPKCs. Major targets of activated PKB are GSK-3 and AS160. PKB mediated inactivation of GSK-3 by phosphorylation leads to glycogen synthesis (61). PKB by means of AS160 regulates the insulin-stimulated translocation of the glucose transporter GLUT-4 at the plasma membrane, resulting in increased glucose uptake. On a parallel pathway, activated IRS1/2 recruit Grb2, which associates to SOS and activates the Erk1/2 MAPK pathway. Insulin mediated activation of MAPK pathway mainly involve in cell growth and differentiation (62).

**Figure 1**




**The insulin signaling pathway.** The insulin binds to insulin receptor (IR) at alpha subunit leads to autophosphorylation on the β subunit of IR and the Tyr phosphorylation of insulin receptor substrate (IRS) proteins and other signaling molecules such as Shc.

Phosphorylated IRS serve as docking proteins for other signaling proteins, such as PI3K and Grb2. PI3K binding to IRS-1 at phosphotyrosine residue induces its activation and the initiation of a downstream cascade of events leading to the phosphorylation and activation of Akt and aPKC. Activation of theses downstream effectors appears to be important for glucose transport, protein, glycogen, and lipid synthesis, whereas activation of Grb2 dependent of Shc, leads to activation of MAPK signaling pathways that control cell proliferation and growth (63).

## 2.2 Organ Crosstalk in metabolic syndrome

### 2.2.1 Adipose tissue and metabolic syndrome

Traditionally, white adipose tissue known to responsible for heat insulation, mechanical cushioning, and storage site for fat in the form of triglycerides (64). However, this view has been dramatically changed with the recognition of the adipose tissue as a key endocrine organ (65). Adipose tissue secretes active endocrine, paracrine and autocrine substances in response to different stimulus (65). Adipose tissue secreted proteins (Adipokines) including leptin and adiponectin involve in different homeostatic functions. Leptin is a signal emitted by adipose tissue and act as a anorexigenic, reduces intracellular lipids and improves insulin sensitivity (by limiting food intake), inhibits glucocorticoids and enhances T4, sex and growth hormones (66). While, adiponectin increases fat deposition, increases fatty acid oxidation in skeletal muscle but promote glucose utilization, reduced hepatic glucose production, resulting in global increase in insulin sensitivity (67). Apart from these, other Adipokines (resistin, visfatin etc) have been identified, an increase in their levels or a resistance to its signal was suggested as the cause of the MetS (68).

### 2.2.2 Skeletal muscle and metabolic syndrome

Skeletal muscle also plays an important role in the pathophysiology metabolic syndrome. This has been much appreciated with the observation that increase in 171% chance of obesity of one friend, if his/her friend become obese in given period of time. This mainly influence by physical inactivity, coin the term “diseasome of physical inactivity”. Importantly physical inactivity associated with systemic inflammation independent of obesity (69). It mainly due to physical inactivity increases abdominal fat (Visceral fat) which is consider detrimental as it is associate with cardiovascular disease, type 2 diabetes and other diseases. Increase in visceral fat due to physical inactivity leads to chronic systemic inflammation caused by infiltration of macrophage in visceral fat (70). Chronic inflammation promotes development of insulin resistance, atherosclerosis, neurodegeneration and tumor growth, and thereby the development of the diseases belonging to the ‘diseasome of physical inactivity’ (69). While many studies have demonstrated the benefits of exercise in preventing all-cause mortality, including cardiovascular disease, metabolic disease, and cancer (71). These exercise-induced benefits are well known to prevent harmful effects of proinflammatory adipokines through skeletal muscle-secreted proteins (myokines). Myokines likely provide beneficial metabolic effects during crosstalk between skeletal muscle and liver, and skeletal muscle and adipose tissue (72). First myokine identified was interleukin (IL)-6 play an anti-inflammatory role by inhibiting TNF-alpha and also improves glucose uptake by stimulating AMP-activated protein kinase (AMPK) signaling. Other exercise-induced myokine which have potential positive effect on metabolic diseases include IL-15, brain-

derived neurotrophic factor (BDNF), leukemia inhibitory factor (LIF), irisin, fibroblast growth factor 21 (FGF-21), and secreted protein acidic and rich in cysteine (SPARC) (72).

### 2.2.3 Brain and metabolic syndrome

Recent advances revive the interest and suggest that a full understanding of the pathogenesis of these disorders must incorporate a role for the brain in metabolic regulation. In case of excess availability of food and ample fat stores, input to key brain areas from afferent signals leads to inhibition of both energy intake and endogenous glucose production, while simultaneously increasing energy expenditure and mobilizing fat stores (73). Conversely, deficiency of stored energy or nutrient availability decreases neuronal input from one or more of these afferent signals and alert the brain to activates responses that promote positive energy balance (increased food intake and decreased energy expenditure) and raise circulating nutrient levels (increased hepatic glucose production) (73). imbalance in this homeostatic system will result in elevated levels of both body fat content and hepatic glucose production predicted to cause weight gain and insulin resistance. In addition, recent finding suggest that brain insulin action is required for intact glucose homeostasis and chronic blockade of hypothalamic insulin receptor signaling was shown to cause hepatic insulin resistance and to increase hepatic glucose production (74, 75). So, progress in understanding and treating diabetes will require an improved understanding of brain systems that control body fuel homeostasis and energy storage.

### 2.2.4 Liver and metabolic syndrome

The liver is known to play an important role in the ongoing epidemics of type 2 diabetes mellitus and cardiovascular disease. Liver diseases mainly non-alcoholic fatty liver

disease (NAFLD) increasingly recognized as a major health burden and its prevalence is estimated to be 20-30% in western population (76). Epidemiological studies indicate that NAFLD may be the hepatic manifestation metabolic syndrome with the insulin resistance as a key pathogenic factor (77). Mainly, the liver is involved in increased glucose production and dysregulated lipoprotein metabolism, conditions that are often found in patients with nonalcoholic fatty liver disease. Recent studies suggested that liver may regulate glucose homeostasis by modulating the sensitivity/resistance of peripheral tissues, by way of the production of secretory proteins, termed hepatokines (78). The first hepatokine that has been proven to have a major pathogenic role in metabolic diseases is α2-HS-glycoprotein (fetuin-A). Fetuin-A strongly associated with insulin resistant, type 2 diabetes and cardiovascular diseases (24).

## 2.3. Fetuin-A

### 2.3.1 Structure and regulation

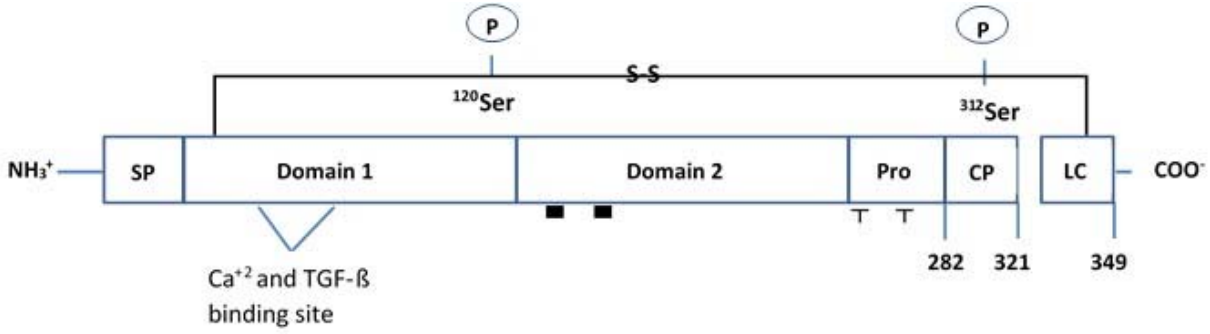
Bovine fetuin-A was first described in 1944 by Pedersen as fetuin (as it found maximum in fetus), the most abundant globular plasma protein in fetal calf serum (26). It is renamed as fetuin A after the discovery of a second fetuin, called fetuin B (27). Fetuin-A, also known as a2-Heremans–Schmid glycoprotein, is an abundant serum protein produced predominantly in the liver (27). The human fetuin-A gene, designated AHSG, resides on chromosome 3 (3q27) and contains seven exons and six introns, spanning an approximately 8.2-kb region (28). Fetuin-A belongs to the cystatin super family of cysteine protease inhibitors and possesses cystatin-like amino-terminal and carboxyl terminal domains (29). However, it lacks cysteine protease-inhibitory capacity because of several post-translational modifications including N-glycosylation, O-glycosylation,

proteolytic processing and phosphorylation on serine residues (79). Human fetuin-A is processed from a single chain precursor to the mature circulating heavy two chain form in which N-terminal heavy chain (312 amino acid residue) is bonded to C-terminal light chain (27 amino acid residue). Further modification and subsequent removal of connecting peptide convert heavy chain into A chain (281 amino acids residue) Fig. (2) (80). Several proteases including chymotrypsin and matrix metalloproteinases was reported to cleave connecting peptides (81, 82). These modifications may regulate protein expression levels, stability, and biological activity. However, process of post-translational modifications is not fully understood. Recently it has been observed that fetuin-A is proteolytically processed by matriptase-2 into a two-chain form, and that the two-chain form lacks the hepcidin expression enhancing activity of the single-chain form (83). In addition, binding site of $Ca^{+2}$ and transforming growth factor-ß (TGF-ß) is also located within domain 1 of fetuin-A (Fig. 2) (84). Fetuin-A shares amino-acid sequence similarity to insulin receptor tyrosine kinase (85) and type-II transforming growth factor-ß receptor (84), and thus been proposed as a natural inhibitor of the insulin-signaling pathway and an antagonist of TGF-ß. As a glycoprotein, fetuin-A carries two N-linked and three O-linked oligosaccharide chains that terminate with sialic acid residues, enabling the binding of cationic $Ca^{2+}$ ions and other anti-inflammatory molecules (e.g. spermine). Accordingly, it has been considered that fetuin A has protective role as an inhibitor of pathological mineralization/calcification, and in inflammatory response including cerebral ischemic injury, endotoxemia and sepsis (86, 37, 87).

During fetus development expression of fetuin A is similarly distributed in several tissues while after birth, fetuin-A is mainly synthesized and secreted from liver and transported

into the target organs (88). However, a recent study in adult human's samples shows, fetuin-A is also produced locally by osteoblasts (89) and adipocyte (33), not only by hepatocyte. Acute inflammation and other types of infection down regulate the expression of fetuin-A and hence, it has been classified as one of the acute-phase proteins (APP). Fetuin-A gene expression is down regulated in rat liver hepatocytes by tumor necrosis factor-α (TNF- α) (90) and by other pro-inflammatory cytokines like IL-6, and IL-1β in human hepatocytes (35). In contrast, fetuin-A gene expression in hepatocytes is up-regulated by high glucose and free fatty acids through ERK1/2 and NFkB respectively or by increase in endoplasmic reticulum (ER) stress (38, 39). In addition, glucocorticoid and estrogen is also found to increase the fetuin-A expression by C/EBP beta and activator protein-1 respectively (40, 41). However, regulation of glycosylated or phosphorylated forms of fetuin-A is not fully known.

**Figure 2**




**Protein structure and posttranslational modification of site of fetuin-A** : N-Linked glycosylation sites, ⊤ :O-Linked glycosylation sites. SP: signal peptide, CP-connecting peptide, LC: light chain, pro: proline rich peptide.

### 2.3.2 Fetuin-A and insulin signaling

Originally Auberger *et al*. provided the first evidence for the role of fetuin-A in insulin signaling. They indicate that phosphorylated N glycoprotein called pp63 secreted from hepatocytes and inhibit insulin receptor tyrosine kinase and receptor autophoshorylation while non-phosphorylated form of protein is inactive, although the protein they isolated was not recognized as fetuin at the time (91). Subsequently, Muller-Esterl *et al.* reported the sequence similarity of protein pp63 with rat fetuin-A which inhibit the insulin-stimulated phosphorylation of IR and IR substrate-1 (IRS-1) (93). In addition, other groups have shown that purified bovine (84) and human fetuin-A (85) also have similar effect indicating common inhibitory function for fetuin-A homolog on insulin signaling. Furthermore, Mathews *et al*. demonstrated that inhibitory action of recombinant fetuin-A on the insulin-stimulated phosphorylation of IR and IRS-1 which is mediated by direct interaction between fetuin-A and IR (30). They also show that fetuin-A did not affect epidermal growth factor (EGF) or insulin-like growth factor I-induced cognate receptor auto-phosphorylation, suggesting the relative specificity of fetuin-A for IR. Although, the precise site of fetuin-A and IR interaction has not been defined, fetuin-A completely inhibited trypsin-activated IR auto-phosphorylation suggesting that fetuin-A does not compete with insulin for binding to IR (30, 94). Recently, fetuin-A also shown to impairs insulin-mediated glucose uptake in C2C12 myotubes by down-regulating GLUT-4 translocation to the plasma membrane through decreased in phosphorylation of Akt and AS160 (45). Moreover, injection of fetuin-A inhibited insulin-stimulated IR auto-phosphorylation and IRS-1 phosphorylation in rats (30) and knocking down of fetuin-A improved the insulin signaling in HFD induced obese mice (31) indicating the physiological relevance of *in vitro* observations. It has been suggested that

phosphorylation is essential for fetuin-A interaction with the insulin receptor. Although the protein kinase(s) catalyzing phosphorylation in fetuin-A have not yet been identified, protein kinase CK2 could be a likely candidate. However, in vivo relevance of these phosphorylation and physiological significance of other proteolytic modifications in relation to insulin signaling is needs to be explored.

### 2.3.3 Fetuin-A and metabolic disease

Fetuin-A was initially described as a growth factor in fetal calf serum (26) and was later identified as an important inhibitor of ectopic calcification (95). More recent studies, however, have shown that fetuin-A could also play a role in the development of the metabolic syndrome. Fetuin-A knockout mice exhibit increased glucose tolerance and insulin sensitivity and are resistant to diet- induced obesity, NAFLD, and age-associated insulin resistance (96). On the other hand, administration of fetuin-A induced the cytokine expression and represses the adiponectin expression indicating the implication of fetuin-A in metabolic disease. Fetuin-A expression and secretion was shown to be up-regulated in liver of high-fat diet induced obese animals (39), patients of NAFLD (44) and exercise and dietary life style interventions results in declines in serum fetuin-A levels commensurate with improvement in NAFLD and decline in body weight (97). Plasma fetuin-A levels was also shown to be associated with gestational diabetes (98), newly onset type 2 diabetes (99), obesity and other complications (100). Recently, it has been demonstrated that short term exercise in NAFLD patients decrease the fetuin-A level without change in hepatic steatosis and body weight (45). Also, Hepatic expression of fetuin-A correlated with key enzymes in glucose and lipid metabolism and status of glucose intolerance and insulin resistance (44). Treatment of fetuin-A to human

adipocytes derived from fat biopsies leads to reduced mRNA expression levels of adiponectin providing evidence that fetuin-A modulates adipocyte function (32). They also found that treatment of mice with fetuin-A, analogously, resulted in a marked increase in adipose tissue tumor necrosis factor (TNF) mRNA as well as interleukin 6 (Il6) expression, accompanied by a decrease in adipose tissue adiponectin mRNA expression and lower circulating adiponectin levels (32). This is also supported by *in vitro* study which shows that fetuin-A is taken up by adipocyte in a calcium dependent manner and affects the cytokine and adiponectin expression (39). In addition, fetuin-A found to diminished lipid uptake, adipogenic factors and insulin signaling in adipocyte suggesting the severe impairment of adipocyte function (39). These data suggest the important role of fatty liver in regulation of hepatokine and their crosstalk with other organs in the pathophysiology of metabolic diseases.

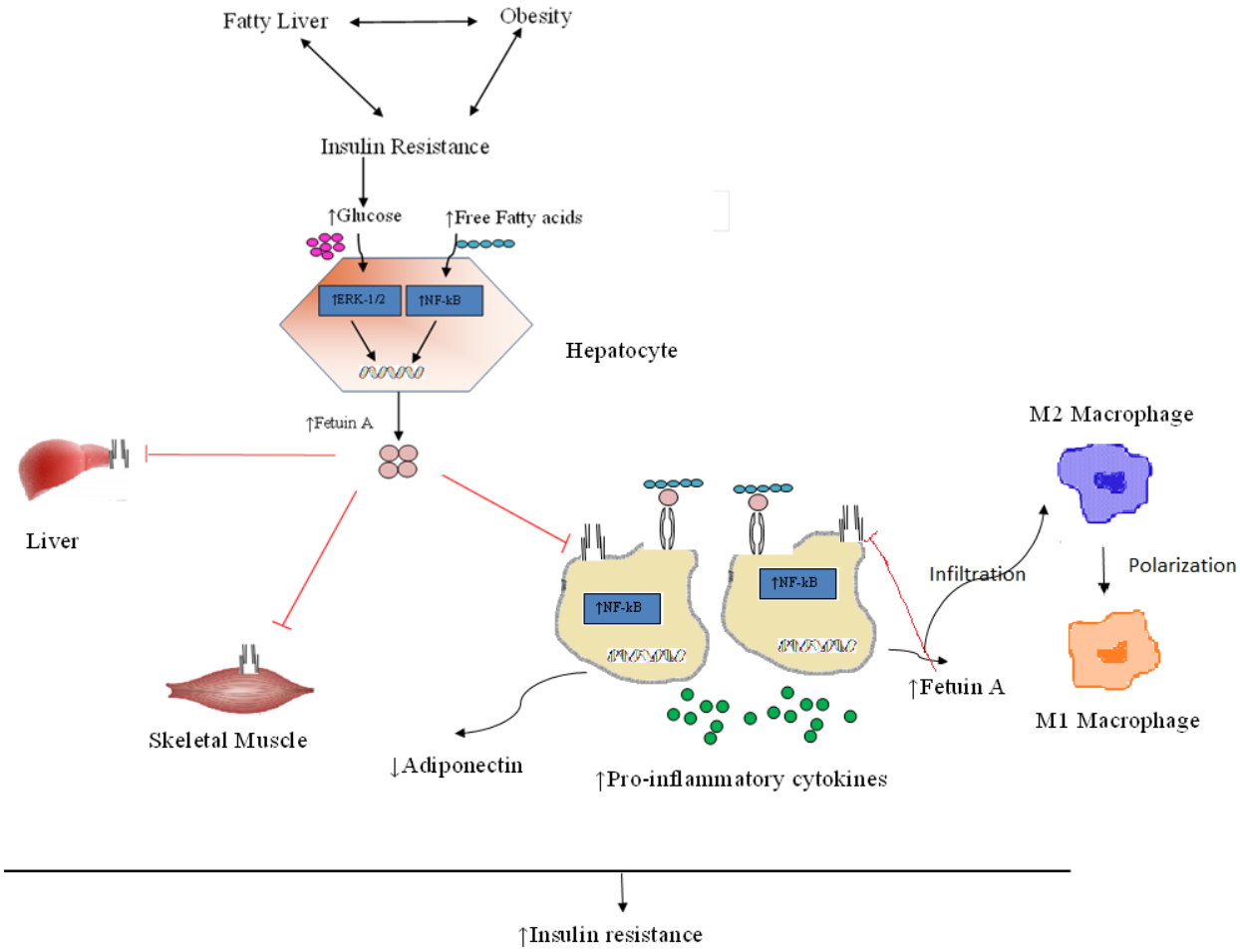
Along with insulin resistance, persons with the metabolic syndrome often have a low-grade systemic inflammation. The new studies by Pal *et al* suggest that fetuin-A act as an endogenous ligand for TLR4 through which lipids induce insulin resistance (31). The authors showed that Knockdown of TLR4 or fetuin-A in obese insulin- resistant mice dramatically improved glucose homeostasis as a result of reduced activation of TLR4-mediated pro-inflammatory signaling cascades. Most convincingly, fetuin-A knockdown mice was devoid of saturated fatty acid (SFA) infusion induced insulin resistance as compared in control mice. The author's findings that fetuin-A knockdown improved glucose homeostasis to a similar degree as Tlr4 knockdown and that fetuin-A was necessary for SFA-dependent inflammatory gene expression suggested an association between SFAs, Fetuin-A and Tlr4 (31, 101). *In vivo* relevance of this observation in

human was established by Norbert et al. by anthropometric and metabolic data from 347 healthy subjects who are at increased risk for type 2 diabetes and CVD (102). They observed strong interaction between FFAs and fetuin-A concentrations to induce insulin resistance but not between adiponectin and FFA (102). Recently, Bhattacharya *et al* reported that fetuin-A is also synthesize and secreted from adipocyte and involved in lipid induced macrophage migration and M2 to M1 polarization into adipose tissue (33). This suggest that the fetuin-A–fatty acid complex induces inflammatory signaling and insulin resistance, important driving forces behind type 2 diabetes and cardiovascular disease (CVD). On the contrary, Dorit S *et al* examined the effects of 28-day overfeeding resulted in significant elevations in circulating levels of fetuin-A correlate with increases peripheral insulin resistance in humans (103). However, this effect is independent of change in FFAs (103) suggesting involvement of some other factors apart from interaction of FFA and fetuin-A in induction of insulin resistance.

Further correlation of fetuin-A and metabolic disease has been outline by identification of metabolic drug which decrease fetuin-A level. Mori *et al* found that pioglitazone decrease serum fetuin-A level as compared to metformin or exercise treatment for 6 months in patients with type 2 diabetes (42). While Haukeland *et al* have shown decrease in fetuin-A secretion in NAFLD patients and in HepG2 cell line after treatment with metformin (44). In addition, salsalate is also found to improved palmitate-induced steatosis and impairment of lipid metabolism in hepatocytes via fetuin-A inhibition through the AMPK-NFkB pathway (46). These data indicate the suppression of fetuin-A production in the liver may be a novel therapeutic target for reducing insulin resistance in type 2 diabetes. However, Jensen MK *et al* increase the possibility that the association between

fetuin-A and type 2 diabetes may not be causal as common variants in the *AHSG* gene are strongly associated with plasma fetuin-A concentrations, but not with risk of type 2 diabetes or glucose concentrations (104).

