Regulation of insulin resistance by Cyp2c44-derived lipids

By

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CHAPTER I

INTRODUCITON

Insulin resistance occurs when the hormone insulin no longer elicits an appropriate response from the cells and tissues insulin acts upon. Three major sites of insulin action include liver, skeletal muscle, and adipose tissue (Chawala et al. 2011). In a healthy adult, a post-prandial rise in glucose levels causes increased secretion of insulin from pancreatic beta cells (Arnoff et al. 2004). At the liver this insulin promotes glucose utilization and storage by increasing glycogenesis, promoting glucose oxidation over fatty acid oxidation, and increasing lipogenesis. In the skeletal muscle, insulin's main effect is to increase glucose uptake, whereas in adipose tissue it decreases lipolysis (Lizcano & Alessi, 2002). When insulin sensitivity decreases and a person becomes insulin resistant nearly all of the insulin's effects described above become comprised except for the increase in lipogenesis in the liver (Solinas et al. 2015). Despite insulin having varying effects in different tissues, the upstream signaling events involved in these processes are all very similar.

Canonical insulin signaling

The PI3K/AKT pathway is a critical pathway which insulin activates (Cheng & White, 2012). Insulin binds and activates the insulin receptor (IR), a tyrosine kinase receptor. Upon activation the IR autophosorylates itself and phosphorylates other proteins such as insulin receptor substrate (IRS). Through a SH2-domain IRS interacts with the regulatory subunit of PI3K bringing the catalytic domain in proximity with its substrate, phosphatidylinositol (4,5)-bisphosphate (PIP2), at the plasma membrane. PI3K phosphorylates PIP2 to form phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 recruits AKT and along with phosphorylation by phosphoinositide-dependent kinase-1 at threonine 308 and phosphorylation at serine 473, AKT becomes activated. AKT regulates many downstream pathways involved in insulin signaling (Saltiel & Kahn, 2001). In liver AKT regulates gluconeogenesis by phosphorylating downstream effectors like forkhead box protein O1 (FoxO1) transcription factor

(Puigserver et al. 2003). In skeletal muscle AKT plays a role in GLUT4 translocation and in adipose tissue decreases lipolysis (Wang et al. 1999). Therefore when this canonical pathway is disrupted it can lead to states of insulin resistance.

Insulin Resistance and disease

Decreased insulin sensitivity is associated with multiple disease states. It has been well-established that insulin resistance is one of the underlying factors in type 2 diabetes (Leahy, 2005). It has also been associated with a variety of cardiovascular diseases such as hypertension, atherosclerosis, and ischemic stroke (Bloomgarden, 2002; Hankey & Feng, 2010; Bornfeldt & Tabas, 2011). Insulin resistance is also associated with obesity and the nonalcoholic fatty liver disease where one has increased ectopic lipid accumulation in the liver (Samuel & Shulman, 2012). Because of insulin associations with type 2 diabetes, cardiovascular diseases, and obesity, it has been suggested that insulin resistance could be a common contributor to all of these diseases. Therefore by treating insulin resistance, one might be able to treat multiple associated diseases. Unfortunately, currently avaialable drugs do not effectively prevent cardiovascular events. For example, 68% of diabetes related deaths mention heart disease as a contributing cause (Gorina & Lentzer, 2008). Before we can answer the question if targeting insulin resistance can be used to treat such comorbidities, we first must understand the mechanisms behind insulin resistance.

Role of Lipids and inflammation in insulin resistance

Both inflammation and changes in lipids have been shown to contribute to insulin resistance (Glass & Olefsky, 2012). Cytokines such as TNF-alpha produced from tissue macrophages, adipose tissue, and other cells activate pathways such as NF-κB and JNK pathways. JNK can directly phosphorylate IRS at serine residues preventing tyrosine phosphorylation of IRS by the insulin receptor. Furthermore NF-κB and JNK provide positive feedback by increasing expression of cytokines. NF-κB also promotes the synthesis of lipids such as ceramide. Ceramide inhibits AKT by activating the phosphatase PP2A that dephosphorylates AKT at important activating residues. At the same time it activates PKC zeta which phosphorylates inhibitory serine residues on AKT (Samuel & Shulman, 2012).

The mechanism above helps illustrate how inflammation influences changes in lipids to contribute to insulin resistance. The reverse also is true, and lipids can affect inflammation. Circulating saturated free fatty acids like palmitate have been shown to activate Tlr4. Tlr4 in turn activates both the JNK and NF-κB pathway which promote insulin resistance (Glass & Olefsky, 2012). Both these examples illustrate how important inflammation and lipid crosstalk can potentially be for regulation of insulin sensitivity.

Another well-known intersection between lipids and inflammation is found in the arachidonic acid pathway, where many fatty acid metabolites may exhibit pro- and anti-inflammatory effects. This thesis focuses on a subset of the arachidonic acid pathway and its potential to regulate insulin resistance.

Arachidonic acid pathway and insulin resistance

Arachidonic acid is 20 carbon omega-6 fatty acid. It can be metabolized into many different downstream compounds that can act both as pro- and anti-inflammatory molecules (Meng et al. 2015). Arachidonic acid metabolism can be divided into three main pathways: Cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome p450 (Cyp450) pathways (Spector, 2009).

The COX pathway produces the well-known group of eicosanoids known as prostaglandins such as PGD2 and PGE2. In 1876, the first case study showing that salicylates and later aspirin, an inhibitor of the COX pathway, could improve or decrease hyperglycemia in individuals who presumably had type 2 diabetes (Shoelson et al. 2006). Now studies have shown that non-steroidal anti-inflammatories like indomethacin, which inhibit COX enzyme, can improve insulin sensitivity in HFD mice (Fjære et al. 2014). But such drugs can have negative cardiovascular effects (Patricio J et al. 2013), therefore they are not ideally used in diabetic patients who have higher risks of cardiovascular problems.

The LOX pathway produces leukotrienes and some hydroxyeicosatetranoic acids like 12-HETE- and 15-HETE. The leukotriene LTB4 has been associated with insulin resistance and inhibition of its receptor increases insulin sensitivity (Li P et al. 2015). A 12/15 LOX knockout mouse showed a defect in GLUT4 translocation (Vahsen et al 2006). This again shows arachidonic acid pathways and lipids can regulate insulin resistance.

The Cyp450 pathway can be divided into two pathways. CYP4 family enzyme produce terminal HETEs like 20-HETE (Zhang & Klaassen, 2013). In a mouse with increased levels of 20-HETE, increased glucose intolerance was observed and mice exhibited severe increases in adiposity (Pandey et al. 2015).

