The Role of the Prostaglandin E₂ EP3 Receptor in Obesity, Insulin Resistance, and Glycemic Control

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To my parents, Joe and Susan Ceddia. Thank you for your love and inspiration.
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<td>15Δ-PGJ₂</td>
<td>15-Deoxy-$\Delta^{12,14}$-Prostaglandin J₂</td>
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<td>AA</td>
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<td>Adipoq</td>
<td>Adiponectin</td>
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<td>AdPLA</td>
<td>Adipose-Specific PLA</td>
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<td>AIS</td>
<td>Adipocyte Incubation Solution</td>
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<td>ALT</td>
<td>Alanine Aminotransferase</td>
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<td>ApoB</td>
<td>Apolipoprotein B</td>
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<td>ATGL</td>
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<td>ΔAUC</td>
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<td>IκB</td>
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<td>Platelet Endothelial Cell Adhesion Molecule-1 (a.k.a. CD31)</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>PPARγ Coactivator-1α</td>
</tr>
<tr>
<td>PGDS</td>
<td>PGD₂ Synthase</td>
</tr>
<tr>
<td>PGES</td>
<td>PGE Synthase</td>
</tr>
<tr>
<td>PGIS</td>
<td>PGI₂ Synthase</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Protein Kinase A</td>
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<tr>
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<td>Perilipin</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator-Activated Receptors</td>
</tr>
<tr>
<td>Ptger3</td>
<td>Prostaglandin E Receptor 3 (subtype EP3)</td>
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<tr>
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<tr>
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<tr>
<td>SNARE</td>
<td>Soluble NSF Attachment Protein Receptor</td>
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<tr>
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<td>Secreted PLA₂</td>
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<tr>
<td>StDev</td>
<td>Standard Deviation</td>
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<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TP</td>
<td>TXA₂ Receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>TXA₂</td>
<td>Thromboxane</td>
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<tr>
<td>TXA2S</td>
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<td>Vascular Endothelial Growth Factor A</td>
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<td>Very-Low-Density Lipoproteins</td>
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<td>WAT</td>
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CHAPTER I
INTRODUCTION

*Obesity – Why is it a problem?*

Obesity is rapidly becoming a major problem for both adults and children in the United States and throughout the world. This condition can be caused by many factors, including genetic, metabolic, behavioral, and environmental influences (1). In fact, obesity has reached epidemic proportions in many countries, including the U.S. Obesity is defined as having a body mass index (BMI) that is greater than 30 kg/m\(^2\) and as of 2012, 35.04% of men and 36.84% of women in the US were considered obese (2). Between 1950 and 2000, the proportion of overweight men and women rose from 21.8% to 35.2% and from 15.0% to 33.1.9%, respectively (3). Even more striking has been the approximately three fold rise in BMI between 1950 and 2000, with men seeing a change from 5.8% to 14.8% for a BMI of \(\geq 30 \text{ kg/m}^2\), and from 0.2% to 5.4% for a BMI \(\geq 35 \text{ kg/m}^2\) (3). Women also saw a rise in the prevalence of obesity with a change from 3.9% to 14% for a BMI of \(\geq 30 \text{ kg/m}^2\), and 1.7% to 4.4% for a BMI \(\geq 35 \text{ kg/m}^2\) (3). Obesity has been estimated to have caused between 95,000 and 163,000 excess deaths in 2004 (4) and it has been estimated to cost the US over $147 billion per year (5).

*Diabetes – Why is it a problem?*

Increased body mass correlates with and increased risk for type 2 diabetes (6-8). Diabetes had a total cost of $218 billion to the United States in 2007 (9). It is the seventh leading cause of death and affects an estimated 25.8 million people in the U.S. or 8.3% of the population (10). Diabetes is a group of metabolic diseases characterized by elevated blood glucose levels caused by a failure to produce enough insulin and/or a reduced response to the insulin produced. Type 1 diabetes results from an autoimmune destruction of insulin producing pancreatic \(\beta\)-cells. Type 2
diabetes results from a reduction in both insulin sensitivity and β-cell function. Type 2 diabetes is a comorbidity of obesity, occurring when the body is unable to produce enough insulin to maintain glycemic control. As fat mass increases, the amount of insulin needed to maintain glycemic control increases due to both the increasing body mass as well as declining insulin sensitivity. β-cell mass and insulin production are initially increased to compensate for the increasing insulin need (11). As the disease progresses, β-cell function begins to decline and a disparity arises between the insulin produced and the insulin needed to maintain glycemic control, which is Type 2 diabetes (12). Type 2 diabetes is the most prevalent form of diabetes and has the highest economic burden of $159.5 billion per year (9). Complications from diabetes can result in kidney damage, vision loss, loss of limbs, and death.

Initial therapies for diabetes primarily focus on either providing more insulin to the patient, by increasing endogenous production or exogenous administration, or improving the function of insulin. Insulin was first administered to a patient with type 1 diabetes in 1922 by Frederick Banting (13). Administration exogenous insulin is still standard for persons with type 1 and advanced Type 2 diabetes. More recently, insulin secretagogues have come onto the market that attempt to reduce the need for regular insulin administration for patients with type 2 diabetes. The first class of drugs to become available in the 1950’s were sulfonylureas which act by increasing insulin release from the pancreatic β-cells (14). Agonists for the glucagon-like peptide-1 (GLP-1) receptor also increase glucose stimulated insulin secretion (GSIS) from pancreatic β-cells. The first commercial GLP-1 receptor agonists to be used in the clinic were liraglutide and exenatide; these are mimetics of human GLP-1 and exendin-4, a peptide found in the venom of the Gila Monster, respectively (15). Exenatide was the first of this class of drug to come onto the market, approved in 2005, and was marketed as Byetta. Aside from insulin and
drugs to improve insulin secretion, some therapies attempt to improve insulin sensitivity, such as metformin, which improves glucose tolerance and reduces hepatic gluconeogenesis (16). Metformin, which was first approved in Canada in 1972 (17), is the first-line drug of choice for patients with type 2 diabetes and may be used as a therapy for prevention in those at risk for diabetes (18). Similar to metformin, thiazolidinediones are insulin-sensitizing drugs, which act through the peroxisome proliferator-activated receptors (PPAR), first saw clinical use in 1997 (19). In addition to improving insulin resistance, thiazolidinediones reduce systemic inflammation (20). Inflammation plays an important role in diabetes and targeting inflammation may become an important mechanism to treat diabetes (21).

*Diabetes as an inflammatory disease*

Inflammation has long been known to be a complicating factor worsening diabetes mellitus. An early review on the subject by Valy Menkin in 1941, discussed the observation that diabetes complications worsen during inflammation or infection (22). Little did Menkin know, the interaction of diabetes and inflammation would become a subject of intense scientific investigation for the rest of the twentieth century and beyond (23-25).

The pathological involvement of the immune system in type 2 diabetes has been well established. During obesity macrophage infiltrate metabolically important tissues, such as adipose tissue, liver, muscle and pancreas (26-31). These proinflammatory macrophages produce cytokines, which are pro-inflammatory signaling molecules. Pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, C-reactive protein (CRP), and tumor necrosis factor (TNF)-α are elevated in obese and diabetic subjects (32-34). These cytokines activate signaling pathways which contribute to insulin resistance (35). Notably, activation of IKK-β (IkB kinase-β) and its downstream target NFκB (nuclear factor κ-light-chain-enhancer of activated B cells), a
transcription factor that regulates genes responsible for the innate and adaptive immune response, leads to insulin resistance (36-38). In addition to causing insulin resistance, IL-1β signaling in islets leads to activation of cyclooxygenase (COX) and inducible nitric oxide synthase (iNOS), which reduces GSIS from pancreatic islets (39,40). COX enzymes generate prostaglandins (PGs), which are themselves important mediators of inflammation (41-45). PGE2 is one of the most prevalent PGs produced and it plays important roles in inflammation and other biological processes (41,44).

The importance of inflammation in the pathogenesis of obesity and diabetes has culminated in numerous clinical trials targeting inflammation as an approach to treat type 2 diabetes (23). Many of these trials utilize mechanisms to block IL-1 or IKK-β/NFκB. Several of these studies resulted in a prolong lowering of blood glucose following the treatment (23). Modulation of PGs has also been investigated as a potential therapy for improving the diabetic phenotype. Aspirin was found to decrease the risk of developing type 2 diabetes, though this was not found with other non-steroidal anti-inflammatory drugs (NSAIDs) (46). NSAIDs act by inhibiting COX mediated PG production (47), though it should be appreciated that some NSAIDS, such as salicylate and aspirin, also block the activity of the pro-inflammatory transcription factor NFκB (48). Recently, much interest has focused on the use of salicylate and related drugs to improve the diabetic phenotype because it has the ability to inhibit both NFκB signaling and COX mediated PG production (23,49,50)

**Historical Overview of Prostaglandins and Metabolism**

Salicylic acid received its name from salicin, from which it was first synthesized in 1838 (51). Salicin, is named after salix, which is Latin for willow, because it was first isolated from the bark of the white willow (Salix alba) in 1828 (52). Willow bark and leaves have been used as
a treatment for headaches and inflammation since ancient times. One of the earliest references for using willow leaves to treat inflammatory rheumatic diseases comes from Assyrian tablets from the Sumerian period (53). The Ebers papyrus, from ancient Egypt circa 1550 B.C., suggests using leaves of the willow to treat an inflamed wound (53). Hippocrates of Kos, circa 460-370 B.C., also recommended an extract of willow bark to treat inflammation (53). The Reverend Edmund Stone gave the first scientific description of the beneficial effects of willow bark in 1763, describing the ability of willow bark to treat agues (a febrile illness involving successive cold, hot, and sweating fits) (54).

There is a long history of salicylate, NSAIDs, and PGs being investigated for their potential therapeutic benefits in the treatment of diabetes and other metabolic complications (Figure 1). The first recorded use of salicylate to treat diabetes was by Wilhelm Ebstein in 1876 who found that it decreased urinary glucose in diabetic patients indicating that the diabetic condition was improved (55). The beneficial effects of salicylate on glucosuria were replicated by other physicians; however, it appears that treatment of diabetes with salicylates never became common (56). In 1957, acetylsalicylic acid, also known as aspirin, was shown to lower blood glucose in diabetic patients (57,58). In 1967 it was shown that salicylate increased plasma insulin explaining the reduction in blood glucose during aspirin treatment that had been previously reported (59). Sodium salicylate and other aspirin-like drugs were shown to block the synthesis of PGs in 1971 (47) which was later shown to be due to inhibition of COX enzymes. PGs had been known for some time prior, with their effect of causing uterine contractions being first described in 1930 (60) and the term “prostaglandin” being first used in 1935 (61). The first prostaglandin isolated was isolated in 1957 and termed “prostaglandin factor (PGF)” (62). At the time of the isolation of PGF, it was noted that at least one other factor was present in the sheep
Figure 1. History of prostaglandins and metabolism
Early studies show that salicylate and other NSAIDs decrease plasma free fatty acids and improve glucose homeostasis. These effects were later shown to be due to blockade of prostaglandin synthesis, namely PGE$_2$. PGE$_2$ inhibits GSIS, lipolysis, and glucagon-induced gluconeogenesis. GSIS and lipolysis inhibition by PGE$_2$ have been shown to occur via the EP3 receptor.
prostate glands. This factor was found in the more lipid soluble ether fractions, whereas PGF was found in the buffer fractions, and was termed PGE (63). In 1959 the biological effects of PGE and PGF were characterized with these PGs being found to affect smooth muscle and have cardiovascular effects (64). The structures of PGE and PGF were determined in 1960 (63,65) and those of other PGs were found about 10-15 years later (66-68). Much later, in 2002, the COX2 selective inhibitor SC58236 was shown to improve GSIS from Ins-1 cells suggesting that salicylate and aspirin’s effect of raising plasma insulin is due to inhibition of the COX2 enzyme (69). Three years after salicylate was shown to block PG synthesis, PGE2 was shown to inhibit GSIS (70). In 1987 PGE2 was shown to reduce cyclic adenosine monophosphate (cAMP) in islets and to inhibit GSIS in a pertussis toxin sensitive manner indicating that PGE2 signaling in islets is mediated through the inhibitory PGE2 receptor (71). This inhibitory, Gi alpha (Gαi) coupled PGE2 receptor was named prostaglandin E receptor 3 (EP3) and was first cloned in 1992 (72). In 2008 a U.S. patent application suggested that EP3 antagonist GW67021B may be used to lower blood glucose (73). It was not until 2013 that the use of an EP3 antagonist, L-798,106, on islets was first published, confirming that PGE2 inhibits GSIS by signaling through EP3 (74). These landmark studies indicate that salicylate and other aspirin-like drugs block PGE2 production via COX2, which leads to reduced PGE2 evoked EP3 signaling in islets, resulting in enhanced GSIS lowering blood glucose and improving glucosuria.

PGE2 also reduces hepatic glucose production in addition to improving GSIS; in 1974 PGE2 was shown to inhibit glucagon-induced production of hepatic cAMP (75) and in 1980 inhibition of glucagon-induced gluconeogenesis was demonstrated (76). An alternative mechanism of improving diabetic phenotype by saliclyate and aspirin was found in 1994 when it was shown that these drugs block the activity of the pro-inflammatory transcription factor NFkB
by inhibiting the activity of IKK-β (77). In 2001 inhibition of IKK-β by salicylates was shown to reverse hyperglycemia by improving insulin sensitivity (78,79). Hence, salicylates may lower blood glucose by both improving insulin sensitivity and increasing GSIS. Unfortunately, the doses of NSAIDs required to have a clinical impact on diabetes by improving GSIS caused negative side effects so their therapeutic use for diabetes was abandoned (80). However, recent studies with salsalate in humans have been promising showing reduced inflammation and increased insulin levels (81-87).

In addition to improving GSIS, salicylate and PGE$_2$ also have a profound effect on adiposity. An effect was first noted in 1961 when salicylate was shown to decrease plasma free fatty acid (FFA) (88), though this result is controversial as it was later shown that salicylate increased FFA (89). Two years later PGE$_2$ was shown to inhibit lipolysis, the breakdown of triglycerides into FFA (90-92). PGE$_2$ was shown to reduce cAMP in adipose tissue, similar to its effect in pancreatic islets; however, the effect in adipose tissue was first reported in 1967, preceding the first report in islets by twenty years (93,94). Despite these early observations that PGE$_2$ inhibits lipolysis, it was not until 1968 that PG release from adipose tissue was first demonstrated (95). The effects of PGE$_2$ in obesity and adiposity received much attention early on. In 1975 Curtis-Prior proposed that metabolic obesity is caused by the over production of PGs (96). He reasoned that because PGs prevented lipolysis, the over production of PGs would cause lipids to be unable to leave the adipose tissue and travel to other tissues to be metabolized. He therefore proposed that NSAIDs would treat obesity by promoting lipolysis and causing lipid redistribution to ectopic tissues where the lipid could be metabolized. Aspirin was not shown to reduce body-weight in people (97), but COX2$^{-/-}$ mice have reduced adiposity (98,99) and the NSAID indomethacin has been shown to prevent high fat diet (HFD) induced obesity in mice
In 2009 the EP3 antagonist L-826,266 was shown to block PGE\(_2\)’s inhibition of lipolysis (101). Hence, the mechanism of PGE\(_2\) action on adipocytes appears similar to its mechanism of action in pancreatic islets. NSAIDs block PGE\(_2\) synthesis, which signals through the EP3 receptor to decrease cAMP, and thereby reduces conversion of triglycerides into FFA. In 2007 it was noted that EP3\(^{-/-}\) mice become obese and insulin resistant when fed a breeder chow diet; this was attributed to hyperphagia and increased feeding during the day (102). More recent understanding of lipid signaling indicates that lipid accumulation in ectopic tissues causes insulin resistance as opposed to simply reducing obesity as Curtis-Prior proposed in 1975 (35). It is possible that these EP3\(^{-/-}\) mice had enhanced lipolysis, which culminated in increased insulin resistance. Curtis-Prior’s proposal for using NSAIDs to promote lipolysis may work to decrease lipid storage in adipose tissue; however, subsequent accumulation of lipid in ectopic tissues will likely not be metabolized as he proposed, but rather stored in those tissues contributing to insulin resistance and thereby promoting the diabetic phenotype.

The drug salsalate has recently been of interest as a possible anti-inflammatory drug that may be beneficial for improving diabetes (50,103,104). Salsalate is a prodrug that is converted into salicylate (salicylic acid). Salicylate has been long known to increase plasma insulin (59) and this effect is shared among most NSAIDs except indomethacin (50,105,106). It is thought that this occurs by inhibiting COX enzymes, which synthesize PGs, and which would normally act to inhibit GSIS. Salicylate also improves insulin sensitivity by inhibition of IKK-\(\beta\) (78,79). Hence, salicylate appears to have a beneficial effect on both reducing insulin resistance and improving insulin secretion.

Thus PGs, though perhaps best known for their role in pain and inflammation, have a long history of modulating metabolism. PGE\(_2\) and EP3 in particular play an important role in
regulating GSIS from pancreatic islets and lipolysis from adipose tissue. With the recent understanding of the role inflammation plays in obesity and diabetes, PGs present an attractive therapeutic target because they modulate insulin secretion, adiposity, and inflammatory processes.

**Prostaglandin Signaling**

PGs are oxidative metabolites of arachidonic acid (AA) that are produced by COX enzymes (COX1 and COX2). They are lipid-signaling molecules that are named for the prostate because they were first detected in seminal fluid and that comes from the prostate (61). AA is liberated from the plasma membrane by phospholipase A2 (PLA2). COX converts AA into PGH2 which is converted into five primary bio-active prostanoids: PGE2, PGF2α, PGD2, prostacyclin (PGI2, and its degradation product 6-keto-PGF1α), and thromboxane (TXA2, and its more stable metabolite TXB2). These prostanoids act locally in an autocrine or paracrine manner. The local action of PGs depends on activation of a family of specific G-protein coupled receptors (GPCRs) designated EP for E-prostanoid receptors, FP, DP, IP and TP receptors, for the other PGs, respectively.

(Reviewed in (43,107-109))

**Phospholipase A2**

PLA2s are enzymes which catalyze the release of a fatty acid from the second carbon group (sn-2 position) of membrane glycerophospholipids. PLA2s can be divided into six families: low-molecular weight secreted PLA2s (sPLA2), calcium-dependent cytosolic (cPLA2), calcium-independent cytosolic (iPLA2), platelet-activating factor acetylhydrolase (PAF-AH), lysosomal PLA2s, and adipose-specific PLA (AdPLA). Of these, cPLA2s have specificity for AA in the sn-2 position hence they are associated with the production of eicosanoids, which include
leukotrienes and PGs. In addition, cPLA$_2$s are important because they are hormonally regulated and are therefore the source of hormonally regulated AA release (110,111). The other PLA$_2$ family members can liberate a wide variety of fatty acids.

