

# Interaction of Integrin and Insulin Actions in the Insulin-Resistant Liver

Jenny Zheng, Ashley Williams, and David H. Wasserman

KEYWORDS. Type 2 diabetes, extracellular matrix, integrin  $\alpha 1$

BRIEF. Investigating the glucose-output pathway and the triglyceride production pathway in the liver and links to T2DM.

**ABSTRACT.** In Type 2 Diabetes (T2DM), an excess in body fat causes unclearly linked defects in pathways responsible for controlling liver glucose output and excess protein buildup in the structural support of cells (Extracellular matrix also known as the ECM). Research has shown that high fat (HF) fed mice lacking a receptor to an ECM protein are insulin resistant and have low liver fat levels. Typical HF fed mice are usually insulin resistant but have high liver fat levels. This makes treatment hard as synthetic increase in insulin can cause liver failure. The project's aim was to investigate how the unique mouse model develops its traits. Activators of this receptor are a great appeal in the potential treatment of T2DM as a drug could be developed that controls blood sugar without causing fatty liver.

## INTRODUCTION.

Type 2 Diabetes is a major concern today. According to the Center for Disease Control (CDC), roughly 8.3% of the population – or 25.8 million - people are diagnosed with diabetes. Type 2 diabetes mellitus (T2DM) generally develops due to an increase in fat mass that causes a low-grade inflammation, a biological response to harmful stimuli [1]. This inflammatory response causes a transformation in the extracellular matrix that surrounds cells so that more collagen is present leading to formation of excess fibrous tissue. Moreover, it results in defects in pathways involved in glucose and fat metabolism inside cells. These effects that occur in response to inflammation create resistance to the hormone produced in the pancreas, insulin. Liver, muscle, and adipose tissue are the bulk of insulin sensitive tissue. In response to feeding, insulin inhibits the entry of glucose from the liver into the blood, promotes the removal of glucose from the blood, and causes storage of glucose and fats in insulin sensitive tissues [2]. Thus people who are insulin resistant have high blood glucose, high blood triglyceride levels, and impaired nutrient metabolism. As mentioned previously, one effect of the inflammatory response triggered by increase in fat mass causes the formation of excess fibrous tissue in extracellular matrix. However, it is still unclear how extracellular remodeling and the other inflammatory response effects are linked.

One key element that remains to be defined is whether the extracellular adaptations to high fat feeding cause changes in insulin signaling and metabolism. The integrin receptors are of particular interest because they convey information about the extracellular environment to the intracellular environment [3]. Integrins are family of transmembrane proteins containing two subunits. The  $\alpha$  subunit confers ligand specificity, while the  $\beta$  subunit is catalytic. Integrin  $\alpha 1\beta 1$ , the Collagen IV (and extracellular matrix protein) binding receptor, is expressed in the liver, among other cell-types [3]. Integrin receptors cross talk with the actions of insulin (and other growth factor-like hormones) as shown in Figure S1 in the supplemental. Insulin has many actions at the liver. The pathways that are most susceptible to insulin resistance are the synthesis of glucose by gluconeogenesis and the synthesis of fatty acids and triglycerides pathway (lipogenesis) [4, 2]. Normally insulin suppresses the glucose synthesis pathway and stimulates the fatty acids synthesis pathway. In the insulin resistant state, these pathways are accelerated and lead to the diabetic symptoms of high glucose and high lipid concentrations in the blood. Preliminary data from our laboratory show that the presence of the integrin  $\alpha 1\beta 1$  receptor improves insulin sensitivity in mice made insulin resistant using high fat fed feeding [3].

Insulin suppresses gluconeogenesis, thereby keeping the blood sugar low, by stimulating the phosphorylation of FoxO1, a transcription factor (controls the expression of mRNA from DNA in the cell nucleus), This prevents FoxO1 from entering the nucleus [6]. FoxO1 downregulates genes required for gluconeogenesis,

notably phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase). The other action insulin takes is to stimulate lipogenesis by increasing the amount of nuclear SREBP-1c, which then signals genes responsible for fatty acid and triglyceride synthesis, most notably acetyl-coenzyme A carboxylase (ACC) and fatty acid synthase (FAS) [2,5]. Figure S2 shows a diagram of the mentioned paths (in the supplemental).