**Figure 3**




**Role of fetuin-A in metabolic disease**. Metabolic dysregulation of liver due to obesity, fatty liver and insulin resistance leads to increase in plasma glucose and free fatty acid level. Glucose and fatty acid increase the synthesis and secretion of hepatokine fetuin-A by ERK-1/2 and NF-kB respectively. Secreted fetuin-A will inhibit the insulin signaling in liver, skeletal muscle and adipocyte. In addition, fetuin-A act as an adaptor protein to fatty acid and activate the TLR4 induced inflammatory signaling. Furthermore, fatty acid

also secretes fetuin-A from adipocyte which can act as chemokine and increase the infiltration and polarization of macrophage to further aggravate the lipid induced inflammation.

### 2.3.4 Fetuin-A and cardiovascular disease

In addition to the regulation of insulin signaling, fetuin-A also acts as a physiological calcification inhibitor shown in *in vitro* calcification system and cell-free salt precipitation assay (105). This was further reinforced by *in vivo* study in which, fetuin-A knockout mice developed severe calcification of various organs when the mice were placed on a diet rich in minerals and vitamin D, or on normal diet when the genetic deficiency was combined with a DBA/2 genetic mouse background with propensity for mineralization. Furthermore, offspring from fetuin-A deficient mouse were suffered from severe ectopic calcification in almost every organ, especially skin, kidney, and testis when backcrossed to the calcification susceptible DBA/2 strain (101). On the other hand, over-expression of fetuin-A can prevent ectopic mineralization of connective tissues in a mouse model of pseudoxanthoma elasticum (PXE) developed by targeted ablation of the Abcc6 gene (106). Mechanistically, independent groups revealed that protective effect is mainly by forming soluble mineral complexes, consisting calcium, phosphate and fetuin-A, termed them calciprotein particles (CPPs) (34, 107). Recently, it has been confirmed by animal study that transiently soluble CPPs mediated the transport and clearance of potentially harmful calcium phosphate mineral in the body (108). In addition to act as inhibitor of ectopic calcification, fetuin-A also involve in preventing spontaneous mineral precipitation in the vasculature (109). Since, vascular calcification and low grade inflammation is the main non-traditional risk factor for cardiovascular disease induced

mortality rate (110), physiological function of fetuin-A is consider as important for cardiovascular health.

Meta-analysis study by Xie *et al* indicated that decreased serum fetuin-A level is correlated with the development of CVDs and fetuin-A might be clinically valuable for reflecting the progression of CVDs (111). In addition, polymorphism at Fetuin-A 742 (C/T) and 766 (C/G) sites was associated with risk of myocardial infarction in patients older than 40 years of age (112). It has been reported that low fetuin–A levels are associated with malnutrition, inflammation and atherosclerosis as well as with increased cardiovascular and other mortality causes (113). Different studies have shown that serum fetuin-A is regulated as a negative acute phase protein and its serum concentration falls during the acute inflammatory response and normalizes when the infection is successfully treated. Decreased in fetuin-A level will predisposed patients to vascular calcification and are significantly linked to all causes of cardiovascular mortality (114, 115, 116). Prospective study from Tarık C *et al* demonstrate the usefulness of a single random CRP and fetuin-A determination in predicting subclinical inflammation, cardiovascular mortality and vascular calcification in peritoneal dialysis patients (117). Also other independent group demonstrated that lower fetuin-A levels are independently correlated with coronary artery calcification in older adults with normal kidney function and no known CVD (114) and with atrial stiffness in patients without type 2 diabetes (116). In addition, a descriptive study in hemodialysis patients shows that a decrease in serum Fetuin-A is significantly associated with development and progression of atherosclerosis in hemodialysis patients and related to Calcium-Phosphorus Homeostasis indicating its protective role for cardiovascular system (118). On the contrary Devuyst O *et al* showed

that circulating fetuin-A levels in renal transplant recipients are determined by variants in the ASHG gene, plasma cholesterol levels, and a history of smoking, independently of inflammation suggesting that a gene–environment interaction regulates circulating fetuin-A levels in this population (119). This suggest the possibility that fetuin-A levels may be determined by inflammation- independent mechanisms, including genetic factors.

Conversely, other studies have reported that high plasma Fetuin-A level is related to an increased risk of myocardial infarction and ischemic stroke, especially in patient with metabolic syndrome or diabetes (120). In community-dwelling individuals, low plasma fetuin-A levels are independently associated with increased risk of CVD mortality among men and women without diabetes, but with reduced risk of CVD death in those with diabetes (121). This is supported by calorie restriction study in obese patients with type 2 diabetes and in obese animal's which shows decreases hepatic fetuin-A expression associated with improving both hepatic steatosis and cardiovascular risk factors (122). Furthermore, for cardiovascular outcomes fetuin-A adds prognostic value among patients with coronary artery disease with moderate calcification (123). These results suggest the relationship of fetuin-A to cardiovascular health is more complex than previously thought. Whether it acts to promote or protect against cardiovascular disease remains unknown. This resembles a double-edged sword in that a low level of fetuin-A increases vascular calcification, but a high level leads to plaque instability and cardiovascular events (123). In addition, prospective and cross-sectional study by Dogru T *et al* demonstrated that circulating fetuin-A in NAFLD is independently associated with endothelial dysfunction and subclinical atherosclerosis (124). Since, atherosclerosis is strongly associated with inflammation which can be effectively counteracted by anti-inflammatory action of

fetuin-A, then this protein may have the potential for prevention and/or retardation of atherogenesis in various diseases, including chronic renal failure. These dual physiological roles of fetuin-A indicate the sufficiency may be necessary to prevent vascular calcification, but fetuin-A excess may lead to insulin resistance and metabolic dysregulation. So, future studies, with larger populations, are required to determine whether measurement of plasma fetuin-A will be useful as a CVD risk stratification tool and whether prediction criteria will differ for those with and without diabetes.

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# Chapter 3: Effects of metabolic mediators on regulation of hepatic fetuin-A and its phosphorylated forms in relation to insulin resistance

## 3.1 Abstract

Fetuin-A (Fet-A) is a liver secreted protein, implicated in metabolic abnormalities including insulin resistance and Type 2 diabetes. Previously we and others suggested that phosphorylation status of Fet-A (pFet-A) is critical to inhibit insulin receptor tyrosine kinase (IR-TK) activity and thus it is associated with insulin resistance. However, regulation of Fet-A and its phosphorylated form by mediators associated with metabolic diseases is not completely understood. Here, we examined the effects of Fet-A treatment on insulin signaling and that of glucose, saturated fatty acid, 5' AMP-activated protein kinase (AMPK) activation and oxidative stress on Fet-A and pFet-A status. High glucose (25mM) increased Fet-A and phosphorylated (Ser312) fetuin-A (pFet-A) expression after 6hr and 12hr of treatment independent of change in osmotic pressure due to high glucose treatment. Interestingly high glucose increases pFet-A secretion but not Fet-A secretion as compare to low glucose (5mM). High glucose-induced increase in Fet-A and pFet-A level is associated with impaired insulin signaling as indicated by inhibition of insulin induced AKT and GSK-3 phosphorylation in human hepatoma HepG2 cells. Similarly, palmitic acid also increases Fet-A and pFet-A expression in HepG2 cells. On the other hand, activation of AMPK by AICAR downregulate high glucose-induced Fet-A expression without affecting pFet-A expression. In addition, treatment with insulin or by

induction of oxidative stress by hypoxia/hydrogen peroxide decreases high glucose-induced Fet-A and pFet-A expression. This study clearly showed complex regulation of Fet-A and pFet-A by mediators associated with metabolic diseases.

### 3.2 Introduction

Metabolic syndrome is defined as a cluster of risk factors, such as central obesity, insulin resistance, dyslipidemia and hypertension that together culminate in the increased risk of type 2 diabetes mellitus and cardiovascular disease (1). Rapid changes in the definition over time make it very difficult to evaluate temporal trends and regional variations in the prevalence of the metabolic syndrome. In addition, use of different criteria by diverse definitions changes the prevalence of metabolic syndrome based on regional differences, ethnicity, ageing and gender. In 2011-12, nearly 35% of the U.S. adults and 50 percent of those 60 years of age or older population estimated to have metabolic syndrome (2).

The major risk factors for developing metabolic syndrome are physical inactivity and a diet high in fats and carbohydrates, contributing to the two central clinical features, i.e. central obesity and insulin resistance (IR) (3). In addition, insulin resistance plays a major role in the development of various diseases such as cardiovascular diseases and nonalcoholic steatohepatitis that can impair insulin action, promotes hepatic glucose production and reduces glucose uptake by peripheral tissues (4). However, it is now well accepted that abnormal secretion of tissue-derived factors such as adipokines, myokines, and hepatokines influences insulin resistance.

Fetuin-A (encoded by AHSG gene) is expressed preferentially by human adult and fetal liver cells and is secreted into the bloodstream (5). The early study using fetuin-A-null mice showed that fetuin-A impaired insulin signaling and is associated with diet-induced

obesity (6). Fetuin-A found to inhibit the insulin receptor tyrosine kinase autophosphorylation by direct binding with the insulin receptor beta subunits (7,8,9). Pal et al. more recently reported that fetuin-A act as an endogenous ligand necessary for free fatty induced TLR4 ligand activation and insulin resistance (10). Same group also shows that fetuin-A secreted from adipose tissue act as chemokine involve in migration of macrophage to adipose tissue and polarization of M2 anti-inflammatory to inflammatory M1 macrophage (11). Previously we and other suggested that phosphorylation status of fetuin-A is critical for insulin signaling impairment and it is associated with insulin resistance (12,13). However, regulation of fetuin-A and its phosphorylated form in relation to metabolic diseases is not completely understood.

High glucose known to increase Fet-A expression by activation of ERK-1/2 promoter activity (14). Similarly, saturated fatty acid (palmitic acid) upregulates fetuin-A secretion by NFkB pathway (15). While, glucocorticoid, including dexamethasone, increases fetuin-A expression by C/EBP-β, a transcription factors responsible for basal level of fetuin-A (16). In addition, estrogen (17) and endoplasmic reticulum stress (18) also found to increases fetuin-A expression. However, status of pFet-A expression by these factors is not known. Also, there are discrepancies in the effect of AMPK agonist, metformin on fetuin-A expression. Mori et al. shows that metformin treatment or exercise for six month have no impact on fetuin-A level in patients with type 2 diabetes (19, 20). On contrary, Haukeland *et al* have shown decrease in fetuin-A secretion in NAFLD patients and *in vitro* after treatment with metformin (21). So, in this study we have examined the effect of important metabolic mediators such as glucose, free fatty acid (palmitic acid), AMPK

agonist (AICAR), insulin and oxidative stress on fetuin-A and its phosphorylated forms (Ser312).

### 3.3 Materials and Methods

#### 3.3.1 Reagents and antibodies

All cell culture materials were obtained from VWR International (Radnor, PA) or Life Technologies (Grand Island, NY). Recombinant protein of Fet-A was procured from BioVendor (Asheville, NC). AICAR was purchased from Cayman Chemical Company (Ann Arbor, MI). Anti-Fet-A antibody was from R&D Systems (Minneapolis, MN). Phosphorylated (Ser312) Fet-A was detected using a custom-generated antibody that specifically recognized phosphorylation on Ser312-Fet-A (22). Anti-pAMPK (Thr172), anti-AMPK, anti-pAKT (Ser473) and anti-pGSK3 (Ser21) antibodies were purchased from Cell Signaling (Danvers, MA). Antibodies against GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or as indicated in each section.

#### 3.3.2 Cell lines and culture

HepG2 human hepatocyte-derived cell lines was purchased from American Type Culture Collection (Manassas, VA). HepG2 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% (v/v) FBS (fetal bovine serum), penicillin, streptomycin and neomycin (1%) in a humidified 5% CO2 atmosphere at $37^0$C. Confluent HepG2 cells were subcultured by trypsinization and subsequently seeded in 6-well culture plates. After serum starvation, cells were treated with either glucose, palmitic acid, AICAR, insulin or combination of substances as indicated doses and times. For induction of oxidative stress, HepG2 cells were incubated in hypoxic condition and/or treated with

hydrogen peroxide (H202) for indicated time and doses on figures. At the end of the incubation period, medium was collected and/or cells were lysed and centrifuged at 10,000 x g for 20 min.

### 3.3.3 Western blot analysis

Following each treatment, the cells were washed twice with ice-cold phosphate buffer saline (PBS) buffer and lysed in the buffer supplemented with 50 mM HEPES, 1% Triton X 100, 10 mM EDTA, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, and protease inhibitor cocktail (Amresco, Solon, OH). The protein content in total cell lysates was determined using the Pierce 660 Protein Assay kit (Life Technologies). Cell lysates and culture medium were mixed with a sample loading buffer and separated on 8 or 4-20% SDS-PAGE gel (NuSep Inc, GA). For immunoprecipitation, cell lysates (500 μg) were diluted in lysis buffer and incubated with 4 μg of primary antibody. The immunoprecipitates were collected with protein A-Sepharose beads (Sigma) overnight at 4 °C and then washed three times with PBS. Samples were boiled in sample buffer and resolved on 4-20% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and incubated with appropriate antibodies. Protein bands were visualized by UVP BioImaging and VisionWorks software package (UVP, Upland, CA) using SuperSignal West Dura Extended Duration substrate (Pierce, Rockford, IL) and SuperSignal West Femto maximum sensitivity substrate (Pierce, Rockford, IL). Relative area densities were quantified using the UN-SCAN IT software package, v.6.1 (Silk Scientific, Orem, UT).

### 3.3.4 Real-time PCR

Serum starved HepG2 cells treated with dexamethasone, insulin and/or Fet-A as indicated on figures. After incubation time, total RNA was extracted from HepG2 cells using RNeasy Mini RNA isolation kit (QIAGEN, Valencia, CA). cDNA was synthesized by using iScript cDNA synthesis kit (BIO-RAD, Hercules, CA). Quantitative real-time PCR was carried out with SYBR green using the MyiQ single-color real-time PCR detection system (Bio-Rad, Hercules, CA). The following primer sets were used, phosphoenol pyruvate carboxy kinase (PEPCK) forward 5'-GGG TGC TAG ACT GGA TCT GC-3', PEPCK reverse 5'-GAG GGA GAA CAG CTG AGT GG-3', β-actin forward primer- 5'-CCT CTA TGC CAA CAC AGT GC-3' and β-actin reverse primer- 5'-CAT CGT ACT CCT GCT TGC TG-3'. Gene expression of PEPCK was normalized to that of β-actin mRNA.

### 3.3.5 Glucose production assay

Glucose production from HepG2 cells was measured as described by Shao et al. (23) using a colorimetric glucose oxidase assay (Sigma, St. Louis, MO). Briefly, after 18 h of treatment with increasing concentrations of Fet-A (0.1-1 uM) in the presence of dexamethasone and cAMP with or without insulin treatment, the cells were washed three times with PBS. Then the cells were incubated for 6 h at 37°C, 5% CO2, in glucose production buffer (glucose-free DMEM, pH 7.4, containing 20 mmol/l sodium lactate, 1 mmol/l sodium pyruvate, and 15 mmol/l HEPES, without phenol red). The glucose assays were conducted in duplicate. The intra-assay coefficient of variation was 5%.

### 3.3.6 Statistical analysis

Results are expressed as Mean ± Standard Error of the Mean (SEM). Comparisons between various treatments and/or groups were carried out using the unpaired Student's t-

test or one-way Analysis of Variance (ANOVA) where appropriate. Statistical analyses were performed using GraphPad Prism 6 (GraphPad, San Diego, CA). Differences were considered statistically significant when $p < 0.05$.

### 3.4 Results

#### 3.4.1 Fet-A inhibits insulin signaling in HepG2 cells

Fet-A is a secretary protein, known to impair adipocyte and skeletal muscle function (15, 24). Previously, we and others also suggested critical role of phosphorylated forms in Fet-A mediated inhibition of insulin signaling (25, 12). So, to determine the effect of Fet-A on insulin signaling in HepG2 human hepatoma cells, confluent cells were treated with varying concentrations of recombinant Fet-A. As depicted in Fig.4A, recombinant Fet-A consists of phosphorylated forms. It inhibited insulin-induced phosphorylation of MAPK and insulin receptor substrates (Fig. 4B) in a dose dependent manner. In a second series of experiments, we have tested whether Fet-A prevent inhibitory effect of insulin on dexamethasone and cAMP-activated PEPCK gene expression in hepatocytes. We have thus cultured HepG2 cells for 18 h with increasing concentrations of Fet-A (0.2-1 uM) in the presence of dexamethasone and cAMP with or without insulin treatment (Fig. 4C). We observed that insulin is highly effective in downregulating dexamethasone-induced PEPCK mRNA expression. While, treatment of Fet-A (1μM) significantly prevents the insulin mediated downregulation of dexamethasone induced PEPCK gene expression (Fig 4C). Next, glucose production from gluconeogenic precursors was assayed in HepG2 cells by measuring the amount of glucose released into the medium, which initially contained gluconeogenic precursors but no glucose. Compare to non-stimulated cells, cAMP and dexamethasone stimulated glucose production in HepG2 cells was

increased by 1.75 fold (Fig 4D). While, treatment of insulin significantly prevent the cAMP and dexamethasone stimulated glucose production in HepG2 cells. However, pre-treatment of Fet-A at 1μM significantly inhibit the suppressive effect of insulin on cAMP and dexamethasone stimulated glucose production in HepG2 cells (Fig 4D). Thus, these data demonstrated that Fet-A inhibit the insulin signaling and its action in human hepatoma HepG2 cells.

**3.4.2 Effect of glucose and palmitate on Fet-A, pFet-A expression**

High glucose and palmitate known to upregulate fetuin-A expression by ERK-1/2 and NF-kB pathways, respectively (14, 15). However, effect on pFet-A is not explored. Here we observed that high glucose (25 mM) increases Fet-A and pFet-A expression after 6hr and 12hr of treatment (Fig 5A). To understand the contribution of osmotic pressure due to high glucose on Fet-A or pFet-A, we also treated HepG2 cells with 25mM mannitol as an osmotic control. We observed that 25mM mannitol treatment for 12hr have minimal effect of Fet-A and pFet-A expression and secretion. In contrast, glucose upregulate Fet-A and pFet-A expression in a concentration dependent manner (Fig 5B). Interestingly, 25mM glucose increases pFet-A secretion but not Fet-A as compared to 5mM glucose (Fig 5B, 5C). High glucose induced increase in Fet-A and pFet-A expression/secretion is associated with impairment of insulin mediated AKT and GSK phosphorylation (Fig 5D). Similarly, palmitic acid was also found to upregulate Fet-A and pFet-A expression after 4hr of treatment in HepG2 cells (Fig 5E). Collectively, these data suggest that high glucose and saturated fatty acid increases Fet-A and pFet-A expression/secretion. This effect is associated with insulin resistant in human hepatoma cells.

### 3.4.3 Effect of insulin and H2O2/hypoxia on high glucose-induced Fet-A, pFet-A expression

Hyperinsulinemia and oxidative stress is also one of the important pathophysiological characteristic of diabetes and insulin resistance (26). So, here we examine the effect of insulin and oxidative stress induced by H2O2 and/or hypoxia on Fet-A and pFet-A expression. Insulin pre-treatment for 24hr before incubation of HepG2 cells in media containing 25mM glucose was found to downregulate the high glucose-induced Fet-A and pFet-A expression (Fig 6A). In addition, treatment with insulin for 15 min after high glucose (25mM, 12 hr) also suppresses the Fet-A and pFet-A expression (Fig 6B). Similarly, we also observed that treatment with hydrogen peroxide (H2O2) alone or in combination with hypoxia for 24hr downregulate Fet-A and pFet-A expression under high glucose conditions (Fig 6C).

### 3.4.4 Effect of AICAR on high glucose-induced Fet-A, pFet-A expression

Role of AMPK in the regulation of Fet-A and pFet-A is not clearly known. To understand the effect of AMPK activation, here we examine the effect of AICAR (AMPK agonist) on high glucose-induced Fet-A and pFet-A expression. As described earlier, high glucose increases Fet-A and pFet-A expression after 12hr of incubation (Fig 7A). While, treatment with AICAR (2mM, 12 hr) under high glucose condition suppresses the high glucose induced Fet-A expression without affecting pFet-A expression. Decrease level of Fet-A by AICAR is associated with the activation of AMPK (Fig 7A). AICAR found to decrease Fet-A level both in high glucose and low glucose conditions (Fig 7B).

## 3.5 Discussion

Fet-A is a liver secreted phosphorylated protein known to be associated with endocrine and metabolic disease including insulin resistance, diabetes and cardiovascular outcomes (27, 28, 29). However, regulation of Fet-A and its phosphorylated forms by metabolic mediators is not completely understood. Here, we report that recombinant Fet-A containing phosphorylation at site of Ser312 (Fig 4A) inhibit the insulin-induced IRS-1 and MAPK phosphorylation in a dose dependent manner (Fig 4B) in human hepatoma HepG2 cells. In addition, we also observed that recombinant Fet-A inhibit insulin mediated downregulation of dexamethasone induced PEPCK gene expression (Fig 4C) and glucose production (Fig 4D) in HepG2 cells. Our finding supports the validity of previous finding of Fet-A inhibit insulin signaling and its downstream action, which required presence of phosphorylation.

Glucose and saturated fatty acid known to increase Fet-A level by ERK1/2 and NFkB pathway respectively (14, 15). As, hyperglycemia and increase free fatty acid level is main characteristic of diabetes, we first treat HepG2 cells with high glucose or palmitic acid to understand its effect on Fet-A and pFet-A. As previously reported, here we observed that high glucose and palmitic acid upregulate the Fet-A expression as compare to control. Importantly, high glucose and palmitic acid found to increases the phosphorylation forms of Fet-A and is not driven by osmotic pressure. Glucose also found to increase Fet-A secretion and pFet-A secretion as compared to osmotic control, mannitol. However, as compare to low glucose, high glucose increases pFet-A secretion which is critical for inhibition of insulin signaling without change in Fet-A secretion. So, previous observation of increase level Fet-A and pFet-A in obese individual might be driven by increased concentration of glucose and free fatty acid.