CYP450-derived EETs and insulin resistance

A second group of CYP450 enzymes are called epoxygenases. They produce metabolites called epoxyeicosatrienoic acids (EETs). Potentially eight different EETs can be formed. This includes 5,6-EETs, 8,9-EETs, 11,12-EETs, and 14,15-EETs. Each of these has two enantiomers. These products are metabolized into less biologically active molecules called dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH) (Bellien et al. 2011). In humans CYP2C8, CYP2C9, and CYP2J2 are the major EET-producing epoxygenases (Joannides & Bellien). In mice, Cyp2c29, Cyp2c37, Cyp2c38, Cyp2c39, Cyp2c40, and Cyp2c44 all can contribute to EET production, although Cyp2c44 is the more enzymatically active epoxygenase (Capdevila & Wang, 2013; DeLozier et al. 2004; Nelson et al. 2004).

EETs were first shown to potentially regulate insulin and glucagon secretion in isolated rat islets (Falk et al. 1983). Since then there is data suggesting they could affect both type 1 and type 2 diabetes animal models. In mice, EETs effects on insulin sensitivity have mainly been studied by decreasing EET degradation using soluble epoxide hydrolase (sEH) inhibitors or increasing EET production by overexpressing CYP450 epoxygenases. Mice given a low dose of streptozotocin to cause pancreatic injury and decreased insulin levels, were protected from insult when sEH was inhibited or genetically deleted (Chen et al. 2012). Rats on a high-fructose diet used to induce insulin resistance, had improved glucose tolerance when CYP2J3 was genetically overexpressed (Xu et al. 2010). Human studies have shown that EET levels positively correlate to insulin sensitivity and that an isoform of a less active sEH enzyme is associated with increased insulin sensitivity (Gangadhariah et al. 2016; Ramirez et al. 2014). Even though studies show EETs potentially regulate insulin sensitivity and glucose homeostasis, very little is known about how they do this.

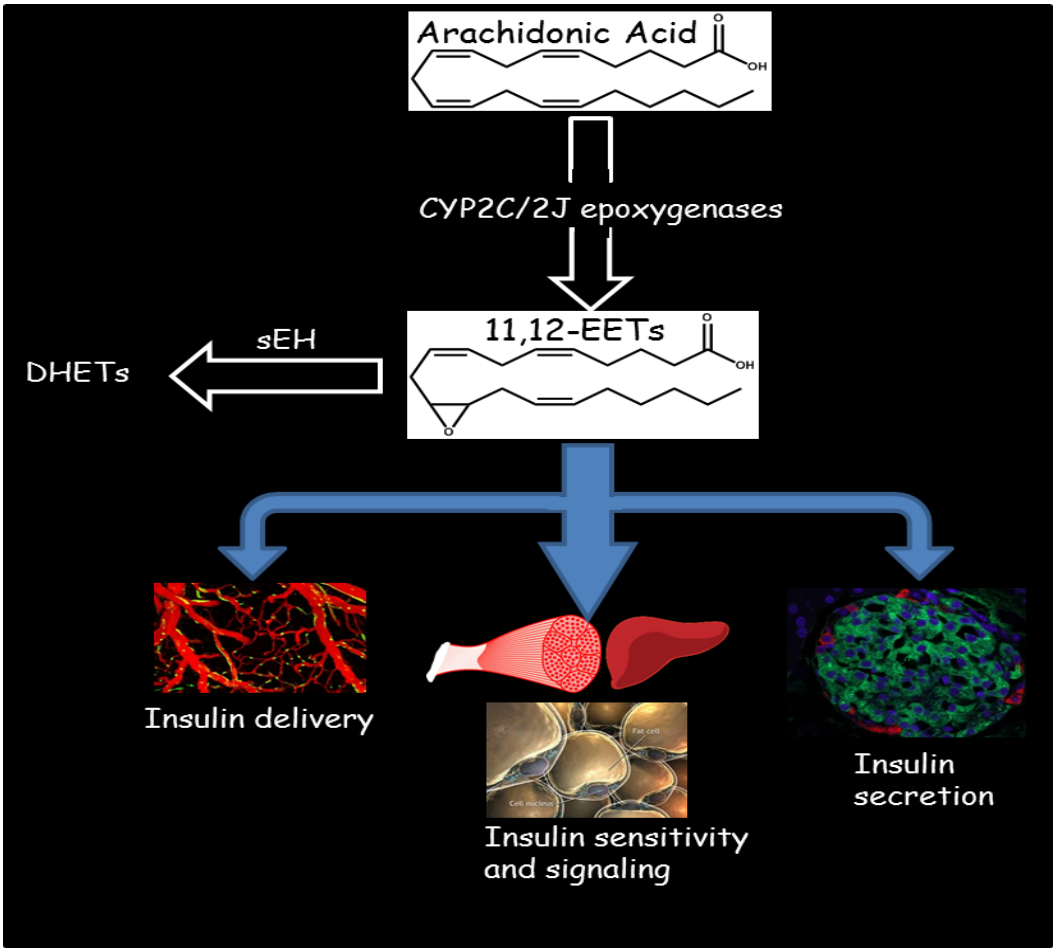

Figure 1: Cyp450-derived EETs potentially regulate glucose homeostasis and insulin sensitivity. CYP450 subfamilies CYP2C and CYP2J produce epoxyeicosatrienoic acids (EETs) from arachidonic acid. They can produce two enantiomers of each of the following EETs: 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET. EETs are degraded into dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH). Through either inhibiting/deleting sEH or overexpressing CYP2C/2J epoxygenases, EET levels were increased and corresponded to improved glucose homeostasis. Improved glucose homeostasis could be the result of EETs regulating insulin delivery, insulin sensitivity and signaling, and/or insulin secretion. This thesis focuses primarily on how EETs affect insulin sensitivity/signaling. It also investigates a potential mechanism for EET regulation of insulin secretion. EETs role in insulin delivery are addressed but not actively investigated.

CHAPTER II

GOALS OF THIS STUDY

Despite there being some indication that CYP450-derived EETs play a role in regulating glucose homeostasis and insulin sensitivity, these studies are confounded by similar flaws and gaps of knowledge. First, *in vivo* studies have only indirectly shown that EETs are regulating glucose homeostasis (Chen et al. 2012; Xu et al. 2010). Both overexpression of epoxygenases and sEH inhibition/deletion will alter other lipid metabolites. For example one study showed that the effect on ischemic/reperfusion injury caused by overexpression of CYP2C8 in endothelial cells was not due to EETs but actually the formation of a linoleic acid metabolite (Edin et al. 2011). Second, the *in vivo* and *in vitro* studies have barely investigated how EETs are causing these effects, and the results from these studies have been inconsistent (Luria et al. 2011; Schafer et al. 2015; Skepner et al. 2011). In liver lysates of *EPHX2(-/-)* mice IR, IRS-1, PI3K, and AKT all had increased activation at basal levels and in insulin treated mice (Luria et al. 2001). But in isolated primary WT hepatocytes and in importalized cell lines, EETs only increased AKT activation consistently (Schafer et al. 2015; Skepner et al. 2011). Therefore the first goal of this research is to develop a simple *in vivo* model system to investigate whether EETs are the specific CYP450 metabolites which alter glucose homeostasis and insulin resistance. The second goal of this study is to determine how EETs are affecting glucose homeostasis.