(Reviewed in (112-117))

*Arachidonic Acid*

AA is a polyunsaturated omega-6 fatty acid, 20:4(ω-6) which was first isolated from liver lipids (118). AA can function as a signaling molecule itself or it can be metabolized into a variety of potent signaling molecules. The enzymes 5-lipoxygenase, 8-lipoxygenase, 12-lipoxygenase, and 15-lipoxygenase convert AA into the hydroperoxyeicosatetraenoic acids (HPETE): 5-HPETE, 8-HPETE, 12-HPETE, and 15-HPETE, respectively. 5-lipoxygenase is notable because leukotrienes, a family of eicosanoid inflammatory mediators, are derived from 5-HPETE. Alternatively, COX metabolism of AA into PGH$_2$ is the first committed step towards PG synthesis. In addition, AA can be metabolized into epoxyeicosatrienoic acids (EET) by cytochrome P450 enzymes.

(Reviewed in (119,120))

*Cyclooxygenase*

The COX1 and COX2 enzymes are encoded by the genes prostaglandin-endoperoxide synthase 1 (*Ptgs1*) and 2 (*Ptgs2*), respectively. Both COX enzymes carry out an identical chemical reaction. The COX enzymes are the key enzymes for catalyzing the conversion of AA into PGG$_2$. PGG$_2$ is an intermediate PG that is quickly converted into PGH$_2$ by COX. PGH$_2$ is converted into the five primary prostanoids by enzymatic conversion or spontaneous rearrangement.
Both COX enzymes are the targets for NSAIDs, such as aspirin, which inhibit the conversion of AA into PGG₂. Selective COX inhibitors have recently been developed which allow specific inhibition of COX1 or COX2. Although there are exceptions, expression of \textit{Ptgs1} is generally considered to be constitutive expressed whereas \textit{Ptgs2} expression is generally thought of as being inducible. For this reason it has been classically thought that COX2 mediates inflammatory reactions whereas COX1 mediates many of the “housekeeping” effects.

(Reviewed in (121-123))

\textit{Prostaglandins}

PGH₂ is converted into the five primary prostanoids by their respective synthases or by spontaneous rearrangement. The five primary bioactive prostanoids are PGE₂, PGF₂α, PGD₂, PGI₂, and TXA₂. Conversion of PGH₂ to PGE₂ is performed by one of three PGE synthases (PGES): cytosolic PGE synthase (cPGES) and two membrane bound PGE synthases (mPGES-1 and mPGES-2). There are two types of PGD₂ synthases (PGDS): the lipocalin-type PGDS (glutathione (GSH)-independent) (L-PGDS) and the hematopoietic PGDS (GSH-dependent). Three different enzymes, PGE 9-ketoreductase, PGD 11-ketoreductase, and PGH 9-, 11-endoperoxide reductase, make PGF from PGE₂, PGD₂, or PGH₂, respectively. PGI₂ is synthesized from PGH₂ by a single enzyme, PGI₂ synthase (PGIS). TXA₂ is also synthesized from PGH₂ by a single enzyme, TXA₂ synthase (TXA2S).

(Reviewed in (124-129))

\textit{Prostaglandin Receptors}

PGs signal through GPCRs which are receptors containing seven-transmembrane spanning domains that couple to heterotrimeric G-proteins that consist of \( \alpha \) and \( \beta\gamma \) subunits. Most \( G\alpha \) subunits can be broadly classified into four main types: \( G\alpha_s \), \( G\alpha_i \), \( G\alpha_q \), and \( G\alpha_{12/13} \). \( G\alpha_s \)
stimulates adenylyl cyclases which increases the second messenger cAMP, while G\(_{\alpha_i}\) inhibits adenylyl cyclase thereby decreasing cAMP. G\(_{\alpha_i}\) activates phospholipase C which increases the second messengers diacyl-glycerol (DAG) and inositol trisphosphate (IP\(_3\)) which in turn activates calcium release from the endoplasmic reticulum. G\(_{\alpha_{12/13}}\) activate RhoGEFs that activate Rho GTPases.

PGE\(_2\) signals through four GPCRs termed EP1-EP4. EP3 has three splice variants in mice (\(\alpha, \beta, \gamma\)); the number of splice variants varies between species with at least ten in human (130) that differ in their carboxy-terminal tails. The different carboxy-terminal tails affects the subcellular localization of the EP3 receptor splice variants and signal transduction pathways by coupling to different G proteins (131,132). Pharmacological studies have defined EP2 and EP4 as G\(_{\alpha_s}\) coupled, EP1 as G\(_{\alpha_q}\) coupled, and EP3 as G\(_{\alpha_i}\) and G\(_{\alpha_{12/13}}\) coupled. Recent studies indicate that in pancreatic islets EP3 is also coupled to G\(_{\alpha_z}\), which is a pertussis toxin insensitive inhibitory G-protein (133,134). Agonist activation of EP2 can cause \(\beta\)-arrestin binding and a subsequent \(\beta\)-arrestin-Src complex, which can activate downstream signaling pathways (135,136). EP3 can be internalized in response to PGE\(_2\); however, the degree and the mechanism of internalization vary between the isoforms (137). EP4 can be phosphorylated and desensitized by G protein–coupled receptor kinases (GRKs) (138). In addition, EP4 can bind \(\beta\)-arrestin (139), which can activate Src and its downstream signaling pathways (140,141). The receptors have varying affinities for PGE\(_2\), and EP3 and EP4 have the highest affinity with \(K_d\)'s of 0.8-5 and 2-3.2 nM, respectively. EP1 and EP2 have lower affinity with \(K_d\)'s of about 20 and 13-40 nM, respectively.

PGF\(_2\alpha\) signals through a single GPCR, FP. FP has two splice variants and is primarily coupled to G\(_{\alpha_q}\) though it also signals through G\(_{\alpha_i}\). FP has been reported to lack associatiation
with β-arrestin (142). FP is very similar in sequence to the EP3 receptor and is located on opposite strands of chromosome 1 in the human or chromosome 3 in the mouse. It is thought that FP arose as a gene duplication of EP3.

PGD₂ signals through two GPCRs: DP1 and DP2 (previously called CRTH2 \{chemoattractant receptor-homologous molecule expressed on Th2 cells\}, PTGDR2, GPR44, or CD294). DP1 is Ga₄ coupled while DP2 is coupled to Gaᵢ. PGD₂ can signal through DP2 to induce β-arrestin phosphorylation and activation of its downstream signaling pathways (143). DP1 is closely related to the other members of the prostanoid GPCR family in sequence. DP2 does not share homology with other prostanoid GPCRs, being more closely related to chemoattractant receptors, though its affinity for PGD₂ is similar to that of DP1.

PGI₂ signals through a single GPCR, IP. IP is primarily Ga₄ coupled, but has been shown to couple to Gaᵢ and Gaₐ. Agonist-induced desensitization of IP occurs through PKC phosphorylation of IP and does not involve GRKs or β-arrestins but instead is mediated at least partially through clathrin-coated vesicles (144,145).

TXA₂ signals through a single GPCR, TP. The TP receptor has two splice variants. Though initially described as being Gaₐ q coupled, TP has been shown to couple to Gaₐ, Gaᵢ, and Ga₁₂/₁₃ also. Both splice variants of TP are desensitized by GRKs and are internalized by arrestins (146,147), but the signaling mechanisms leading to desensitization differ between the two splice variants (148). (Reviewed in (43,44,107,109,149-151))

The Role of Prostaglandins in Metabolism

Eicosanoids play an important role in metabolism (152). PGs have been implicated in roles as diverse as pancreatic insulin secretion and energy homeostasis. They are also important modulators of the immune system which itself plays a critical role in obesity and diabetes.
Regulation of Insulin Secretion

Insulin is an important regulator of metabolic homeostasis. Insulin acts on tissues to cause uptake of glucose from the blood. It also has a variety of tissue specific effects. In the liver, insulin inhibits gluconeogenesis, the production of glucose from glycogen stores, while stimulating glycogen synthesis. It also fosters energy storage by increasing de novo lipogenesis. Similarly, in skeletal muscle, insulin also promotes glycogen synthesis. In adipose tissue, insulin inhibits lipolysis, which is the conversion of stored triglycerides into FFA and the release of the FFA into the bloodstream.

Insulin is secreted from β-cells, which reside in the islets of Langerhans in the pancreas. In addition to β-cells, islets also contain α-, δ-, PP-, and ε-cells, which secrete glucagon, somatostatin, pancreatic polypeptide, and ghrelin, respectively (153). Glucose is the primary regulator of insulin secretion. Glucose metabolism increases intracellular ATP, raising the ATP-ADP ratio, which closes the ATP-sensitive potassium channels. The resulting membrane depolarization allows voltage-operated calcium channels to open, resulting in a rise in intracellular calcium, the main trigger for insulin exocytosis. Exocytosis occurs when an insulin-containing secretory granule fuses to the plasma membrane. This process is catalyzed by the formation of a SNARE (Soluble NSF Attachment Protein Receptor) complex, which is formed by vesicle-associated membrane protein 2 (VAMP-2)/synaptobrevin2 from the vesicle interacting with syntaxin-1 and synaptosomal-associated protein-25 (SNAP-25) on the plasma membrane (154). Vesicle fusion and insulin release occur when calcium binds synaptotagmin activating the SNARE complex (154,155).

In addition to the canonical glucose mediated pathway, there are multiple alternative mechanisms that serve to modulate GSIS or can even alter insulin release in the absence of
changes in glucose concentration. For example, cytokines and nitric oxide both decrease GSIS (156), GSIS can be enhanced by estrogen signaling through its cognate nuclear hormone receptors (157), and signaling through the insulin receptors promote β-cell proliferation and insulin synthesis (158). One of the most prevalent class of GSIS modulators are GPCRs (159,160). GPCRs modulate GSIS through their heterotrimeric G-proteins. Gaq mobilizes internal calcium stores to promote insulin secretion via activation of protein kinase C (PKC), a serine and threonine kinase, and calcium responsive calcium channels, such as ryanodine receptors. cAMP increases insulin release via multiple pathways, hence Ga activation usually increases insulin release while Gi signaling has an opposing effect. cAMP directly activates Epac, which is a guanine nucleotide exchange factor (GEF) for Rap, a Ras-related GTPase. Rap activates phospholipase C-ε resulting in the production of IP3 and thereby calcium. Epac also directly interacts with SNAP-25 (161) and indirectly with other secretory granule-associated proteins and SNARE proteins that control exocytosis (162). cAMP also activates protein kinase A (PKA), which is a serine/threonine kinase. PKA phosphorylates SNAP-25 increasing secretory capacity (163). PKA sensitizes intracellular calcium release channels to stimulatory second messengers such as IP3 and calcium which also increases insulin secretion (162). In addition to Ga, GPCRs affect insulin release through Gβγ (164-167) and β-arrestin (168) mediated signaling.

PGs play an important role in regulating GSIS. Glucose stimulation rapidly increases AA concentration in islets (169,170), which appears to be caused by decreasing intracellular calcium concentrations, following the glucose stimulated rise in intracellular calcium, activating iPLA2 (171). Both COX1 and COX2 are expressed in pancreatic islets; however, it is COX2 that mediates the classical inhibition of GSIS by PGs (69,172). Expression of COX2 has been
reported to be higher in β-cells than α-cells indicating that β-cells may produce the PGs that regulate GSIS (173). PGD₂, PGE₂, PGF₂α, PGI₂ and TXB₂ are produced in pancreatic islets in response to glucose and may function as a way to fine-tune GSIS (74,169,172,174-182). PGD₂ and PGF₂α are typically associated with increasing insulin release while PGE₂ inhibits insulin release (71,74,177,183-197).

Prostaglandin E₂

PGE₂ is typically thought to inhibit GSIS (198-200). Glucose stimulates PGE₂ release from islets (169,174,178,180,182). During islet perifusion, the peak release of PGE₂ occurs about 4 minutes after the peak release of insulin (169). This suggests that PGE₂ may function to prevent the overproduction of insulin during GSIS. Though many studies indicate that PGE inhibits GSIS, the results remain controversial as some studies find that PGE₂ has no effect or even increases GSIS while others report specific culture conditions or phenotypes of the islet source being necessary to observe PGE₂’s effects (Table 1).

The effects of PGE on insulin secretion in a whole animal were the first studies to establish a link between PGE₂ and GSIS. Multiple researchers have utilized continuous infusion of PGE₂ into different model organisms to determine the effect of PGE₂ on plasma insulin. In a whole animal setting, continuous infusion of PGE is used because over 90% of PGs are metabolized by a single pass through the lungs that removes 97% of intravenously administered PGE₂ after 1.5 minutes (201,202). Most of these studies indicate that in vivo PGE₂ decreases GSIS. These studies lack target organ specificity; hence, PGE₂ could be affecting plasma insulin levels through mechanisms other than inhibiting GSIS in pancreatic islets. For example, PGE₂ injected into the third cerebral ventricle causes hyperglycemia (203). In addition to affecting
<table>
<thead>
<tr>
<th>Method</th>
<th>Species</th>
<th>Ligand</th>
<th>Concentration</th>
<th>Change in GSIS</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>in vivo Bolus Injection</td>
<td>mouse (white)</td>
<td>PGE₁</td>
<td>2.5 µg</td>
<td>↑</td>
<td>(204)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.0 µg</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>sheep (Merino)</td>
<td>PGE₁</td>
<td>20 µg/kg</td>
<td>No Change</td>
<td>(205)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>15(S)-15-methyl PGE₂ methyl ester</td>
<td>1.0 µg/kg</td>
<td>↓</td>
<td>(206)</td>
</tr>
<tr>
<td>in vivo Continuous Infusion</td>
<td>rat (albino)</td>
<td>PGE₂</td>
<td>2 µg/min</td>
<td>↓</td>
<td>(193)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>PGE₂</td>
<td>10 µg/min</td>
<td>↓</td>
<td>(185)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>PGE₂</td>
<td>10 µg/min</td>
<td>↓</td>
<td>(192)</td>
</tr>
<tr>
<td></td>
<td>human (diabetic)</td>
<td>PGE₂</td>
<td>10 µg/min</td>
<td>↓ (response to arginine)</td>
<td>(207)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>PGE₁</td>
<td>0.2 µg/kg/min</td>
<td>↓</td>
<td>(185)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>PGE₁</td>
<td>0.2 µg/kg/min</td>
<td>↓</td>
<td>(208)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>PGE₁</td>
<td>0.2 µg/kg/min</td>
<td>No Change</td>
<td>(209)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>PGE₁</td>
<td>0.2 µg/kg/min</td>
<td>No Change</td>
<td>(210)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>PGE₁</td>
<td>0.2 µg/kg/min</td>
<td>No Change</td>
<td>(211)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>PGE₁</td>
<td>0.5 µg/kg/min</td>
<td>↓</td>
<td>(212)</td>
</tr>
<tr>
<td></td>
<td>human (diabetic)</td>
<td>PGE₁</td>
<td>0.5 µg/kg/min</td>
<td>No Change</td>
<td>(213)</td>
</tr>
<tr>
<td></td>
<td>rat (Sprague-Dawley)</td>
<td>PGE₁</td>
<td>0.5 µg/kg/min</td>
<td>No Change</td>
<td>(193)</td>
</tr>
<tr>
<td></td>
<td>dog (mongrel)</td>
<td>PGE₁</td>
<td>0.5 µg/kg/min</td>
<td>No Change</td>
<td>(190)</td>
</tr>
<tr>
<td></td>
<td>dog (mongrel)</td>
<td>PGE₁</td>
<td>0.5 µg/kg/min</td>
<td>No Change</td>
<td>(70)</td>
</tr>
<tr>
<td></td>
<td>dog (mongrel)</td>
<td>PGE₁</td>
<td>10 µg/min</td>
<td>↓</td>
<td>(215)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>15(S)-15-methyl PGE₂</td>
<td>0.5 g/kg-hr</td>
<td>↓</td>
<td>(206)</td>
</tr>
<tr>
<td></td>
<td>rat (Sprague-Dawley)</td>
<td>PGA₁</td>
<td>0.5 µg/min</td>
<td>No Change</td>
<td>(213)</td>
</tr>
<tr>
<td></td>
<td>rat (albino)</td>
<td>PGA₁</td>
<td>2 µg/min</td>
<td>↓</td>
<td>(193)</td>
</tr>
<tr>
<td></td>
<td>dog (mongrel)</td>
<td>PGB₁</td>
<td>10 µg/min</td>
<td>No Change</td>
<td>(216)</td>
</tr>
<tr>
<td>Method</td>
<td>Species</td>
<td>Ligand</td>
<td>Concentration</td>
<td>Change in GSIS</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>--------------------------------</td>
<td>---------------------------------</td>
<td>------------------------</td>
<td>----------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Perfused Pancreas</td>
<td>rat (Sprague-Dawley)</td>
<td>PGE$_2$</td>
<td>$\geq$ 0.14 $\mu$M</td>
<td>↑</td>
<td>(217)</td>
</tr>
</tbody>
</table>
|                               | rat (albino)                  | PGE$_2$                         | 0.1 $\mu$M
1.0 $\mu$M
10 $\mu$M | ↑              | (183)     |
|                               | rat (Sprague-Dawley)          | PGE$_2$                         | 0.28 $\mu$M
1.4 $\mu$M | ↑              | (189)     |
|                               | rat (Wistar)                  | PGE$_2$                         | 3 $\mu$M               | ↑              | (218)     |
|                               | rat (Sprague-Dawley)          | PGE$_2$                         | 1 $\mu$M
10 $\mu$M | No Change | (219)     |
|                               | rat (Sprague-Dawley)          | PGE$_1$                         | 0.28 $\mu$M | ↑              | (189)     |
|                               | rat (Wistar)                  | PGE$_1$                         | 3 $\mu$M               | ↑              | (218)     |
|                               | rat (Lewis)                   | 16,16-dimethyl PGE$_2$-methyl-ester | 10 $\mu$g/kg
100 $\mu$g/kg | ↓              | (220)     |
| Isolated Islet - Perfusion    | human                         | PGE$_2$                         | 1 $\mu$M               | NS (↑)         | (221)     |
|                               | rat (Sprague-Dawley)          | PGE$_2$                         | 1 $\mu$M
10 $\mu$M | No Change | (222)     |
|                               | hamster (Syrian Golden)       | PGE$_2$                         | 10 $\mu$M              | ↑              | (223)     |
|                               | rat (Wistar)                  | PGE$_1$                         | 0.85 $\mu$M            | ↑              | (224)     |
|                               |                                |                                 | $\uparrow$ 50 mg/dl glucose
$\downarrow$ 300 mg/dl glucose |                |           |
<p>| Isolated Islet - Static Incubation Insulin Secretion | mouse (BTBR$^{ob/ob}$) | PGE$_2$                         | 0.05 $\mu$M          | ↓              | (74)      |
|                               | rat (Wistar)                  | PGE$_2$                         | 0.1 $\mu$M            | ↓              | (196)     |
|                               | human                         | PGE$_2$                         | 0.1 $\mu$M            | No Change      | (225)     |
|                               | rat                           | PGE$_2$                         | 1 $\mu$M              | ↓              | (226)     |
|                               | rat (Sprague-Dawley)          | PGE$_2$                         | 1 $\mu$M              | ↓              | (227)     |
|                               | mouse (ICR)                   | PGE$_2$                         | 1 $\mu$M              | ↓              | (228)     |
|                               | mouse (C57B6/J)               | PGE$_2$                         | 1 $\mu$M              | ↓              | (177)     |
|                               | mouse (C57B6/J PGES$^{-1/-}$) | PGE$_2$                         | 1 $\mu$M              | ↓              | (177)     |
|                               | rat (Sprague-Dawley)          | PGE$_2$                         | 10 $\mu$M             | ↓              | (184)     |
|                               | rat (Wistar)                  | PGE$_2$                         | 100 $\mu$M            | ↓              | (197)     |
|                               | rat (Wistar)                  | PGE$_2$                         | 100 $\mu$M            | ↑              | (229)     |
|                               | rat (Sprague-Dawley)          | PGE$_2$ (40 hours)              | 1 $\mu$M              | No Change      | (225)     |
|                               | rat (Wistar)                  | PGE$_1$                         | 0.01 - 10 $\mu$M      | ↑              | (229)     |
|                               | rat (Wistar)                  | PGE$_1$                         | 2.8 $\mu$M            | NS (↑)         | (230)     |</p>
<table>
<thead>
<tr>
<th>Method</th>
<th>Species</th>
<th>Ligand</th>
<th>Concentration</th>
<th>Change in GSIS</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated Islet - Static Incubation Insulin Secretion - continued</td>
<td>mouse (BTBR)</td>
<td>PGE&lt;sub&gt;1&lt;/sub&gt;</td>
<td>10 µM</td>
<td>No Change</td>
<td>(74)</td>
</tr>
<tr>
<td></td>
<td>mouse (BTBR&lt;sup&gt;ob/ob&lt;/sup&gt;)</td>
<td>PGE&lt;sub&gt;1&lt;/sub&gt;</td>
<td>10 µM</td>
<td>No Change</td>
<td>(74)</td>
</tr>
<tr>
<td></td>
<td>mouse (BTBR&lt;sup&gt;ob/ob&lt;/sup&gt;)</td>
<td>Sulprostone</td>
<td>0.001 µM</td>
<td>↓</td>
<td>(74)</td>
</tr>
<tr>
<td></td>
<td>rat (Wistar)</td>
<td>Sulprostone</td>
<td>0.1 µM</td>
<td>↓</td>
<td>(197)</td>
</tr>
<tr>
<td></td>
<td>Mouse (C57BL/6&lt;sup&gt;ob/ob&lt;/sup&gt;)</td>
<td>Sulprostone</td>
<td>0.6 µM</td>
<td>↓</td>
<td>(134)</td>
</tr>
<tr>
<td></td>
<td>rat (Wistar)</td>
<td>Sulprostone</td>
<td>0.1 µM</td>
<td>↓</td>
<td>(197)</td>
</tr>
<tr>
<td></td>
<td>rat (Wistar)</td>
<td>Misoprostol</td>
<td>0.3 µM</td>
<td>↓</td>
<td>(197)</td>
</tr>
<tr>
<td></td>
<td>Cell Line</td>
<td>PGA&lt;sub&gt;1&lt;/sub&gt;</td>
<td>100 µM</td>
<td>No Change</td>
<td>(229)</td>
</tr>
<tr>
<td></td>
<td>guinea pig, HIT</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; = 1 nM</td>
<td>↓</td>
<td>(71)</td>
</tr>
<tr>
<td></td>
<td>rat, Ins-1 (832/3)</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~50 nM</td>
<td>↓</td>
<td>(74)</td>
</tr>
<tr>
<td></td>
<td>mouse, βHC13</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.1 µM</td>
<td>↓</td>
<td>(196)</td>
</tr>
<tr>
<td></td>
<td>guinea pig, HIT-T15</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.1 µM</td>
<td>↓</td>
<td>(196)</td>
</tr>
<tr>
<td></td>
<td>guinea pig, HIT-T15</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1 µM</td>
<td>↓</td>
<td>(227)</td>
</tr>
<tr>
<td></td>
<td>guinea pig, HIT-T15</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1 µM</td>
<td>↓</td>
<td>(228)</td>
</tr>
<tr>
<td></td>
<td>guinea pig, HIT</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1 µM</td>
<td>↓</td>
<td>(194)</td>
</tr>
<tr>
<td></td>
<td>rat (Sprague-Dawley), fetal pancreas cells</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>10 µM</td>
<td>↓</td>
<td>(195)</td>
</tr>
<tr>
<td></td>
<td>rat, Ins-1 (832/13)</td>
<td>PGE&lt;sub&gt;1&lt;/sub&gt;</td>
<td>10 µM</td>
<td>↓</td>
<td>(133)</td>
</tr>
<tr>
<td></td>
<td>rat (Sprague-Dawley), neonatal pancreas cells</td>
<td>PGE&lt;sub&gt;1&lt;/sub&gt;</td>
<td>10 µM</td>
<td>↓</td>
<td>(231)</td>
</tr>
<tr>
<td></td>
<td>rat (Sprague-Dawley), fetal pancreas cells</td>
<td>PGE&lt;sub&gt;1&lt;/sub&gt;</td>
<td>10 µM</td>
<td>↓</td>
<td>(195)</td>
</tr>
<tr>
<td></td>
<td>rat (Sprague-Dawley), neonatal pancreas cells</td>
<td>PGE&lt;sub&gt;1&lt;/sub&gt; + sodium salicylate</td>
<td>10 µM</td>
<td>↑</td>
<td>(232)</td>
</tr>
<tr>
<td></td>
<td>rat, Ins-1 (832/3)</td>
<td>Sulprostone</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~10 nM</td>
<td>↓</td>
<td>(74)</td>
</tr>
</tbody>
</table>