Wild type mice on a high fat diet recapitulate the human insulin resistant state. Excess extracellular matrix proteins (collagens etc.), and defects in insulin intracellular signaling characterize both the dietary mouse model and insulin resistant humans. As in insulin resistant humans, insulin is less effective at suppressing glucose production in the high fat fed mouse. Paradoxically, triglyceride accumulation continues unimpeded. The reason for this is that the FoxO1 pathway becomes insensitive while the SREBP-1c pathway retains its sensitivity [4,5,6]. Thus, insulin resistance is selective since it has differential effects on gluconeogenesis and lipogenesis. If the pathways of the liver were uniformly insulin resistant (i.e. total insulin resistance) an increase in production of glucose but a decrease in triglyceride accumulation would be the predicted outcome. From a therapeutic standpoint this is undesirable as high both blood glucose and lipid result. These are risk factors for cardiovascular disease and metabolic disorders.

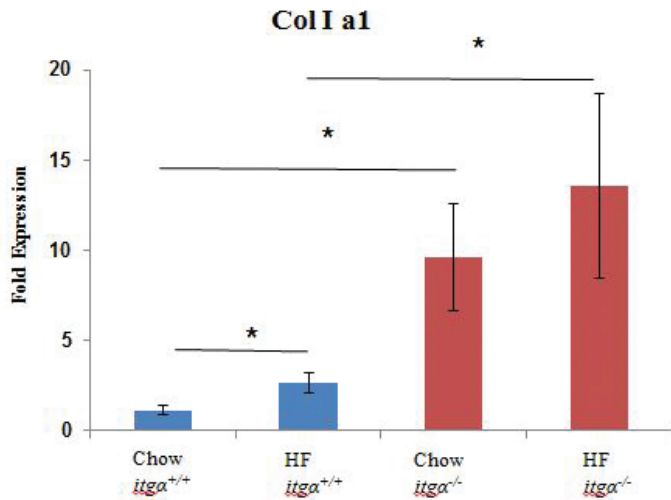
A high fat fed mouse model with genetic deletion of the  $\alpha 1$  subunit of the integrin receptor (*itga1<sup>-/-</sup>*) exhibits an exaggerated insulin resistance compared to wild type littermates (*itga1<sup>+/+</sup>*) that is total, and not selective, as it causes insulin resistance to both signs typical of total insulin resistance [2,7]. The data in Figure S3 shows that high fat fed *itga1<sup>-/-</sup>* mice exhibit increased liver glucose output during an insulin clamp coupled with decreased liver triglyceride (TG) accumulation. The implication of this is that the presence of the integrin  $\alpha 1\beta 1$  receptor is protective against insulin resistance by affecting a pathway that is common to both insulin-regulated gluconeogenesis and lipogenesis.

The purpose of this project is to test whether extracellular matrix remodeling due to high fat feeding causes enhanced collagen signaling through the integrin  $\alpha 1\beta 1$  receptor that is protective against a more severe insulin resistance. Preliminary data (Figure S3) has led to the hypothesis that the integrin receptor enhances insulin action on genes for key enzymes of both the gluconeogenic and lipogenic pathways. This hypothesis will be tested using the *itga1<sup>-/-</sup>* mouse and biochemical indices of insulin action on gluconeogenesis and lipogenesis in the liver.