Since hyperinsulinemia and oxidative stress is also a characteristics of type 2 diabetes (26), next we determine the effect of insulin and oxidative stress on high glucose-induced Fet-A and pFet-A expression. Insulin pre-treatment or posttreatment suppress high glucose-induced increase in Fet-A and pFet-A expression. Similarly, induction of oxidative stress by hydrogen peroxide and/or hypoxia also downregulate Fet-A and pFet-A expression. This effect probably due to negative feedback regulation by insulin and oxidative stress which is impaired in diabetic conditions resulting in higher level of Fet-A and pFet-A.

Finally, we determine the effect of AMPK agonist on high glucose-induced Fet-A and pFet-A expression. The AMPK is a highly conserved sensor of cellular energy being negatively regulated by exposure to high glucose. Here, we observed that activation of AMPK by treatment of AICAR for 12hr in high glucose condition downregulate Fet-A expression without affecting pFet-A level. Treatment of AICAR also downregulate the basal expression of Fet-A in low glucose condition. This observation is contradicting with the previous finding by Mori et al. which shows that metformin treatment or exercise did not change the Fet-A level (19, 20). So, further study to confirm the negative regulation of AICAR on Fet-A as well as its mechanism of action is warranted.

**3.6 Figure Legends**

**Fig. 4.** Effect of recombinant Fet-A on insulin signaling and insulin action: (A) Different concentrations of recombinant Fet-A were analyzed by western blotting for presence of Fet-A and pFet-A levels. (B) HepG2 cells were pre-treated with insulin in the presence or absence of recombinant Fet-A and cell lysates were subjected to immunoblotting for MAPK and IRS-1 phosphorylation status. (C) HepG2 cells were maintained in DMEM

containing 10% FBS. Twenty-four hours after cells reached confluence, the cells were incubated in serum free media containing Dexa+cAMP and insulin pretreated with either Fet-A (0.2 μM, 0.5 μM, 1μM) or vehicle. Total RNA was isolated after 18hr of treatment, and PEPCK mRNA levels were analyzed by real time PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were measured for internal controls (D) HepG2 cells were incubated with DMEM for 24 h and were washed three times with PBS and subsequently cultured in serum and glucose-free medium supplemented with lactate and pyruvate with Dexa+cAMP and insulin pretreated with either Fet-A (0.2 μM, 0.5 μM, 1μM) or vehicle. Six hours after hormone treatment, the medium was collected for glucose measurement. The bars depict the mean ± SE of at least three independent assays. *$P < 0.05$ vs. vehicle-treated cells.

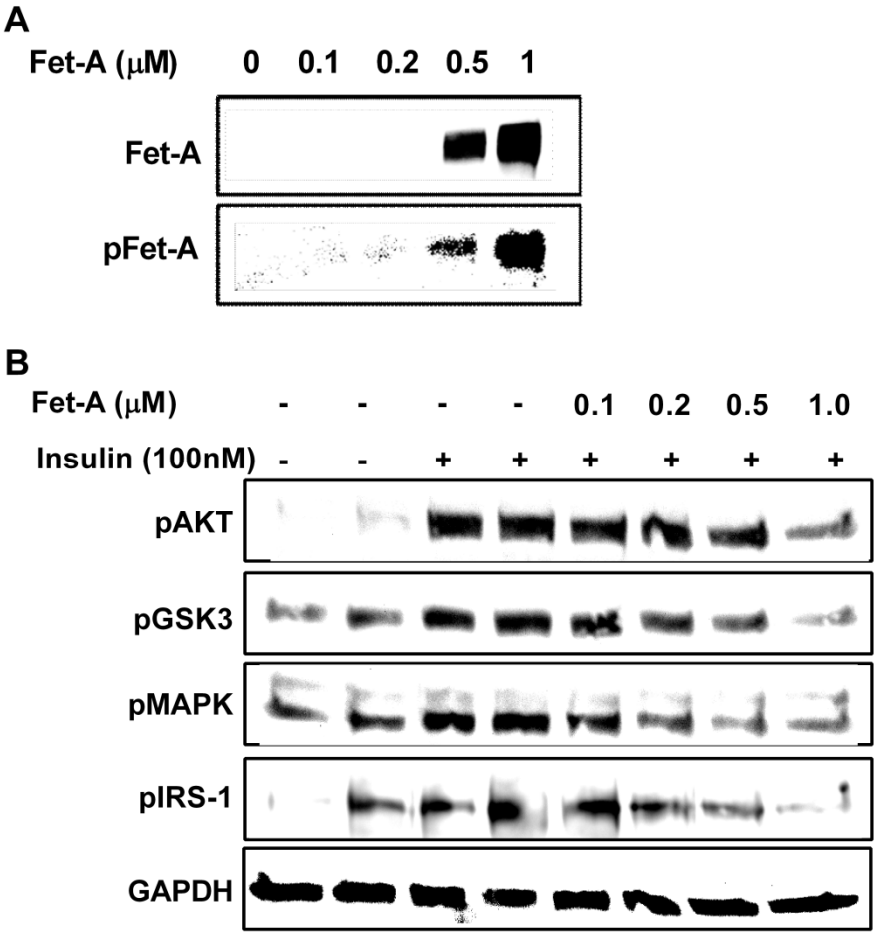
**Fig. 5.** Glucose and palmitate upregulate Fet-A and pFet-A level. (A) HepG2 cells were incubated in low or high glucose media for 1,6, and 12 hours, and cell lysates were subjected to immunoblotting for Fet-A and phosphorylated Fet-A. (B) HepG2 cells were incubated in a media containing either mannitol, low glucose or high glucose for 12 hours, and cell lysates/media were subjected to immunoblotting for Fet-A and phosphorylated Fet-A. (C) HepG2 cells were incubated in low or high glucose media for 12 hours, and media were subjected to immunoblotting for Fet-A and phosphorylated Fet-A. (D) HepG2 cells were incubated in low (5 mM) or high glucose (25 mM) media for 12 h followed by insulin treatment for 15 min and cell lysate were subjected to immunoblotting for AKT, GSK3, MAPK and IRS-1 phosphorylation status. (E) HepG2 cells were treated with palmitic acid (0.25, 0.5, 1mM) for 4 hours, and cell lysates were

subjected to immunoblotting for Fet-A and phosphorylated Fet-A. GAPDH levels were measured for internal controls.

**Fig. 6.** Insulin and oxidative stress downregulate high glucose-induced Fet-A and pFet-A level. (A) Insulin were treated to HepG2 cells for 24hr followed by incubation of cells with media containing low glucose or high glucose. At the end of treatment cell lysates were subjected to immunoblotting for Fet-A and phosphorylated Fet-A. (B) HepG2 cells were exposed to low glucose or high glucose containing media for 12 hr. In the end, insulin was treated for 15 min and cell lysates were subjected to immunoblotting for Fet-A and phosphorylated Fet-A. (C) HepG2 cells were treated with H2O2 (50μM) alone or in combination with hypoxia for 4hr and cell lysates were subjected to immunoblotting for Fet-A and phosphorylated Fet-A.

**Fig. 7.** AICAR downregulates Fet-A but not pFet-A expression in HepG2 cell. (A) & (B) HepG2 cells were incubated with either low- or high-glucose in the absence or presence of AICAR for 12 h and cell lysate were analyzed by western blotting. The blots were analyzed with antibodies against pAMPK, AMPK, Fet-A and pFet-A.

**Figure 4**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Fet-A (μM) - - - - 0.1 0.2 0.5 1.0
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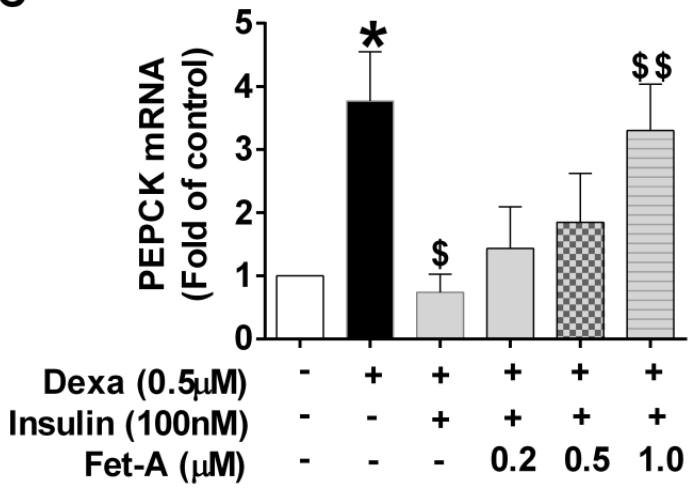
pMAPK
pIRS-1
GAPDH

C


PEPCK mRNA
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Fet-A (μM) - - - 0.2 0.5 1.0

D

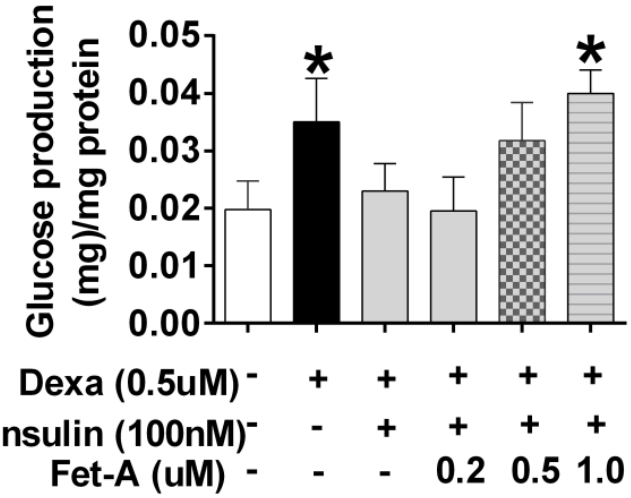
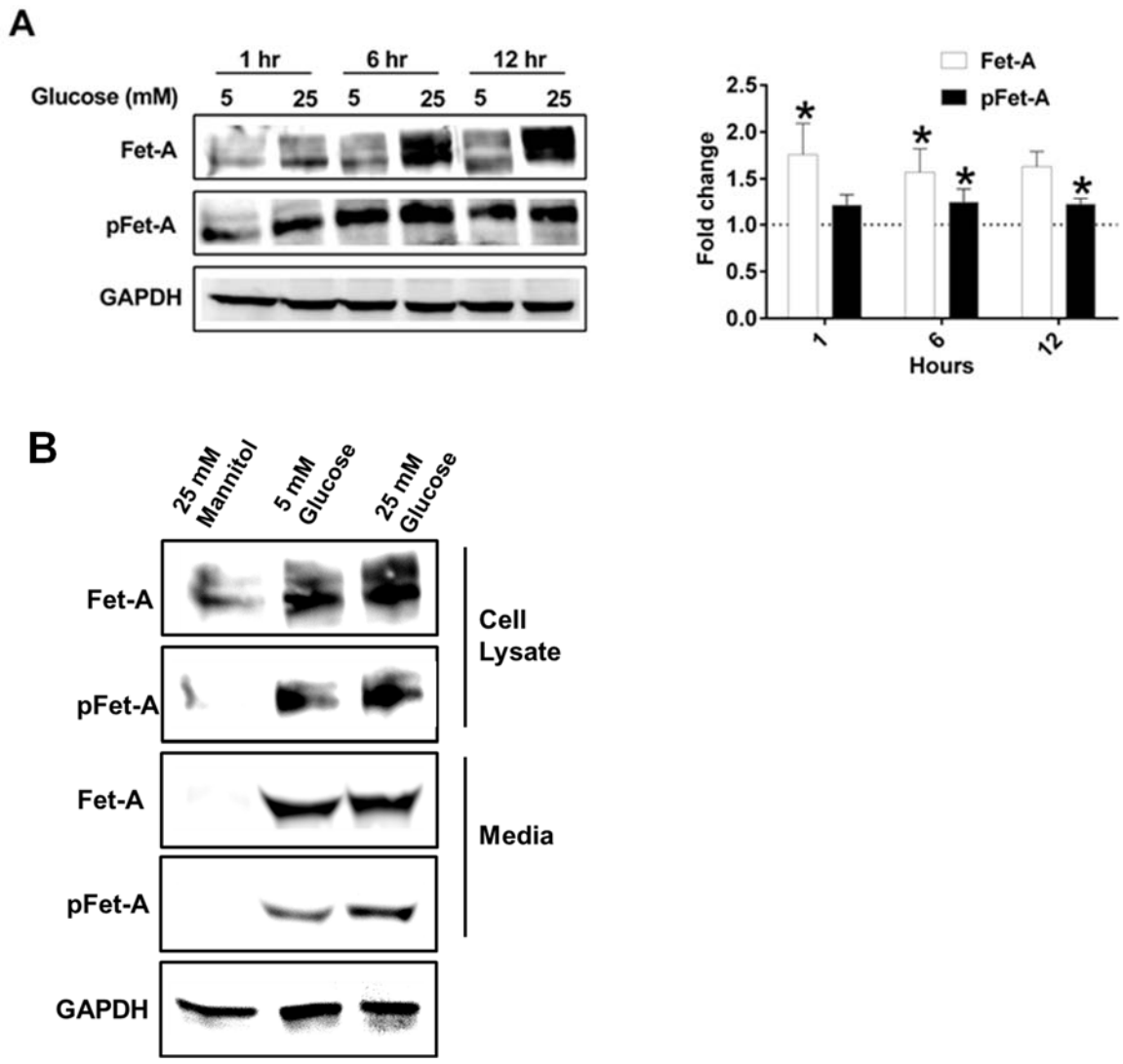

Glucose production
(mg)/mg protein
0.00
0.01
0.02
0.03
0.04
0.05
*
*
Dexa (0.5uM) - + + + + +
Insulin (100nM) - - + + + +
Fet-A (uM) - - - 0.2 0.5 1.0

**Figure 5**


A
1 hr
6 hr
12 hr
Glucose (mM)
5
25
5
25
5
25
Fet-A
pFet-A
GAPDH
Fet-A
pFet-A
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
1
6
12
Hours
B
25 mM Mannitol
5 mM Glucose
25 mM Glucose
Fet-A
pFet-A
Cell Lysate
Fet-A
pFet-A
Media
GAPDH

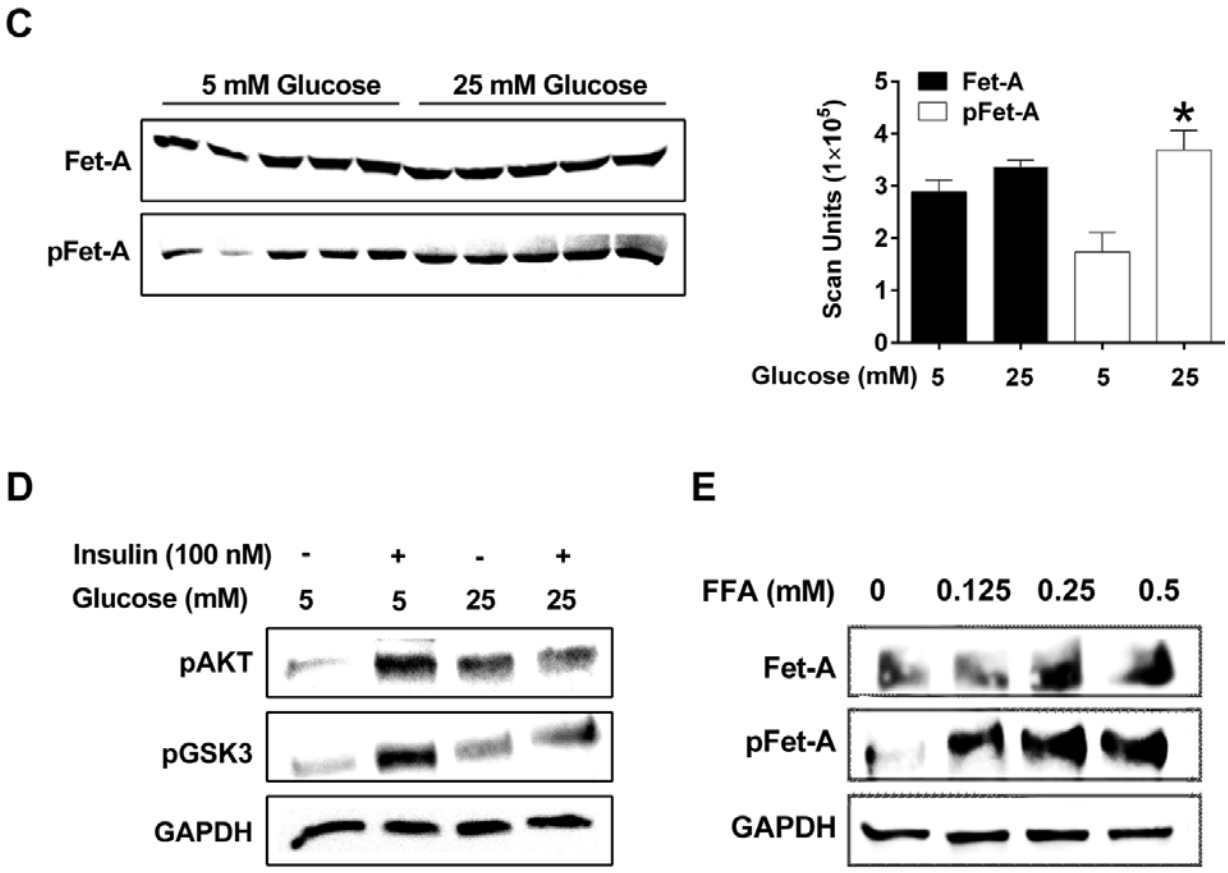

C
5 mM Glucose
25 mM Glucose
Fet-A
pFet-A
Fet-A
pFet-A
Scan Units (1×10^5)
5
4
3
2
1
0
*
Glucose (mM) 5 25 5 25
D
Insulin (100 nM) - + - +
Glucose (mM) 5 5 25 25
pAKT
pGSK3
GAPDH
E
FFA (mM) 0 0.125 0.25 0.5
Fet-A
pFet-A
GAPDH

**Figure 6**

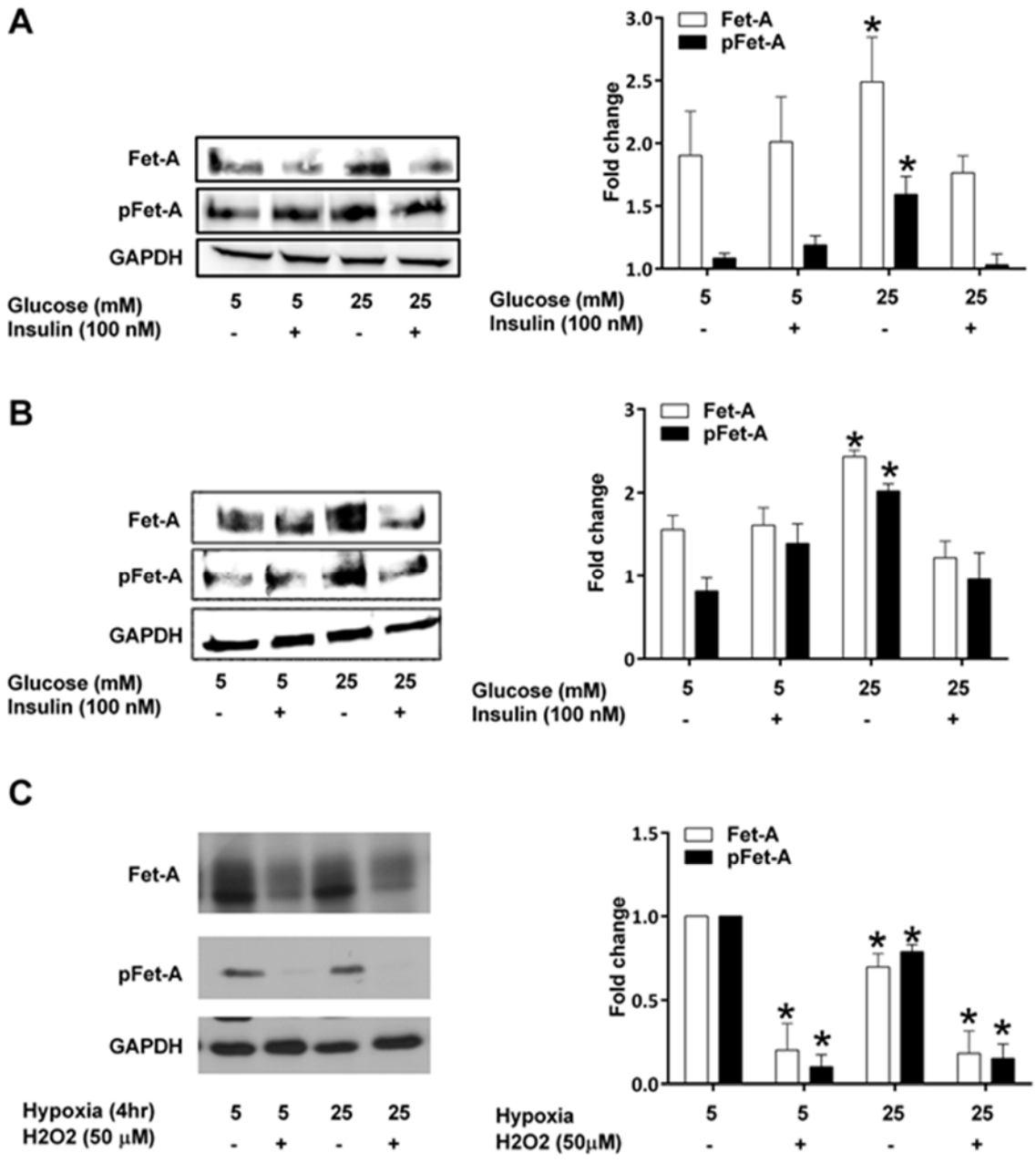
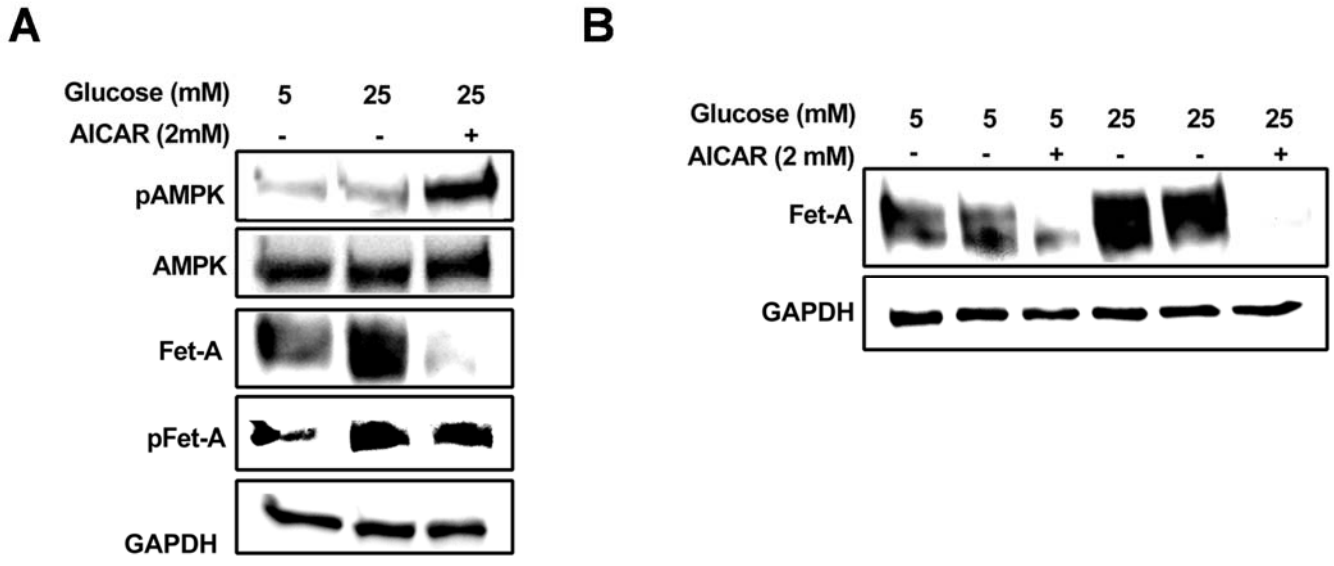