**Table 1. Summary of the effects of E prostanoid receptor agonists on glucose stimulated insulin secretion**

The effects of PGE<sub>2</sub> and PGE<sub>2</sub> analogs on insulin secretion have been extensively studied. The majority of studies have found that PGE<sub>2</sub> and PGE<sub>2</sub> analogs decrease GSIS, being frequently seen in studies utilizing whole animals and cell lines. Studies utilizing isolated islets have produced discordant results, but decreased GSIS in response to PGE<sub>2</sub> is frequently observed. Contradicting these results, studies utilizing pancreas perfusion often show that PGE<sub>2</sub> increases GSIS.

NS = not significant, parenthesis indicates direction of change
<table>
<thead>
<tr>
<th>Method</th>
<th>Species</th>
<th>Ligand</th>
<th>Receptor</th>
<th>Concentration</th>
<th>Change in GSIS</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated Islet - Static Incubation Insulin Secretion</td>
<td>rat (Wistar)</td>
<td>AH-6809</td>
<td>EP1</td>
<td>0.1 – 10 µM</td>
<td>No Change</td>
<td>(197)</td>
</tr>
<tr>
<td></td>
<td>mouse (C57BL/6)</td>
<td>L-798,106</td>
<td>EP3</td>
<td>10 µM</td>
<td>↑</td>
<td>(233)</td>
</tr>
<tr>
<td></td>
<td>mouse (BTBR)</td>
<td>L-798,106</td>
<td>EP3</td>
<td>20 µM</td>
<td>No Change</td>
<td>(74)</td>
</tr>
<tr>
<td></td>
<td>mouse (BTBR&lt;sup&gt;ob/ob&lt;/sup&gt;)</td>
<td>L-798,106</td>
<td>EP3</td>
<td>10 – 20 µM</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>L-798,106</td>
<td>EP3</td>
<td>20 µM</td>
<td>No Change</td>
<td>(74)</td>
</tr>
<tr>
<td></td>
<td>human (type 2 diabetic)</td>
<td>L-798,106</td>
<td>EP3</td>
<td>&gt; 100 nM</td>
<td>↑</td>
<td>(74)</td>
</tr>
</tbody>
</table>

**Table 2. Summary of the effects of E prostanoid receptor antagonists on glucose stimulated insulin secretion**

Selective EP receptor agonists suggest that EP3 is a negative regulation of GSIS.
GSIS, PGE$_2$ has also been shown to increase glucagon secretion in whole animal and perfused pancreas models, this may alter in insulin or glucose utilization (183,189,208-210,217,218,234).

The effects of PGE$_2$ in perfused pancreata have also been examined. This method keeps the entire pancreas with islets and exocrine tissue intact, but still removes it from the effect of other organs and innervation. The majority of studies show that PGE$_2$ increases GSIS during pancreas perfusion. These results are contrary to what has been observed in β-cell lines and some islet studies where PGE$_2$ decreased GSIS. It is possible that PGE$_2$ acts upon non-β-cell cell types in the intact pancreas, which in turn, raises GSIS.

Isolated islets, which contain a mixture of all islet cell types but lack innervation and pancreatic exocrine tissue, give more varied results, though the majority of studies still found that PGE$_2$ decreased GSIS. β-cell line models give consistent results with PGE reducing GSIS in all published reports that were reviewed, which clearly indicates that PGE decreases GSIS in β-cells.

Several groups have speculated upon the reason for the varied results in isolated islets. One group has reported that islets must be taken from obese or diabetic subjects in order to observe the PGE-mediated inhibition of GSIS (74,134). This suggests that PGE can only inhibit GSIS in islets from obese animals; however, most other studies used islets derived from normal animals (184,196,197,224). Robertson and colleagues emphasized that in order to see the inhibitory effects of PGE$_2$, islets must be cultured in the presence a phosphodiesterase inhibitor, such as IBMX (177) or theophylline (229). It is thought that in the presence of active phosphodiesterases, cAMP is quickly degraded mitigating the effect of Ga$_i$ signaling. Similarly, Sharp and colleagues used forskolin and 12-O-tetradecanoylphorbol 13-acetate (184) while Kimple and colleagues used GLP-1 (74) to keep cAMP at super maximal concentrations in order
to maximize insulin release allowing the detection of inhibitory \( \Gamma_{i} \) signaling by PGE\(_2\) or sulprostone, respectively. The contradictory nature of these data makes it difficult to definitively determine the effect of PGE\(_2\) on GSIS from isolated islets. From the experiments discussed, it is clear that the islet source and culture conditions can have a profound effect on the results.

These studies indicate that PGE\(_2\) is most likely inhibits GSIS by causing a reduction in intracellular cAMP in the pancreatic \( \beta \)-cell. The \( \Gamma_{i} \) coupled EP3 receptor is a good candidate for the PGE\(_2\) evoked inhibition of GSIS in islets. Gene expression of EP receptors has been shown in the mouse (181,235) and human pancreas (236), in human pancreatic stellate cells (237), in human (221), mouse (74,181), rat (196,197,238), and guinea pig islets (71), mouse \( \beta \)-cells (239), HIT and Min6 \( \beta \)-cell lines (71,74,227,240), 832/13 rat insulinoma cell line (238), and in the \( \alpha \)-cell line, \( \alpha \)TC1 (74). These data suggest that EP3, the \( \Gamma_{i} \) coupled receptor, has the highest expression of the four EP receptors in islets. EP3 is not highly expressed in embryonic \( \beta \)-cells; but in adult \( \beta \)-cells, it is the sixth highest expressed \( \Gamma_{i/o} \) coupled GPCR (239). The diabetic phenotype increases the expression of all three mouse EP3 splice variants and PGE\(_2\) production in islets (74).

Selective agonists were first used to identify the EP receptors mediating PGE\(_2\)’s inhibition of GSIS. Several studies have shown that PGE\(_2\) inhibition of GSIS is pertussis toxin sensitive, indicating that PGE\(_2\) inhibition of GSIS occurs through a \( \Gamma_{i} \) coupled receptor (71,197,226). Sulprostone, an EP1/EP3 selective agonist, and misoprostol, an EP2, EP3, and EP4 selective agonist, inhibit GSIS in a pertussis toxin-sensitive manner in rat islets which implicates the \( \Gamma_{i} \) coupled EP3 receptor as the primary mediator of PGE\(_2\) signaling (197). Selective antagonists have also been used to elucidate PGE\(_2\)’s signaling mechanisms in islets (Table 2). The involvement of EP3 was further confirmed by the use of EP3 antagonist L-798,106 which
blocked PGE$_2$ inhibition of GSIS and cAMP production in diabetic mouse islets (74). L-798,106 was shown to increase GSIS in Min6 cells, mouse islets, and diabetic human islets (74,233). In summary, PGE$_2$ appears to signal through the EP3 receptor on β-cells to cause a decrease in intracellular cAMP reducing GSIS (Figure 2). These results indicate that EP3 blockade may be a therapeutically useful mechanism to increase GSIS.

Few studies that examine the effects of PGE$_2$ and EP receptors on β-cell function, *in vivo*. Transgenic mice that have increased β-cell PGE$_2$ production by expressing COX2 and mPGES-1 in their β-cells using the rat insulin-2 gene promoter had reduced plasma insulin and displayed a poorer glucose tolerance (188). This effect was confounded by the observation that these transgenic mice had reduced β-cell proliferation and fewer β-cells. Other studies have shown that PGE$_2$ inhibits β-cell proliferation (195) and that salicylate prevents β-cell death (241). PGE$_2$ has also been shown to decrease cell viability in HIT cells, but it has no significant effect on cell cycle phase or apoptosis (227). However, a study using Min6 cell showed that PGE$_2$ reduces apoptosis (240). In a setting of streptozotocin (STZ)-induced diabetes, COX2$^{−/−}$ mice and EP2$^{−/−}$ mice treated with an EP4 antagonist have decreased survival; while mice treated with EP2 and EP4 agonists concurrently have increased survival (181). The EP4-selective agonist, ONO-AE1-329 improves glucose homeostasis and insulin resistance in *Lepr*$_{db/db}$ (leptin receptor diabetes mutation) mice (242). These studies indicate that PGE$_2$ signaling through an EP2/EP4 mediated pathway is protective for β-cells. It could be hypothesized that EP3$^{−/−}$ mice would have increased survival because the signaling pathway of EP3 classically opposes that of EP2/EP4. Furthermore, signaling through G$_{αi/z}$ coupled GPCRs restrict β-cell expansion (134,239). Though this study did not report survival of STZ treated EP3$^{+/−}$ mice, STZ treated EP3$^{+/−}$ mice were reported to have no difference in blood glucose indicating that β-cell function was not improved
Figure 2. Prostaglandin signaling in the β-cell

Glucose import into the β-cell is the main trigger of insulin secretion. Glucose triggers insulin release by opening voltage dependent calcium channels thereby increasing the concentration of free cytoplasmic calcium. PKA and PKC phosphorylate proteins required for exocytosis. PGE$_2$ signals through its four cognate G-protein coupled receptors to modulate the activity of regulators of exocytosis, such as PKA, PKC, and Epac. EP3 is one of the most prominently expressed prostaglandin receptors on the β-cell and mediates the inhibition of insulin secretion by PGE$_2$. 
(181). Contradicting this lack of change in EP3<sup>-/-</sup> STZ treated mice, an U.S. patent application demonstrated that EP3 antagonist GW671021B ameliorated hyperglycemia in mice when diabetes was induced with diet induced obesity in combination with STZ treatment (73). It is possible that the beneficial effects of EP3 inhibition can only be seen in a setting of obesity. The EP3 antagonist L-798,106 has been reported to only have beneficial effects in islets from diabetic patients or Leptin<sup>ob/ob</sup> mice (74). This may be because the EP3 receptor functions to decrease intracellular cAMP; hence, the lack of EP3 signaling has no effect in physiological states when cAMP is already low. In another study, EP3<sup>-/-</sup> mice have been reported to have elevated plasma insulin; however, these results are confounded by the fact that this study also reported that their EP3<sup>-/-</sup> mice were obese and insulin resistant (102). In a setting of insulin resistance, higher plasma insulin is necessary to compensate for the declining efficacy and therefore does not indicate differences in GSIS. These data indicate that PGE<sub>2</sub> has both beneficial and deleterious effects on the pancreatic β-cell in vivo. PGE<sub>2</sub> signaling through EP2 and EP4 promote β-cell survival whereas signaling through EP3 appears to reduce β-cell proliferation and function.

**Prostaglandin D<sub>2</sub>**

The effects of other PGs in the islet have received little attention. PGD<sub>2</sub> is found in the pancreatic islet (186) and its production is increased in the presence of high glucose (174). Unlike PGE<sub>2</sub>, PGD<sub>2</sub> has no or a very minimal effect on insulin release but is a potent stimulator of glucagon secretion (183,186,187,218). The PGD<sub>2</sub> receptor, DP1, does not appear to be expressed in islets, so PGD<sub>2</sub> signaling in islets may occur through DP2 which exhibits somewhat ubiquitous expression including the pancreas and islets of Langerhans (235). However, it has been reported that within the islet, DP2 is primarily expressed on β-cells and not on α-cells.
In vivo the contradictory effects of PGD₂ have been reported. One study reported L-PGDS⁻/⁻ mice to have worse glycemic control due to impaired insulin sensitivity on both control and HFD (244). Another group reported no differences in L-PGDS⁻/⁻ mice fed control diet, but glucose utilization being improved in L-PGDS⁻/⁻ mice when fed HFD (245,246). Two independent groups found no differences in insulin sensitivity in L-PGDS⁻/⁻ mice (245-247). These conflicting results give no clear answer as to the effect of PGD₂ on glucose homeostasis necessitating further studies.

**Prostaglandin F₂α**

PGF₂α is found in the pancreatic islet (176,186) and its production is increased in the presence of high glucose (174). PGF₂α increases GSIS in isolated rat islets and NIT-1β cells (229,248). In perfused pancreata, PGF₂α increases GSIS at a concentration of 1 µM, but the effect is diminished at 10 µM (183,219). In contrast, PGF₂α infused into humans at 0.2 and 0.5 µg/kg/min had no effect on plasma glucose or insulin (208). PGF₂α has also been reported to increase glucagon levels (183,189). FP has low expression in islets (235), and 1 µM PGF₂α is a sufficient concentration to activate other PG receptors indicating that any effect of PGF₂α on GSIS may not be through FP but rather is an off target effect.