Cyp2c44(-/-) mice and insulin sensitivity

To accomplish these two goals, our lab uses a mouse model with disrupted endogenous EET production. The important mouse epoxygenase, Cyp2c44, has been knocked out. Cyp2c44 is a major epoxygenase in mice (Imig, 2012). Its major metabolite is 11,12-EET followed by 8,9-EET and 14,15-EET (DeLozier et al. 2004). It is expressed in important tissues that are targets of insulin such as the liver, skeletal muscles, endothelial cells, and pancreas (Delozier et al. 2004; Gangadhariah et al. 2016). Cyp2c44 regulates blood pressure by altering ENaC activity (Capdevila et al. 2014), but the role of

Cyp2c44 in insulin sensitivity and glucose homeostasis is unknown. Our lab showed that streptozotocin induced pancreatic injury in *Cyp2c44(-/-)* mice was exacerbated, determined by increased fasting glucose levels compared to WT mice. This indicated either a protective role in the pancreas or regulation of insulin action by EETs. To assess whether insulin sensitivity was altered by disruption of endogenous EET production, hyperinsulinemic-euglycemic clamps were employed. *Cyp2c44(-/-)* mice developed insulin resistance on a regular chow diet while WT mice did not (Gangadhariah et al. 2016).

Glucose Tolerance tests provide a simple protocol to evaluate glucose homeostasis

While the clamps definitely demonstrate that *Cyp2c44(-/-)* mice have decreased insulin sensitivity, using this method to carry-out for the needed follow-up studies to test if stable EET analogs rescue these mice is expensive and technically challenging. An alternative protocol to test the effect of stable EET analogs on changes in glucose homeostasis and indirectly for insulin sensitivity is a glucose tolerance test (GTT). I hypothesize that *Cyp2c44(-/-)* mice will have decreased glucose tolerance compared to WT mice. If glucose tolerance is decreased in *Cyp2c44(-/-)* mice, then this will provide a cost-effective and technically facile method for future studies to test the effect of EET analogs in *Cyp2c44(-/-)* mice.

Three ways to examine EETs regulation of glucose homeostasis

The second goal of determining how EETs are regulating glucose homeostasis will be addressed in three ways.

First, canonical insulin signaling will also be assessed in mice. EETs regulate insulin signaling at the level of the kinase AKT (Luria et al. 2011; Schafer et al. 2015; Skepner et al. 2011). Whether or not they effect signaling upstream of AKT is unclear. Therefore I will begin with testing if AKT and a downstream target, FoxO1, are regulated by EETs. Insulin promotes phosphorylation of these two proteins. I hypothesize that insulin resistance in *Cyp2c44 (-/-)* mice will reduce insulin-stimulated phosphorylation of AKT and FoxO1 compared to WT mice. Both liver and skeletal muscles will be assessed. Impaired insulin signaling would correspond to decreased insulin sensitivity seen in the clamp studies, especially in the liver.

Second, tissue-specific effects will be studied by generating conditional knockout mouse lines of Cyp2c44. This will investigate the paracrine versus systemic effects of EETs produced by a particular tissue. It could help reveal tissue-specific mechanisms behind EET regulation on insulin sensitivity. I will generate liver-specific knockout mice or hep*Cyp2c44(-/-)* mice. I hypothesize that hep*Cyp2c44(-/-)* mice will be glucose intolerant and have decreased insulin sensitivity in the liver. The combined high expression of Cyp2c44 and high vascularization of the liver could allow EETs derived here to have both a paracrine and systemic effect that would be seen in GTTs.

Finally it has been shown that Cyp2c44 alters insulin secretion (Gangadhariah et al. 2016). This effect was subtle but could possibly play a role in glucose homeostasis. We hypothesize that EETs alter insulin secretion by increasing activity of K_{ATP} channels and in turn decreasing insulin release. Therefore when treated with a K_{ATP} channel inhibitor, insulin levels in *Cyp2c44 (-/-)* mice should remain near pre-treated levels, while WT mice should have an increase in insulin levels.

Therefore by completing these three separate avenues into EETs regulation of insulin resistance, we will have a wider breadth of understanding on the mechanisms behind EETs role in glucose homeostasis and insulin sensitivity.

Significance

There is relatively little data known about the EET pathway's role in glucose homeostasis and insulin resistance, but there is a plethora of knowledge associating insulin resistance with diabetes and cardiovascular risk. Insulin resistance is the underlying feature of type 2 diabetes, which affects 28 million people in the United States alone (Diabetes report card, 2014). It also is associated with cardiovascular disease, ischemic stroke, hypertension, and non-alcoholic fatty liver disease (Bloomgarden, 2002; Bornfeldt & Tabas, 2011; Hankey & Feng, 2010; Leahy, 2005; Samuel & Shulman, 2012). Because insulin resistance is a common thread between these diseases, intervention at the point of insulin resistance could prove as a common treatment. Therefore learning more about EETs regulation of insulin action could help show whether targeting the EET pathway could be used to improve insulin sensitivity and if it might be suitable as a potential treatment for such diseases as type 2 diabetes.

Methods

*Animals***:** Male mice aged 10-12 weeks were used for all experiments. GTTs were performed one week prior to insulin signaling experiments. *Cyp2c44(-/-)* mice were on 129SvJ background. hep*Cyp2c44(-/-)* were on a C57BL/6J background. All mice had free access to water and housed in a temperature-controlled facility with a 12-hour light/dark cycle. All mice were on a normal chow diet.

*Glucose tolerance tests***:** 10-12 week old male mice were fasted in the morning for five hours. Tail-vein blood was collected to measure baseline glucose levels using ACCU-CHECK glucometer (Roche Diagnostics, Basel Switzerland). Then mice were injected i.p. with 2g dextrose/kg of whole body mass (20% dextrose solution, Hospira Inc., Lake Forest, IL). Blood glucose was measured at 15, 30, 45, 60, 90, and 120 minutes.