A
Fet-A
pFet-A
GAPDH
Glucose (mM) 5 5 25 25
Insulin (100 nM) - + - +
Fold change
Fet-A
pFet-A
B
Fet-A
pFet-A
GAPDH
Glucose (mM) 5 5 25 25
Insulin (100 nM) - + - +
Fold change
Fet-A
pFet-A
C
Fet-A
pFet-A
GAPDH
Hypoxia (4hr) 5 5 25 25
H2O2 (50 μM) - + - +
Fold change
Fet-A
pFet-A
Hypoxia 5 5 25 25
H2O2 (50μM) - + - +

**Figure 7**


A
Glucose (mM) 5 25 25
AICAR (2mM) - - +
pAMPK
AMPK
Fet-A
pFet-A
GAPDH
B
Glucose (mM) 5 5 5 25 25 25
AICAR (2 mM) - - + - - +
Fet-A
GAPDH

# Chapter 4: AICAR Negatively Regulates Hepatic Fetuin-A Expression through AMPK→p38 MAPK→C/EBPβ/E3 Ubiquitin Ligase Signaling Pathway

## 4.1 Abstract

Fetuin-A (Fet-A) is a liver-secreted phosphorylated protein, known to impair insulin signaling, and is associated with obesity, insulin resistance, and incident diabetes. Fet-A interacts with the insulin-stimulated insulin receptor (IR) and inhibits IR tyrosine kinase activity and glucose uptake. Recent studies have shown that high glucose increases Fet-A expression through the ERK1/2 signaling pathway. However, factors that downregulate Fet-A expression and their potential mechanisms are unclear. We examined the effect of AMP-activated protein kinase (AMPK) activator, AICAR, on Fet-A expression in HepG2 cells, Hep3B cells and primary rat hepatocytes. High glucose increased Fet-A and phosphorylated (Ser312) fetuin-A (pFet-A) expression. While AICAR treatment significantly down-regulated high glucose-induced Fet-A expression without affecting pFet-A expression, which is associated with activation of AMPK and p38 MAPK. Treatment with Compound C (AMPK inhibitor), SB202190 (p38 MAPK inhibitor) or p38 MAPK siRNA transfection prevented AICAR-induced downregulation of Fet-A expression. In addition, activation of p38 MAPK, by anisomycin, decreased the hepatic expression of Fet-A. Further, we demonstrated that short term effect of AICAR on Fet-A expression was mediated by proteosomal degradation. While long term treatment of AICAR associated with decrease in hepatic expression of C/EBP beta, important

transcription factor involves in Fet-A regulation. Taken together, our studies implicate a critical role for AMPK-p38 MAPK axis in the regulation of the expression of hepatic Fet-A. Effect of AICAR is mediated by combination of increase in degradation of Fet-A and decrease in synthesis of Fet-A through C/EBP beta.

**4.2 Introduction**

The liver is known to play an important role in the ongoing epidemic of type 2 diabetes, nonalcoholic fatty liver disease (NAFLD), and cardiovascular disease. In these conditions, the liver is involved in increased glucose production and dysregulated lipoprotein metabolism (30, 31). Analogous to the adipokines and myokines, recent studies have suggested that the liver may regulate glucose homeostasis by modulating the sensitivity/resistance of peripheral tissues, by way of the production of secretory proteins, termed hepatokines (32, 33, 34). Fet-A (also known as alpha-2-HS-glycoprotein in humans) is a major hepatokine, originally identified in 1944; but its importance in physiology has only recently been appreciated (35). Fet-A functions as an important component of diverse normal and pathological processes, including insulin resistance, vascular calcification and bone metabolism (36).

Fet-A shares amino-acid sequence similarity to insulin receptor tyrosine kinase (9) and type-II transforming growth factor-ß (TGF-ß) receptor (37), and thus been proposed as a natural inhibitor of the insulin-signaling pathway and an antagonist of TGF-ß. It has been suggested that phosphorylation status of Fet-A is critical to inhibit insulin receptor tyrosine kinase (IR-TK) activity (25). Previously, we demonstrated that Fet-A interacts with the activated insulin receptor, and inhibits IR and IRS-1 phosphorylation in liver and skeletal muscle. Fet-A showed relative specificity for IR inhibition, without affecting

epidermal growth factor or insulin-like growth factor1-induced cognate receptor autophosphorylation and TK activity (7, 8). Fet-A impairs insulin-mediated glucose uptake in C2C12 and L6-GLUT4myc myotubes by down-regulating GLUT-4 translocation to the plasma membrane through decreased in phosphorylation of Akt and AS160 (24, 13).

Fet-A level was shown to be up-regulated in high-fat diet induced obese animals (15) and patients of NAFLD (21). While, exercise and dietary life style interventions results in declines in serum Fet-A levels commensurate with improvement in NAFLD and decline in body weight (38). Fet-A null mice show improved insulin signaling and prevent weight gain when fed a high-fat diet, indicating the physiological relevance of *in vitro* observations (6). Epidemiological studies have shown that elevated levels of serum Fet-A is associated with obesity, type 2 diabetes mellitus, and metabolic syndrome (21, 38). Recent studies have implicated Fet-A in adipocyte dysfunction (15), toll-like receptor 4 activation (10), migration and polarization of macrophage (11), and hepatocyte triacylglycerol accumulation (21). However, regulation of Fet-A and its phosphorylated forms in relation to insulin resistance is not fully understood.

Fet-A gene expression has been shown to be downregulated by tumor necrosis factor-α (39) and by other pro-inflammatory cytokines, including IL-6 and IL-1β, in rat and human hepatocytes (40). In contrast, high glucose (14) and free fatty acids (15) upregulate hepatic Fet-A gene expression via ERK1/2 and NFkB pathways, respectively. Further, glucocorticoids (16), endoplasmic reticulum (ER) stress (17) and estrogen (18) have been shown to increase Fet-A expression. However, factors that downregulate Fet-A expression and their potential mechanisms are unclear. Recent studies examining the

effect of AMPK activation on Fet-A synthesis and secretion are somewhat equivocal. Haukeland *et al* demonstrated that metformin treatment significantly decreased plasma Fet-A compared to placebo in NAFLD patients and dose-dependently decreased Fet-A secretion in HepG2 cells (21). Further, Jung *et al* demonstrated that salsalate inhibit Fet-A expression through the AMPK-NFkB pathway and improving palmitate-induced steatosis. (41). On the other hand, Mori *et al* found that while pioglitazone decreased serum Fet-A, no changes in serum Fet-A was observed in type 2 diabetic patients receiving metformin treatment for 6 months (19). Further, these authors also report that, unlike metformin, pioglitazone acts directly to downregulate Fet-A in Fao rat hepatoma cells (20).

To clarify the role of AMPK activation on Fet-A expression, we examined the effect of AICAR and metformin on high glucose-induced Fet-A and pFet-A expression in HepG2 cells, Hep3B cells and rat hepatocytes. We also investigated the pathway by which AICAR can regulate the Fet-A expression. Here, we demonstrate that AICAR regulate high glucose mediated hepatic Fet-A expression through AMPK/p38MAPK pathways. Further, we observed that effect of AICAR mediated by combination of increase in degradation of Fet-A and decrease in synthesis of Fet-A through C/EBP beta.

### 4.3 Materials and Methods

#### 4.3.1 Reagents and antibodies

All cell culture materials were obtained from VWR International (Radnor, PA) or Life Technologies (Grand Island, NY). AICAR and chloroquine (autophagy inhibitor) were purchased from Cayman Chemical Company (Ann Arbor, MI) and BioVision Inc (Milpitas, CA) respectively. Human ELISA for Fet-A was procured from BioVendor

(Asheville, NC). Metformin, Compound C (AMPK inhibitor), SB202190 (p38 MAPK inhibitor), PD98059 (MEK1 inhibitor), SP600125 (JNK inhibitor), MG-132 (proteasome inhibitor) were obtained from Enzo Life Science. Anti-pAMPK (Thr172), anti-AMPK, anti-pERK1/2 (The202/Tyr204) and anti-pJNK antibodies were purchased from Cell Signaling (Danvers, MA). Antibodies against C/EBPβ (C-19), p38 MAPK; pP38 MAPK (Thr 180/Tyr 182), ubiquitin and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Fet-A antibody was from R&D Systems (Minneapolis, MN). Phosphorylated (Ser312) Fet-A was detected using a custom-generated antibody that specifically recognized phosphorylation on Ser312-Fet-A (22). p38 MAPK siRNA (short interfering RNA; Entrez-Gene ID#1432) was purchased from OriGene Technologies (Rockville, MD). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**4.3.2 Cell lines and primary culture**

HepG2 and Hep3B human hepatocyte-derived cell lines were purchased from American Type Culture Collection (Manassas, VA). HepG2 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% (v/v) FBS (fetal bovine serum), penicillin, streptomycin and neomycin (1%) in a humidified 5% CO2 atmosphere at $37^0$C. Hep3B cells were cultured in MEM (minimal essential medium) containing non-essential amino acids supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% (v/v) FBS and antibiotics. Fresh primary rat hepatocytes, plated as a monolayer in 6-well plates, were procured from Triangle Research Labs (Research Triangle Park, NC) and maintained in hepatocytes maintenance media.

**4.3.3 Cell culture treatment**

Confluent HepG2 or Hep3B cells were subcultured by trypsinization and subsequently seeded in 6-well culture plates. Cells were serum-starved overnight and incubated with low glucose (5.5mM) or high glucose (25.5 mM) for 1, 6, or 12 h. Concomitantly, cells were treated with AICAR (0, 0.5, 1, 2 mM), metformin (2 mM), or anisomycin (0.5 μg/ml). When inhibitors were used, cells were pre-incubated with Compound C (10 μM), SB202190 (25 μM), PD98059 (25 μM), SP600125 (25 μM), chloroquine (10 μM) or MG-132 (0.5 μM) for 1 h before treatment with high glucose and AICAR. Primary rat hepatocytes were treated with either AICAR or anisomycin at the indicated dose and time. At the end of the incubation period, medium was collected and cells were lysed and centrifuged at 10,000 x g for 20 min.

**4.3.4 Immunoprecipitation and Western blot analysis**

Following each treatment, the cells were washed twice with ice-cold phosphate buffer saline (PBS) buffer and lysed in the buffer supplemented with 50 mM HEPES, 1% Triton X 100, 10 mM EDTA, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, and protease inhibitor cocktail (Amresco, Solon, OH). The cells were lysed with SDS lysis buffer to detect covalent interaction of ubiquitin and Fet-A (Triton lysis buffer containing 2% SDS). The protein content in total cell lysates was determined using the Pierce 660 Protein Assay kit (Life Technologies) except those samples containing SDS, which were estimated by DC assay (Bio-Rad). Cell lysates or culture supernatant medium were mixed with a sample loading buffer and separated on 8 or 4-20% SDS-PAGE gel (NuSep Inc, GA). For immunoprecipitation, cell lysates (500 μg) were diluted in lysis buffer and incubated with 4 μg of primary antibody. The immunoprecipitates were collected with protein A-Sepharose beads (Sigma) overnight at

4 °C and then washed three times with PBS. Samples were boiled in SDS-PAGE sample buffer and resolved on either 4-20% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and incubated with appropriate antibodies. Protein bands were visualized by UVP BioImaging and VisionWorks software package (UVP, Upland, CA) using SuperSignal West Dura Extended Duration substrate (Pierce, Rockford, IL) and SuperSignal West Femto maximum sensitivity substrate (Pierce, Rockford, IL). Relative area densities were quantified using the UN-SCAN IT software package, v.6.1 (Silk Scientific, Orem, UT).

**4.4.5 ELISA**

The total amount of Fet-A secreted to the culture media was determined using a Human Fet-A (BioVendor R&D, Asheville, NC) ELISA kits following the manufacturer´s instructions.

**4.4.6 Real-time PCR**

Total RNA was extracted from HepG2 cells treated with or without AICAR (0.5–2.0 mM). Quantitative real-time PCR was carried out with SYBR green using the MyiQ single-color real-time PCR detection system (Bio-Rad, Hercules, CA). The following primer sets were used, Fet-A forward primer- 5'-ACG TGG TCC ACA CTG TCA AA-3', Fet-A reverse primer- 5'-CGC AGC TAT CAC AAA CTC CA-3', β-actin forward primer- 5'-CCT CTA TGC CAA CAC AGT GC-3' and β-actin reverse primer- 5'-CAT CGT ACT CCT GCT TGC TG-3'. Gene expression of Fet-A was normalized to that of β-actin mRNA.

**4.5.7 Transfection**

For siRNA studies, HepG2 cells were plated in 12-well culture plate dishes and transfected with 10nM siRNA with siTran 1.0 (OriGene) according to the manufacturer's instructions. After 36 h of transfection, cells were treated with AICAR (2 mM) for 12 h. Following this cells were lysed and used for Western blot analysis.

### 4.4.8 Statistical analysis

Results are expressed as Mean ± Standard Error of the Mean (SEM). Comparisons between various treatments and/or groups were carried out using the unpaired Student's t-test or one-way Analysis of Variance (ANOVA) where appropriate. Statistical analyses were performed using GraphPad Prism 6 (GraphPad, San Diego, CA). Differences were considered statistically significant when $p < 0.05$.

## 4.5 Results

### 4.5.1 AICAR downregulates Fet-A expression through AMPK in HepG2 cells

Evidence for the role of AMPK activation on Fet-A expression is currently unclear. In addition, effect of AMPK on high glucose induced Fet-A expression is not known. To address this, we treated HepG2 cells with AICAR, an AMPK activator, in the presence of low or high glucose. AICAR increases AMPK phosphorylation and suppresses high glucose-induced increase in Fet-A synthesis (Fig 8A) and secretion (Fig 8C, 8D). AICAR dose-dependently decreased Fet-A expression in cell lysates, but not pFet-A (Fig.8A). However, AICAR decreased the secretion of Fet-A and pFetA into the media (Fig.8C, 8D). Fet-A expression was downregulated progressively starting from 2 h (similar time-frame compared to activation of AMPK by AICAR) through 24 h of treatment (Fig 8B). pFet-A concentrations were not affected during this treatment period, except for 24 h (Fig 8B). Furthermore, Compound C, an inhibitor of AMPK, reversed the effect of AICAR on

Fet-A expression (Fig 8E). Treatment of AICAR also significantly inhibited Fet-A gene expression in a dose-dependent manner (Fig 8F). Additionally, metformin also inhibited the Fet-A mRNA and protein expression (Fig 8E, 8F).

### 4.5.2 AICAR downregulates Fet-A expression through p38 MAPK in HepG2 cells

Previously, high glucose was shown to increase the transcriptional activation of Fet-A through ERK 1/2 pathway (14). To determine the effect of AICAR on high glucose induced ERK 1/2 expression, we treated HepG2 cells with AICAR in the presence of low- or high-glucose. As shown previously (14), high glucose increased the phosphorylation of ERK 1/2 compared to low glucose (Fig. 9A). AICAR treatment further increased ERK 1/2 phosphorylation suggesting that ERK 1/2 pathway may not be involved in AICAR-mediated lowering of Fet-A expression. We observed that AICAR also increased the phosphorylation of other MAP kinases, including JNK and p38 MAPK in a dose-dependent manner (Fig. 9B). Pretreatment with PD98059 (MEK1 inhibitor) and SP600125 (JNK inhibitor) did not reverse the high glucose-induced Fet-A expression (Fig 9C, 9D). However, pretreatment with p38 MAPK inhibitor SB202190, significantly reversed the effect of AICAR on high glucose-induced expression of Fet-A in cell lysates and its secretion into the media (Fig 9E). Treatment with Compound C reversed AICAR-induced p38 MAPK activation (Fig. 9F) suggesting that AMPK may regulate Fet-A expression through p38 MAPK. To further confirm the role of p38 MAPK in AMPK-induced reduction in Fet-A expression, we used siRNA to knockdown the expression of p38 MAPK in HepG2 cells. As expected, AICAR treatment decreased Fet-A expression in cells transfected with scrambled siRNA. However, knockdown (~60-70%) of p38

MAPK reversed AICAR-mediated inhibition of Fet-A expression (Fig.9G). Interestingly, we observed that AICAR-treatment increased AMPK phosphorylation in both scrambled and p38 MAPK siRNA transfected cells, suggesting that AICAR-induced downregulation of Fet-A is mediated through p38 MAPK.

### 4.5.3 Direct activation of p38 MAPK inhibits Fet-A expression in HepG2 cells

To understand the role of p38 MAPK in the regulation of Fet-A expression, we treated HepG2 cells with p38 MAPK activator, anisomycin. We found that anisomycin treatment, at concentrations previously shown to activate p38 MAPK (42), increased p38 MAPK phosphorylation status and inhibited Fet-A expression in a dose- (Fig 10A) and time-dependent manner (Fig 10B) without affecting pFet-A expression. However, pre-treatment of p38 MAPK inhibitor, SB 202190, partially prevented the anisomycin-induced downregulation of Fet-A (Fig 10C). Since anisomycin is also a protein synthesis inhibitor, we screened other agents including puromycin and cycloheximide, which are known to inhibit protein synthesis [without activating p38 MAPK (43)]. Unlike anisomycin, the other protein synthesis inhibitors, puromycin and cycloheximide, neither activated p38 MAPK nor inhibited Fet-A expression, suggesting the involvement of p38 MAPK in the regulation of Fet-A (Fig 10D).

### 4.5.4 AICAR or anisomycin inhibits Fet-A expression in Hep3B cells and primary rat hepatocytes

To further probe our findings that AMPK and p38 MAPK is involved in the negative regulation of Fet-A, we carried out similar experiments in Hep3B human hepatoma cells and primary rat hepatocytes. Similar to HepG2 cells, treatment with AICAR (Fig 11B) or anisomycin (Fig 11C) decreased Fet-A expression in a dose-dependent manner, without

affecting pFet-A expression in Hep3B cells. In Hep3B cells, high glucose significantly increases Fet-A and pFet-A expression as compared to low glucose. While AICAR treatment downregulate Fet-A and pFet-A expression (Fig 11A). In addition, AICAR treatment led to the downregulation of Fet-A and its secretion into the media, in a concentration dependent manner in primary rat hepatocytes (Fig 11D). This was correlated with an increase in phosphorylation of AMPK and p38 MAPK (Fig 11D). Further, anisomycin-induced increase in p38 MAPK phosphorylation was associated with a decrease in Fet-A expression (Fig 11E).

### 4.5.5 Proteosomal degradation pathway is involved in AICAR induced Fet-A downregulation

To check the involvement of degradation pathway in Fet-A down-regulation, we analyzed the effect of proteasome inhibition on Fet-A expression. MG132, a proteasome inhibitor, blocked AICAR-induced Fet-A down-regulation at 2 and 4 hr (Fig. 12A). This effect was not observed by MG-132 at 12hr treatment (Fig. 12B), suggesting the involvement of the ubiquitin-proteasome pathway in short term effect of AICAR on Fet-A expression. To check the involvement of lysosomal degradation pathway in Fet-A down-regulation, we analyzed the effect of autophagy inhibition on Fet-A expression. Chloroquine, an autophagy inhibitor, did not blocked AICAR-induced Fet-A down-regulation at 4 hr (Fig. 12C). Next, we tested whether Fet-A was ubiquitinated in cells. We immunoprecipitated Fet-A from cell lysates, and then analyzed with antibody against ubiquitin. As can be seen, the multi-ubiquitination of Fet-A was significantly increased in the immunoprecipitated sample treated with AICAR (Fig. 12D). These results

demonstrate that proteasome pathway is involved in AICAR-mediated Fet-A down-regulation.