**Prostacyclin**

Production of PGI₂ is mediated by PGIS which is expressed in islets in response to glucose (249). The IP receptor is expressed in islets, but the level of expression is not high (235). The effects of PGI₂ on insulin secretion vary with both PGI₂ and glucose concentration. At low glucose, PGI₂ stimulates cAMP in rat islets in a dose dependent manner but increases insulin secretion following a bell shaped curve, with maximal stimulation occurring at 1 µM PGI₂ (250). At high glucose concentrations, high levels of PGI₂ still increase cAMP, but have an inhibitory
effect on insulin release (250,251). PGI₂ leads to varying responses in GSIS (251). Perfusion of the rat pancreas with 100 nM – 10 µM PGI₂ also had no effect on insulin or glucagon secretion (183). PGIS over-expression promotes GSIS by increasing intracellular cAMP and signaling through the Epac2 pathway (249). Consistent with PGI₂ improving GSIS during periods of low glucose, IP knockout mice have decreased blood insulin and increased blood glucose during the fasting state (252). Exogenous administrations of PGI₂ or the PGI₂ analog, Beraprost, have been reported to reduced blood insulin (253-255), though this has not been noted every study (256). Most studies noticed a concomitant increase in blood glucose following PGI₂ administration (255,257); however, Beraprost treatment tended to lower blood glucose (253,254). It should also be noted that PGI₂ has significant effect on gluconeogenesis which impacts blood glucose levels in vivo (252). PGI₂ agonists Beraprost and Iloprost both improve islet viability (258,259), which may explain in part why long-term Beraprost treatment lowers blood glucose and improves glycated hemoglobin (HbA1c) (253,254). It should also be noted that Beraprost might improve HbA1c due to increasing circulation, which is known to improve insulin sensitivity (254,260).

**Thromboxane**

TXA₂ does not appear to have an effect on islet insulin production. The TP receptor has low expression in islets (235) and TXA₂ is produced by islets as evidenced by low levels of TXB₂ found in islet arachidonic acid metabolites (179). With these low levels of expression, it is not surprising that TXA₂ has no effect on insulin secretion in the perfused rat pancreas (183).

(Reviewed in (39,40,50,105,108,119,198-200,261-274))

**Obesity and Adiposity**

Obesity and adiposity are greatly influenced by insulin. Insulin is an anabolic hormone and hence acts to increase body mass (275,276). In addition, insulin activates phosphodiesterase
3B in adipocytes to reduce cAMP levels and lipolysis (277,278). Insulin sensitivity declines during obesity and the concomitant decline of β-cell function ultimately leads to reduced insulin production in obese individuals (11,279). In addition to regulating insulin secretion, PGs have also been found to be involved with the development and progression of obesity. There are several reports of PLA enzyme and PG signaling disruption that cause a reduction in obesity. Knockout mouse models of a cPLA2 (pla2g4a) (280,281), AdPLA (101), COX2 (98,99,282), and IP (283) all show resistance to obesity. In addition to knockout models, obesity can also be reduced pharmacologically with indomethacin, an NSAID (100), and 5-(4-benzylxoyphenyl)-(4S)-(phenyl-heptanoylamino)-pentanoic acid [KH064], a selective sPLA2 (pla2g2a) inhibitor (284). In contrast, group X sPLA2 (pla2g2a) (285), LPGDS (247), EP3 (102), and mice treated with indomethacin (286) have been reported to be more prone to obesity. Obesity is a complex disease that is mediated by numerous physiological processes. PGs play a role in regulating some of these processes, such as energy homeostasis, adipocyte proliferation, and lipolysis, all of which may be involved in contributing to the role of PGs in obesity.

**Energy Homeostasis**

**Feeding Behavior**

PGE2 is involved in stress responses which signal through hypothalamic-pituitary-adrenal (HPA) axis which affects a variety of behaviors including feeding and social behavior (287). PGE2 and PGF2α are known to inhibit food intake in rats (288-291). Anorexia can also be promoted by arachidonic acid and this is reversible by NSAID administration implicating PG action in this effect (292,293). Experiments in sheep reveal a more complex role of hypothalamic PGs and feeding behavior. PGE1 decreases feeding when injected into the medial and anterior loci of the hypothalamus but increases feeding when injected into the lateral loci (294). This
indicates that PGE$_2$ may elicit different feeding responses depending on the area of the brain in which it is produced. The anorexic actions of PGE$_2$ may be mediated through the EP4 receptor in mice; EP4 agonist ONO-AE1-329 mimicks the anorexic action of PGE$_2$ while EP4 antagonist ONO-AE3-208 blocks PGE$_2$’s effect (295). However, a different study has reported no effect of the ONO-AE1-329 on food intake or body weight in $Lepr^{db/db}$ mice (242). PGE$_2$ also stimulates leptin release from adipocytes (296,297). Leptin acts mainly on the central nervous system to reduce food intake and energy expenditure (298); therefore, systemic PGE$_2$ may also have anorexic effects via leptin. Conversely, PGD$_2$ stimulates feeding behavior through the DP1 receptor in mice, but it does so indirectly requiring the neuropeptide Y1 receptor, which is a Ga$_{i/o}$ coupled GPCR (299). In human cerebral spinal fluid, the L-PGDS protein levels correlates with neuropeptide Y levels and visceral adiposity (300).

The EP3$^{-/-}$ mouse has been reported to be obese due to hyperphagia, which was attributed to disrupted sleep patterns and increased “night eating” (daytime feeding for these nocturnal animals) (102). Numerous studies have shown that PGD$_2$ promotes sleep (301-303), while PGE$_2$ has been shown to promote wakefulness (304-307). EP3$^{-/-}$ mice demonstrated continuous feeding during the dark period which is in contrast to EP3$^{+/+}$ which had periods of increased feeding at the beginning and end of the dark cycle. Conversely, another study found that EP3 knockout in neuronal and glial cells had no effect of on sleep-wake rhythm (308). These data indicate that EP3 may be responsible for some of PGE$_2$’s anorexic effects (102), despite the reported lack of effect of EP3 agonists on feeding behavior (295). It is possible that EP3 and EP4 mediate feeding behavior in different parts of the brain. Alternatively, it is possible that the effects of EP3 loss primarily affect circadian rhythms, which in turn result in hyperphagia.
Thermoregulation

PGE has been long known to induce fever (309). In the first study examining the effect of PGE$_2$ infusion in humans, a “feeling of warmth” was among the first symptoms reported (64). This pyretic response of PGE$_2$ is mediated through EP3 (310). PGE$_2$ activates hypothalamic neurons in the preoptic nucleus in order to increase thermogenic activity of brown adipose tissue (BAT) and thereby raise the body temperature (311,312). Though PGE$_2$ plays an important role in acute thermoregulation during fever, it does not appear to play a similar role in normal temperature regulation, and body temperature change does not alter PG levels in the cerebrospinal fluid (313,314). PGI$_2$ also appears plays a role in thermoregulation; PGI$_2$ analog Beraprost increases temperature two hours after injection (254). When PGF$_{2 \alpha}$ is injected into the preoptic area of the anterior hypothalamus, it also causes a rise in temperature and this rise is curare sensitive indicating that the rise in temperature is due to shivering (203,315). The effect of intracerebroventricular PGD$_2$ on thermogenesis is variable; high doses produce mild hyperthermia whereas low doses reduce body temperature (203,316,317). Injections of PGE$_2$, PGD$_2$, and PGF$_{2 \alpha}$ into the third cerebral ventricle also increase hepatic glucose production, with the effect of PGF$_{2 \alpha}$ being especially pronounced, providing fuel for the increased thermogenesis (203).

PGs also influence energy utilization by playing an important role in BAT recruitment and development (Figure 3). BAT is adipose tissue that contains a large amount of mitochondria, which imparts its characteristic brown color, and functions primarily to burn energy and generate heat as opposed to white adipose tissue (WAT) which functions primarily to store energy. Cold exposure causes sympathetic nerves to release norepinephrine activating β$_3$-adrenergic receptors thereby inducing COX2 expression and activity (282,318). COX2 derived PGE$_2$ signals through
Figure 3. PGE$_2$ regulation of thermogenesis

The sympathetic nervous system is stimulated by cold exposure to release norepinephrine which binds to the $\beta_3$ adrenergic receptors to stimulate thermogenesis in adipose tissue. Stimulation of the $\beta_3$ receptor up regulates COX2 in white adipose tissue leading to increased PGE$_2$ production. PGE$_2$ signals in an autocrine or paracrine manner through the EP4 receptor to further promote $Ucp1$ expression and thermogenesis.

In response to inflammatory stimuli, PGE$_2$ in the brain vasculature binds to EP3 receptors in the preoptic area to inhibit warm-sensitive, GABAergic inhibition of sympathoexcitatory neurons thereby promoting pro-thermogenic signaling of the sympathetic nervous system leading to fever.
EP4 to induce uncoupling protein 1 (UCP1) in adipose tissue (282,286). UCP1, a protein found exclusively in mitochondria of adipose tissue, uncouples respiration from ATP synthesis by causing the electron gradient to dissipate without the generation of ATP resulting in a loss of energy (318). Transcription of Ucp1 is promoted by activation of PKA by Ga<sub>s</sub> coupled receptors, such as β<sub>3</sub>-adrenergic receptor and EP4, resulting in the activation of p38α mitogen-activated protein kinase (MAPK) which in turn phosphorylates regulators of Ucp1 transcription, such as PPARγ coactivator-1α (PGC-1α) (319). This “browning” of adipose tissue by PGE<sub>2</sub> induction of UCP1 would be expected to increase thermogenesis and energy expenditure. Indeed, mice over-expressing COX2 have increased oxygen consumption, increased energy expenditure, and reduced body fat due to increased BAT (282). COX2<sup>−/−</sup> mice have reduced body temperature and indomethacin treatment of mice reduces energy intake leading to increased adiposity (286).

BAT also expresses L-PGDS and this expression is positively correlated with thermogenesis (245). L-PGDS<sup>−/−</sup> mice preferentially utilize glucose for thermogenesis indicating that PGD<sub>2</sub> plays an important role in fat metabolism (245). The regulation of BAT recruitment indicates an important role of PGs in thermoregulation and energy expenditure. Though acute effects of PGs on body temperature appear to be primarily related to fever, PGs also have a chronic role in thermoregulation increasing BAT recruitment and long-term energy expenditure.

**Adipose Tissue**

Lipid signaling molecules, including PGs and other inflammatory mediators, play an important role in adipose tissue physiology (320). Altering PG signaling has been reported to either prevent or predispose animals to obesity depending on which part of the pathway is disrupted. As discussed above, PGs clearly affect energy homeostasis through their roles in feeding behavior and BAT mediated thermogenesis. These effects on global metabolic profiles
affect the amount of energy stored contributing to or reducing obesity. Changes in obesity are reflected by changes in adipocyte cell size or numbers. PGs play a crucial role regulating many of these changes.

Adipose tissue is an important site of PG action in obesity. Adipose tissue has been shown to express constitutively express COX1 while COX2 is inducible (282,286,321-336). Expression of all three PGES isoforms (324,327,334,335,337,338), L-PGDS (326,327,332,334,339-343), PGIS (327), and PGF synthase (335) has been reported in adipose tissue. Aldo-keto reductases have also been reported to function as PGF synthases in adipocytes (328,330,332,344,345). PGE\(_2\), PGD\(_2\), PGF\(_{2\alpha}\), and PGI\(_2\) are all produced by adipocytes, pre-adipocytes, or have been found in adipose tissue.

**Adipocyte Differentiation and Proliferation**

Transcriptional regulation of PG biosynthesis and adipogenesis are regulated by a common transcription factor; CCAAT/enhancer-binding protein (C/EBP) \(\beta\) promotes the transcription of both COX2 (346-348) and the critical transcription factors for adipogenesis, PPAR\(\gamma\) and C/EBP\(\alpha\) (349,350). PGs are generally thought to play an inhibitory role in WAT adipogenesis. Inhibition of COX1, COX2, and PGES activity increases adipogenesis and triglyceride accumulation (321,324,331,333,336,351). Consistent with this, expression of both COX enzymes and PGES are decreased during adipogenesis (323,327,330,331,336). Other studies suggest that only COX2 inhibition increases adipogenesis with no effect of COX1 inhibition being observed (323,352). In contrast, aspirin and indomethacin have been reported to reduce the formation of adipose cells (326,341,353,354). Analysis of PG receptor expression shows prominent expression of EP1-4, DP2, FP, and IP, but low expression of TP in WAT, BAT, and adipocytes (197,235,327,330,338). EP3 has the highest expression of the four EP
receptors in mouse epididymal fat pads, and EP2, EP3, and EP4 expression are all increased by high fat diet feeding (338). PGES expression has been reported to be both decreased during obesity (337) and unchanged by high fat diet feeding (338). Radioligand binding studies confirm that EP (355,356) and FP (357) proteins are present on adipocytes. WAT, BAT, and adipocytes are some of the highest EP3 expressing tissues in the body (235); however, all three splice variants of EP3 are expressed more in mature adipocytes suggesting that EP3 is more important for mature adipocyte function than for differentiation (327,352,358). EP1 and EP4 are expressed on pre-adipocytes implicating their involvement in adipogenesis (352,358). FP and aldo-keto reductase 1B expression are transiently increased during the early stages of adipogenesis but their expression is significantly lower in differentiated adipocytes compared to undifferentiated cells (327,330,352,358). IP is not expressed in pre-adipocytes, but is expressed shortly after cells begin to differentiate and in fully mature adipocytes (358,359).

Some studies have shown that PGE2 inhibits adipocyte differentiation (324,335), though this has not been shown in every study (351,360,361). This may be due to PGE2 only exerting its inhibitory effects during the later stages of differentiation (362). Alternatively, the effect may be concentration dependent as 280 nM PGE1 was unable to affect differentiation, but 28 µM stimulated differentiation (363). cAMP analog, dibutyril cyclic AMP, similarly promotes adipogenesis during early stages, but inhibits at later time points (362). PGE2 signals through the EP4 receptor to inhibit adipogenesis of WAT (352,364) and to promote Ucp1 expression, which is indicative of beige adipose tissue (286,324). Further evidence that PGE2-EP4-cAMP signaling is anti-adipogenic comes from studies that found that PGE2, EP4 agonist AE1-392, and dibutyril cyclic AMP, all suppress the expression of the key adipogenic genes Pparg and Cepba during 3T3-L1 adipocyte differentiation (365). This suppression of differentiation is dependent upon
PKA activity (331). Conflictingly, EP4−/− mice have reduced fat mass without differences in adipocyte size (338).

PGI2 is well known to promote adipogenesis (366). PGI2 and its stable analog carbacyclin are both known to stimulate differentiation of adipocytes (283,353,354,367-369). PGI2 promotes differentiation in adipocytes by raising intracellular cAMP (283,353,370-372). However, there is at least one report that PGI2 inhibits adipogenesis by raising cAMP (373). This increase in cAMP mediated by PGI2 and other IP agonists increases the expression of adipogenic transcription factors PPARγ, C/EBPβ, and C/EBPδ (253,370). Consistent with these findings, IP−/− mice are resistant to diet induced obesity and have reduced fat mass (283).

PGF2α is another potent inhibitor of adipose tissue differentiation (351,357,360-362,374-381), although at least one study has found that PGF2α stimulates differentiation (363). PGF2α analogs fluprostenol, cloprostenol, latanoprost, and travoprost likewise inhibit differentiation (357,375,380,382,383). However, there is a report of fluprostenol having no effect on differentiation in mouse embryonic fibroblasts (MEF) (352). Furthermore, at sub-maximal concentrations of PGI2, PGF2α potentiates PGI2 induced adipocyte differentiation (366). PGF2α inhibits adipogenesis via a Gαq-Calcium-Calcinurin dependent signaling pathway that inhibits the expression of PPARγ and C/EBPα (379). In addition, activation of MAPK and subsequent PPARγ phosphorylation by PGF2α signaling also reduces adipogenesis (380). More evidence supporting PGF2α’s role in inhibiting adipogenesis comes from mice lacking aldo-keto reductase 1B which are more obese because they have reduced PGF2α production (345).

There are conflicting reports on the role of PGD2 in adipogenesis. Knockdown of L-PGDS has been shown to either promote (339) or to inhibit adipogenesis (340). Over expression of L-PGDS in cell culture inhibits adipogenesis (341). However, transgenic mice over
expressing L-PGDS have increased adipogenesis (384). PGD₂ has also been reported to inhibit adipocyte differentiation, as evidenced by stimulation of Stearoyl-CoA desaturase-1 (SCD1) mRNA in 3T3-L1 preadipocytes (375). PGD₂ metabolite, 15-deoxy-Δ¹²,¹⁴-prostaglandin J₂ (15Δ-PGJ₂), is an agonist for PPARγ that stimulates adipogenesis (326,341,380,385-387). While it is unclear whether PGD₂ itself has an effect on adipogenesis, PGD₂ production promotes adipogenesis via its metabolite 15Δ-PGJ₂.

In summary, COX metabolites negatively influenced differentiation of adipocytes (321,331,336,352). The PGs, PGE₂ and PGF₂α, that play an important role in inhibiting WAT adipogenesis. PGE₂ and promotes the inhibition of WAT adipogenesis in part by promoting the “browning” of WAT and recruiting brown adipocytes. PGF₂α also inhibits adipose tissue development. 15Δ-PGJ₂ opposes the role of the aforementioned primary eicosanoids, promoting adipogenesis via PPARγ activation.

(Reviewed in (388-390))

Adipocyte Morphology

Several models of in vivo PG inhibition have reported differences in adipocyte cell size. COX₂⁻/⁻, cPLA₂⁻/⁻, and AdPLA⁻/⁻ mice have reduced adipocyte cell sizes (101,280,281,391) whereas L-PGDS⁻/⁻ and group X sPLA2⁻/⁻ mice have larger adipocytes (244,285). Treatment of mice with EP4 agonist CAY10580 decreases adipocyte size, but adipocyte size is unchanged in EP4⁻/⁻ mice (338). Indomethacin treatment of mice fed a high fat and high salt diet also reduces adipocyte cell size (100); however, when fed a high fat diet, indomethacin caused a small increase in adipocyte cell size (286). AdPLA⁻/⁻ mice have increased ectopic lipid accumulation accounting for the triglyceride, consistent with redistribution from the adipose tissue (101). However, COX₂⁻/⁻, cPLA₂⁻/⁻, and mice treated with indomethacin have reduced ectopic lipid
accumulation (100,280,281,391). These changes in adipocyte size may be indicative of changes in energy homeostasis or adipocyte function.

Several studies have investigated the effects of PGE\(_2\) signaling on adipose tissue inflammation. Signaling through the EP4 receptor has been shown to reduce crown-like structure formation, macrophage infiltration, and chemokine production in adipose tissue (242,338). Reduced prostaglandin production in AdPLA\(^{-/-}\) mice had no effect on the expression of inflammatory markers (101).