*Microsome preparation and Cyp2c44 western blot***:** Frozen tissue was homogenized in dounce homogenizer (100mg tissue/ 1ml buffer, 20 strokes, buffer: .25 sucrose, 0.1M phosphate, pH = 7.4). Mixture was spun down at 5000g for 20 minutes, supernatant was saved. Homogenate was spun down twice at 10,000g, supernatant was saved. Homogenate was spun down at 100,000g for 90 minutes. Supernatant was discarded. Pellet was resuspended in above buffer, protein concentration was determined, and samples were aliquoted and stored at -80 degrees Celsius until further use. 30 ug of liver microsome lysates were separated on a 10% SDS-page gel, either stained with coomassie or transferred overnight to pvdf membrane, and immunoblotted for Cyp2c44.

*Insulin signaling experiments***:** Male SV129 Mice were fasted for 5 hours in the morning. Next they were injected with 1U insulin/kg body mass (Humulin R, Eli Lilly). 15 minutes later gastrocnemius and liver were harvested, snap frozen in liquid nitrogen, and stored at -80 degrees Celsius until further use. Gastrocnemius and liver lysates were made, separated out on an 8% sds-page gel, transferred to nitrocellulose, immunoblotted for AKT and FoxO-1 were performed, and visualized using chemiluminescene. For all liver lysates 30 ug of lysate was used. 30 ug of gastrocnemius lysate was loaded for AKT, pAKT, and FoxO1. 40 ug of gastrocnemius lysate was loaded for pFoxO1. The following primary antibodies (1:1000) from cell signaling were used: Total AKT (#9272), pAKT (S473,

#9271), total FoxO1 (C29HC, #2880), and pFoxO1 (S256, #9461). 1:5000 dilution of peroxidase AffiniPure Donkey Anti-rabbit Ig (Jackson Immuno research, #711-035-152).

K_{ATP} inhibition experiment: Male SV129 Mice were fasted for 5 hours in the morning. After 5 hours blood was collected from the saphenous vein. Glyburide, K_{ATP} channel blocker, was injected i.p. at 1.25mg/kg. 15 minutes after injection blood was collected again. Blood samples were centrifuged for 10 minutes at 5000 rpm on a table top centrifuge. Plasma was isolated, put into a clean tube, frozen on dry ice, stored at -80 degree Celsius, and sent to the Vanderbilt Mouse Metabolic Phenotyping Center for analysis of plasma insulin levels. (Gangadhariah, 2016)

CHAPTER III

CYP2C44 ALTERS GLUCOSE HOMEOSTASIS

Hyperinsulinemic-euglycemic clamps are the gold standard in assessing insulin sensitivity. They eliminate confounding variables such as body composition, basal insulin levels, and insulin secretion all while providing the opportunity to gather information on insulin sensitivity at different tissues (Hughey et al. 2014). Unfortunately clamps are technically difficult and costly. Glucose tolerance tests (GTTs) are a simple and cost-effective alternative to assess glucose homeostasis and indirectly insulin sensitivity. The confounding factors that the clamp technique evades are found in GTTs. Therefore such factors could mask a decrease in insulin sensitivity. In *Cyp2c44 (-/-)* mice fasting insulin levels were no different than WT on a normal chow diet. They had increased body mass that arose from an increase in lean mass from muscle and decrease in fat mass (Gangadhariah et al. 2016). Increased muscle mass is associated with increased insulin sensitivity (Srikanthan & Karlamangla, 2011). Increase in lean mass could mask the decreased insulin sensitivity in the KO if glucose dose is normalized to total mass. Despite this, if a difference is observed, GTTs will allow our lab to easily perform further experiments in the future to test for ways to recover insulin sensitivity in *Cyp2c44(-/-)* mice.

Results

To determine if Cyp2c44 disruption altered glucose homeostasis, glucose tolerance tests (GTTs) were performed. Two independent experiments were performed and results were combined in Figure 1. As expected. Cyp2c44(-/-) mice had significantly greater exposure to glucose over the 120 time period (**Figure 2B)**.

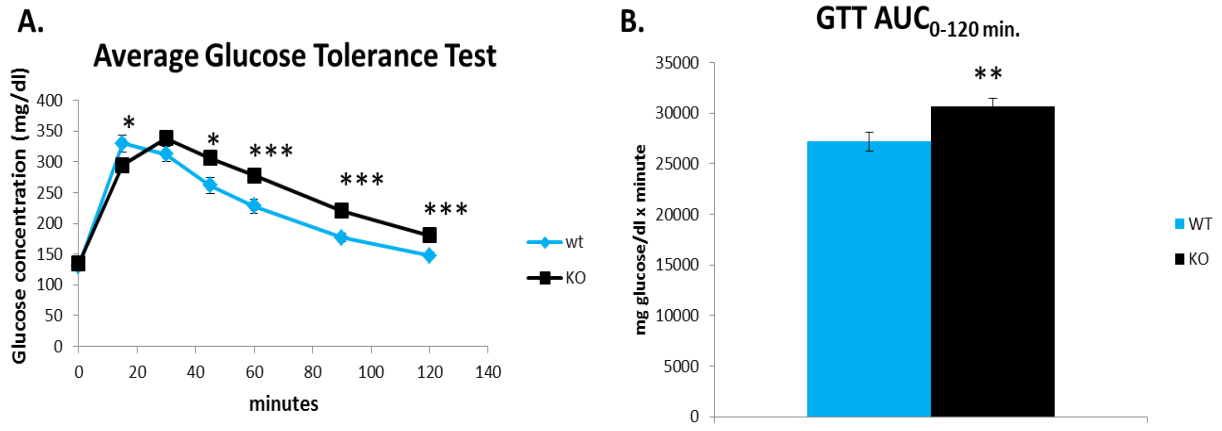

Figure 2: Glucose tolerance is impaired with global Cyp2c44 deletion. After glucose administration, Cyp2c44(-/-) mice had increased blood concentrations of glucose compared to WT mice at 45, 60, 90, and 120 minute time points (**A)** and overall increased exposure to glucose (**B**). *p<0.04, **p<0.007. ***p<0.004. Error bars represent SEM. N=15 for both groups.

The observed difference could be possibly caused by impaired insulin signaling or by impaired insulin delivery resulting in decreased insulin signaling. Our lab has shown that *ex vivo* in mesenteric vessels of *Cyp2c44(-/-)* mice have impaired vascular reactivity (Gangadhariah et al. 2016), supporting that this difference in glucose tolerance could possibly result from a differences in vascularity as well as a difference in intracellular signaling.

The main purpose using GTTs was to see if they could be used in future studies as a surrogate for clamps to test if the insulin resistant phenotype of *Cyp2c44(-/-)* mice could be rescued under certain manipulations such as treatment with a stable EET analog. Therefore GTTs would be an appropriate surrogate for clamps in rescue experiments where manipulations would affect both the vasculature and insulin signaling at tissue (e.g. EET analog). Manipulations that mainly effect insulin signaling and not vasculature reactivity might be better evaluated using clamp studies.