### 4.5.6 AICAR-induced downregulation of Fet-A is associated with decrease in C/EBP*β* expression

Next, we considered the role of C/EBP*β* on AICAR's inhibitory effect on Fet-A expression, since previous reports have shown several binding sites for C/EBP*β* on the promoter region of Fet-A, and that binding of C/EBP*β*, augmented Fet-A expression (17). Here, we observed that 12 hr treatment of AICAR significantly decrease expression of C/EBP*β* in dose dependent manner in HepG2 cells, (Fig 13A). This effect was also observed in primary rat hepatocytes (Fig. 13B), suggesting that effect of AICAR mediated by combination of increase in degradation of Fet-A by proteosomal pathway and decrease in synthesis of Fet-A through C/EBP beta.

## 4.6 Discussion

In this study, we have explored mechanisms that mediate the hepatic regulation of Fet-A using pharmacological and siRNA-mediated knockdown approaches. While previous studies have shown that high glucose, elevated free fatty acids, dexamethasone, estrogen, and ER stress increase Fet-A expression (15, 14, 16, 17, 18), this is the first study to examine mechanisms that mediate the downregulation of Fet-A expression.

In the present study, we have established that AICAR- or metformin-induced activation of AMPK downregulates Fet-A expression in HepG2 and Hep3B human hepatoma cells, and in primary rat hepatocytes. Our studies have also identified p38 MAPK as a critical determinant of hepatic Fet-A expression. Inhibition of p38 MAPK activity using SB202190 or its knockdown with p38 MAPK siRNA blocked AICAR's effect on Fet-A

expression. Further, we have shown that direct activation of p38 MAPK with anisomycin inhibited Fet-A expression in HepG2 cells, Hep3B cells and rat primary hepatocytes. Unlike anisomycin, other protein synthesis inhibitors, including cycloheximide and puromycin, had no effect on p38 MAPK and Fet-A expression. A critical question that stems from this study is whether the AICAR/anisomycin-mediated downregulation of Fet-A expression is due to a decrease in its synthesis or due to its degradation. Our studies suggest that while this may affect both synthesis and degradation, there may be differences in acute versus long-term regulation. First, our studies show that Fet-A expression was significantly decreased (50-80%) within 2-6 h of AICAR treatment. Second, treatment with protein synthesis inhibitors, puromycin and cycloheximide did not alter Fet-A expression in short-term. Third, inhibition of proteosomal degradation prevent the short term but not long term effect of AICAR. This suggests that, acutely, Fet-A undergo degradation within the cell. However, in a more long-term manner (over 12 hours), AICAR treatment also decreased Fet-A gene expression (synthesis) and its secretion into the media. Since basal levels of Fet-A synthesis in the liver are regulated by C/EBP$\beta$ and NF-1-binding to AHSG promoter (17), we examined long term effect of AICAR on C/EBP$\beta$ expression. We observed that AICAR decreases C/EBP$\beta$ expression in HepG2 cells and primary rat hepatocytes, suggesting that C/EBP$\beta$ may be involved in AICAR-induced Fet-A downregulation.

Our findings that AICAR-treatment downregulates Fet-A expression is supported by a few indirect studies. Recently, salicylate and adiponectin were shown to inhibit palmitate-induced Fet-A expression through AMPK-NFkB pathway in HepG2 cells (41). Further, short term exercise training, which activates AMPK, was shown to significantly

decrease plasma Fet-A in subjects with NAFLD (24). However, Mori *et al* reported that metformin treatment did not affect Fet-A expression in Fao hepatoma cells and in patients with type 2 diabetes (19, 20). While we have not replicated this study in Fao cells, it is possible that this may be cell-specific effects and/or potential differences in the activation state of AMPK induced by metformin. It is well established that AMPK plays an important role in the regulation of glucose and lipid metabolism and that a low activation state of AMPK has been correlated with metabolic disorders, including insulin resistance and obesity (44, 45, 46).

These studies also shed light on the potential differences of secreted Fet-A versus pFet-A. Our studies have shown that high glucose increases pFet-A secretion, but not Fet-A. Recent studies from our laboratory (13), implicate a critical role of phosphorylation status of Fet-A in mediating the inhibitory effects of Fet-A on insulin signaling. Our findings raise the possibility that in insulin resistant or hyperglycemic conditions, that pFet-A, the physiologically active form of the inhibitor, may be preferentially secreted by hepatocytes. In this regard, it is of significant interest that our studies show that AICAR treatment decreased the secretion of both Fet-A and pFet-A in HepG2 cells. Additionally, Haukeland et al, demonstrate that metformin treatment for six month decreased serum Fet-A levels in individuals with fatty liver (21). These results suggest that activation of AMPK can lower elevated Fet-A and potentially pFet-A levels. However, potential mechanisms catalyzing phosphorylation of Fet-A is still unclear.

There are several limitations to this study. Signaling proteins upstream of AMPK and downstream of p38 MAPK that mediate the negative regulation of Fet-A remain to be identified. Secondly, while it may be assumed that activation of AMPK and or p38

MAPK pathway may lead to decrease Fet-A synthesis through C/EBP beta, such mechanisms need to fully characterized. Also, activation of AMPK known to inhibit the mammalian target of rapamycin complex 1 (mTORC1) signaling which is involve in regulation of autophagy and protein synthesis (47). Since autophagy inhibitor did not reverse the effect of AICAR on Fet-A expression, mTORC1 induced autophagy is not involve in Fet-A regulation. However, recent studies show regulation of C/EBPβ-isoform expression by mTORC1 (48). So, it is important to determine role of mTORC1 in AICAR induced downregulation of hepatic C/EBPβ-isoform and their relation with Fet-A expression. Future studies will include characterization of the proposed mechanisms shown above. Additionally, these will require validation in a relevant animal model.

In conclusion, our studies provide strong biochemical evidence for a novel AMPK→ p38 MAPK pathway in the regulation of hepatic Fet-A expression in both low- and high glucose conditions Effect of AICAR mediated by combination of increase in proteosomal degradation of Fet-A and decrease in synthesis of Fet-A through C/EBP beta. (see model in Fig. 14). This is the first characterization of the mechanisms that downregulate Fet-A, a protein shown to be associated with obesity, liver fat, insulin resistance, metabolic syndrome, and incident diabetes (49, 50, 51, 52).

**4.7 Figure Legends**

**Fig. 8.** AICAR-treatment downregulates Fet-A expression through AMPK: (A) HepG2 cells were incubated with different concentration of AICAR for 12 h and cell lysate were analyzed by western blotting for indicated proteins. (B) The effect of AICAR (2 mM) on HepG2 cells at different periods was observed by western blot analysis. (C) cells were incubated with different concentration of AICAR for 12 h and media were analyzed by

western blotting for Fet-A and pFet-A. (D) HepG2 cells were incubated in low or high glucose media for 12 hours, and media were used to detect Fet-A through ELISA. (E) HepG2 cells were incubated with AICAR/metformin in the presence or absence of Compound C, an AMPK inhibitor; cell lysates were immunoblotted for pAMPK, Fet-A, and phosphorylated Fet-A (F) Real-time gene expression analysis was carried out for Fet-A following AICAR and metformin treatment.

**Fig. 9.** AICAR-treatment downregulates Fet-A expression through p38 MAPK: (A) HepG2 cells were incubated with either low- or high-glucose in the absence or presence of AICAR for 12 h and used for Western blot analysis for ERK1/2 phosphorylation expression. (B) HepG2 cells were incubated with different concentration of AICAR for 12 h and cell lysates were analyzed by western blotting for ERK1/2, p38MAPK and JNK phosphorylation. HepG2 Cells were treated with (C) ERK inhibitor (PD98059), (D) JNK inhibitor (SP600125), (E) p38 MAPK inhibitor (SB202190), or (F) AMPK inhibitor (Comp C) before treatment of AICAR for 12 hr. Cell lysate or media were analyzed by Western blotting for indicated proteins. (G) Knockdown of p38 MAPK was performed using MAPK14 (p38 MAPK) small interfering RNA (siRNA) in HepG2 cells. Following AICAR treatment for 12 h, cell lysates were analyzed by Western Blotting for expression of p38 MAPK, phosphorylated p38 MAPK, Fet-A, and pAMPK.

**Fig. 10.** Anisomycin treatment decreases Fet-A expression: (A) HepG2 cells were treated with various concentrations of anisomycin for 0.5 h or (B) at different time intervals, with or without (C) SB202190 (p38 MAPK inhibitor) to analyze phosphorylated p38 MAPK, Fet-A and pFet-A expression. (D) The effect of protein synthesis inhibitors,

cycloheximide and puromycin, were compared with anisomycin, also a protein synthesis inhibitor, for effects on Fet-A and phosphorylated p38 MAPK expression.

**Fig. 11.** Effect of AICAR or anisomycin on Fet-A expression in Hep3B cells and primary rat hepatocytes: (A) Hep3B cells were incubated in low or high glucose media with or without AICAR for 12 hours, and cell lysates were subjected to immunoblotting for Fet-A and phosphorylated Fet-A. (B) Hep3B cells or primary rat hepatocytes (C) were incubated with different concentration of AICAR for 12 h and cell lysate or media were analyzed by western blotting for indicated proteins. (D) Hep3B cells or primary rat hepatocytes (E) were treated with various concentrations of anisomycin for 0.5 h to analyze phosphorylated p38 MAPK, Fet-A and pFet-A expression**.**

**Fig. 12.** Proteosomal degradation pathway is involved in AICAR induced Fet-A degradation: (A) HepG2 cells were treated with AICAR (2mM) at 2 and 4 hr in the presence or absence of MG-132 and used for Western blot analysis of pAMPK, pP38MAPK, Fet-A expression. (B) HepG2 cells were treated with AICAR (2mM) in the presence or absence of different concentration of MG-132 for 12 hr and used for Western blot analysis Fet-A expression. (C) HepG2 cells were pretreated chloroquine for 1 h and incubated with AICAR for 12 h. The cell lysates were analyzed via Western blotting for Fet-A antibody. (D) HepG2 cells were treated with AICAR (2mM, 2, 4 and 12hr) and immunoprecipitated with anti-Fet-A antibody. The immunoprecipitates were analyzed via Western blotting for anti-ubiquitin and Fet-A antibodies.

**Fig. 13.** Long term effect of AICAR is associated with C/EBP beta downregulation: (A) HepG2 cells were incubated with different concentration of AICAR or metformin for 12 h and cell lysates were analyzed by western blotting for C/EBP beta. (B) Primary rat

hepatocytes were treated with different concentration of AICAR for 12 h and lysates were used to immunoblotting for C/EBP beta.

**Fig. 14.** Scheme for AICAR mediated regulation of hepatic Fet-A expression**.** In this model, high glucose increases the expression of Fet-A through ERK-1/2 pathway. High glucose also observed to decrease AMPK activation. AICAR increase the phosphorylation of AMPK, which can then activate p38 MAPK to downregulate Fet-A expression. Inhibition of AMPK or p38MAPK activation prevents the effect of AICAR on Fet-A expression. Whereas activation of p38MAPK by anisomycin decreases Fet-A expression. Short term effect of AICAR prevented by Proteosomal degradation inhibitor (MG-132). Long term effect is associated with downregulation of C/EBP beta, an important transcription factor involved in Fet-A regulation.

**Figure 8**

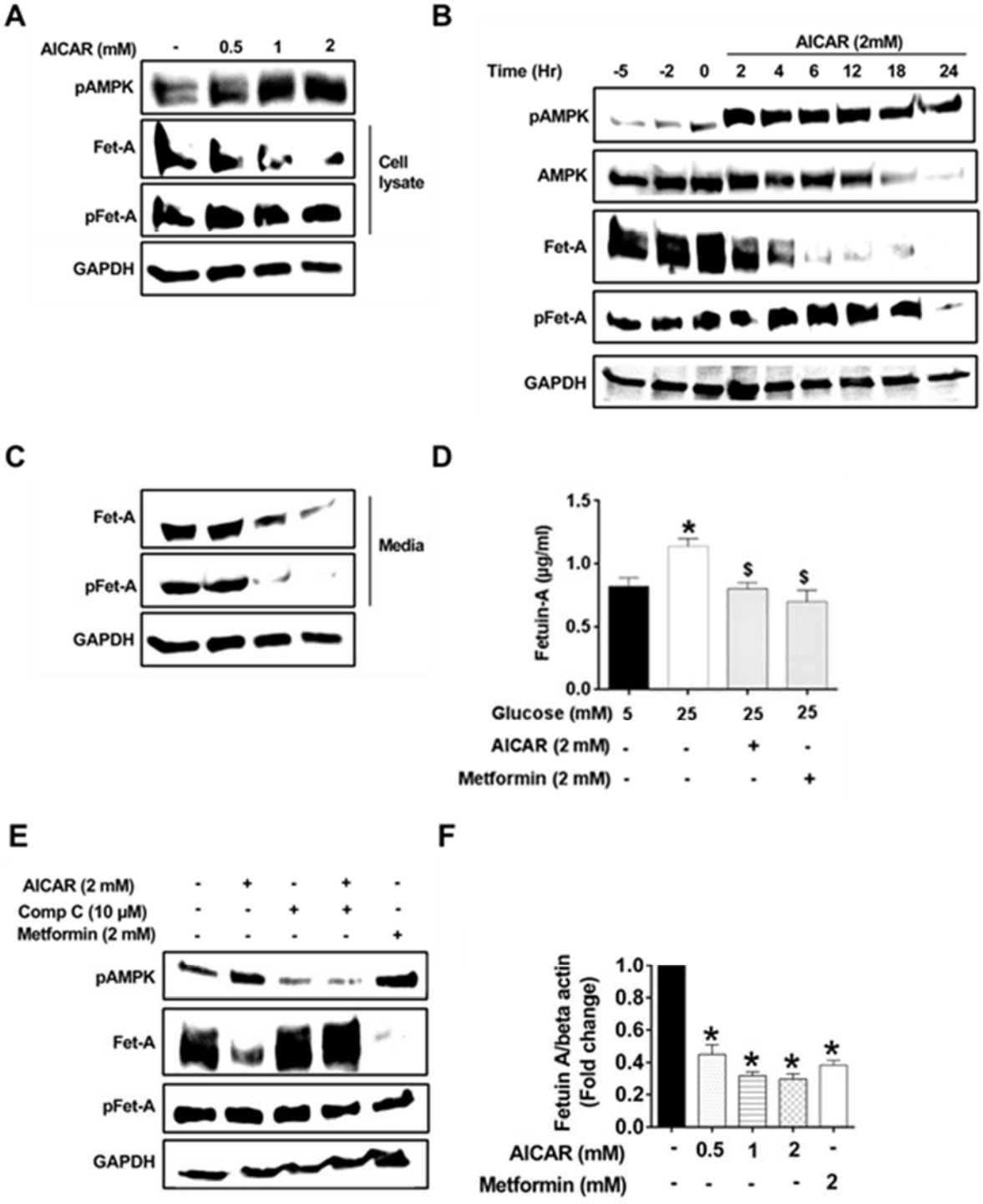
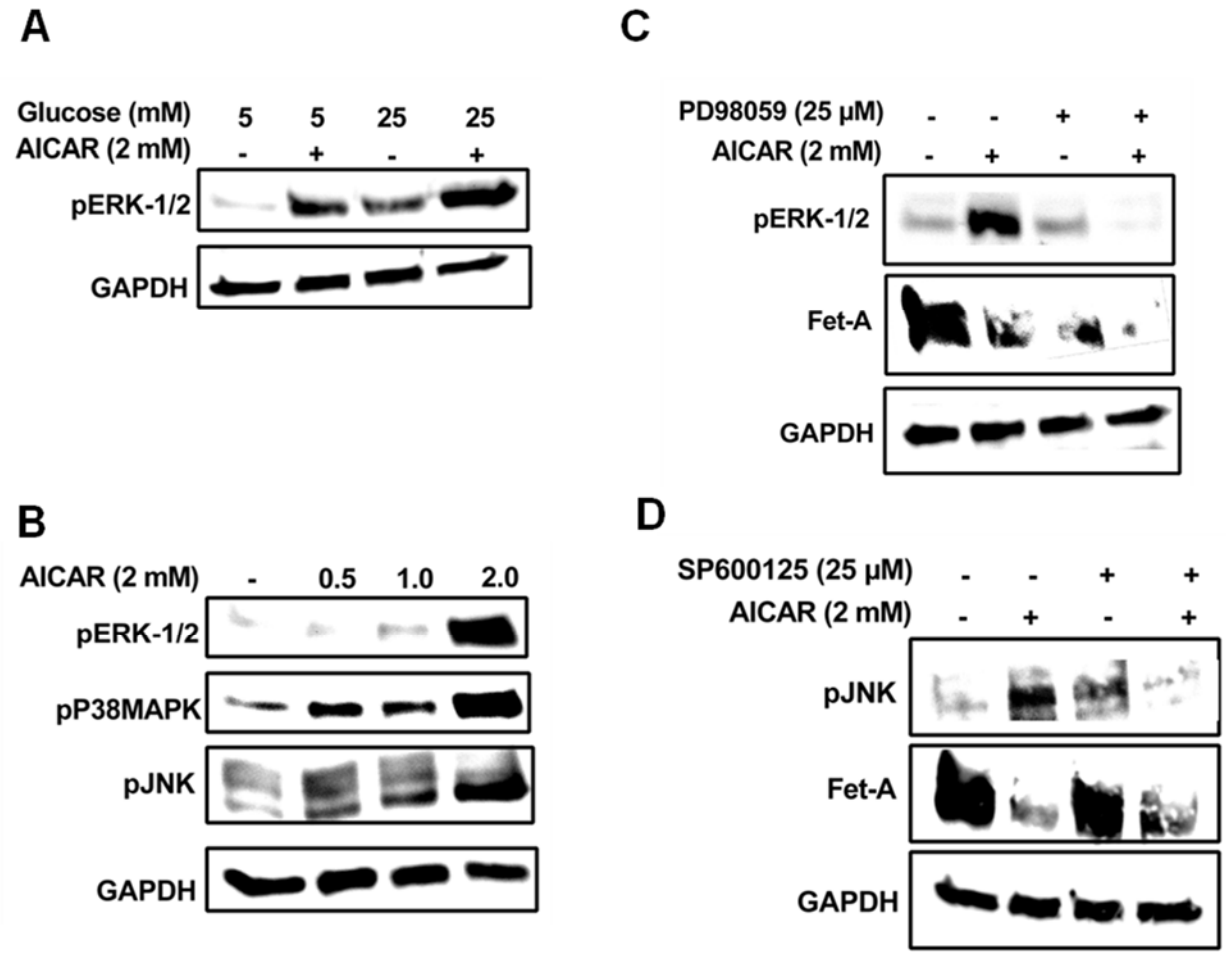
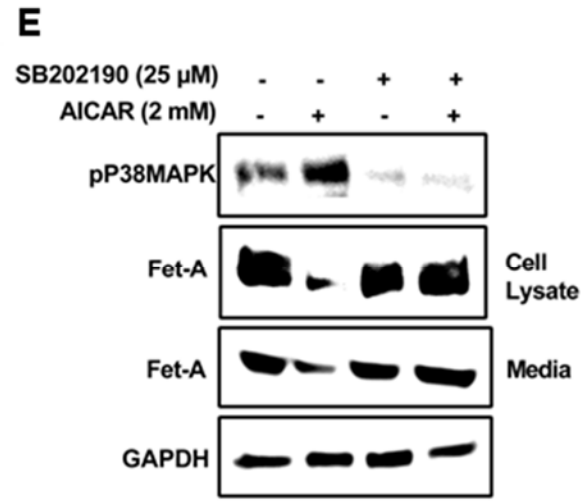
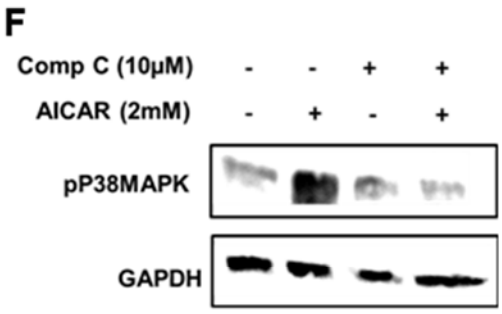

A
AICAR (mM) - 0.5 1 2
pAMPK
Fet-A
pFet-A
GAPDH
Cell lysate
B
AICAR (2mM)
Time (Hr) -5 -2 0 2 4 6 12 18 24
pAMPK
AMPK
Fet-A
pFet-A
GAPDH
C
Fet-A
pFet-A
GAPDH
Media
D
Fetuin-A (µg/ml)
1.5
1.0
0.5
0.0
*
$
$
Glucose (mM) 5 25 25 25
AICAR (2 mM) - - + -
Metformin (2 mM) - - - +
E
AICAR (2 mM) - + - + -
Comp C (10 µM) - - + + -
Metformin (2 mM) - - - - +
pAMPK
Fet-A
pFet-A
GAPDH
F
Fetuin A/beta actin (Fold change)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
AICAR (mM) - 0.5 1 2 -
Metformin (mM) - - - - 2

**Figure 9**


A
Glucose (mM) 5 5 25 25
AICAR (2 mM) - + - +
pERK-1/2
GAPDH
B
AICAR (2 mM) - 0.5 1.0 2.0
pERK-1/2
pP38MAPK
pJNK
GAPDH
C
PD98059 (25 μM) - - + +
AICAR (2 mM) - + - +
pERK-1/2
Fet-A
GAPDH
D
SP600125 (25 μM) - - + +
AICAR (2 mM) - + - +
pJNK
Fet-A
GAPDH


E
SB202190 (25 μM) - - + +
AICAR (2 mM) - + - +
pP38MAPK
Fet-A
Cell Lysate
Fet-A
Media
GAPDH