**Adipocyte Function**

Production of PGE\(_2\), PGD\(_2\), PGF\(_{2\alpha}\), and PGI\(_2\) by adipocytes has been well characterized. Numerous studies have reported that PGE\(_2\) inhibits lipolysis (90-92,101,296,297,321,328-400). Lipolysis is the generation of FFA from triglycerides (401-405). Triglycerides are first converted into diacylglycerol by adipose triglyceride lipase (ATGL). Hormone-sensitive lipase (HSL) converts diacylglycerol into monoacylglycerol. Monoacylglycerol is then converted into glycerol and three FFA by monoacylglycerol lipase (MGL). Prior to lipolysis, non-activated Perilipin (PLIN) and CGI-58 (comparative gene identification-58) binds to a triglyceride while unstimulated HSL is in the cytoplasm. External stimuli increase cAMP, which activates PKA that phosphorylates HSL and PLIN. Phosphorylation of PLIN causes CGI-58 to be released from the triglycyeride droplet, which then activates ATGL initiating lipolysis. Phosphorylated HSL translocates from the cytosol to the lipid droplet and interacts with PLIN. HSL’s role as a diacylglyceride hydrolase is the rate-limiting step in lipolysis. MGL completes the final hydrolysis reaction producing the third FFA and glycerol. It should be noted that ATGL, HSL, and MGL will hydrolize all three acylglycerols, but not with equal activity.
Insulin is a major inhibitor of lipolytic activity. This serves to reduce the amount of FFA available to cells during periods of elevated insulin, which normally occurs when blood glucose is elevated. Insulin reduces lipolytic activity by reducing intracellular cAMP in adipocytes (406). Insulin causes the degradation of intracellular cAMP by activating phosphodiesterase 3B via PI3K-AKT signaling (277,278). In addition to inhibiting lipolytic activity, insulin is a driver of glucose uptake and fatty acid synthesis in adipocytes. PGE$_2$ has an additive effect with insulin on increasing glucose uptake and metabolism opposing the actions of adrenergic agonists (407-409), though PGE$_1$ was not found to have this effect in at least one study (377).

Many early studies of PG signaling in adipocytes compared the effect of PGE$_2$ to that of catecholamines. Isoproterenol and the endogenous catecholamines, epinephrine and norepinephrine, stimulate lipolysis; this stimulation of lipolysis is inhibited by PGE$_2$ (91,92,393-399,410-419). Isoproterenol, epinephrine, and norepinephrine are all agonists for the $\beta$-3 adrenergic receptor, which is highly expressed in adipose tissue, is $G\alpha_s$ coupled, and promotes cAMP production. PGE$_2$ has been repeatedly shown to cause a marked reduction in cAMP in adipocytes (93,94,284,393,395,396,400,413,415,420-425). PGE$_2$ directly opposes the actions of $\beta$-adrenergic agonists on lipolysis and cAMP accumulation. PGE$_2$ also opposes the actions of other drugs which raise cAMP, such as forskolin (284,421), which activates adenylyl cyclase, and phosphodiesterase inhibitors such as theophylline (393,398,412,417-419,426) and IBMX (416). The pronounced effect of PGE$_2$ reducing cAMP levels and opposing the action of agonists for $G\alpha_s$ coupled receptors, suggests that PGE$_2$ signals through the $G\alpha_i$ coupled EP3 receptor in adipocytes. Sulprostone, an EP1 and EP3 selective agonist, has also been shown to reduce cAMP in adipocytes and to exhibit anti-lipolytic effects similar to PGE$_2$ (396,421,427). EP3 antagonist L-798,106 has been shown to block PGE$_2$ mediated reduction in cAMP in
adipocytes (284) and EP3 antagonist L-826,266 has been shown to block PGE₂’s inhibition of
lipolysis (101). These data clearly indicate that PGE₂ signals through the EP3 receptor to reduce
cAMP in adipocytes and thereby inhibit lipolysis (Figure 4).

Administration of PGE in vivo produces mixed results on FFA levels; with reports of
plasma FFA being decrease (410,428) or increased (209,210). Epinephrine causes an increase in
FFA release in vivo, which is blocked by PGE₁ administration though PGE₁ administration alone
showed only a minimal effect (92). This indicates that PGE does inhibit lipolysis in vivo, but
other stimuli also heavily influence lipolysis and could potentially overwhelm the effects of
PGE.

Opposing the action of PGE₂ on lipolysis is PGI₂, which is a stimulator of lipolysis (392).
This is not surprising because PGI₂ increases intracellular cAMP in adipocytes (283,366,370-
372). However, some pre-adipocyte cell lines lose the ability to respond to PGI₂ after
differentiation and hence do not have a lipolytic response to PGI₂ (372). Some studies have
reported that PGE₂ has biphasic effects on adipocyte adenylyl cyclase activity, inhibiting
adenylyl cyclase at low concentrations but stimulating adenylyl cyclase and increasing cAMP
levels at concentrations greater than 1 µM (413,421). PGE₂ has an affinity for the IP receptor of
~4 µM (429), hence PGE₂ is possibly stimulating IP, in addition to EP2 and EP4 which have
Kd’s ~1-5 nM, when used at 10 µM and greater. PGI₂ analog Beraprost decreases adipocyte cell
size, possibly due to increased lipolysis and increased thermogenesis, improving insulin
sensitivity and other symptoms of metabolic syndrome (253,254).

PGF₂α signaling in adipocytes primarily raises intracellular calcium (357,430-433),
though there is at least one report of PGF₁α inhibiting cAMP production in adipose tissue (94).
PGF₂α causes adipose tissue to increase basal glucose uptake in a PKC dependent manner which
Prostaglandins primarily regulate lipolysis in adipocytes by signaling through the EP3 and IP receptors. PGI\textsubscript{2} signals through the G\textsubscript{\alpha}s coupled IP receptor to increase lipolysis. PGE\textsubscript{2} signals through the G\textsubscript{\alpha}i coupled EP3 receptor to reduce adenylyl cyclase activity that is augmented by the pro-lipolytic signaling of catecholamines, PGI\textsubscript{2}, and other G\textsubscript{\alpha}s dependent signaling pathways in adipose tissue.

Figure 4. Prostaglandin regulation of lipolysis in adipocytes

Prostaglandins primarily regulate lipolysis in adipocytes by signaling through the EP3 and IP receptors. PGI\textsubscript{2} signals through the G\textsubscript{\alpha}s coupled IP receptor to increase lipolysis. PGE\textsubscript{2} signals through the G\textsubscript{\alpha}i coupled EP3 receptor to reduce adenylyl cyclase activity that is augmented by the pro-lipolytic signaling of catecholamines, PGI\textsubscript{2}, and other G\textsubscript{\alpha}s dependent signaling pathways in adipose tissue.
was synergistic with increasing cAMP (377,434,435). Due to the general lack of effect of PGF$_{2\alpha}$ on cAMP levels, it is not surprising that PGF$_{2\alpha}$ has no effect on lipolysis (376).

Inhibition of PG synthesis in vivo has mixed effects on lipolysis. These conflicting results during global PG inhibition may be due, in part, to increased insulin which inhibits lipolysis. Knockout models of cPLA$_2$ (pla2g4a) (280,281), AdPLA (101), and COX2 (98,99) or treatment of mice with the NSAID indomethacin for seven weeks (100) causes an increase in plasma FFAs. Administration of aspirin for four days to humans or eight days to rats decreases plasma FFA in diabetic subjects (436,437). However; in normal subjects aspirin decreased FFA in non-diabetic humans (436) but increased FFA in control rats (437). Most studies indicate that salicylate decreases FFA release (83,88,438-443), but it has also been reported to have no effect (59,444,445) or to cause an increase (89).

(Reviewed in (390,401,446))

Liver

The liver is a key regulator of energy homeostasis. The liver regulates glucose homeostasis, being the site of glycogen synthesis, the conversion of excess glucose into glycogen for storage during times of hyperglycemia, glycogenolysis, the conversion of glycogen into glucose into for immediate use during times of hypoglycemia, and gluconeogenesis, the conversion of non-carbohydrate carbon substrates into glucose (447). These processes are primarily regulated by the opposing actions of the hormones insulin, promoting glycogenolysis and glycogen synthesis, and glucagon, promoting gluconeogenesis. In addition to being the site of carbohydrate storage, the liver also plays a role in storing excess triglycerides (448). The liver is the secondary site of lipid storage in the body, being the preferential place to store excess lipid that is unable to be stored in adipose tissue. Excess lipid storage in the liver can lead to insulin
resistance and fatty liver disease. Fatty liver disease can progress into steatohepatitis and eventually lead to cirrhosis. Lipids are capable of altering hepatic gene expression and lipid content (449). Among the many factors that affect the liver, PGs play an important role regulating hepatic function and disease (450-452).

Hepatic tissue expresses COX and PGES genes that are necessary for production of PGs (252,391,453-459). Kupffer cells, which are hepatic macrophages, have been reported to be the primary source of hepatic PGs (460). Kupffer cells can be induced to produce excess PGE\textsubscript{2} by stressors such as lipopolysaccharide (LPS) (460-464), alcohol (456,464,465), carbon tetrachloride (466), anaphylatoxin (467-470), or liver resection (471-473). Hepatic endothelial and Kupffer cells produces multiple PGs: PGD\textsubscript{2} >> TxB\textsubscript{2} > PGF\textsubscript{2α} > PGE\textsubscript{2} > 6-Keto-PGF\textsubscript{1α}, with PGD\textsubscript{2} being the primary eicosanoid produced by both (474,475). Parenchymal cells primarily generate PGD\textsubscript{2} and PGF\textsubscript{2α} with lesser quantities of TxB\textsubscript{2}, PGE\textsubscript{2}, and 6-Keto-PGF\textsubscript{1α} being made (474,476). Providing free arachidonic acid to liver tissue changes the relative ratios of PGs produced with PGE\textsubscript{2} >> PGF\textsubscript{2α} > PGD\textsubscript{2} (477). Obesity and other models of liver damage, such as alcohol and inflammation, also increase hepatic PGE\textsubscript{2} production and expression of PGE\textsubscript{2} generating enzymes (252,454,462,465,478).

Radioligand binding studies show that PG receptors are expressed in the liver (479-488). Studies present conflicting reports on the relative expression of PG receptors in the liver. Most studies agree that EP1 is highly expressed, relative to the other PG receptors, with all other PG receptors being detected in liver (197,235,236,489-495). Hepatocytes are known to express EP3\textbeta and a splice variant of FP containing a unique 5’ untranslated region, because both have been cloned from rat hepatocytes (496,497). Expression of hepatic PG receptors is not constitutive; the inflammatory cytokine, IL-6, can induce the expression of EP2, EP4, and DP (491,495).
Kupffer cells express EP1-4 and TP (490,498). Sinusoidal endothelial cells have high expression of EP1-4, IP, and TP (490). Hepatic stellate cells have high expression of EP1, EP3, and IP (490). Diabetic stress can increase the expression of the IP receptor (252).

**Glycogen Synthesis, Glycogenolysis, & Gluconeogenesis**

PGs play an important role in regulating hepatic glycogen stores and glucose production (Figure 5). Gluconeogenesis is classically promoted by glucagon which signals through a G\(_{\alpha_s}\) coupled GPCR; promoting glucose production by raising cAMP (499). The glycogen phosphorlase enzyme catalyzes the rate-limiting step in gluconeogenesis; this enzyme is activated by phosphorylase kinase, which in turn is activated by increases in intracellular cAMP/PKA or an increase in intracellular calcium/calmodulin (500-504). Glucagon has been shown to induce PG formation (505). PGE\(_2\) inhibits the glucagon mediated rise in cAMP and thereby reduces hepatic glycogenolysis and gluconeogenesis (75,76,482,494,505-513). This inhibition of hepatic glucose production by PGE\(_2\) during periods of elevated cAMP is pertussis toxin sensitive (514-516). Sulprostone, an EP1/EP3 selective agonist, and misoprostol, an EP2, EP3, and EP4 selective agonist, inhibit glucagon stimulated cAMP formation in primary culture rat hepatocytes with a similar potency (494,516). These results are consistent with EP3 mediating a reduction in cAMP. In the absence of glucagon, PGE\(_2\) increases intracellular IP\(_3\), calcium, and cAMP in hepatocytes and thereby increases glucose output (75,479,517-522). Sulprostone also increases IP\(_3\), but misoprostol only produces a minimal response (494). Sulprostone is more potent than misoprostol at increasing glycogen phosphorylase activity (516). These results are consistent with PGE\(_2\) signaling through EP1 to activate PLC and thereby raise intracellular calcium. Together these studies indicate that EP1 and EP3 have opposing roles in hepatic glucose production. EP1 causes a rise in intracellular calcium, which promotes glycogen breakdown and
Figure 5. Prostaglandin regulation of hepatic glucose output
A. PGE₂ affects gluconeogenesis predominately through the EP1 and EP3 receptors. EP1 promotes gluconeogenesis in an IP₃- and calcium-dependent manner. EP3 inhibits glucagon-induced gluconeogenesis by reducing intracellular cAMP accumulation. B. PGF₂α signals through both Ga₉ and Gaᵢ to promote and inhibit gluconeogenesis, respectively. C. PGD₂ signals through a Ga₃ coupled receptor, presumably DP, to promote gluconeogenesis. D. PGI₂ promotes gluconeogenesis through Ga₃ and Gβγ mediated signaling pathways. Figure D modified from (252).
gluconeogenesis. Signaling through EP3 reduces cAMP accumulation and thereby inhibits gluconeogenesis. Similar to what has been seen in pancreatic islets and adipose tissue, the inhibitory effect of PGE$_2$ on cAMP and gluconeogenesis cannot be seen in the absence of stimuli such as glucagon or β-adrenergic stimulation which raise cAMP above basal levels.

PGF$_{2α}$ signals through the FP receptor to regulate hepatic gluconeogenesis. PGF$_{2α}$ causes a reduction in glucagon stimulated cAMP and glucose production in a pertussis toxin sensitive manner, implicating a G$_{α_i}$ coupled signaling pathway (494,508,509,514). PGF$_{2α}$ also signals through a G$_{α_q}$ dependent signaling pathway to increase IP$_3$ and intracellular calcium, and thereby promote glycogen phosphorylase activity and hepatic gluconeogenesis (485,494,517,519,521,523). Similar to what was observed with PGE$_2$, the inhibitory action of PGF$_{2α}$ is only noticed during periods of elevated cAMP, such as during glucagon stimulation. Another similarity to hepatic PGE$_2$ signaling is that PGF$_{2α}$ also increases glycogenesis (524).

PGD$_2$, which is the primary PG produced by the liver, functions to oppose the actions of PGE$_2$ and PGF$_{2α}$. PGD$_2$ enhances hepatic glucose production (474,525). Furthermore, it does not play a role in promoting glycogen production (524).

PGI$_2$ also acts to promote gluconeogenesis. IP activation stimulates cAMP accumulation, thereby activating PKA, which phosphorylates CREB to promote the expression of gluconeogenic genes (252,479). In addition, IP signals through G$βγ$ subunits to block activation of AKT, an inhibitor of gluconeogenic gene expression (252).

**Hepatic Lipolysis**

In addition to regulating circulating glucose levels, the liver also plays an important role in regulating the extracellular transport of lipids in the form of lipoproteins. The liver packages triglycerides and cholesterol into very-low-density lipoproteins (VLDL) for transport to other
tissues via the circulatory system (526). PGE\textsubscript{2} and PGD\textsubscript{2} both suppress VLDL secretion from hepatocytes (506,527). Apolipoprotein B (ApoB) and microsomal triglyceride transfer protein (MTTP) are involved in packaging lipoproteins. PGE\textsubscript{2} acts synergistically with insulin to decrease the expression of Apob and Mtp (454,478). PGD\textsubscript{2} and PGF\textsubscript{2a} also decrease expression Apob only, but do not affect Mtp (527). PGE\textsubscript{2}, PGD\textsubscript{2} and PGF\textsubscript{2a} reduce the lipid to ApoB ratio in VLDL resulting in smaller particles with less lipid content (527). The reduced lipids in VLDL particles may be due to reduced lipogenesis because fatty acid synthase gene expression is reduced by PGE\textsubscript{2} (454,455,506,527). Cholesterol biosynthesis has been reported to be reduced by PGE\textsubscript{2}, PGD\textsubscript{2} and PGF\textsubscript{2a} (527) or unaffected by PGE\textsubscript{2} and PGD\textsubscript{2} (506). The reduced secretion of lipoprotein in hepatocytes treated with PGE\textsubscript{2}, PGD\textsubscript{2} or PGF\textsubscript{2a} leads to an increase in cellular triglyceride content (506).

**Fatty Liver Disease**

Hepatic lipid accumulation is well known to be a major contributing factor to nonalcoholic fatty liver disease and hepatosteatosis, which can lead to insulin resistance (448,528-531). PGE\textsubscript{2} promotes hepatic lipid accumulation in an EP2/4 dependent mechanism (465). Consistent with this, EP3\textsuperscript{-/-} mice were observed to have increased liver weight when fed a breeder chow diet; however, progression of hepatic lipid contents were not evaluated (102). Beraprost, an IP agonist, reduces hepatic steatosis (253,254), whereas IP\textsuperscript{-/-} mice were reported to have reduced hepatic triglycerides and steatosis (252), presenting conflicting conclusions on the role of IP in the development of fatty liver disease. Mice treated with the NSAID indomethacin, COX2 inhibitors, or lacking a cPLA\textsubscript{2} (pla2g4a) have decreased hepatic triglycerides providing further evidence that PGE\textsubscript{2} promotes hepatic lipid accumulation (100,280,281,391,459).
The major source of hepatic triglycerides is derived from adipose tissue lipolysis (528). The liver is also an important source of de novo lipogenesis, which also accounts for a significant portion of hepatic lipids (532). A large portion of hepatic triglycerides are packaged into VLDL and secreted; inhibition of this process can increase hepatic triglycerides content (533). Reduced mitochondrial β-oxidation due to mitochondrial dysfunction can also lead to lipid accumulation in the liver (534). As has already been discussed, PGs play an important role in adipose tissue and hepatic lipolysis, which affects the amount of lipids in the liver. PGE₂ has been reported to decrease de novo lipogenesis in the liver (454,455,506,527). PGE₂ also decreases triglyceride breakdown and reduce β-oxidation of fatty acids (454). Taken together, these data indicate that PGE₂ increase hepatic triglyceride accumulation by increasing lipolysis in adipose tissue, increasing de novo hepatic lipogenesis, reducing lipid secretion, and reducing β-oxidation.