Liver-specific Cyp2c44 KO mice (hep*Cyp2c44(-/-)*)

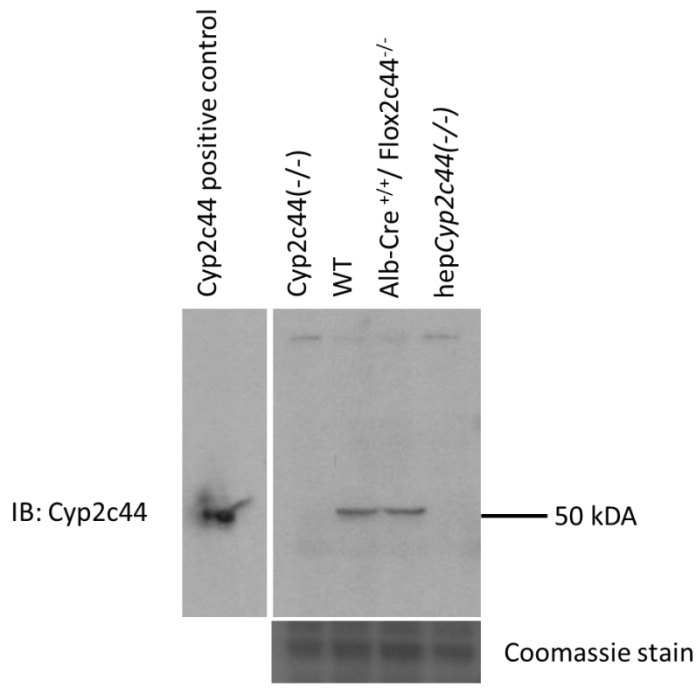

Figure 3: Generation of liver-specific Cyp2c44 knockout mice [hep*Cyp2c44(-/-)*]. Albumin-cre mice were crossed with *Cyp2c44(flox/flox)* mice on a C57 background to make Albumin-cre/*Cyp2c44(flox/+)* heterozygotes and subsequently hep*Cyp2c44(-/-)* mice. Western blots of Cyp2c44 in liver lysates confirmed successful breeding of hep*Cyp2c44(-/-)* mice.

Eicosanoids including Cyp450 metabolites like EETs are generally considered paracrine hormones but have been shown to have systemic effects on vasculature and inflammation (Hales et al. 1986; Shearer & Newman, 2009). Furthermore tissue-specific overexpression of the same Cyp450 in rodents can result in different phenotypical outcomes (Edin et al. 2011). Hence the location of a Cyp450 epoxygenase can dictate function. By investigating tissue-specific deletion of Cyp2c44, we can identify important sites of EET production, associate locations of production with a specific phenotype, and mechanism of action. We already have evidence that supports skeletal muscle insulin resistance could arise from impaired vasculature while hepatic insulin resistance might result from impaired signaling (Gangadhariah et al. 2016). Creation of tissue specific knockout allows us to address such questions. Therefore we successfully generated a liver-specific knockout or hep*Cyp2c44(-/-)* mice (**Figure 3**) and

performed glucose tolerance tests on these mice to investigate whether or not liver-produced EETs had a discernible effect on glucose homeostasis.

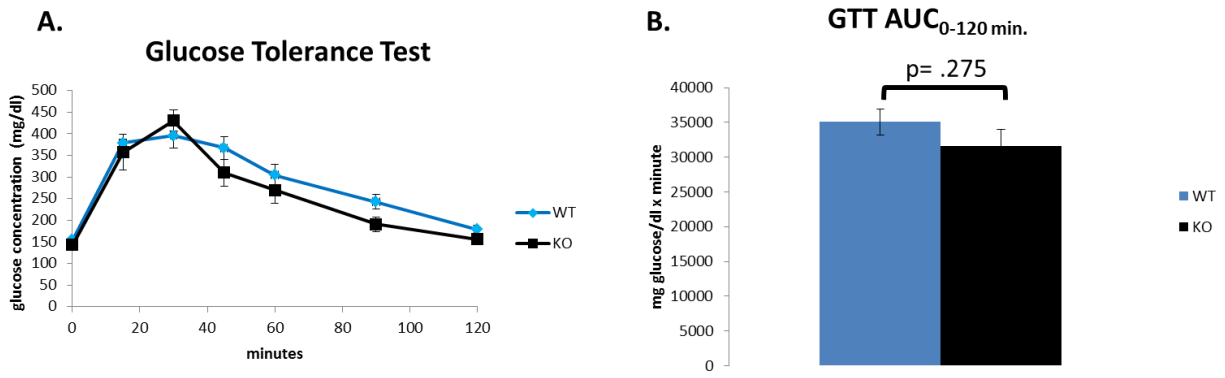

Figure 4: Glucose tolerance is not impaired in liver-specific Cyp2c44 deletion. After glucose administration, hep*Cyp2c44(-/-)* mice had no significant difference in blood concentrations of glucose compared to WT mice at all time points (**A)** and overall exposure to glucose was also similar (**B**). Error bars represent SEM. N=10 for WT and n=6 for hep*Cyp2c44(-/-)* mice.

GTTs showed there was no difference in glucose tolerance in the hep*Cyp2c44(-/-)* mice (**Figure 4)**. There are many potential reasons for this. Despite the liver expressing the highest amount of Cyp2c44 of any tissue and being highly vascularized, EETs produced here might only be acting locally. Therefore even if they regulate insulin resistance in the liver this might not translate to a difference in peripheral glucose homeostasis because there are many factors that influence glucose homeostasis. Cyp2c44 is also expressed in skeletal muscle, islet of Langerhans, and endothelial cells (Gangadhariah et al. 2016; Yang et al. 2009). All three of these regulate glucose homeostasis and insulin sensitivity (Leahy, 2005). Production of EETs at these sites could correct for a disruption of liver-derived EETs. Testing whether or not the liver is still insulin resistant would be the next logical step in determining if and how liver-derived EETs are affecting insulin sensitivity.

CHAPTER IV

EET REGULATION OF INSULIN SIGNALING

There have only been a few studies that have studied how EETs affect the insulin signaling pathway. The results of these studies have not been consistent. Some have shown that EETs can affect insulin signaling by altering IR activity while others have not. The one consistent node in the canonical insulin pathway that EET activity seems to regulate is AKT (Luria et al. 2011; Schafer et al. 2015; Skepner et al. 2011). An important downstream effector of AKT is the transcription factor FoxO1. In the liver it is a key factor in promoting expression of gluconeogenic genes and AKT blocks this through direct phosphorylation (Puigserver et al. 2003). In skeletal muscle when over-activated in insulin resistant states, FoxO1 can lead to muscle atrophy (Cheng & White, 2011). By studying these two proteins, we want to confirm that insulin signaling is altered by EETs through AKT and that this results in typical downstream effects like decrease phosphorylation of FoxO1 in both the liver and the skeletal muscle.