F
Comp C (10μM) - - + +
AICAR (2mM) - + - +
pP38MAPK
GAPDH

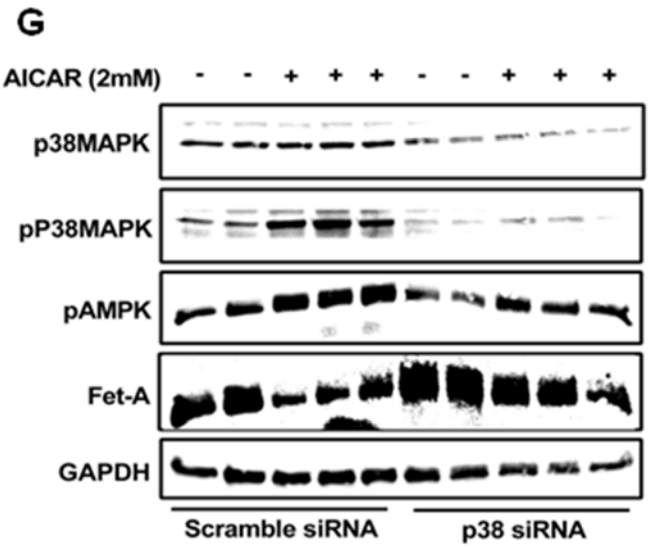

G
AICAR (2mM) - - + + + - - + + +
p38MAPK
pP38MAPK
pAMPK
Fet-A
GAPDH
Scramble siRNA
p38 siRNA

**Figure 10**

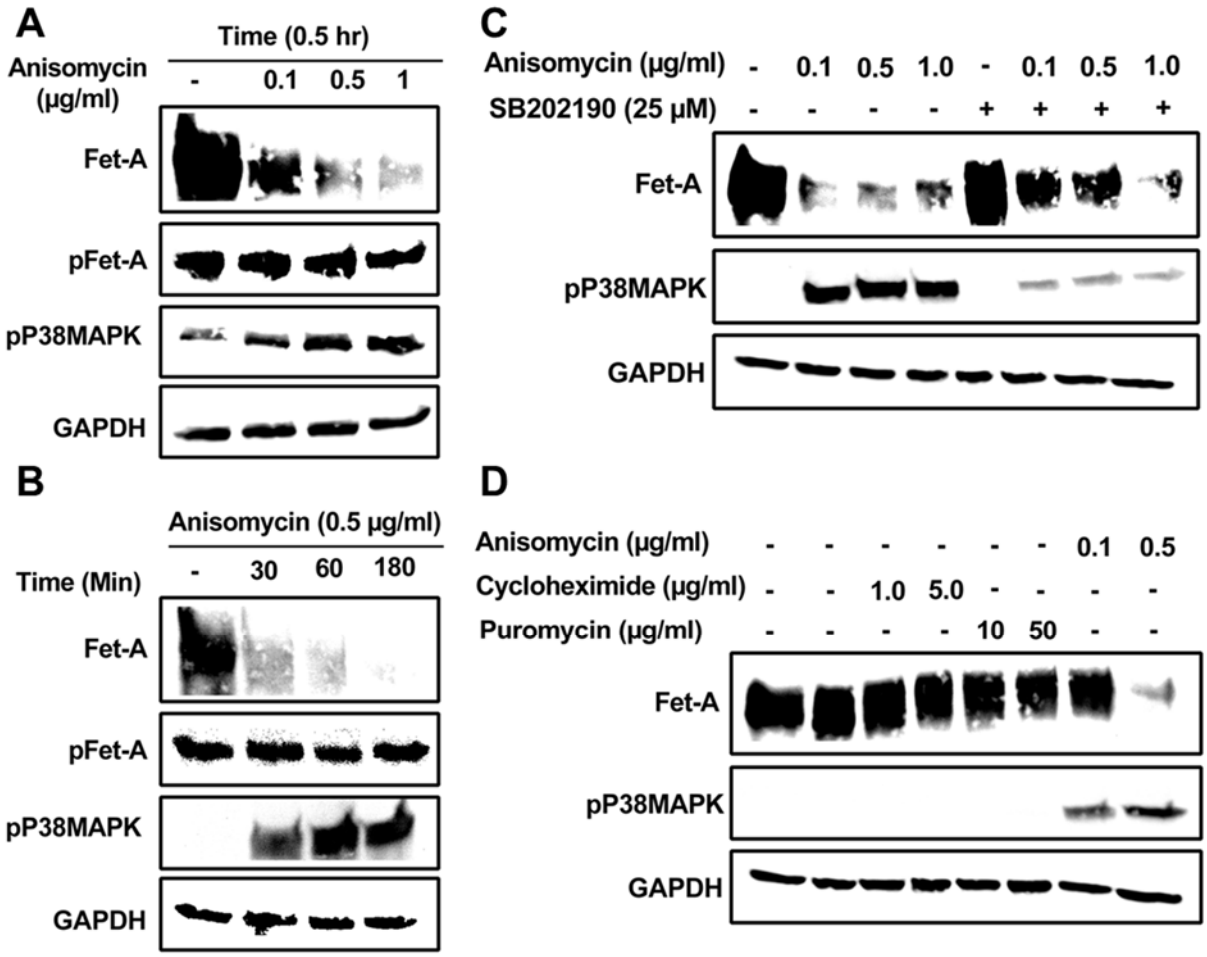

A
Time (0.5 hr)
Anisomycin (μg/ml)
- 0.1 0.5 1
Fet-A
pFet-A
pP38MAPK
GAPDH
B
Anisomycin (0.5 μg/ml)
Time (Min)
- 30 60 180
Fet-A
pFet-A
pP38MAPK
GAPDH
C
Anisomycin (μg/ml) - 0.1 0.5 1.0 - 0.1 0.5 1.0
SB202190 (25 μM) - - - - + + + +
Fet-A
pP38MAPK
GAPDH
D
Anisomycin (μg/ml) - - - - - - 0.1 0.5
Cycloheximide (μg/ml) - - 1.0 5.0 - - - -
Puromycin (μg/ml) - - - - 10 50 - -
Fet-A
pP38MAPK
GAPDH

**Figure 8**

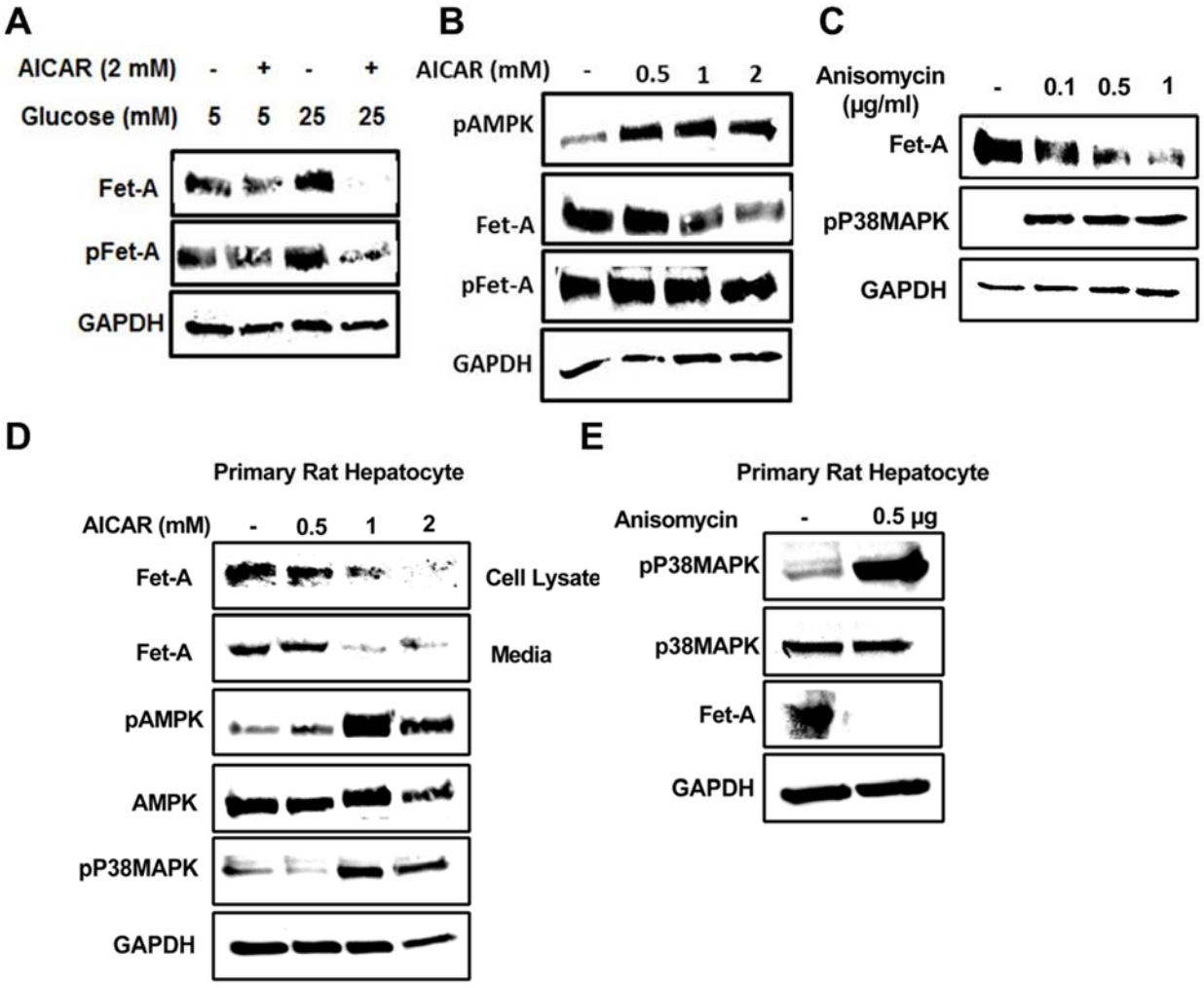

A
AICAR (2 mM) - + - +
Glucose (mM) 5 5 25 25
Fet-A
pFet-A
GAPDH
B
AICAR (mM) - 0.5 1 2
pAMPK
Fet-A
pFet-A
GAPDH
C
Anisomycin (μg/ml) - 0.1 0.5 1
Fet-A
pP38MAPK
GAPDH
D
Primary Rat Hepatocyte
AICAR (mM) - 0.5 1 2
Fet-A
Cell Lysate
Fet-A
Media
pAMPK
AMPK
pP38MAPK
GAPDH
E
Primary Rat Hepatocyte
Anisomycin - 0.5 μg
pP38MAPK
p38MAPK
Fet-A
GAPDH

**Figure 9**

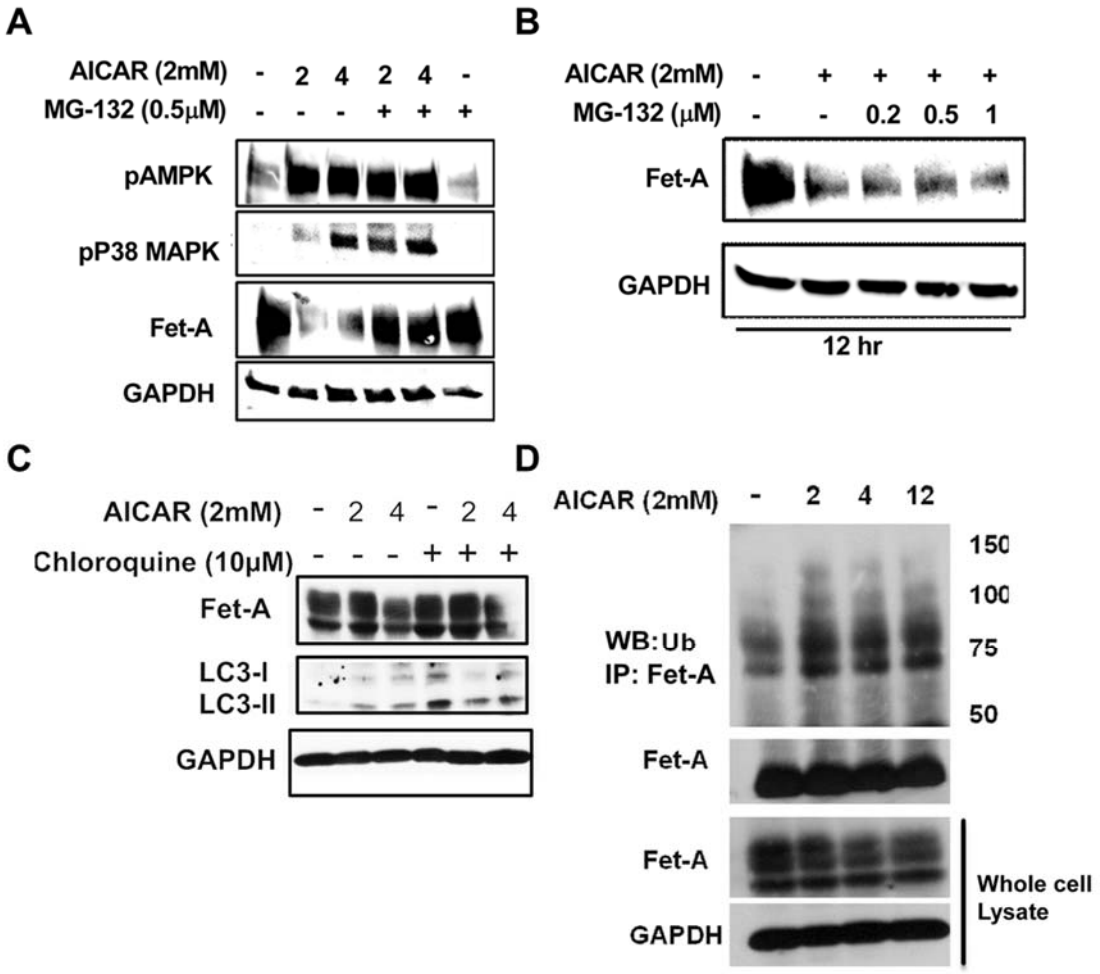

A
AICAR (2mM) - 2 4 2 4 -
MG-132 (0.5μM) - - - + + +
pAMPK
pP38 MAPK
Fet-A
GAPDH
B
AICAR (2mM) - + + + +
MG-132 (μM) - - 0.2 0.5 1
Fet-A
GAPDH
12 hr
C
AICAR (2mM) - 2 4 - 2 4
Chloroquine (10μM) - - - + + +
Fet-A
LC3-I
LC3-II
GAPDH
D
AICAR (2mM) - 2 4 12
150
100
75
50
WB: Ub
IP: Fet-A
Fet-A
Fet-A
GAPDH
Whole cell Lysate

**Figure 103**

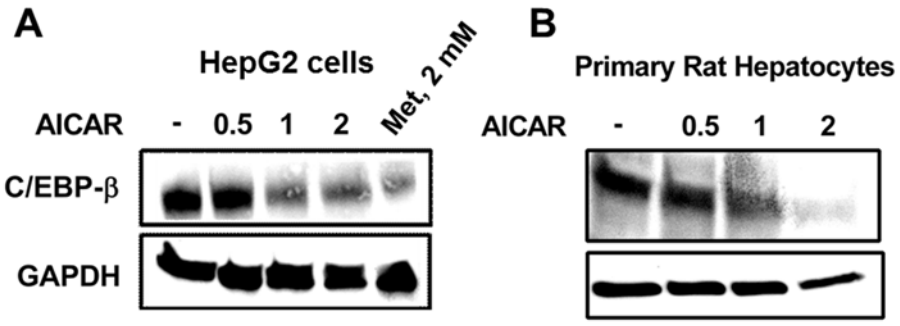
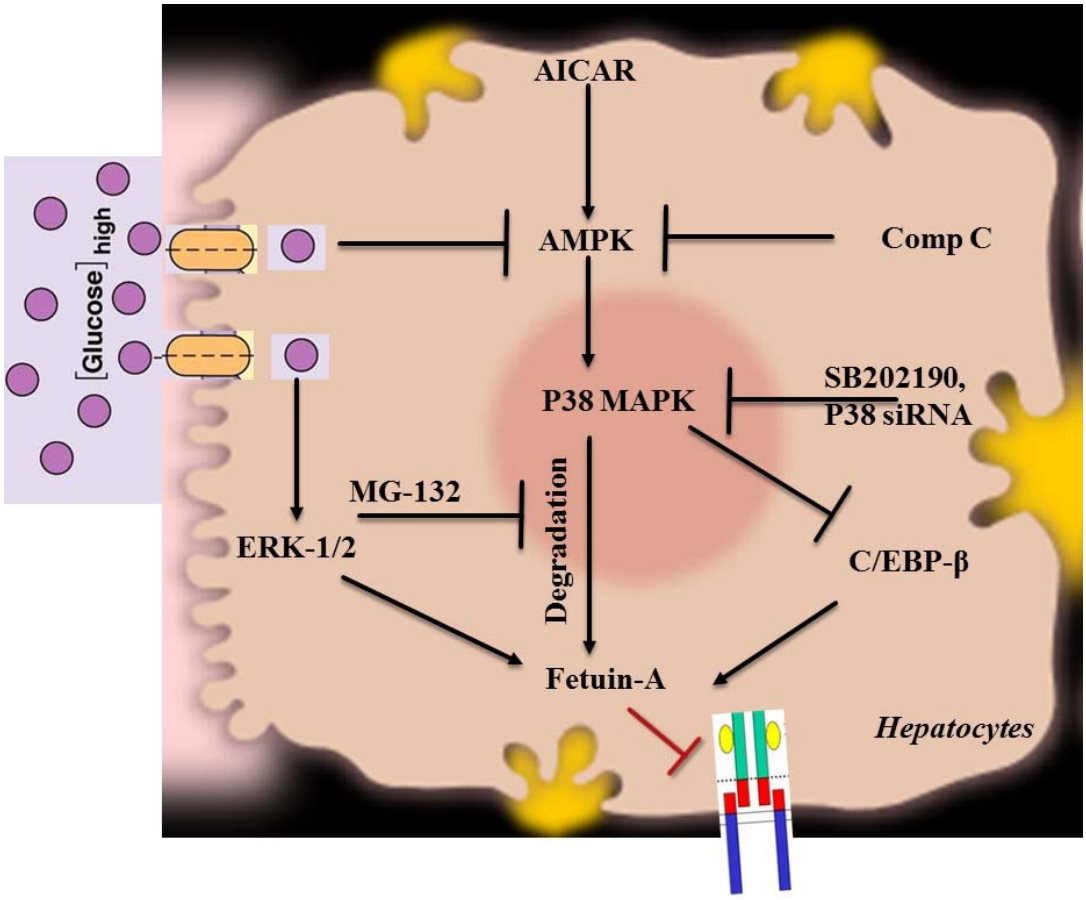

A
HepG2 cells
AICAR - 0.5 1 2 Met, 2 mM
C/EBP-β
GAPDH
B
Primary Rat Hepatocytes
AICAR - 0.5 1 2

**Figure 114**


AICAR
AMPK
Comp C
[Glucose]high
P38 MAPK
SB202190,
P38 siRNA
MG-132
ERK-1/2
Degradation
C/EBP-β
Fetuin-A
Hepatocytes

# Chapter 5: Regulation of hepatic fetuin-A expression: differential effect of lipopolysaccharide and tumor necrosis factor-α

## 5.1 Abstract

Fetuin-A is a hepatokine, known to impair insulin signaling, and is associated with obesity, insulin resistance, and non-alcoholic fatty liver disease. Fetuin-A synthesis is divergently regulated in injury versus infection, and acute versus chronic low-grade chronic inflammation, classifying it as a negative or positive acute-phase protein. Several pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-1, and IL-6 have been shown to decrease fetuin-A expression, while others including high-mobility group protein-1 (HMGB1) increase fetuin-A expression, suggesting that cytokines differently regulate hepatic fetuin-A expression. However, there is a significant gap in our knowledge of the mechanisms underlying this differential regulation of fetuin-A. We examined the effect of recombinant fetuin-A (Fet-A) on insulin signaling and its correlation with lipopolysaccharide (LPS)-induced insulin resistance. HepG2 or Hep3B cells were incubated with either LPS or TNF-α for different time/dosage before analyzing Fet-A and Ser312-phosphorylated fetuin-A (pFet-A) expression. We also characterized mechanistic basis of LPS or TNF-α effect on Fet-A expression in HepG2 cells. We observed that LPS increased the expression and secretion of Fet-A and its phosphorylated form, while TNF-α decreased Fet-A expression in a dose dependent manner. The effect of LPS on Fet-A was associated with the inhibition of insulin-induced AKT and GSK3

phosphorylation. To identify the mechanisms that potentially mediate the divergent effects of LPS and TNF-α, we determined their effect on C/EBP beta, a key transcription factor involved in the regulation of Fet-A expression. We observed that while TNF-α decreased the expression of C/EBP beta, LPS increased its expression. Moreover, LPS was found to increase the interaction of C/EBP beta with Fet-A. Taken together, our studies implicate a critical role of C/EBP beta in the differential effects of LPS- and TNF-α mediated regulation of hepatic Fet-A expression.

**5.2 Introduction**

Diabetes is a global health problem with a prevalence of more than 415 million cases and the incidence that expected to increase to 642 million in 2040 (52). The vast majority of diabetes patients (90-95%) suffer from type 2 diabetes, whereas type 1 diabetes account for only 5-10% (53). The underlying metabolic causes of type 2 diabetes are the combination of impairment in insulin mediated glucose disposal (insulin resistance) and defective secretion of insulin by pancreatic β –cells. While, type 1 diabetes is a chronic condition in which the pancreas produces little or no insulin (54).

Recently, a chronic low-grade inflammation and an activation of the immune systems are well recognized in the pathogenesis of insulin resistance and type 2 diabetes (55). Among several pathways involved in the pathogenesis of the type 2 diabetes, an altered secretory pattern of the in various tissues is thought to be important for the regulation of insulin sensitivity and subclinical inflammation. In this respect adipose tissue has gained much attention in the past years because the circulating levels of this adipokines/cytokines are not only markers of type 2 diabetes risk, but also because of strong involvement in its progression (56). Analogous to the adipose tissues, dysregulated secretory pattern from

liver, which can be referred to as hepatokines are also involved in its pathophysiology (27). Among them Fet-A gained much attention during the recent years because of its association with type 2 diabetes and cardiovascular disease risk and its important role in the pathogenesis of insulin resistance and subclinical inflammation (28, 29).