A progressive deterioration of the liver characterizes nonalcoholic fatty liver disease (NAFLD) (448,535). The liver begins to become diseased when it accumulates lipid and develops simple steatosis, which is frequently associated with insulin resistance. This disease state progresses into non-alcoholic steatohepatitis, which is characterized by inflammation. Advanced stages progress to cirrhosis, which is characterized by fibrosis, i.e. the replacement of liver tissue with scar tissue. Hepatocellular carcinoma may develop as the final stage of the disease. COX2 is expressed primarily in earlier stages of chronic liver disease and in hepatocellular carcinoma its expression is inversely correlated with tumor grade (453).

PGs play a protective role in the liver. PGs are well known to be cytoprotective and to promote regeneration in the liver (536-540). Metabolites of COX2 are proliferative and anti-apoptotic in hepatic tissues (492,494,541-544). These protective effects of PGE₂ are mediated through the EP4 receptor (492,493,545-549). IP agonists also decrease markers of liver injury.
and fibrogenic gene expression (550,551). PGE$_2$ has anti-inflammatory effects in the liver that leads to immunosuppression (466). PGE$_2$ reduces TNF-α and other pro-inflammatory cytokines in an EP2 and EP4 dependent manner (498,552,553). TXA$_2$ synthetase inhibitors and receptor antagonists decrease markers of liver injury indicating that TXA$_2$ causes adverse effects in the liver (550).

**Summary**

Obesity and diabetes are a major health concern. Inflammatory signaling pathways represent an attractive therapeutic target for both diabetes and obesity. PGs are one of the many signaling molecules that play a role in inflammation and immune response and as have been noted play many diverse roles in obesity, diabetes, and metabolism. The roles these PGs play in metabolism are complex as the same molecules are oftentimes critical mediators of GSIS, feeding behavior, adipogenesis, adipocyte function, and hepatic function. The overlapping nature of the PGs and their receptors, with multiple PGs/receptors controlling the same physiological process and multiple physiological processes being modulated by the same PG/receptor, oftentimes necessitates investigation of the PG receptors individually. Due to the fact that the EP3 receptor has been shown to play an important role in the pancreatic islet, adipose tissue, and liver, I chose to investigate the physiological role of the EP3 receptor in a mouse model of diet induced obesity and diabetes. To accomplish this, I proposed the following aims: 1. Evaluate the physiological effects of EP receptor inhibition on diabetes *in vivo*, 2. Compare EP receptor expression in the pancreas and 3. Elucidate the signaling pathway by which PGE$_2$ inhibits insulin release *ex vivo*. 
CHAPTER II

THE ROLE OF THE PROSTAGLANDIN E\textsubscript{2} EP3 RECEPTOR IN INSULIN ACTION

Introduction

Type 2 diabetes has recently been recognized as a disease of low-grade chronic inflammation (reviewed in (24,25)). This state of chronic inflammation contributes to the development of insulin resistance seen in type 2 diabetes and prediabetes. Antidiabetic drugs such as thiazolidinediones reduce systemic inflammation and improve insulin resistance (20), while inflammatory cytokines such as interleukin 1-\textbeta\ (IL1-\textbeta) have deleterious effects on pancreatic islet \textbeta-cell function and proliferation (40,177,196,197). Circulating inflammatory cytokines increase in obesity and type 2 diabetes. It is now appreciated that pancreatic islets respond to inflammatory signals, and inflammatory ligands likely contribute to the etiology of \textbeta-cell dysfunction in type 2 diabetes. Thus, inflammatory mediators negatively affect both insulin production as well as insulin action.

PGs are important mediators of inflammation (reviewed in (41-45)). These oxidative metabolites of arachidonic acid (AA), are produced by cyclooxygenases (COX1 and COX2), and act locally in an autocrine or paracrine manner (reviewed in (43)). Inhibition of COX activity and PG production by nonsteroidal anti-inflammatory drugs (NSAIDs) has long been appreciated to have beneficial effects on glucose homeostasis, consistent with inhibition of insulin secretion by PGs (105).

PGE\textsubscript{2} mediates its effects by activation of four GPCRs, designated \textit{E-Prostanoid (EP)1 through EP4} (43). EP1 elevates intracellular calcium, while EP2 activates adenylyl cyclase. The EP4 receptor was initially identified as activating adenylyl cyclase, but has been demonstrated to signal through a number of other pathways as well (151). Similarly, although EP3 activation was
first described to inhibit adenylyl cyclase, leading to decreased intracellular cyclic adenosine monophosphate (cAMP) levels by a Ga<sub>i</sub> coupled mechanism, it has also been appreciated to couple to multiple signal transduction pathways (see (43)). Pancreatic tissue expresses mRNA encoding each of the four EP GPCR types (181,196). Thus, PGE<sub>2</sub> is hypothesized to act in an autocrine or paracrine fashion in pancreatic islets through endogenous EP receptors. A more thorough analysis of GPCR expression across a panel of mouse tissues revealed that the EP3 receptor was most highly expressed in the pancreas, kidney and white adipose tissue, an expression pattern consistent with regulation of metabolic homeostasis (235). Recent studies have shown that EP3 is expressed in pancreatic β-cells and that signaling through inhibitory G proteins, Ga<sub>i</sub>/Ga<sub>o</sub>/Ga<sub>z</sub>, within pancreatic β-cells negatively regulates glucose stimulated insulin secretion (GSIS) and β-cell proliferation (134,239,554).

Based upon its signal transduction properties, pharmacological blockade or genetic deletion of the EP3 receptor might be expected to increase GSIS, perhaps improving glycemic control. However, studies by Bartfai and co-workers utilizing global EP3<sup>−/−</sup> mice maintained on breeder chow were remarkable in that they observed an increase in insulin resistance and body weight, which was ascribed to altered circadian activity patterns and an increase in feeding during the normal daytime sleeping period (102). This phenotype had not been noted in previous descriptions of the EP3 null animals (310,555). These studies also demonstrated an increase in serum insulin levels that may have been a consequence of increased body weight and adiposity in the EP3<sup>−/−</sup> mice.

We have not previously observed obesity in our EP3<sup>−/−</sup> mice when maintained on a standard rodent chow diet. We predicted that EP3<sup>−/−</sup> mice would have enhanced GSIS resulting
from the loss of the EP3-evoked inhibition of insulin secretion. We hypothesized that EP3<sup>−/−</sup> mice would have improved glucose handling when fed a normal chow diet.

**Materials and Methods**

**Materials**

PGE<sub>2</sub> was obtained from Cayman Chemical Company (Ann Arbor, MI) and [³H] PGE<sub>2</sub> was obtained from PerkinElmer (Boston, MA). DG-041, an EP3 antagonist (556), was provided by the Vanderbilt Institute of Chemical Biology Synthesis Core. Recombinant human IL-1β was obtained from R&D Systems (Minneapolis, MN, Cat. No. 201-LB). Formaldehyde 10% (v/v), buffered at pH 7.0 was obtained from Ricca Chemical Company (Arlington, TX). OCT was obtained from Tissue-Tek (Sakura Finetek U.S.A., Torrance, CA).

**Animal procedures**

Mice utilized for these experiments were generated from homozygous breeding of EP3<sup>+/+</sup> or EP3<sup>−/−</sup> mice on a C57BL/6 background at Vanderbilt University unless otherwise stated. Mice were maintained *ad libitum* on chow, Laboratory Rodent Diet 5001 (LabDiet, Richmond, IN). Mice were maintained on a 12-hour light/dark cycle and housed with three to five animals per cage on corncob bedding except during fasting when placed on Pure-o’Cel™ bedding (The Andersons, Maumee, OH). Body composition of live mice was measured by pulsed NMR with Minispec Model mq7.5 (Bruker Instruments, The Woodlands, TX). Mice were euthanized by isoflurane (Baxter Healthcare Corporation, Deerfield, IL) overdose for collection of tissues. All procedures were approved by the Institutional Animal Care and Use Committee at Vanderbilt University.
Radioligand binding

Pancreata for radioligand binding were obtained from 11.5 to 18.5 week old chow fed male mice. Prior to pancreas dissection, mice were perfused with 0.9% saline to remove blood from the pancreas. Pancreata were flash frozen in liquid nitrogen and ground with a mortar and pestle and stored at -80°C. Ground pancreata were further lysed with an electric homogenizer in a buffer containing the following: HEPES 15 mM, EDTA 5 mM, EGTA 5 mM, indomethacin 40 µM, PMSF 2 mM at pH 7.6. Pancreata lysates were placed on top of a 60% sucrose gradient and centrifuged at 32,000 RCF for 1.5 hours at 4°C. The layer of pancreas membranes was removed and further homogenized with an 18-26 gauge needle. Binding was performed on 250 µg pancreas membranes pooled from five EP3+/+ or five EP3−/− mice. All dilutions and washes were performed using a chilled buffer containing the following substances: K₂HPO₄ 15.8 mM, KH₂PO₄ 6.14 mM, EDTA 1 mM, MgCl₂ 10 mM. Radioligand binding was performed using 5 nM [³H] PGE₂ with nonspecific binding determined in the presence of 1 µM unlabeled PGE₂. Membranes were incubated with PGE₂ ligands for 1 hour at 30°C while shaking. Bound ligand was separated from free by vacuum filtration onto 24 mm GF/F Whatman filters and placed in scintillation vials with Ultima Gold scintillation cocktail (PerkinElmer, Boston, MA). Analysis [³H] PGE₂ binding was measured, following overnight incubation at room temperature, by counting each sample for 10 minutes with a Beckman LS 6500 multi-purpose scintillation counter.

Autoradiography

The radiohistochemical technique was originally described by Eriksen, et al. (557). Briefly, 9 to 11 week old male mice were euthanized and subsequently perfused with 0.9% saline followed by 10% formaldehyde to fix the tissue. Pancreata were dissected and embedded in
OCT. Sections were cut 10 microns thick on a cryostat and thaw-mounted onto gelatinize slides (Lab Scientific, Livingston, NJ). Tissue sections were washed in 0.9% saline to remove OCT. The sections were then incubated for one hour at room temperature in a humidified chamber with 5 nM $[^{3}H] \text{PGE}_2 \pm 1 \mu M \text{PGE}_2$ in buffer containing the following substances: HEPES 10 mM, NaCl 135 mM, KCl 4.8 mM, MgSO$_4$ 1.7 mM, CaCl$_2$ 0.5 mM, NaH$_2$PO$_4$ 1.0 mM, and BSA 0.5% at pH 7.4. Free PGE$_2$ was separated from tissue-bound PGE$_2$ by three consecutive five-minute washes in ice-cold 0.9% saline. Sections were vacuum dried overnight at 4°C. Sections were imaged for 6-hours on a Micro Imager™ (Biospace Lab, Nesles-la-Vallée, France) at Vanderbilt University Institute of Imaging Science.

**Mouse islet perifusion**

Pancreatic islets were isolated from male C57BL/6 EP3$^{+/+}$ or EP3$^{-/-}$ mice and perifusion assays were performed at the Vanderbilt Islet Procurement & Analysis Core as previously described (558). Studies comparing EP3$^{+/+}$ or EP3$^{-/-}$ islets utilized fresh islets. For studies employing the EP3 antagonist DG-041, islets isolated from 9 week old mice were cultured for 24 hours following isolation in RPMI-1640 containing 10% fetal bovine serum and 5.6 mM glucose at 37°C. Islet preparations were equilibrated and stable baseline response established at 5.6 mM glucose, and insulin secretion was stimulated with 16.7 mmol/l glucose. For some studies, 1 µM DG-041 was added to the islets at the indicated times. The studies utilizing DG-041 contained 100 µM 3-Isobutyl-1-methylxanthine (IBMX) and 0.01% DMSO throughout the perifusion. Statistical analyses for differences in insulin secretion were performed on the difference of the area under the curve.
**Mouse static incubation insulin secretion**

Pancreatic islets were isolated from 8.5 to 9.5 week old male C57BL/6 EP3\(^{+/+}\) or EP3\(^{-/-}\) mice and static incubation insulin secretion assays were performed at the Vanderbilt Islet Procurement & Analysis Core as previously described (558). Briefly, isolated islets were cultured overnight in RPMI with 5.6 mM glucose. The following day islets were incubated in DMEM media with the indicated glucose and drug concentrations for one hour. For studies utilizing DG-041, islets were cultured for one hour in 5.6 mM glucose ± DG-041 prior to indicated conditions. 200 µl of acid ethanol was used to disrupt islets for total insulin content. Mice used for experiments containing IL-1β were purchased from The Jackson Laboratory (Bar Harbor, ME).

**Intraperitoneal glucose tolerance tests (IP-GTT)**

Male C57BL/6 EP3\(^{+/+}\) or EP3\(^{-/-}\) mice were fasted overnight for 16 hours and fasting blood glucose was measured from a drop of tail vein blood with an Accu-Check Aviva glucometer and glucose test strips (Roche Diagnostics, Indianapolis, IN). Fasting insulin was measured from plasma collected by saphenous vein blood draw. Animals received an intraperitoneal injection of filter-sterilized glucose in PBS (2 mg dextrose/g body weight) and blood glucose was measured at 15, 30, 60, 90, and 120 minutes while plasma for insulin analysis was collected at 15 and 30 minutes after injection. Statistical analyses for differences in glucose handling were performed on the difference from baseline of the area under the curve (ΔAUC). Insulin content was analyzed in duplicate by RIA by Vanderbilt University Hormone Assay & Analytical Services Core.
**Intraperitoneal insulin tolerance tests (ITT)**

Male EP3^{+/+} or EP3^{-/-} mice were fasted for 6 hours during the light cycle and fasting blood glucose level were measured with the Accu-Check Aviva blood glucose meter using blood obtained from the tail. Following intraperitoneal injection of 34.1 µg/kg insulin (human recombinant insulin, Sigma, St. Louis, MO, Cat. No. I9278) in 0.9% normal saline, blood glucose levels were measured at 15, 30, 60, 90, and 120 minutes post-injection.

**Statistics**

Data are means ± SEM, using GraphPad Prism version 6.0e for Mac OS X (GraphPad Software, La Jolla, CA). All analyses were performed with an unpaired t-test unless otherwise stated.

**Results**

**Prostaglandin E\(_2\) receptors are expressed in the mouse pancreas**

Ligand binding studies were performed on pancreata from EP3^{+/+} and EP3^{-/-} mice to determine if EP receptors are expressed in the mouse pancreas. Total cell membranes prepared from pancreata of EP3^{-/-} mice showed a decrease, but not a total elimination, of [\(^3\)H] PGE\(_2\) binding (Figure 6A). This finding suggests EP3 constitutes a large proportion of PGE\(_2\) receptors in the pancreas and that other EP receptors, in addition to EP3, are expressed in the pancreas.

In order to examine the location of EP3 protein expression in the mouse pancreas, autoradiography was performed on mouse pancreatic tissue sections (Figure 6B). Specific [\(^3\)H] PGE\(_2\) binding was found throughout the EP3^{+/+} pancreatic tissue section. Because specific [\(^3\)H] PGE\(_2\) binding was global and not restricted to punctae, as islets constitute only 1-2% of pancreatic mass (559), it can be assumed that EP receptors are expressed in the exocrine tissue in the pancreas. The presence, or lack of, EP expression in islets cannot be determined because of
Figure 6. $[^3]H\text{ PGE}_2$ binds to EP3 receptors in the mouse pancreas

A. Specific $[^3]H\text{ PGE}_2$ binding to pancreata membranes pooled from five EP3$^{+/+}$ and five EP3$^{-/-}$ mice with each sample assayed in triplicate. Membranes from EP3$^{-/-}$ pancreata had less specific $[^3]H\text{ PGE}_2$ binding ($P = 0.015$). B. PGE$_2$ receptor localization was performed using autoradiography. $[^3]H\text{ PGE}_2$ had specific binding to wild-type pancreas that was greatly reduced in EP3$^{-/-}$ tissue. White is tissue, color is $[^3]H$ counts with blue being lowest and red being highest. Binding data are representative samples of three independent experiments. Values are expressed as mean ± SEM.
the overwhelming nature of \[^{3}H\] PGE\(_{2}\) binding to the surrounding tissue. Tissue sections from EP3\(^{-/-}\) mice showed less \[^{3}H\] PGE\(_{2}\) binding suggesting that the majority of \[^{3}H\] PGE\(_{2}\) binding seen in EP3\(^{+/+}\) sections is due to the EP3 receptor.

**PGE\(_{2}\)-EP3 signaling does not affect glucose stimulated insulin secretion**

Pancreatic islets isolated from EP3\(^{+/+}\) and EP3\(^{-/-}\) mice were assessed for alterations in GSIS by islet perifusion and static culture. EP3 genotype did not affect GSIS (Figure 7A,B). Furthermore, neither first nor second phase insulin secretion was altered by disruption of EP3 (Figure 7A). 1 \(\mu\)M PGE\(_{2}\) failed to suppress GSIS in both EP3\(^{+/+}\) and EP3\(^{-/-}\) islets (Figure 7A,C). Moreover, treatment of islets with the EP3 antagonist DG-041 had no effect on GSIS (Figure 7D,E). In addition, IL-1\(\beta\), which has previously been shown to suppress GSIS in a PGE\(_{2}\) dependent manner (177,196,560), also had no significant effect on GSIS (Figure 7E).

**EP3\(^{-/-}\) mice do not have altered body or tissue mass**

Male EP3\(^{+/+}\) and EP3\(^{-/-}\) mice fed a chow diet did not differ in body weight over the period observed, from four weeks of age to 38 weeks of age (Figure 8A). Post-mortem analysis found no difference in epididymal fat pad or liver weight (Figure 8B,C). *In vivo* body composition analysis at 40 weeks of age also found no difference in fat mass or lean body mass (Figure 8D,E). Similar results were found in female mice, which also exhibited no difference in body mass during the observed period (Figure 8F). Similarly, post-mortem analysis found no difference in gonadal fat pad or liver weight (Figure 8G,H).

**EP3\(^{-/-}\) mice are not glucose intolerant**

EP3\(^{+/+}\) and EP3\(^{-/-}\) male mice fed chow had no difference in glucose tolerance at 20 weeks of age (Figure 9A), though a modest improvement was observed in EP3\(^{-/-}\) mice at 40 weeks of age (Figure 9B). This difference in glucose tolerance could not be attributed to increased plasma
**Figure 7. Glucose-stimulated insulin secretion is not affected by PGE\textsubscript{2}-EP3 signaling**

**A.** Perfusion of islets from EP3\textsuperscript{+/+} and EP3\textsuperscript{−/−} mice showed no effect of EP3 genotype on glucose-stimulated insulin secretion (GSIS). Both first phase (P = 0.741) and second phase (P = 0.945) insulin secretion were unchanged. PGE\textsubscript{2} administration during perfusion did not affect GSIS. N = 4 mice for each genotype. Values are expressed as mean ± SEM.