## MATERIALS AND METHODS.

### *Collagen, Gluconeogenic, and Lipogenic Gene Expression.*

Because integrin receptors convey information about the extracellular environment to the intracellular environment, the activity of specific extracellular matrix proteins was investigated. Bio-rad Sybr Green qPCR was used to measure gene activity of one of the collagen versions: Col I  $\alpha 1$ . qPCR has three main steps: isolation of mRNA, reverse-transcription of mRNA to cDNA, and finally measurement of mRNA levels. First, liver was excised by my mentor, snap frozen in liquid nitrogen and stored at -80C freezer until analysis. Total RNA was isolated from the liver using Rneasy Tissue Kit (Qiagen). Total RNA (1 ug) was reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad) in order to stabilize the samples (mRNA is easily degraded). Syber Green qPCR was then performed by a real time thermal cycler, CFX96 machine (Bio-rad). Specific genes for enzymes of the gluconeogenic and lipogenic pathways were measured using Taqman qPCR. Taqman Real-time PCR was then performed by a real time thermal cycler, CFX96 machine (Bio-rad) for PEPCK and G6Pase of the gluconeogenic pathway, SREBP-1c, FAS, ACC1 and ACC2 of the lipogenic pathway. The qPCR is illustrated in Figure S4 in the supplementary.



**Figure 1.** Collagen Ia1 gene expression is significantly increased in *itga*<sup>-/-</sup> HF mice compared to Chow fed *itga*<sup>+/+</sup> littermates. Increased collagen I a1 is also apparent in Chow *itga*<sup>-/-</sup> compared to WT chow *itga*<sup>+/+</sup> mice. “\*” denotes a p-value of less than 0.05 and is statistically significant.

#### Plasma Triglycerides.

To explore a possible reason for the unexpected increase of lipogenic gene expression in HF *itga*<sup>-/-</sup> mice, the amount of triglycerides circulating in the blood was measured. This was done to test if the liver was exporting triglycerides faster than it could produce them. To measure triglyceride levels in blood plasma, blood was collected via cardiac puncture. Blood plasma is the pale-yellow liquid component of blood that holds blood cells in the whole blood in suspension. The whole blood samples were then placed in tubes containing an anticoagulant, EDTA (20 ul of 0.5 M). The tubes were then spun at 13,000 rpm for 1 minute to separate the blood cells and blood plasma. The pale liquid, blood plasma, at the top of the tube was then drawn off and stored at -80C until use. Plasma TGs were measured colorimetrically using Triglycerides GPO reagent (Raichem). The procedure involves enzymatic hydrolysis by lipase of the triglycerides to glycerol and free fatty acids. The glycerol produced is then measured by coupled enzyme reactions. The absorbance is then read at 540 nm. A standard curve provided a way to interpret absorbance (540 nm) versus Triglycerides (mg/dl). Figure S5 in the supplementary illustrates the process.

#### RESULTS.

##### Collagen Expression.

Increased gene expression for Col I a1 (Figure 1 shown below), Col III a3 (Figure S6 in the supplementary), and Col I a2 (Figure S7 in the supplement) is seen increased in HF *itga*<sup>-/-</sup> mice compared to HF *itga*<sup>+/+</sup> littermates.

##### Gluconeogenic and Lipogenic Pathway.

The first experiment conducted was to evaluate gene expression levels of PEPCK and G6Pase through qPCR in order to evaluate whether failure of insulin to suppress liver glucose production is due to increased gluconeogenic gene expression. SREBP-1c, Fas, ACC1 and ACC2 were evaluated to understand insulin's effect on this specific mouse model's lipogenic pathway. Significantly increased PEPCK (shown in Figure 2 below), SREBP-1c and Fas (Figure S8 in the supplementary), and ACC1 (Figure S9 in the supplementary) gene expressions are seen in HF *itga*<sup>-/-</sup> mice compared to HF *itga*<sup>+/+</sup> littermates.

##### Plasma Triglycerides.

Plasma TGs are increased in HF *itga*<sup>-/-</sup> mice compared to HF *itga*<sup>+/+</sup> littermates as shown in Figure 3.