Fetuin-A, also known as a2-Heremans–Schmid glycoprotein, is an abundant serum protein produced predominantly in the liver (57). The human Fet-A gene, designated AHSG, resides on chromosome 3 (3q27) which is mapped as a type 2 diabetes and metabolic syndrome susceptibility locus and contains seven exons and six introns, spanning an approximately 8.2-kb region (58). Fet-A shares amino-acid sequence similarity to insulin receptor tyrosine kinase (9) and type-II transforming growth factor-ß receptor, and thus been proposed as a natural inhibitor of the insulin-signaling pathway and an antagonist of TGF-ß (37). Originally Auberger *et al*. provided the first evidence for the role of fetuin A in insulin signaling. They indicate that phosphorylated N glycoprotein called pp63 secreted from hepatocytes and inhibit insulin receptor tyrosine kinase and receptor autophoshorylation while non-phosphorylated form of protein is inactive, although the protein they isolated was not recognized as fetuin at the time (25). Subsequently, Muller-Esterl *et al.* reported the sequence similarity of protein pp63 with rat fetuin A which inhibit the insulin-stimulated phosphorylation of IR and IR substrate-1 (IRS-1) (59). In addition, other groups have shown that purified bovine (9) and human fetuin A (7) also have similar effect indicating common inhibitory function for fetuin A homolog on insulin signaling. Furthermore, Mathews *et al*. demonstrated that inhibitory action of recombinant fetuin A on the insulin-stimulated phosphorylation of IR and IRS-1 which is mediated by direct interaction between Fet-A and IR (8). They also show that

Fet-A did not affect epidermal growth factor (EGF) or insulin-like growth factor I-induced cognate receptor auto-phosphorylation, suggesting the relative specificity of Fet-A for IR. Although, the precise site of Fet-A and IR interaction has not been defined, Fet-A completely inhibited trypsin-activated IR auto-phosphorylation suggesting that fetuin-A does not compete with insulin for binding to IR (8, 60). Recently, Fet-A also shown to impairs insulin-mediated glucose uptake in C2C12 myotubes by down-regulating GLUT-4 translocation to the plasma membrane through decreased in phosphorylation of Akt and AS160 (24). Moreover, injection of Fet-A inhibited insulin-stimulated IR auto-phosphorylation and IRS-1 phosphorylation in rats (8) and knocking down of fetuin A improved the insulin signaling in HFD induced obese mice indicating the physiological relevance of *in vitro* observations (10).

Further correlation of fetuin A and metabolic disease has been outline by discovery of fetuin A as an endogenous ligand for TLR4 through which lipids induce insulin resistance. This new studies by Pal *et al* showed that Knockdown of TLR4 or fetuin A in obese insulin- resistant mice dramatically improved glucose homeostasis as a result of reduced activation of TLR4-mediated pro-inflammatory signaling cascades suggested an association between SFAs, Fetuin A and Tlr4 (10). *In vivo* relevance of this observation in human was established by Norbert et al. by anthropometric and metabolic data from 347 healthy subjects who are at increased risk for type 2 diabetes and CVD. They observed strong interaction between FFAs and Fet-A concentrations to induce insulin resistance but not between adiponectin and FFA (61). Recently, Bhattacharya *et al* reported that fetuin A is also synthesize and secreted from adipocyte and involved in lipid induced macrophage migration and M2 to M1 polarization into adipose tissue (11). This

suggest that the Fet-A–fatty acid complex induces inflammatory signaling and insulin resistance, important driving forces behind type 2 diabetes and cardiovascular disease (CVD).

Post-translational modification of fetuin A at different glycosylation and phosphorylation sites considered to regulate protein expression levels, stability, and biological activity. However, process of post-translational modifications is not fully understood. It has been suggested that phosphorylation is essential for Fet-A interaction with the insulin receptor (25). Fet-A gene expression in hepatocytes is up-regulated by high glucose and free fatty acids through ERK1/2 and NFkB respectively or by increase in endoplasmic reticulum (ER) stress (15, 16). In addition, glucocorticoid and estrogen is also found to increase the fetuin A expression by C/EBP beta and activator protein-1 respectively (17, 18). While, acute inflammation and other types of infection down regulate the expression of Fet-A and hence, it has been classified as one of the acute-phase proteins (APP). Fet-A gene expression is down regulated in rat liver hepatocytes by tumor necrosis factor-α (TNF- α) and by other pro-inflammatory cytokines like IL-6, and IL-1β in human hepatocytes (39, 40). In contrast, other late inflammatory mediators including high-mobility group protein-1 (HMGB1) increases Fet-A expression, suggesting that cytokines differently regulate hepatic Fet-A expression (62). However, there is a significant gap in our knowledge of the mechanisms underlying this differential regulation of Fet-A. In addition, regulation of phosphorylated forms of fetuin A is not known.

To clarify the effect of lipopolysaccharide (LPS) and TNF-α on Fet-A expression, we examined the effect on expression of Fet-A and its phosphorylated forms in HepG2 cells and Hep3B cells. Here, we demonstrate that LPS increase Fet-A expression, while TNF

decrease it through changes in through C/EBP beta. Further, we also observed that AICAR downregulate LPS induced Fet-A expression through AMPK activation.

### 5.3 Materials and Methods

#### 5.3.1 Reagents and antibodies

All cell culture materials were obtained from VWR International (Radnor, PA) or Life Technologies (Grand Island, NY). Recombinant protein of Fet-A was procured from BioVendor (Asheville, NC). Compound C (AMPK inhibitor) were obtained from Enzo Life Science. AICAR was purchased from Cayman Chemical Company (Ann Arbor, MI). Anti-Fet-A antibody was from R&D Systems (Minneapolis, MN). Phosphorylated (Ser312) Fet-A was detected using a custom-generated antibody that specifically recognized phosphorylation on Ser312-Fet-A (22). Anti-NFkB, anti-pNFkB, anti-pAMPK (Thr172), anti-AMPK, anti-pAKT (Ser473) and anti-pGSK3 (Ser21) antibodies were purchased from Cell Signaling (Danvers, MA). Antibodies against C/EBPβ (C-19) and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

#### 5.3.2 Cell lines and primary culture

HepG2 and Hep3B human hepatocyte-derived cell lines were purchased from American Type Culture Collection (Manassas, VA). HepG2 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% (v/v) FBS (fetal bovine serum), penicillin, streptomycin and neomycin (1%) in a humidified 5% CO2 atmosphere at $37^0$C. Hep3B cells were cultured in MEM (minimal essential medium) containing non-essential amino acids supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% (v/v) FBS and antibiotics.

### 5.3.3 Cell culture treatment

Confluent HepG2 or Hep3B cells were subcultured by trypsinization and subsequently seeded in 6-well culture plates. After serum starvation, cells were treated with either LPS or TNF-α at the indicated dose and time. When inhibitors were used, cells were pre-incubated with Compound C (10 μM) or NS-50 (50 μg/ml) for 1 h before treatment with LPS. At the end of the incubation period, medium was collected or cells were lysed and centrifuged at 10,000 x g for 20 min.

### 5.3.4 Immunoprecipitation and western blot analysis

Following each treatment, the cells were washed twice with ice-cold phosphate buffer saline (PBS) buffer and lysed in the buffer supplemented with 50 mM HEPES, 1% Triton X 100, 10 mM EDTA, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, and protease inhibitor cocktail (Amresco, Solon, OH). The protein content in total cell lysates was determined using the Pierce 660 Protein Assay kit (Life Technologies). Cell lysates or culture supernatant medium were mixed with a sample loading buffer and separated on 8 or 4-20% SDS-PAGE gel (NuSep Inc, GA). For immunoprecipitation, cell lysates (500 μg) were diluted in lysis buffer and incubated with 4 μg of primary antibody. The immunoprecipitates were collected with protein A-Sepharose beads (Sigma) overnight at 4 °C and then washed three times with PBS. Samples were boiled in sample buffer and resolved on 4-20% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and incubated with appropriate antibodies. Protein bands were visualized by UVP BioImaging and VisionWorks software package (UVP, Upland, CA) using SuperSignal West Dura Extended Duration substrate (Pierce, Rockford, IL) and SuperSignal West Femto maximum sensitivity substrate (Pierce,

Rockford, IL). Relative area densities were quantified using the UN-SCAN IT software package, v.6.1 (Silk Scientific, Orem, UT).

### 5.3.5 Real-time PCR

Total RNA was extracted from HepG2 cells treated with or without AICAR (0.5–2.0 mM). Quantitative real-time PCR was carried out with SYBR green using the MyiQ single-color real-time PCR detection system (Bio-Rad, Hercules, CA). The following primer sets were used, Fet-A forward primer- 5'-ACG TGG TCC ACA CTG TCA AA-3', Fet-A reverse primer- 5'-CGC AGC TAT CAC AAA CTC CA-3', β-actin forward primer- 5'-CCT CTA TGC CAA CAC AGT GC-3' and β-actin reverse primer- 5'-CAT CGT ACT CCT GCT TGC TG-3'. Gene expression of Fet-A was normalized to that of β-actin mRNA.

### 5.3.6 Statistical analysis

Results are expressed as Mean ± Standard Error of the Mean (SEM). Comparisons between various treatments and/or groups were carried out using the unpaired Student's t-test or one-way Analysis of Variance (ANOVA) where appropriate. Statistical analyses were performed using GraphPad Prism 6 (GraphPad, San Diego, CA). Differences were considered statistically significant when $p < 0.05$.

## 5.4 Results

### 5.4.1 Lipopolysaccharide upregulate Fet-A and its phosphorylated forms

Previously, it has been observed that Fet-A expression in human HepG2 hepatoma cells reduced by pro-inflammatory cytokines such as TNF, IL-1, IL-6, and IFN-γ (39, 40). In contrast, HMGB1, a late pro-inflammatory mediator of lethal systemic inflammation, elevated Fet-A expression levels by 2-3 folds in HepG2 cells (62), suggesting that

different cytokines divergently regulate hepatic Fet-A expression. To have additional information on the association of inflammation with Fet-A and its phosphorylated forms, we selected HepG2 cells as Fet-A can inhibit the insulin effect/action in this cell line (Fig 5). We incubated HepG2 cells with different concentration of LPS for 4 hr (Fig 16A). LPS treatment increases NFkB phosphorylation at 0.5-5 µg/ml, indicating activation of inflammatory pathway at the indicated concentrations (Fig 16A). Activation of NFkB with LPS was found to be associated with significantly increased in expression of Fet-A and its phosphorylated forms in a dose-dependent manner as compare to control. Effect of LPS starts at concentration as low as 0.5 µg/ml and peaking at concentration around 1µg/ml (Fig 16A). Subsequently, effect of LPS (10ng/ml to 5 µg/ml) on secretion of Fet-A and pFet-A has been tested after 4 hr of treatment (Fig 6B). As shown in Figure 2B, activation of NFkB was correlated with increase in LPS mediated secretion of Fet-A and pFet-A into the media. Interestingly, LPS treatment increases more pFet-A secretion as compare to Fet-A levels at tested concentrations (Fig 16B). Moreover, pulse chase analysis at 1.0 µg/ml suggesting LPS increases Fet-A and its phosphorylated forms starting from 30 minutes till 8 hr (Fig 16C). To further confirm these finding, we also tested effect of LPS in human hepatoma Hep3B cells. As shown in figure 17A and 17B, similar to HepG2 cells, treatment of LPS increases Fet-A and pFet-A level in a dose and time dependent manner in Hep3B cells. Interestingly, after 8hr of LPS treatment downregulate Fet-A expression in HepG2 and Hep3B cells (Fig 17C, 17B). Similar to the protein level, gene expression of Fet-A is increase with LPS treatment at 2 and 4 hr, while decrease at 6 and 12 hr (Fig 16D). In addition, increase in expression/secretion of Fet-A and its phosphorylated forms with LPS treatment for 4 hr is also correlated with

impairment of insulin signaling as indicated by decrease in AKT and GSK3 phosphorylation (Fig 16F). These data demonstrated that 4 hr treatment of LPS upregulate Fet-A and pFet-A levels in human hepatoma cells which is associated with impairment of insulin signaling.

### 5.4.2 TNF-α downregulate Fet-A and its phosphorylated forms

To further understand the inflammation mediated regulation of Fet-A and pFet-A, we also tested the effect of TNF-α (endogenous) in human hepatoma cells. As shown in figure 18A and 18B, TNF-α activate inflammatory pathway in HepG2 and Hep3B cells as indicated by NFkB phosphorylation after 4hr of treatment. However, contrary to LPS, treatment of TNF-α in HepG2 and Hep3B cells for 4hr down-regulated Fet-A and pFet-A expression in a dose dependent manner (Fig 18A, 18B). In addition, TNF-α also decreased Fet-A and pFet-A secretion in HepG2 and Hep3B cells (Fig 18A and 18B). Despite activation NFkB, downregulation of Fet-A and pFet-A by TNF-α indicate NFkB pathway is not involve in inflammation mediated regulation of Fet-A and pFet-A. This is further supported by increase in Fet-A expression by LPS, despite inhibition of NFkB activation (Fig 19D).

### 5.4.3 Differential effect of LPS and TNF-α on C/EBP-β

The transcriptional activity of the Fet-A gene is mediated by several CCAAT enhancer-binding proteins (C/EBP)-β and NF-1 binding sites in its promoter region (63). Basal transcription of Fet-A mainly maintained by long isoforms (LAP) of C/EBP-beta, while short isoforms (LIP) counter the basal transcription of Fet-A. Negative regulation of Fet-A by inflammation is mediated by cytokine-induced replacement of long C/EBP-β isoforms by short isoforms, which were unable to trans-activate Fet-A gene transcription

(63). To understand the differential effect of LPS and TNF-α on hepatic Fet-A expression, we check its effects on C/EBP-β long isoforms. As shown in Fig 19A, treatment of LPS increased expression of C/EBP-β long isoforms starting from 0.1 μg/ml to 5 μg/ml. While, treatment of TNF-α downregulates the expression of C/EBP-β long isoforms (Fig 19B). In addition, treatment of LPS was found to upregulate the Fet-A interaction with C/EBP-β long isoforms in HepG2 cells (Fig 19C). Thus, these data suggesting divergent effect of LPS and TNF-α on Fet-A and pFet-A is mediated by differential effect on C/EBP-β long isoforms expression/interaction in human hepatoma cells.

### 5.4.4 AICAR downregulate LPS induced Fet-A expression through AMPK

Previously, we observed that AICAR negatively regulate high glucose-induced Fet-A expression by AMPK-p38 MAPK kinase mediated degradation of Fet-A in short term treatment. While, in long term treatment AICAR also regulate Fet-A expression at transcription level by downregulation C/EBP beta. To understand its effect on LPS mediated upregulation of Fet-A and pFet-A expression, we treated HepG2 cells with LPS in presence or absence of AICAR for 4 hr. As observed before, LPS treatment significantly increases Fet-A and pFet-A expression in HepG2 cells associated with NFkB activation (Fig 20A). While pre-treatment of AICAR activate AMPK and downregulate the LPS induced Fet-A and pFet-A expression with decrease in NFkB phosphorylation (Fig 20A). However, blocking of AMPK activation by Comp C, prevent the effect of AICAR on LPS-induced Fet-A upregulation (Fig 20B). These results suggest that AICAR downregulate LPS-induced Fet-A and pFet-A expression through AMPK.

## 5.5 Discussion

On the basis of several reports that have accumulated for several years, Fet-A, a secreted hepatokine is emerge as an independent risk predictor of Type 2 diabetes and associated with insulin resistance, diabetes, cardiovascular outcomes and sub-inflammation (27, 28, 29). Fet-A is known to inhibit the insulin receptor tyrosine kinase and inhibition of insulin-stimulated downstream signaling (8). However, certain factors related to its regulation are still unclear. Fet-A is classifying as an acute phase protein, divergently regulate during injury and infection. Early inflammatory mediators negatively regulate, while late inflammatory mediators positively regulate Fet-A expression. However, mechanism of this differential regulation is not completely understood. In addition, regulation of phosphorylation forms of Fet-A, which is consider as a critical for inhibition of insulin signaling is not known. In the present study, we wanted to add new information, which may fill some gaps in our understanding of Fet-A's regulation during inflammation. Since Fet-A can impair insulin action in HepG2 cells; we initially used this cell line to understand the regulation of hepatic Fet-A and its phosphorylation forms by exogenous (bacterial endotoxin) and endogenous (TNF-α) inflammatory stimuli. Previously it has been shown that Fet-A is a negatively regulated during acute inflammation and its synthesis decreased in HepG2 cells in the presence of LPS treated condition medium (40). Similarly, treatment of LPS decreases circulating fetuin A level in Balb/C mice at 24hr and 48hr of injection, considering it as negative APP (62). In contrary, we observed that LPS increases Fet-A and pFet-A protein expression as well as secretion in HepG2 cells (Fig 16A, B). The effect of LPS on upregulation of Fet-A peak at 1µg/ml and started from 30 min till 8hr in HepG2 cells (Fig 16C). Importantly, LPS also found to increase Fet-A and pFet-A protein expression in human hepatoma Hep3B cells as well at 4 hr of

treatment (Fig 17A). While similar to the previous reports, at 8hr and 24 hr of LPS treatment Fet-A level decrease significantly as compare to basal (Fig 17B) without downregulation in phosphorylation status of Fet-A. This observation also correlated at mRNA level, as LPS increase Fet-A gene expression at 2 and 4hr, while decreases significantly at 6 and 12hr as compare to control. In addition, increase level of Fet-A and pFet-A (at 4hr) is correlated with inhibitory effect of LPS on insulin signaling (Fig 16E). So, here we propose that Fet-A synthesis and secretion upregulated with LPS treatment acutely. While, long term treatment of LPS downregulate Fet-A synthesis as described previously probably due to feedback regulation. On the other hand, treatment of TNF-α decreases Fet-A and pFet-A synthesis/secretion in human hepatoma HepG2 and Hep3B cells (Fig 18A, 4B).

Since, LPS and TNF-α differently regulate Fet-A and pFet-A expression, it is important to identify factors involved in it. First, we analyzed the expression of NFkB, as activation of NFkB is involved in Fet-A upregulation (16). As expected, NFkB activated by treatment of both LPS and TNF-α, despite differential effect on Fet-A and its phosphorylated forms. In addition, inhibition of NF-kB activation does not prevent the effect of LPS on Fet-A, indicating noninvolvement of NF-kB in exogenous and endogenous inflammatory stimuli mediated regulation of Fet-A. Second possibility we consider is the role of late inflammatory mediator HMGB1, as it is known to upregulate Fet-A expression. However, LPS and TNF-α reported increases release of HMGB1 after 12hr of treatment (65) at which, we found LPS decrease Fet-A and pFet-A expression. So, HMGB1 is also not involve in LPS and TNF mediated Fet-A regulation. Third possibility we explore is the contribution of C/EBP beta, a transcription factor involved in basal

expression of Fet-A (64). It is known that glucocorticoid upregulate Fet-A expression through C/EBP beta and downregulation of Fet-A expression was observed during inflammation due to loss of interaction of C/EBP beta isoforms with neighboring sites. Loss of interaction was mainly due to replacement of long C/EBP beta isoforms with short C/EBP beta isoforms (64). Here, we observed that LPS upregulate, while TNF-α downregulate the expression of C/EBP beta long isoform. In addition, LPS found to increases interaction of C/EBP beta with Fet-A in HepG2 cells. So, the contrary effect on Fet-A and pFet-A after 4hr treatment of LPS and TNF-α may be due to differential effect on C/EBP beta. Given the important various functions of Fet-A in different systems, the present data pave the way to understand the pleotropic (sometimes even contradictory) effects such as pro-inflammatory and anti-inflammatory attributes of Fet-A.

**5.6 Figure Legends**

**Fig. 15.** Effect of LPS on Fet-A, pFet-A levels and its association with insulin signaling in HepG2 cell. (A) HepG2 cells were incubated with different concentration of LPS for 4 h and cell lysate or (B) media were analyzed by western blotting for Fet-A, pFet-A, pNFkB and GAPDH. (C) HepG2 cells were incubated with LPS at 1µg/ml for different time and cell lysate were analyzed by western blotting. The blots were analyzed with antibodies against Fet-A, pFet-A, pNFkB and GAPDH. (D) HepG2 cells were incubated in serum free DMEM media containing 1µg/ml LPS for 2, 4, 6 and 12 hours. Total RNA was isolated after treatment, and Fet-A mRNA levels were analyzed by real time PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were measured for internal controls (E) HepG2 cells were incubated in low (5 mM) glucose media containing either vehicle or LPS (1µg/ml) for 4h followed by insulin treatment for 15 min

and cell lysate were subjected to immunoblotting for AKT and GSK3 phosphorylation status.

**Fig. 16.** LPS-treatment upregulate Fet-A and pFet-A expression in Hep3B cells: (A) Hep3B cells were incubated with different concentration of LPS for 4 h and cell lysate were analyzed by western blotting for Fet-A, pFet-A, pNFkB and GAPDH. (B) Hep3B cells were incubated with LPS at 1µg/ml for different time and cell lysate were analyzed by western blotting. The blots were analyzed with antibodies against Fet-A, pFet-A, pNFkB and GAPDH.

**Fig. 17.** TNF-α treatment decreases Fet-A and pFet-A expression: (A) HepG2 or Hep3B cells were maintained in DMEM containing 10% FBS. Twenty-four hours after cells reached confluence, the cells were incubated overnight in the same medium without FBS. Then HepG2 or Hep3B cells (B) with TNF-α at different concentration for 4 h and cell lysate or media were analyzed by western blotting for Fet-A, pFet-A, NFkB, pNFkB and GAPDH.