**B.** Static incubation insulin secretion in the presence of 100 µM IBMX found no difference in GSIS from EP3\textsuperscript{+/+} and EP3\textsuperscript{−/−} islets (P = 0.592). N=5 mice per genotype, each sample assayed in duplicate. Values are expressed as mean ± SEM.

**C.** Static incubation insulin secretion found no differences in GSIS when EP3\textsuperscript{+/+} islets were treated with PGE\textsubscript{2}. (P = 0.309). N = 2 mice. Values are expressed as mean ± StDev.

**D.** Perfusion of islets from EP3\textsuperscript{+/+} mice in the presence of 100 µM IBMX demonstrated that addition of the EP3 antagonist DG-041 does not alter GSIS. N = 4 mice. Values are expressed as mean ± SEM.

**E.** Static incubation insulin secretion in the presence of 100 µM IBMX found no difference in GSIS when EP3\textsuperscript{+/+} islets were treated with EP3 antagonist, DG-041, IL-1β, or DG-041 + IL-1β (P = 0.212). Statistical significance was determined by one-way ANOVA. N = 5 mice. Values are expressed as mean ± SEM. Within each panel, islets from each mouse were utilized for every condition.
Figure 8. Chow fed EP3\(^{-/-}\) mice have no overt morphological phenotype

A. Male EP3\(^{+/+}\) and EP3\(^{-/-}\) mice fed standard chow were weighed weekly from age 4 weeks to age 38 weeks. No difference in body weight was observed. N = 11 EP3\(^{+/+}\) and 10 EP3\(^{-/-}\). B. Epididymal fat pads weighed post mortem at ~40 weeks of age showed no difference between genotypes (P = 0.282). N = 9 EP3\(^{+/+}\) and 10 EP3\(^{-/-}\). C. Male livers weighed post mortem at ~40 weeks of age showed no difference between genotypes (P = 0.886). N = 9 EP3\(^{+/+}\) and 10 EP3\(^{-/-}\). Body composition was assessed around 40 weeks of age by pulsed NMR. D. No difference in body fat in males was observed (P = 0.619). N = 5 EP3\(^{+/+}\) and 4 EP3\(^{-/-}\). E. No difference in lean mass in males was observed (P = 0.367). N = 5 EP3\(^{+/+}\) and 4 EP3\(^{-/-}\). F. Female EP3\(^{+/+}\) and EP3\(^{-/-}\) mice fed standard chow were weighed weekly from age 4 weeks to age 38 weeks. No difference in body weight was observed. N = 5 for each genotype. G. Gonadal fat pads weighed post mortem at ~40 weeks of age showed no difference between genotypes (P = 0.637). N = 5 for each genotype. H. Female livers weighed post mortem at ~40 weeks of age showed no difference between genotypes (P = 0.203). N = 5 for each genotype. Values are expressed as mean ± SEM.
Figure 9. Chow fed EP3\(^+\) male mice have no overt metabolic phenotype
A. Glucose tolerance was not different between 20 week old EP3\(^{+/+}\) and EP3\(^{-/-}\) male mice (P = 0.362). N = 10 EP3\(^{+/+}\) and 18 EP3\(^{-/-}\). B. Assessment of glucose tolerance in 40 week old EP3\(^{+/+}\) and EP3\(^{-/-}\) mice showed a modest improvement in the change in the area under the curve (29300 vs. 24600 min.\(\times\)mg/ml; P = 0.0244). N = 24 each group. C. Measurement of plasma insulin levels during the first 30 minutes of the GTT of 40 week old chow fed mice showed no difference in insulin secretion between EP3\(^{+/+}\) and EP3\(^{-/-}\) mice (P = 0.759). N = 13 each group. D. Insulin tolerance was not different between EP3\(^{+/+}\) and EP3\(^{-/-}\) mice at 20 weeks of age. N = 10 EP3\(^{+/+}\) and 19 EP3\(^{-/-}\). E. Assessment of insulin tolerance in 40 week old EP3\(^{+/+}\) and EP3\(^{-/-}\) was also unchanged. N = 18 EP3\(^{+/+}\) and 19 EP3\(^{-/-}\). F. The homeostatic model assessment of insulin resistance (HOMA-IR), a function of fasting blood glucose (from B) and plasma insulin (from C), showed no difference between 40 week old chow fed EP3\(^{+/+}\) and EP3\(^{-/-}\) mice (P = 0.609). N = 13 each group. Values are expressed as mean ± SEM.
insulin levels (Figure 9C). Insulin sensitivity was also not altered in male EP3<sup>−/−</sup> mice at either 20 (Figure 9D) or 40 weeks of age (Figure 9E,F). Female EP3<sup>−/−</sup> mice also showed no difference in glucose or insulin tolerance at 40 weeks of age (Figure 10).

**Discussion**

Blockade of PGE<sub>2</sub> action at the inhibitory EP3 receptor has been hypothesized to be a therapeutic target to improve GSIS, which would be beneficial for the treatment of type 2 diabetes. Studies have shown that *ptger3* mRNA is highly expressed in the pancreas (181,235), pancreatic islets (74,181), and β-cells (239) of mice. The present studies suggest that EP3 protein is highly expressed in mouse pancreata. Prior studies have implicated EP3 function in β-cells of pancreatic islets (71,74,194,197,233). Autoradiography was performed to visualize EP3 expression predominantly in pancreatic islets; however, EP3 was globally expressed in the pancreas indicating that is also expressed in exocrine tissue, which makes up 98-99% of the pancreas (559).

There are conflicting data in the literature regarding the effects of EP3 receptor activation on GSIS. Some studies have suggested an improvement in GSIS upon EP3 receptor blockade or inhibition of PG production (74,233), while others have not found any effect of PGE<sub>2</sub> signaling on GSIS in isolated islets (222). Data from rat pancreatic islets showed that GSIS was inhibited by EP3 receptor agonists in a pertussis toxin sensitive manner (197). *Ex vivo* studies using human islets from individuals with or without diabetes found that in islets derived from non-diabetic humans no effect in GSIS was observed in agreement with data shown here (74). It is of interest that in these studies, the effect of EP3 receptor blockade on islets became apparent only when the subjects were obese and/or diabetic. Our studies using an EP3 antagonist in mouse islet perifusion studies, as well as perifusion of EP3<sup>−/−</sup> islets, did not find any effect on GSIS. The
Figure 10. Chow fed EP3+/− female mice have no overt metabolic phenotype
observed differences among these studies may represent species differences between rat, mouse and human, off-target effects of high antagonist concentrations, or the physiologic status of the individuals at the time of islet harvest.

Consistent with EP3 gene deletion or blockade having no effect on GSIS ex vivo, we also found no change in GSIS in vivo. We did note a small improvement in glucose homeostasis in 40 week old male EP3−/− mice, but we could not attribute this to either improved GSIS or insulin sensitivity. Despite a previous study that reported that EP3−/− mice are obese (102), we found that both male and female mice maintain similar body and tissue weights. Results from the present studies are consistent with the first studies reporting the generation of EP3−/− mice, which also found no apparent morphological abnormalities (310,555). It is possible that EP3−/− mice do not develop obesity when fed a standard chow diet, which contains 13.5% calories from fat as compared to the 29% calories from fat found in breeder chow diet (102). If dietary fat significantly modulates the phenotype of the EP3−/− mouse, it would be of interest to examine the physiology of EP3−/− mice when fed matched, defined diets with varying fat content.
CHAPTER III

THE PGE\textsubscript{2} EP3 RECEPTOR REGULATES DIET-INDUCED ADIPOSY IN 20 WEEK OLD MALE MICE

Introduction

Obesity and ectopic lipid accumulation are associated with insulin resistance, and contribute to the pathology of a number of diseases, notably type 2 diabetes. Obesity has been recognized as a disease of low-grade chronic inflammation, and circulating inflammatory cytokines increase in obesity and type 2 diabetes (reviewed in (26,320,388,561,562)). PGs are important mediators of inflammation that have been recognized to play a significant role in metabolic diseases (reviewed in (41,44,152,198)). These oxidative metabolites of arachidonic acid, are produced by cyclooxygenases (COX1 and COX2), and act locally in an autocrine or paracrine manner (reviewed in (43)). PGE\textsubscript{2} is a major COX metabolite, which mediates its effects by activation of four GPCRs, designated E\textsubscript{-}Prostanoid (EP)1 through EP4 (43). Analysis of GPCR expression across a panel of mouse tissues revealed that the EP3 receptor was most highly expressed in the pancreas, kidney and white adipose tissue, an expression pattern consistent with regulation of metabolic homeostasis (235).

PGs and other inflammatory mediators play an important role in adipose tissue physiology (320,388,390). EP3 has the highest expression of the four EP receptors in mouse epididymal fat pads (338). Pharmacological studies suggest that PGE\textsubscript{2} inhibits lipolysis and reduces intracellular cAMP in adipocytes via the EP3 receptor (101,284). cAMP-protein kinase A (PKA) signaling is a central regulator of lipolysis, being important for activating both hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) (401,403-405). Loss of PG production in adipose tissue by deletion of adipocyte phospholipase, AdPLA, increases lipolysis causing lipids to be stored in ectopic tissues (101). These AdPLA\textsuperscript{-/-} mice are resistant to
obesity but are insulin resistant due to the ectopic triglyceride storage (101). Taken together, these data suggest that the EP3 receptor plays a role in facilitating adipose tissue lipid accumulation by inhibiting lipolysis. Thus, a loss of EP3 signaling may limit adipose tissue lipid deposition and facilitate ectopic lipid distribution, which could exacerbate diet induced insulin resistance.

Contrary to what would be expected in a model of increased adipocyte lipolysis, mice with a global gene disruption of the EP3 receptor have increased body weight and fat pad mass when fed breeder chow (102). This report contrasts with a previous report that EP3\(-/-\) mice have normal growth rates (310) and our data from EP3\(-/-\) mice fed a standard chow diet (Chapter II). (310). To more completely characterize the interaction of the EP3 receptor with diet and its role in metabolism, EP3\(-/-\) mice were challenged with a high fat diet (HFD). We show that EP3\(-/-\) mice have no difference in body weight on a control or chow diet. However, when fed a HFD, EP3\(-/-\) mice gained more weight than did EP3\(+/-\) fed the same diet, similar to what was previously found when the EP3\(-/-\) mice were fed breeder chow (102). As with EP3\(-/-\) mice fed breeder chow, we noted insulin resistance and elevated blood glucose in EP3\(-/-\) mice fed HFD. We found that the insulin resistance in EP3\(-/-\) mice fed a HFD is accompanied by lipid redistribution from the adipose tissue to ectopic, insulin sensitive tissues such as liver and skeletal muscle. Fat pads in EP3\(-/-\) HFD fed mice were relatively smaller, had increased necrosis, increased inflammatory cytokine expression and failed to suppress lipolysis in response to PGE\(_2\). Although EP3\(-/-\) mice were not hyperphagic, they were less active, became obese, had increased ectopic lipid accumulation and were insulin resistant.
Materials and Methods

Animal procedures & high fat diet feeding

Mice utilized for these experiments were maintained as previously described (Chapter II). Beginning at age 4 weeks, male C57BL/6 EP3+/+ or EP3-/- mice were fed a control diet (10% calories from fat; 4.3% fat by weight, D12450Bi, Research Diets, New Brunswick, NJ) or high fat diet (HFD) (45% calories from fat; 24% fat by weight, D12451i, Research Diets) for 16 weeks. All procedures were approved by the Institutional Animal Care and Use Committee at Vanderbilt University.

Energy balance

A separate cohort of mice utilized for energy balance studies were fed HFD beginning at 7.5 weeks of age for 10.5 weeks until ~18 weeks of age. Energy balance studies were performed in a Promethion system (Sable Systems International, North Las Vegas, NV) for five days by the Vanderbilt Mouse Metabolic Phenotyping Center.

Plasma chemistry

Plasma was collected from blood obtained by terminal cardiac puncture. Plasma triglyceride quantification was performed by the Vanderbilt Translational Pathology Shared Resource. Plasma FFA quantification was performed by the Mouse Metabolic Phenotyping Center Lipid Lab.

Intraperitoneal glucose tolerance tests

IP-GTT was performed on male C57BL/6 EP3+/+ or EP3-/- mice as previously described (Chapter II).
Intraperitoneal insulin tolerance tests

Male EP3+/+ or EP3-/- mice were fasted for 6 hours during the light cycle and fasting blood glucose level were measured with the Accu-Check Aviva blood glucose meter using blood obtained from the tail. Following intraperitoneal injection of 0.75 U/kg insulin (human recombinant insulin, Novolin R, Novo Nordisk, Princeton, NJ) in 0.9% normal saline, blood glucose levels were measured at 15, 30, 60, 90, and 120 minutes post-injection.

Mouse static incubation insulin secretion

Pancreatic islets were isolated from 10.5 week old C57BL/6 mice, purchased from The Jackson Laboratory (Bar Harbor, ME), which had been fed control (D12450Bi) or HFD (D12451i) since 3.5 weeks of age. Briefly, isolated islets were cultured overnight in RPMI with 5.6 mM glucose. The following day islets were incubated in DMEM media for one hour in 5.6 mM glucose ± DG-041. Following the one-hour culture, islets were then incubated in DMEM media with the indicated glucose and drug concentrations and 100 µM IBMX for one hour. 200 µl of acid ethanol was used to disrupt islets for total insulin content. Islet isolation and culture and insulin and glucagon assays were performed at the Vanderbilt Islet Procurement & Analysis Core as previously described (558).

Prostanoid quantification

PG content of islet secretion media and epididymal fat pads were measured by the Vanderbilt Eicosanoid Core Laboratory as previously described (563).

Plasma Chemistry

At the end of the control and HFD feeding study, 20 weeks of age, plasma was collected from blood obtained by terminal cardiac puncture post-mortally from three to four ad libitum fed mice per group. Leptin and adiponectin were measured with a Luminex 100 system (Luminex
Corporation, Austin, TX) by the Vanderbilt University Hormone Assay & Analytical Services Core.

*Skeletal muscle capillary density*

Combined gastrocnemius and soleus skeletal muscle were fixed by freezing in OCT medium and sectioned by the Vanderbilt Translational Pathology Shared Resource. Frozen slides were allowed to equilibrate to room temperature and excess OCT was removed from slides prior to the beginning of staining. Slides were fixed in HPLC grade acetone for 10 minutes at room temperature then allowed to air dry for 10 minutes. Slides were washed in PBS three times for 3.3 minutes each. Slides were blocked for one-hour in a humidified chamber at room temperature in 3% BSA in PBS. Following blocking, slides were washed one time in PBS for 5 minutes. Slides were incubated with 1:200 rat anti-mouse CD31 antibodies (BD Pharmingen, San Diego, CA, Cat. No. 550274) in PBS with 0.1% Triton X-100 and 1% BSA overnight at 4°C in a humidified chamber. The next morning, slides were washed in PBS three times for five minutes each. Slides were incubated with 1:250 donkey anti-rat-rhodamine red (Jackson ImmunoResearch Laboratories, West Grove, PA, Cat. No. 712-259-150) and 1:10,000 DAPI in PBS with 0.1% Triton X-100 and 1% BSA for 1.5 hours at room temperature in a humidified chamber. Slides were washed in PBS three times for five minutes each and subsequently mounted with Vectashield (Vector Laboratories, Burlingame, CA, Cat. No. H-100). Slides were imaged with an Olympus BX51 microscope (Olympus America, Melville, NY) using a 20× objective with an exposure of 1.5 ms for DAPI and 50 ms for CD31. Three tissue sections from three mice from each diet×genotype group were analyzed with ImageJ 1.43u (National Institutes of Health, USA). Statistics were performed on the average staining from each mouse, which was an average of all three sections, which in turn was an average of at least 10 images.
Histology

Liver and epididymal fat pads from at least three individual mice per group were fixed in 10% formaldehyde overnight at 4°C and subsequently stored in 70% ethanol at 4°C prior to processing routinely, paraffin embedded, sectioned at 4 µm, and stained with hematoxylin and eosin (H&E) or immunohistochemistry. Liver samples from three individual mice per group were fixed by freezing in OCT medium for Oil Red O (ORO) staining. Histology was performed by the Vanderbilt Translational Pathology Shared Resource. All slides were imaged at 20× with a Leica SCN400 Slide Scanner (Leica Microsystems, Wetzlar, Germany) by the Vanderbilt Digital Histology Shared Resource. Whole slides were imaged at 20X magnification to a resolution of 0.5 µm/pixel. Adipocyte cell sizes were measured from 10 images per mouse with ImageJ using Adipocytes Tools (564). Epididymal fat pads were examined by an experienced veterinary pathologist (Kelli L. Boyd). Fat pad necrosis and inflammation (steatitis) were scored on a scale of zero to four, with zero being no steatitis or necrosis, one being minimal steatitis and necrosis not present, two being mild steatitis with necrosis of individual adipocytes, three being moderate steatitis with extensive areas of adipocyte necrosis, and four being severe steatitis with diffuse necrosis of the fat pad. The numbers of positive (brown) and negative (blue) nuclei were determined by analysis of 10 images per mouse in the Ariol® software (Leica Microsystems) at the Vanderbilt Digital Histology Shared Resource.

Mouse adipocyte lipolysis assay

Adipocytes were isolated from epididymal fat pads of ten week old male EP3+/+ and EP3−/− mice that were maintained on a standard chow diet. Lipolysis was assessed as described (565). Each assay was performed in duplicate for each mouse. Briefly, fat pads were placed in an Adipocyte Incubation Solution (AIS) containing 1.8 g/L glucose, 0.1 g/L Magnesium Chloride
hexahydrate, 0.34 g/L Potassium Chloride, 7.0 g/L Sodium Chloride, 0.1 g/L Sodium Phosphate Dibasic, 0.18 g/L Sodium Phosphate Monobasic, 0.84 g/L Sodium Bicarbonate, 30 mM HEPES, and 3% (w/v) fatty acid free bovine albumin fraction V (MP Biomedical, Solon, OH) at pH 7.4. Fat pads were minced and digested in AIS with 500 nM adenosine (Sigma A4036) and 3 mg/ml type 1 collagenase (Worthington Biochemical Corporation, Lakewood, NJ) for one hour at 37°C while shaking at 200 rpm. Digested fat pads were filtered through 250 µm Pierce® Tissue Strainers (Thermo Scientific, Rockford, IL) and washed three times in AIS with adenosine. Adipocytes were resuspended in AIS and counted with Scepter™ Handheld Automated Cell Counter (Millipore Corporation, Billerica, MA). Lipolysis assays were performed on 6,000 to 9,000 adipocytes in 650 µl in 15 ml conicals at 37°C. At the start of the assay, adipocytes were incubated in the presence or absence of DG-041 or human recombinant insulin (Sigma I9288). PGE₂ (Cayman Chemical, Ann Arbor, MI) was added after 15 minutes to account for the slow binding of DG-041 (566,567). After 30 minutes a 50 µl sample was collected from each sample to establish baseline and isoproterenol (Sigma I6504) was added. Isoproterenol stimulated lipolysis was assessed one hour after the addition of isoproterenol. Glycerol was measured with a Glycerol Assay Kit (Sigma MAK117-1KT).