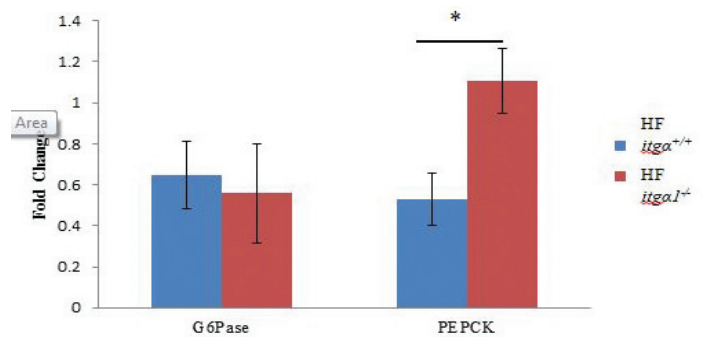
#### DISCUSSION.

Here we show that normal chow fed *itga*<sup>-/-</sup> mice diet have similar insulin-stimulated responses compared to *itga*<sup>+/+</sup> littermates. In contrast, we demonstrate

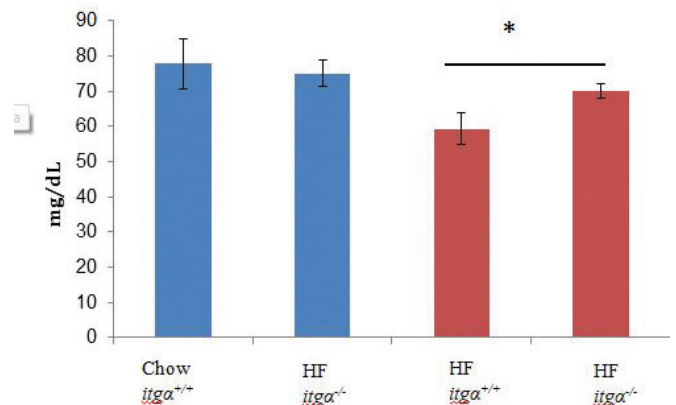
for the first time that collagen expression is increased in the liver of high fat fed mice in Figures 1 and S6, in a total insulin resistant model and that this excess collagen signals through integrin  $\alpha$ 1 $\beta$ 1 receptors to regulate insulin action. High fat fed *itga*<sup>+/+</sup> have a partially impaired insulin suppression of liver glucose production and no detectable effect on insulin-stimulated lipogenesis. In these wild type mice, the insulin resistance due to high fat feeding is selective to glucose production and only partial in magnitude (50% suppression). In contrast, *itga*<sup>-/-</sup> mice have complete resistance to both insulin's suppressive effect on liver glucose production and remarkably insulin's stimulatory effect on lipid accumulation. In these mutant mice, insulin did not suppress glucose production at all and liver triglycerides were only slightly higher than chow fed mice. These mice exhibited total insulin resistance. Thus, *itga*<sup>-/-</sup> mice exhibit total insulin resistant when on a high fat diet. Thus, the normal presence of collagen binding to integrin  $\alpha$ 1 $\beta$ 1 receptors in mice on a high fat diet is protective, enhancing insulin's action on both gluconeogenesis and lipogenesis.

In this study, the aim was to determine the mechanisms whereby collagen-integrin  $\alpha$ 1 $\beta$ 1 receptor signaling affects insulin action in the insulin resistant state. As a first step we examined the transcriptional regulation of genes in the gluconeogenic and lipogenic pathways in high fat fed *itga*<sup>-/-</sup> mice. Liver expression of PEPCK and G6Pase, two gluconeogenic enzymes, are suppressed by insulin. *itga*<sup>-/-</sup> mice have increased PEPCK mRNA, but not G6Pase mRNA. This supports the hypothesis that increased expression of gluconeogenic enzymes is responsible for the failure of insulin to suppress liver glucose production.

Although high fat fed *itga*<sup>-/-</sup> mice had a marked reduction in liver triglyceride concentration, the expression of genes that encode enzymes of the lipogenic pathway was markedly enhanced. Gene expression of ACC2 mRNA, FAS mRNA, and SREBP-1c mRNA were paradoxically increased (Figure 1, S5-6). This strongly suggests that the reduction in liver triglyceride accumulation in



**Figure 2.** PEPCK gene expression is increased in HF *itga*<sup>-/-</sup> mice compared to HF *itga*<sup>+/+</sup> littermates and G6Pase expression showed no significant change. Data are presented as mRNA expression relative to 18S mRNA. All values are expressed as mean + SEM (standard error), n=6, \*p<0.05.