**Fig. 18.** Effect of LPS and TNF-α on C/EBP-β in HepG2 cells. (A) Cells were incubated with different concentration of LPS for 4 h and cell lysate were analyzed by western blotting for C/EBP-β and GAPDH. (B) HepG2 cells were treated with LPS or TNF-α for 4 hr and cell lysate were analyzed by western blotting for C/EBP-β and GAPDH. (C) HepG2 cells were treated with LPS (1µg/ml, 4hr) and immunoprecipitated with anti-C/EBP-β antibody. The immunoprecipitates were analyzed via Western blotting for anti-Fet-A and C/EBP-β antibodies.

**Fig. 19.** AICAR downregulate LPS-induced Fet-A and pFet-A expression in HepG2 cells. (A) HepG2 cells were maintained in DMEM containing 10% FBS. Twenty-four hours

after cells reached confluence, the cells were incubated overnight in the same medium without FBS. Then cells were treated with LPS (1μg/ml) for 4h pre-treated with vehicle or AICAR and cell lysate were subjected to immunoblotting for Fet-A, pFet-A, pNFkB, NFkB, pAMPK, AMPK and GADH status. (B) HepG2 cells were incubated with AICAR in the presence or absence of Compound C, an AMPK inhibitor; cell lysates were immunoblotted for Fet-A and GAPDH.

**Figure 12**

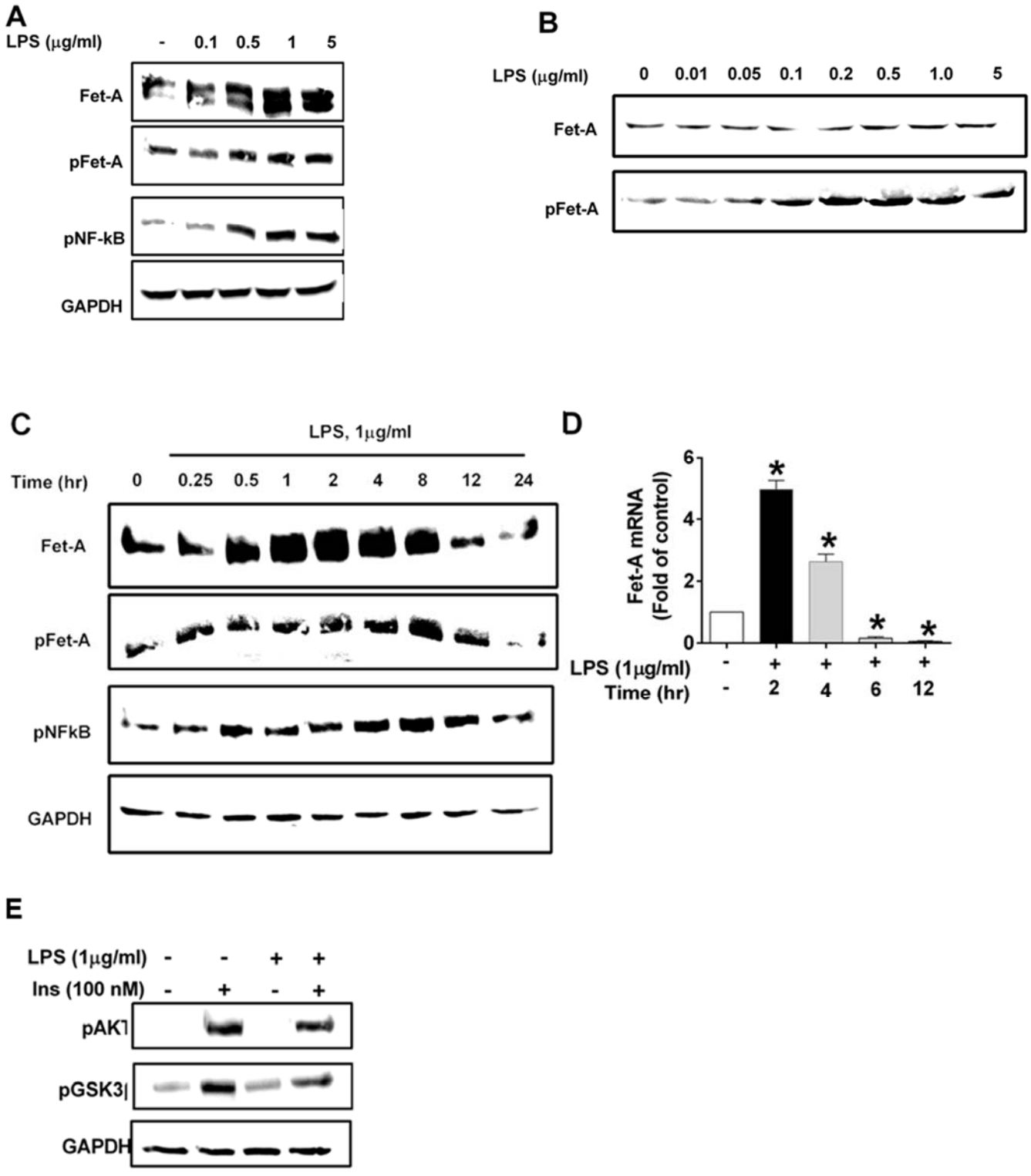

A
LPS (μg/ml) - 0.1 0.5 1 5
Fet-A
pFet-A
pNF-kB
GAPDH
B
LPS (μg/ml) 0 0.01 0.05 0.1 0.2 0.5 1.0 5
Fet-A
pFet-A
C
LPS, 1μg/ml
Time (hr) 0 0.25 0.5 1 2 4 8 12 24
Fet-A
pFet-A
pNFkB
GAPDH
D
Fet-A mRNA (Fold of control)
6
4
2
0
LPS (1μg/ml) - + + + +
Time (hr) - 2 4 6 12
E
LPS (1μg/ml) - - + +
Ins (100 nM) - + - +
pAKT
pGSK3β
GAPDH

**Figure 13**

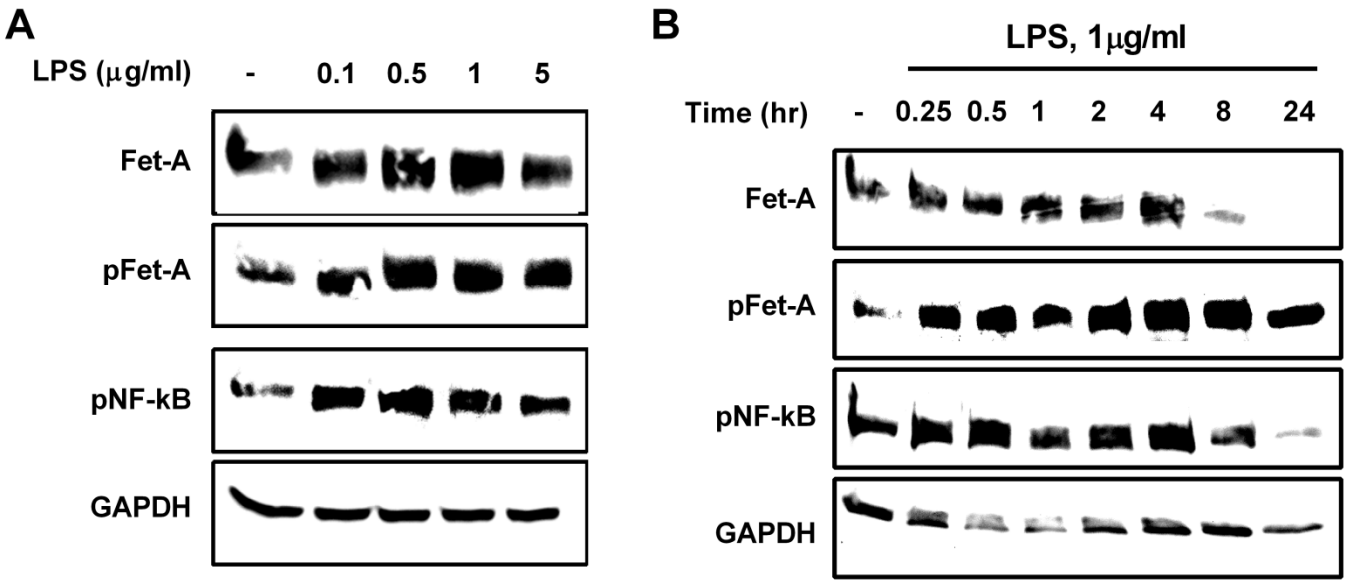

A
LPS (μg/ml)
-
0.1
0.5
1
5
Fet-A
pFet-A
pNF-kB
GAPDH
B
LPS, 1μg/ml
Time (hr)
-
0.25
0.5
1
2
4
8
24
Fet-A
pFet-A
pNF-kB
GAPDH

**Figure 14**

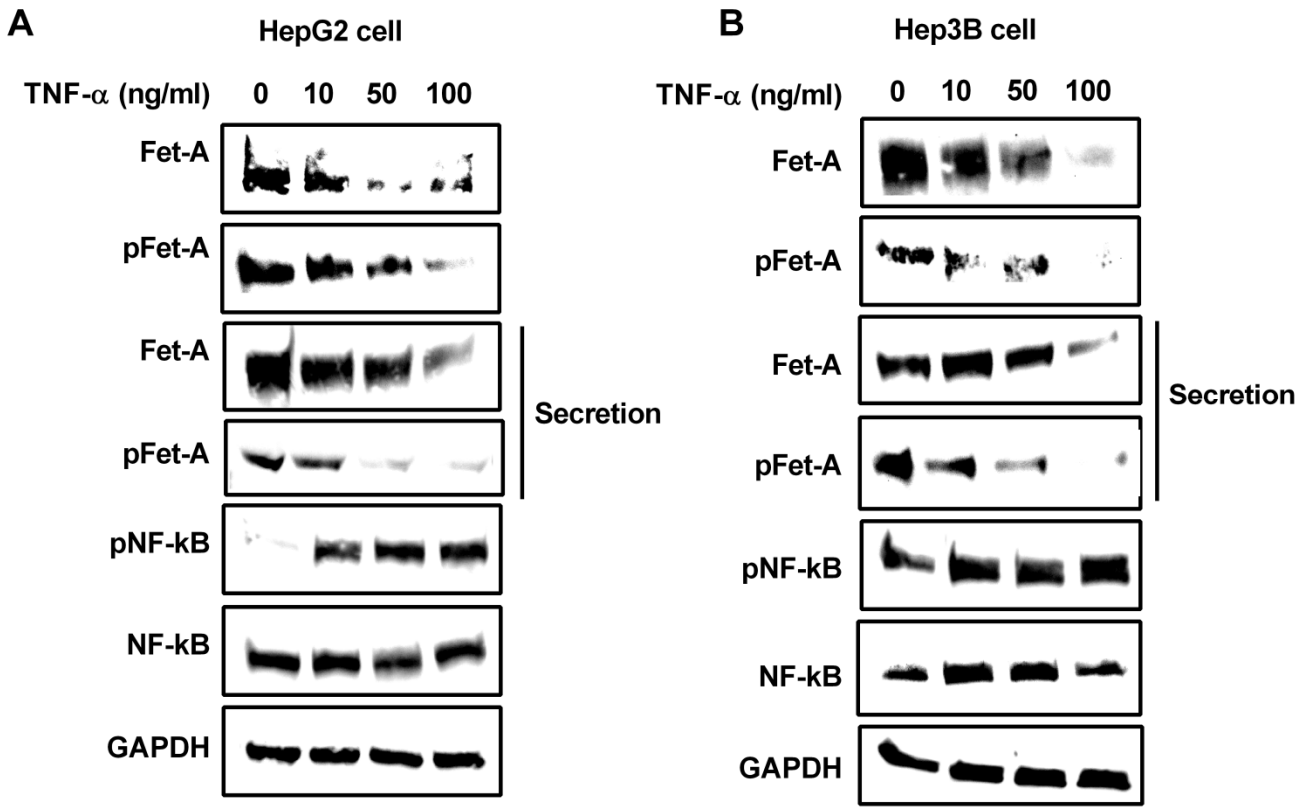
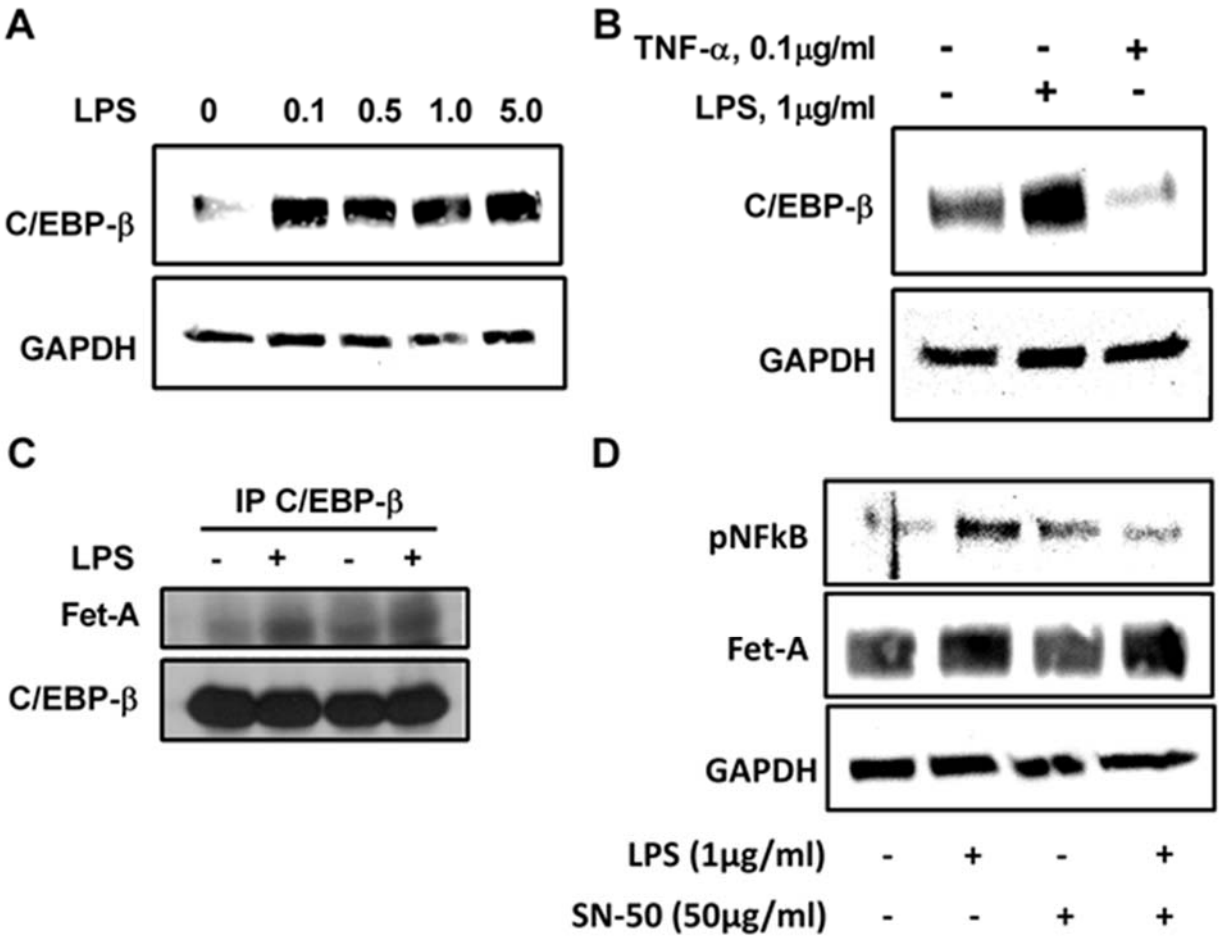

A
HepG2 cell
TNF-α (ng/ml) 0 10 50 100
Fet-A
pFet-A
Fet-A
pFet-A
Secretion
pNF-kB
NF-kB
GAPDH
B
Hep3B cell
TNF-α (ng/ml) 0 10 50 100
Fet-A
pFet-A
Fet-A
pFet-A
Secretion
pNF-kB
NF-kB
GAPDH

**Figure 15**


A
LPS 0 0.1 0.5 1.0 5.0
C/EBP-β
GAPDH
B
TNF-α, 0.1μg/ml - - +
LPS, 1μg/ml - + -
C/EBP-β
GAPDH
C
IP C/EBP-β
LPS - + - +
Fet-A
C/EBP-β
D
pNFkB
Fet-A
GAPDH
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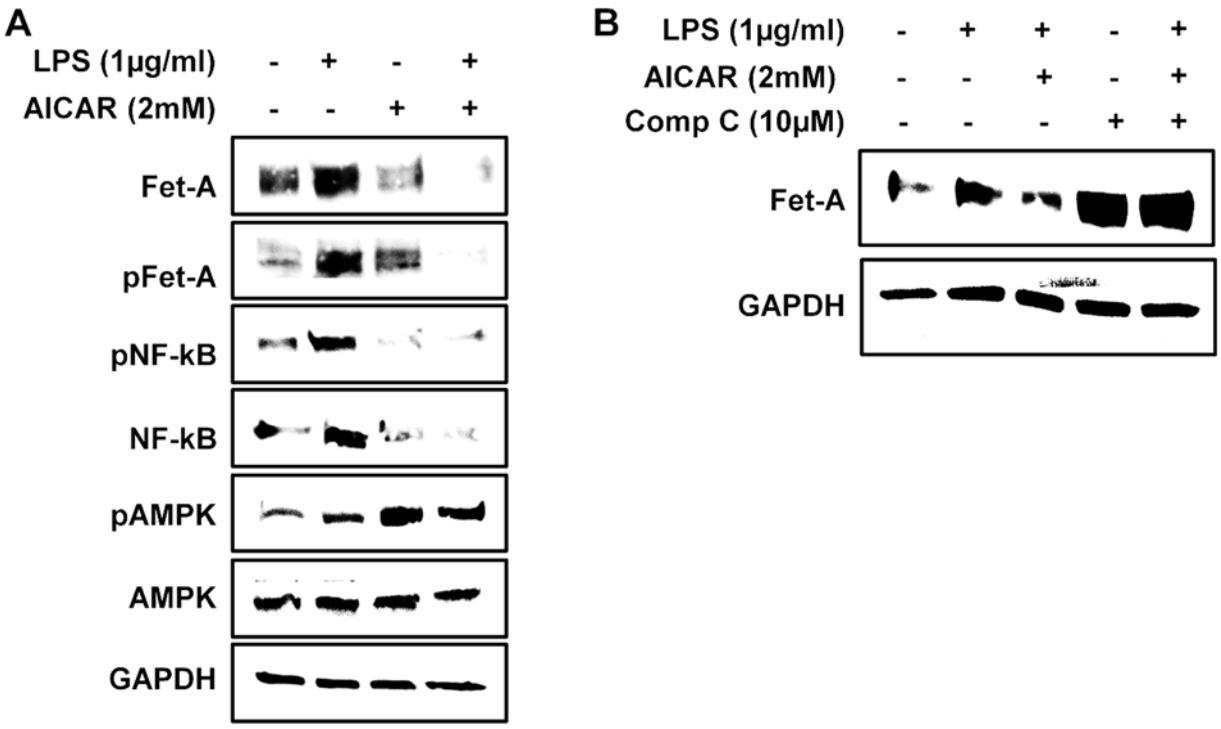
SN-50 (50μg/ml) - - + +

**Figure 16**


A
LPS (1μg/ml) - + - +
AICAR (2mM) - - + +
Fet-A
pFet-A
pNF-kB
NF-kB
pAMPK
AMPK
GAPDH
B
LPS (1μg/ml) - + + - +
AICAR (2mM) - - + - +
Comp C (10μM) - - - + +
Fet-A
GAPDH

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# Chapter 6: Summary and conclusion

Fetuin-A, a liver secreted protein, promotes insulin resistance directly by binding with the insulin receptor beta subunits and inhibiting the insulin-induced tyrosine kinase auto-phosphorylation. In addition, fetuin-A acting as an endogenous ligand necessary for fatty acid-induced TLR4 activation and polarization of anti-inflammatory macrophage (M2) to inflammatory macrophage (M1) in adipocytes increases pro-inflammatory cytokine secretion and indirectly increased insulin resistance. We and other previously suggested that phosphorylation status of fetuin-A is critical for inhibition of insulin signaling and it is positively associated with obesity and other metabolic mediators.

Our findings reveal that recombinant fetuin-A containing Ser312 phosphorylation sites inhibit insulin signaling (phosphorylation of AKT, MAPK, GSK, IRS-1) and insulin action (gluconeogenesis and glucose production) in human hepatoma HepG2 cells. We also report that metabolic mediators including glucose and saturated fatty acid positively regulating Fet-A and pFet-A expression as well as secretion. While, insulin and AMPK activation downregulating high glucose-induced Fet-A and pFet-A expression/secretion. For the first time, we have pursued pharmacological approaches along with siRNA-mediated knockdown methods to study the molecular mechanisms involved in AMPK mediated negative Fet-A regulation. In these studies, we have explored the downstream modulators of agonist-mediated AMPK phosphorylation and have identified a novel AMPK→ p38MAPK pathway that functions as a critical determinant of

hepatic Fet-A expression, both in low glucose and high glucose condition. We also observed that activation of AMPK by AICAR leads to downregulation of fetuin-A by combination of increase in proteosomal degradation of Fet-A and decrease in synthesis of Fet-A through C/EBP beta.

Fetuin-A is an acute phase protein, divergently regulate by early and late inflammatory mediators during injury and infection. Here we observed that acute treatment (4hr) of exogenous endotoxin (LPS) upregulate Fet-A and pFet-A expression/secretion in HepG2 cells, while endogenous inflammatory stimuli (TNF-α) downregulate it. We found that LPS increases C/EBP-β expression and its interaction with fetuin-A, while TNF-α downregulate the C/EBP-β expression after acute treatment in HepG2 cells. Taken together, all of these evidence pave the way to understand the negative and positive regulatory effect of various metabolic mediators on Fet-A and pFet-A, a hepatokine associated with obesity, insulin resistance, and non-alcoholic fatty liver disease.