Liver Results

There was no difference in either AKT or FoxO1 phosphorylation status between genotypes treated with insulin (**Figure 5)**. One would expect that if there was no difference in AKT that FoxO1 would also not be affected by deletion of Cyp2c44. But it is completely unexpected for there to be no difference in AKT phosphorylation between the WT and *Cyp2c44(-/-)* mice. Every other paper has come to the opposite conclusion, that EETs increase insulin-stimulated AKT phosphorylation (Luria et al. 2011; Schafer et al. 2015; Skepner et al. 2011).

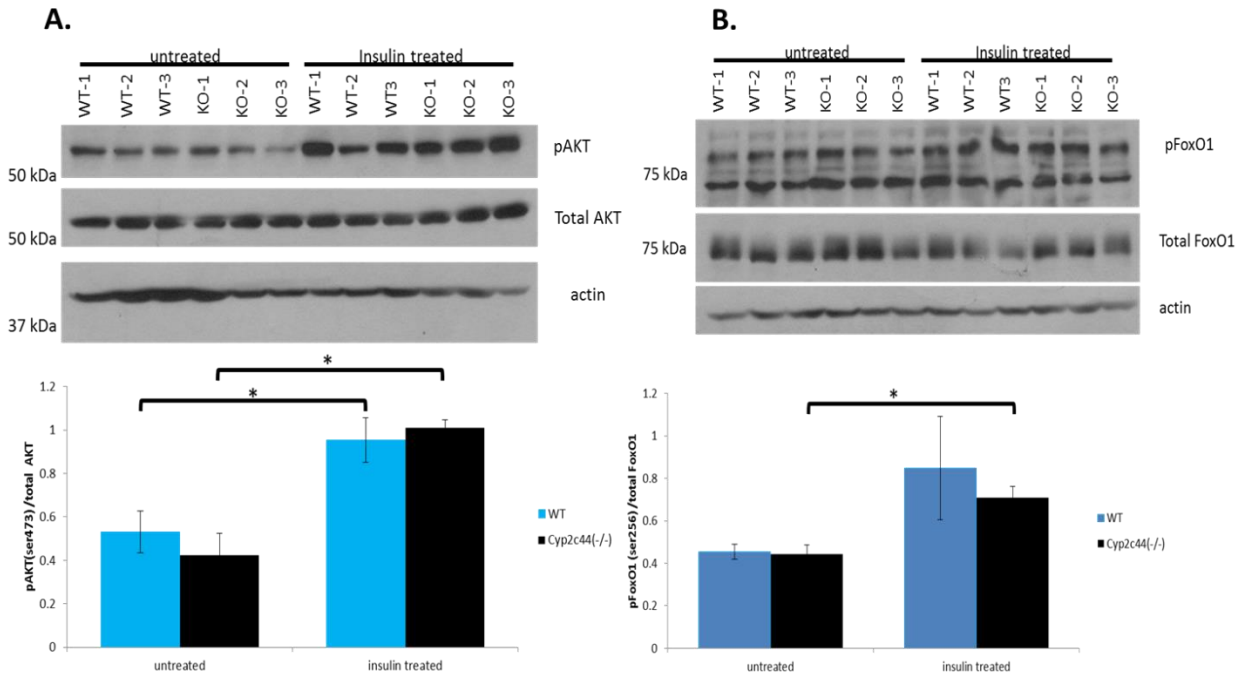

Figure 5: Global Cyp2c44 deletion does not impair hepatic AKT or FoxO1 phosphorylation. After a five hour fast, mice were injected with insulin (1U/kg), sacrificed, and organs were collected to analyze AKT and FoxO1 phosphorylation states. Western blots for both AKT and FOXO1 phosphorylation in liver lysates (30 ug) were performed and ratio of phosphorylated to total protein was reported (**A and B**). There was no difference between genotypes in any condition for AKT and FoxO1. There was a significant increase in phosphorylation in treated mice vs untreated mice. T-tests were performed and error bars show SEM. Two independent experiments for AKT were performed, both having similar results. Only one experiment was performed for FoxO1. *P< 0.05

This same test had previously been performed in our laboratory, and demonstrated a decrease in AKT phosphorylation in the insulin treated *Cyp2c44(-/-)* mice compared to WT. Two possible explanations account for this result. First something has changed with the mice, reagents, and materials used in the experiment. The mice that were used were from the same colony as before. They were the same mice that GTTs were performed on and these GTTs showed the *Cyp2c44(-/-)* mice were glucose intolerant. The mice are an unlikely source of error. Another potential source of error is the insulin. The insulin was kept under appropriate conditions, injected at the same concentration as before, came from the same company, and was not expired. WT mice had an appropriate response to insulin injection furthermore indicating that the problem lies elsewhere. The other explanation for the results is that they are different than before

because of user error. This is the most likely situation but the step or steps where this user error came from could not be determined.