**Figure 3.** Plasma TGs are increased in in HF *itga*<sup>-/-</sup> mice compared to HF *itga*<sup>+/+</sup> littermates. All values are expressed as mean + SEM, n=6, \*p<0.05.

high fat fed *itga1<sup>-/-</sup>* mice is not due to decreased deposition of triglycerides, but rather increased removal. This could be due to either increased lipid oxidation or increased export of triglycerides from the liver. Consistent with the latter possibility is the observation that high fat fed *itga1<sup>-/-</sup>* mice have increased plasma triglyceride concentrations. It is possible based on the increased expression of mRNA for lipogenic enzymes that lipogenesis is increased in high fat fed *itga1<sup>-/-</sup>* mice, but that triglyceride export is accelerated to a greater extent.

Future directions include performing a tyloxapol assay to assess the rate of liver triglyceride secretion [8,9]. Moreover, to further investigate the mechanism whereby integrin  $\alpha1\beta1$  receptors enhance insulin action in high fat fed mice, the activation of transcription factors FoxO1 and SREBP-1c will be investigated. FoxO1 regulates transcription of gluconeogenic enzymes, while SREBP-1c regulates lipogenic enzymes [10]. Other forms of collagen such as col1  $\alpha2$  and col III  $\alpha1$  will also be quantified. These proteins were not investigated this time due to limited time constraints. Another point of interest is to increase collagen samples for the col1  $\alpha1$  qPCR study to minimize the collagen data.

In summary, the results show that the deletion of integrin  $\alpha1$  subunit leads to increased liver insulin resistance. This implies that the presence of integrin  $\alpha1\beta1$  is protective. The ability of integrin  $\alpha1\beta1$  to increase insulin action, suggests that integrin activators may have great appeal as potential therapeutic agents in the treatment of T2DM.

**ACKNOWLEDGMENTS.** Li Kang and the wonderful Wasserman Lab for all their support and guidance. The project described was supported by Award Number R25RR024261 from the National Center For Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center For Research Resources or the National Institutes of Health.

#### SUPPORTING INFORMATION.

**Figure S1.** Integrin role as a transmembrane protein

**Figure S2.** Minimal model of transcriptional regulation of gluconeogenic and lipogenic pathways.

**Figure S3.** Total insulin resistance

**Figure S4.** qPCR process

**Figure S5.** Triglyceride assay enzymatic reactions

**Figure S6.** Collagen III  $\alpha1$  gene expression

**Figure S7.** Collagen III  $\alpha2$  gene expression

**Figure S8.** SREBP-1c and FAS gene expression

**Figure S9.** ACC1 gene expression

#### REFERENCES.

1. Brown, MS., Goldstein, J.L. *Cell.* 7, 95 (2008).
2. Brown, MS., Goldstein, J.L. *Cell.* 89, 331 (1997).
3. Biddinger, S.B., and Kahn, C.R. *Annu. Rev. Physiol.* 68, 123 (2006).
4. Horton, J.D., Goldstein, J.L., and Brown, M.S. *J. Clin. Invest.* 109, 1125 (2002).
5. Kamagate A, Qu S, Perdomo G, et al. *J Clin Invest.* 118, 2347 (2008).
6. Kang L, Ayala JE, et al., *Diabetes.* 60, 416 (2011).
7. Livak KJ, Schmittgen TD. *Applied Biosystems.* 25, 402 (2001).
8. Matsumoto, M., Han, S., et al., *J. Clin.* 116, 2464 (2006).
9. Michael, M.P., Kulkarni, R.N., et al., *Mol. Cell.* 6, 87 (2000).
10. Shimomura, I., Matsuda, M., et al., *Mol. Cell.* 6, 77 (2000).



Jenny Zheng is a student at Martin Luther King Jr. Magnet High School in Nashville, Tennessee, and enrolled in the School for Science and Math at Vanderbilt.