Gastrocnemius results

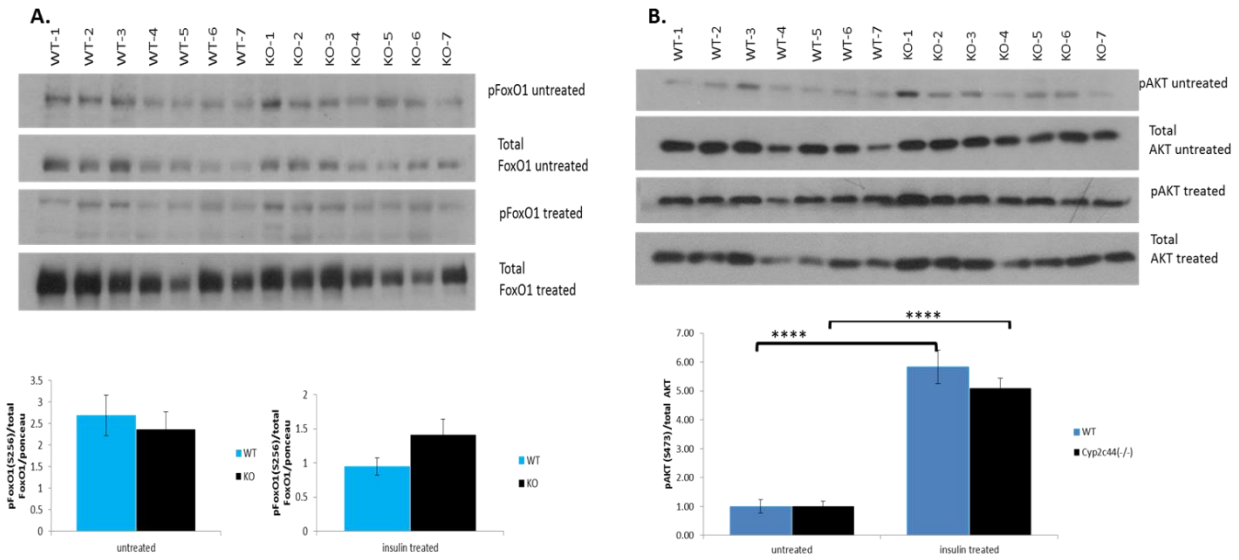

Figure 6: Global Cyp2c44 deletion does not impair AKT or FoxO1 phosphorylation in the gastrocnemius.. Western blots for both AKT and FOXO1 phosphorylation in gastrocnemius lysates (30 or 40 ug) were performed and ratio of phosphorylated to total protein was reported (**A and B**). There was no difference between genotypes in any condition for AKT and FoxO1. There was a significant increase in AKT phosphorylation in treated mice vs untreated mice. T-tests were performed. results are mean± SEM.

There was no difference between genotypes in either untreated or insulin treated groups. There was a significant increase in AKT phosphorylation in both genotypes when treated with insulin. The treatment effect could not be evaluated for FoxO1. This follows the same results as in the liver tissue. This is less surprising than the results seen in the liver but still unexpected. Our lab has shown that despite skeletal muscles displaying insulin resistance in a hyperinsulinemic-euglycemic clamp that ex vivo uptake of glucose is not impaired in Cyp2c44 (-/-) mice. Isolated mesenteric blood vessels had impaired vascular reactivity (Gangadhariah et al. 2016). Therefore impaired vasculature could be a more important contributing factor to insulin resistance at skeletal muscles in *Cyp2c44 (-/-)* mice than intracellular signaling. But one could argue that even if impaired vasculature is the important contributing factor that there would still be impaired signaling due to decrease delivery of insulin to the skeletal muscle.

CHAPTER V

EETS REGULATE INSULIN SECREATION THROUGH K_{ATP} CHANNELS

EETs have been shown to potentially affect hormone secretion such as insulin and glucagon in from islet cells (Falck et al. 1983).

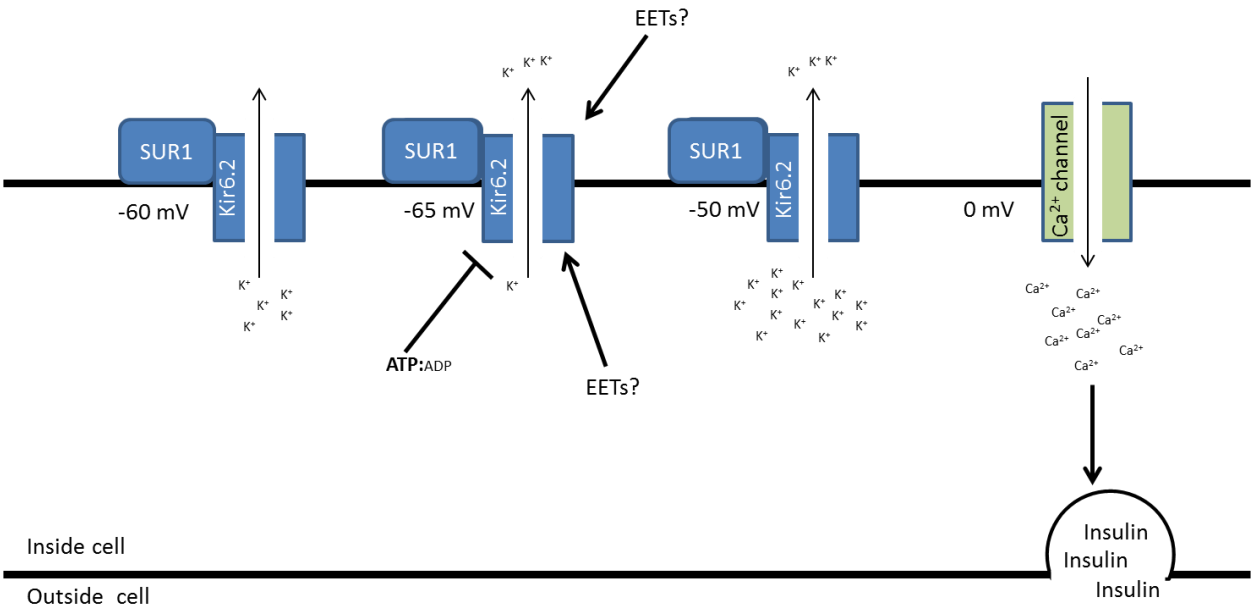

Figure 7: EETs may alter insulin secretion via K_{atp} channels. K_{ATP} channels help maintain resting membrane potential in beta cells preventing. ATP negatively regulates K_{ATP} channels. Upon increase in ATP levels K_{ATP} channels are blocked, intracellular potassium levels rise, and the cell begins to depolarize. After reaching a threshold potential (-50—55 mV), the cell rapidly depolarizes, resulting in an influx of calcium ions and increased insulin secretion. EETs potentially regulate K_{ATP} channel activity through both intracellular and extracellular mechanisms (Braun et al. 2008; Koster et. al. 2005)

In *Cyp2c44(-/-)* mice, fasting levels of insulin are similar on a normal chow diet and unaffected in a hyperglycemic clamp. But on a high fat diet KO mice have higher fasting levels of insulin and increased insulin secretion in the first 20 minutes of hyperglycemic clamps, indicating that EETs could be decreasing insulin secretion in the WT mice (Gangadhariah et al. 2016). A potential mechanism for this

could be decreased activation of ATP-sensitive potassium (K_{ATP}) channels in *Cyp2c44(-/-)* mice **(Figure 7).**

EETs have been shown to regulate K_{ATP} channels in both cardiac and vascular smooth muscle tissues through intracellular and extracellular mechanisms (Lu et al. 2006). K_{ATP} channels in smooth muscle are mainly composed of Kir6.1/SUR2A subunits and Kir6.2/SUR2A in ventricular myocytes (Flagg et al. 2010). Therefore K_{ATP} channel subunit composition could affect EET action at these channels. The pancreas primarily expresses Kir6.2/SUR1 in islets (Flagg et al. 2010), therefore if and how EETs act this particular K_{ATP} isoform needs to be investigated.

Results

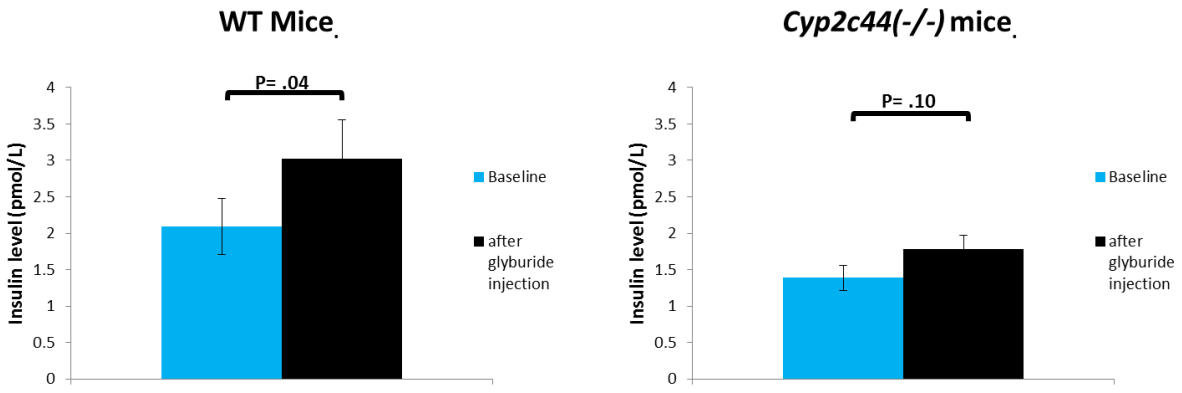

Figure 8: K_{ATP}-dependent insulin secretion is impaired in *Cyp2c44(-/-)* mice. Plasma levels of insulin were measured before and 15 minutes after treatment with the K_{ATP} inhibitor glyburide (1.25mb/kg). Glyburide significantly increased insulin levels in WT mice but failed to do so in *Cyp2c44(-/-)* mice. Paired t-tests were performed. Results are mean ± SEM. n=6 for both WT and *Cyp2c44(-/-)* groups.

Inhibiting Katp channels in WT mice caused a significant increase in insulin plasma levels as expected. However, the insulin response to glyburide in *Cyp2c44(-/-)* mice was blunted **(Figure 8**). This supports that Katp channels can be regulated by EETs.

This mechanistic evidence is contradictory to a study focusing on the insulinotropic drug BL11282. Investigators showed that BL11282 increased insulin in a K_{ATP}-independent mechanism (Sharoyko et al. 2007). But this study focused on glucose stimulated insulin release, while ours focuses on basal levels. Therefore EETs are potentially regulating insulin levels at multiple sites of action and at

different phases of insulin release. But none of this answers whether or not EETs regulation on insulin secretion is important in diseases such as type 2 diabetes. In our model on a HFD, despite WT mice having increased levels of pancreatic Cyp2c44 and lower insulin levels, presumably caused by EETs, there is no difference in insulin resistance from *Cyp2c44(-/-)* mice (Gangadhariah et al. 2016). One explanation for this is differential changes to Cyp2c44 expression in other tissues. HFD has been shown to decrease Cyp450 expression in the liver (Schafer et al. 2015). If Cyp2c44 expression does decrease in the liver and potentially other insulin sensitive organs like skeletal muscle on a HFD, it could indicate that defects in EET production in these organs play a more important role in EET regulation on insulin action than EET regulation of insulin secretion. HFD essentially could be acting like a conditional knockout of Cyp2c44 in tissues like the liver, therefore explaining why there is no difference between insulin sensitivity between WT and *Cyp2c44 (-/-)* mic on a HFD.

EETs may play an important protective role in the pancreas in type 1 diabetes. Streptozotocin is a chemical that destroys beta cells and is used in mice to model type 1 diabetes. In *Cyp2c44(-/-)* mice, treatment with low dose streptozotocin caused increase levels of glucose compared to WT indicating a potential problem with glucose homeostasis (Gangadhariah et al. 2016). Conversely when sEH has been knocked out in mice, increasing the EET pool, mice treated with streptozotocin have lower levels of fasting glucose and are less insulin resistant than WT mice (Chen et al. 2012). Therefore investigating if and how EETs are providing protection to the pancreas through their established anti-inflammatory and/or anti-apoptotic mechanisms might be more important than EETs actions on insulin secretion.

Chapter VI

CONCLUSIONS/FUTURE DIRECTIONS

At the end of this study I established that *Cyp2c44(-/-)* mice have a mild glucose intolerance. GTTs can be used to test further manipulations to glucose homeostasis in these mice, offering a cost-effective alternative to clamps. I generated a liver-specific knockout (hep*Cyp2c44(-/-)*) and showed they did not display any glucose intolerance unlike the global knockout. Finally we showed that a potential mechanism behind EETs regulation of insulin secretion occurs through K_{ATP} channels. Insulin signaling at AKT and FoxO1 were unaltered in the present studies and additional studies are warranted.

Isolated primary hepatocytes may provide atool to better define EETs effects on insulin signaling independent of humoral or neural factors . Cells are easier to manipulate and less variable than animals. This might allow for clearer more consistent results.

The first experiment that must be done to confirm that primary hepatocytes are a suitable model to investigate Cyp2c44 and its affects is to measure Cyp2c44 levels over time. Primary hepatocytes quickly lose their functionality over time including CYP450 expression. Many initial experiments could be done in these hepatocytes as long as they express Cyp2c44 at detectable levels within the first 24 hours of isolation. If Cyp2c44 expression can still be detected within 24 hours then this model would be suitable for many experiments. First, it could be used to test insulin signaling as mentioned above. Next one could also test glucose production. Assuming that it mirrors the *in vivo* system, one would predict increased glucose production in the *Cyp2c44(-/-)* hepatocytes. This would confirm the functionality of this system. Once the *in vitro* system has been characterized and shown to compliment the in vivo results, then the important rescue experiment can be performed. EETs and EET analogs can be tested in the knockout hepatocytes to see if they improve insulin sensitivity and signaling. EETs are not the only metabolites Cyp2c44 produces, so this would give direct evidence that EETs and not some other metabolite are regulating insulin sensitivity.

In the mouse models, again, the effects endogenous disruption of EETs on insulin signaling needs to be investigated further. Other parts in the signaling pathway need to be analyzed such as the insulin receptor, PI3K, and IRS-1. This can be done in both the global and liver-specific knockout mice. Furthermore, the lack of glucose intolerance in the hep*Cyp2c44(-/-)* mice needs to be further investigated. Signaling studies could help reveal whether or not the liver still displays insulin resistance.

Through these further studies we can learn more about how EETs are affecting insulin resistance and hopefully better validate the EET pathway as a potential therapeutic target in diseases associated with insulin resistance, like type 2 diabetes and associated cardiovascular diseases.

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