Tissue fatty acid composition

Lipids were extracted from ~100 mg flash frozen liver or combined gastrocnemius and soleus skeletal muscle tissue (568). The extracts were filtered, and lipids recovered in the chloroform phase. Individual lipid classes were separated by thin layer chromatography using Silica Gel 60 A plates developed in petroleum ether, ethyl ether, acetic acid (80:20:1) and visualized by rhodamine 6G. Phospholipids, diglycerides, triglycerides and cholesteryl esters were scraped from the plates and methylated using BF3/methanol (569). The methylated fatty
acids were extracted and analyzed by gas chromatography. Gas chromatographic analyses were carried out on an Agilent 7890A gas chromatograph equipped with flame ionization detectors, a capillary column (SP2380, Supelco, Bellefonte, PA). Helium was used as a carrier gas. Fatty acid methyl esters were identified by comparing the retention times to those of known standards. Triglycerides were quantified by the Mouse Metabolic Phenotyping Center Lipid Lab.

**Quantitative real-time RT-PCR (qPCR)**

Total RNA was isolated from approximately 50-100 mg frozen liver or 100-250 mg frozen epididymal fat pad using TRIzol Reagent (Invitrogen, Carlsbad, CA). Liver RNA was precipitated with isopropanol and ethanol from the aqueous phase of the TRIzol extraction then dissolved in RNase-free water and subsequently purified with RNeasy Mini Kit (Qiagen, Valencia, CA). Epididymal fat pad RNA was immediately purified from the aqueous phase of the TRIzol extraction with RNeasy Mini Kit. One µg RNA per mouse was reverse transcribed at a concentration of 50 ng/µl using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Approximately 6.25 ng cDNA was utilized per 20 µl quantitative PCR reaction. Quantitative real-time RT-PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems) for *Adipoq* (Mm00456425_m1), *ApoB* (Mm01545156_m1), *CebpB* (Mm00843434_s1), *Ccl2* (Mm00441242_m1), *Cpt1a* (Mm01231183_m1), *Emr1* (Mm00802529_m1), *Fasn* (Mm00662319_m1), *Gapdh* (Mm99999915_g1), *Il6* (Mm00446190_m1), *Lep* (Mm00434759_m1), *Mttp* (Mm00435015_m1), *PECAM1* (Mm01242584_m1), *Ptger3* (Mm01316856_m1), *Ptgs2* (Mm00478374_m1), *Tnf* (Mm00443258_m1), *Ucp1* (Mm01244861_m1), and *Vegfa* (Mm01281449_m1). All assays were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems) at the Vanderbilt Technologies for Advanced Genomics. Gene expression data were collected from three
replicates of each sample and four replicates of each standard curve point. Replicate samples were averaged and analyzed using the Pfaffl method (570). Gene expression was normalized to Gapdh and the individual with the lowest relative gene expression was considered to have a fold change of 1.0.

**Measurement of Systolic Blood Pressure**

Systolic BP was determined in conscious mice by using tail-cuff plethysmography (Visitech systems BP-2000 Blood Pressure Analysis System, Apex NC). Mice were trained for three days to minimize stress. Each measurement is the average of at least 10 consecutive readings after stabilization on the last day.

**Statistical analysis and calculations**

Data are means ± SEM, using GraphPad Prism version 6.0e for Mac OS X (GraphPad Software, La Jolla, CA). All analyses were performed with a 2-way ANOVA comparing EP3 genotype and dietary treatment unless otherwise stated. Multiple comparison tests were performed with the Bonferroni correction for EP3 genotype only and are indicated on figures by asterisks corresponding to *P < 0.05, **P < 0.01, ***P < 0.001. For analyses of plasma insulin, fat mass, and lean mass over time, 2-way ANOVA with repeated measures was performed comparing the factors EP3 genotype and time keeping the dietary treatment consistent. HOMA-IR was calculated as the fasting insulin level (µU/mL) × blood glucose level (mg/dL) / 405. For all studies, P < 0.05 was considered statistically significant.

**Results**

**EP3<sup>−/−</sup> mice are obese when fed a HFD**

EP3<sup>+/−</sup> or EP3<sup>−/−</sup> male mice were fed either HFD (45% calories from fat) or a micronutrient matched control diet (10% calories from fat). Body weight increased in each of the
four groups over the course of the study (Figure 11A). Body weight in both EP3<sup>+/+</sup> and EP3<sup>-/-</sup> HFD fed mice increased at a greater rate than in animals fed control diet, with a divergence in body weight becoming apparent after eight weeks of age. Moreover, by 14 weeks of age the EP3<sup>-/-</sup> mice fed HFD were heavier than HFD-fed EP3<sup>+/+</sup> animals. EP3<sup>-/-</sup> mice continued to gain weight throughout the remainder of the study leading to a greater difference in body weight at 20 weeks of age (P < 0.0001).

**EP3<sup>-/-</sup> mice are have increased adiposity and epididymal fat pad mass**

Body composition analyses over the course of the study revealed that HFD fed EP3<sup>-/-</sup> mice had no difference in lean mass (Figure 11B) but had increased fat mass as compared to HFD fed EP3<sup>+/+</sup> (Figure 12A). A small but statistically significant increase in body fat was also observed at 20 weeks of age in EP3<sup>-/-</sup> mice vs. EP3<sup>+/+</sup> fed control diet. Post-mortem analysis of epididymal fat pads confirmed that, when fed a HFD, fat pads from EP3<sup>-/-</sup> mice were heavier than those from EP3<sup>+/+</sup> (Figure 12B).

When total fat mass determined from body composition analysis at 20 weeks of age was plotted as a function of body weight for HFD fed animals, the expected linear relationship was observed (Figure 12C); and although EP3<sup>-/-</sup> mice fed HFD were heavier, they had the same overall linear relationship of total fat mass to body weight (P = 0.88, Figure 12C). In both genotypes, heavier mice had more fat mass than did the lighter mice, as determined by the body composition scanner. However, when epididymal fat pad weight was plotted as a function of body weight, a striking difference was observed between genotypes (Figure 12D). The heaviest EP3<sup>-/-</sup> HFD-fed mice had smaller epididymal fat pads than did the lightest EP3<sup>-/-</sup> HFD-fed mice. In control diet fed animals, genotype did not affect the relationship between body weight and
Figure 11. EP3−/− mice are obese when fed a HFD
A. Male EP3+/+ and EP3−/− mice fed control diet or HFD were weighed between 4 weeks of age and 20 weeks of age. No difference in body weight was observed between genotypes in the control diet fed animals. EP3−/− mice fed HFD were significantly heavier than EP3+/+ animals beginning around 14 weeks of age (P = 0.0041) and continuing until the end of the experiment at 20 weeks of age (P < 0.0001). B. Body composition of lean mass was assessed by pulsed NMR. C. Representative images of EP3+/+ and EP3−/− mice fed control or HFD at 20 weeks of age. For all figures N = 9 EP3+/+ control, 10 EP3−/− control, 7 EP3+/+ HFD, 9 EP3−/− HFD. Values are expressed as mean ± SEM.
Figure 12. EP3<sup>−/−</sup> mice proportionately increase adiposity but not epididymal fat pad mass

A. Body composition of fat was assessed at four week intervals by pulsed NMR. EP3<sup>−/−</sup> mice had increased fat mass when fed HFD (P = 0.0004). B. Epididymal fat pads were weighed post mortem at the conclusion of the study. Epididymal fat pads mice were heavier from EP3<sup>−/−</sup> HFD fed mice (P = 0.0038). C. Mice fed HFD showed no difference in the total body fat to body weight ratio between genotypes (P = 0.875). D. The relationship between epididymal fat pad weight and body weight differ between EP3<sup>−/−</sup> and EP3<sup>+/+</sup> mice (P < 0.0001). E. Total fat mass determined by NMR plotted showed no correlation to body weight in EP3<sup>−/−</sup> and EP3<sup>+/+</sup> mice fed control diet (R<sup>2</sup> < 0.04 for both). F. Epididymal fat pad weight showed no correlation to body weight in EP3<sup>+/+</sup> and EP3<sup>−/−</sup> mice fed control diet (R<sup>2</sup> < 0.06 for both). For all figures N = 9 EP3<sup>+/+</sup> control, 10 EP3<sup>−/−</sup> control, 7 EP3<sup>+/+</sup> HFD, 9 EP3<sup>−/−</sup> HFD. Values are expressed as mean ± SEM.
either fat mass or epididymal fat pad weight (Figure 12E,F). These data are consistent with abnormal lipid accumulation in fat depots in EP3\(^{-/-}\) mice when challenged with HFD feeding.

**EP3\(^{-/-}\) mice have changes in energy balance**

A separate cohort of mice was fed HFD, beginning at 7.5 weeks of age, for energy balance studies (Figure 13A). Energy balance studies demonstrate that EP3\(^{-/-}\) mice do not have changes in food consumption (Figure 13B). EP3\(^{-/-}\) mice were found to have a significant decrease in movement during the dark cycle of the day (Figure 13C). This was accompanied by more time spent inactive (Figure 13D).

Changes in energy expenditure were also measured. EP3\(^{-/-}\) mice were not found to have significant changes in energy expenditure (Figure 14A). Oxygen consumption was unchanged in these mice (Figure 14B); however, carbon dioxide production was increased in EP3\(^{-/-}\) mice (Figure 14C). Because an increase in respiration contrasts with the decrease in ambulatory activity, expression of a gene involved in oxidative metabolism was measured. UCP1 is a protein found in the mitochondria of adipose tissue that uncouples respiration from ATP synthesis resulting in a loss of energy (318). Expression of Ucp1 was increased in the epididymal fat pads of EP3\(^{-/-}\) mice when fed a HFD (Figure 14D).

**EP3\(^{-/-}\) mice have increased adipocyte hypertrophy**

Adipocytes from mouse epididymal fat pads were visualized by H&E staining (Figure 15A). Both HFD feeding and EP3\(^{-/-}\) genotype were associated with an increase in average adipocyte cell size (Figure 15B). When adipocyte cell size was plotted as a function of body weight, cell size to body weight ratios for EP3\(^{-/-}\) HFD fed mice were significantly different than those observed for EP3\(^{+/+}\) HFD fed mice (Figure 15C). The size distribution showed that EP3\(^{-/-}\) mice have a greater number of large adipocytes regardless of which diet they were fed (Figure
Figure 13. EP3⁻/⁻ mice have decreased movement when fed a HFD

A. Male EP3⁺/⁺ and EP3⁻/⁻ mice were fed HFD between 7.5 weeks of age and 19 weeks of age. EP3⁻/⁻ mice fed HFD were heavier than EP3⁺/⁺ animals (P = 0.029). B. Total food intake was measured in male mice that had been fed HFD for 10.5 weeks. Mice consumed more food during the dark cycle (P = 0.0026), but no significant difference between EP3 genotypes (P = 0.35) or interaction between light cycle and genotype (P = 0.67) were observed. C. Total movement was measured in the same mice. EP3⁻/⁻ mice were found to move significantly less than EP3⁺/⁺ mice during the dark cycle. D. Mice were still for a greater amount of their time in the light cycle (P = 0.0053). EP3⁻/⁻ mice spend a greater proportion of their time inactive (P = 0.014). For all figures N = 6 EP3⁺/⁺ HFD, 4 EP3⁻/⁻ HFD. All values are expressed as mean ± SEM.
Figure 14. EP3\(^{-/-}\) HFD fed mice have increased CO\(_2\) production and uncoupling protein gene expression

A. Total energy expenditure was measured in male mice that had been fed HFD for 10.5 weeks. Mice had greater energy expenditure during the dark cycle (P < 0.0001), but no significant differences between EP3 genotype (P = 0.315) or interaction between light cycle and genotype (P = 0.242) were observed. B. Average oxygen consumption was measured in the same mice. Mice had greater oxygen during the dark cycle (P < 0.0001), but no significant differences between EP3 genotype (P = 0.349) or interaction between light cycle and genotype (P = 0.142) were observed. C. Average CO\(_2\) production was also measured in these mice. Mice had greater CO\(_2\) production during the dark cycle (P < 0.0001). EP3\(^{-/-}\) mice demonstrated increased CO\(_2\) production (P = 0.0252), but a significant interaction between genotype and light cycle was not observed (P = 0.325). For A-C, N = 6 EP3\(^{+/+}\) HFD, 4 EP3\(^{-/-}\) HFD. D. Gene expression of uncoupling protein 1 (Ucp1) in epididymal fat pads was measured in a separate cohort of 20 week old mice that had been fed control or HFD for 16 weeks. Ucp1 expression was increased in EP3\(^{-/-}\) mice (P < 0.0001). HFD feeding increased Ucp1 expression (P = 0.0008) and interacted synergistically with EP3 genotype (P = 0.0007). Ucp1 gene expression was plotted as a function of body weight. The relationships between Ucp1 gene expression and body weight are not different in HFD fed mice (P = 0.650). N = 7 EP3\(^{+/+}\) control, 9 EP3\(^{-/-}\) control, 7 EP3\(^{+/+}\) HFD, 8 EP3\(^{-/-}\) HFD. For all figures values are expressed as mean ± SEM.
Figure 15. EP3⁻/⁻ mice have increased epididymal fat pad adipocyte cell size
A. H&E staining of epididymal fat pads. B. Average epididymal fat pad adipocyte cell sizes were calculated from measurements of adipocytes made using ImageJ software. EP3⁻/⁻ mice were found to have larger adipocytes (P = 0.0002). C. Average adipocyte cell size from HFD fed mice was plotted as a function of body weight. The relationship between epididymal fat pad adipocyte cell size and body weight are significantly different (P = 0.0055). D. Distribution of adipocyte size from mice fed control diet. E. Distribution of adipocyte size from mice fed HFD. For all figures N = 8 EP3⁺/⁺ control, 7 EP3⁻/⁻ control, 7 EP3⁺/⁺ HFD, 9 EP3⁻/⁻ HFD. Images are a representative sample of those used to calculate adipocyte cell size. For B & C, values are expressed as mean ± SEM; for D & E, values are expressed as mean only.
15D,E). These data suggest that the altered correlation between epididymal fat pad mass and body weight observed in EP3<sup>−/−</sup> HFD fed animals is due to an inability to proportionally increase adipocyte size in heavier animals.

*Adipose tissue from EP3<sup>−/−</sup> mice does not have changes in gene expression related to prostaglandin biosynthesis but does have changes in genes associated with increased adipogenesis*

Gene expression was measured in the epididymal fat pads of EP3<sup>−/−</sup> mice. As expected, the EP3 gene (*Ptger3*) was not expressed in EP3<sup>−/−</sup> tissue (Figure 16A). Dietary fat did not have an effect on EP3 gene expression. Expression of the inducible COX2 gene (*Ptgs2*) was also measured and was found to be unaffected by dietary fat or EP3 genotype (Figure 16B). The expression of CCAAT/enhancer-binding protein β (*Cebpb*) was measured because it plays an important role promoting both adipogenesis (349,350) and COX2 transcription (346-348). The expression of *Cebpb* was increased in the adipose tissue of EP3<sup>−/−</sup> HFD fed mice (Figure 16C).

*EP3<sup>−/−</sup> mice have increased adipocyte necrosis when fed HFD*

H&E stained epididymal fat pads from HFD fed EP3<sup>+/+</sup> and EP3<sup>−/−</sup> mice were scored for necrosis on a scale of 0 to 4. EP3<sup>−/−</sup> mice showed areas of increased necrosis in the epididymal fat pads, which was largely absent in EP3<sup>+/+</sup> mice (Figure 17A). EP3<sup>−/−</sup> HFD fed mice showed areas of increased necrosis in the epididymal fat pads (Figure 17B). Necrosis was increased with increasing body weight in both genotypes of mice (Figure 17C). Thus, in the EP3<sup>−/−</sup> HFD cohort, the heaviest mice have the smallest epididymal fat pads (Figure 12D) and these were the most necrotic. Signaling through EP3 is known to promote the production of vascular endothelial growth factor (VEGF) and angiogenesis (571-573). EP3<sup>−/−</sup> might have decreased vascularization leading to ischemia and necrosis in the epididymal fat pads. Expression of *Vegfa* and platelet
Figure 16. Adipose tissue from EP3⁻/⁻ mice fed HFD expresses genes associated with adipocytes
Quantitative real-time RT-PCR analysis of EP3 RNA levels from epididymal fat pads from 20 week old EP3⁺/+ and EP3⁻/⁻ mice fed control or HFD. A. Diet did not have a significant effect on Ptger3 expression in adipose (P = 0.160). B. COX-2 gene (Ptgs2) expression was not affected by dietary fat (P = 0.640) or EP3 genotype (P = 0.386). An interaction between diet and genotype also did not affect Ptgs2 gene expression (P = 0.226). The relationships between Ptgs2 gene expression and body weight are not different in HFD fed mice (P = 0.488). C. CCAAT/enhancer-binding protein β (Cebpb) gene expression was increased in EP3⁻/⁻ mice (P = 0.0007). HFD feeding increased Cebpb expression (P < 0.0001) and interacted synergistically with EP3 genotype (P = 0.0027). Cebpb gene expression was plotted as a function of body weight. The relationships between Cebpb gene expression and body weight are not different in HFD fed mice (P = 0.195). N = 8 EP3⁺/+ control, 10 EP3⁻/⁻ control, 7 EP3⁺/+ HFD, 9 EP3⁻/⁻ HFD. For all figures values are expressed as mean ± SEM.
Figure 17. Epididymal fat pads from EP3−/− HFD fed mice have increased necrosis
A. Representative necrotic sections of H&E stained epididymal fat pads. B. For mice fed a HFD, Epididymal fat pads from EP3−/− mice had more necrosis than EP3+/− (P < 0.0001). No necrosis was observed in epididymal fat pads from control diet fed mice. C. The necrosis score of HFD fed mice was plotted as a function of body weight. A similar correlation between body weight and necrosis score was observed between the EP3+/+ and EP3−/− genotypes (P = 0.1). For all figures N = 9 EP3+/+ control, 10 EP3−− control, 7 EP3+/+ HFD, 9 EP3−− HFD.
endothelial cell adhesion molecule-1 (Pecam1), markers of angiogenesis, were measured by qPCR (Figure 18A,B). Neither Vegfa nor Pecam1 were significantly altered by either EP3 genotype or dietary fat demonstrating that lack of adequate vascularization does not correlate with the observed necrosis.

Necrosis is associated with macrophage accumulation (574). Macrophage infiltration of epididymal fat pads was assessed by qPCR for F4/80 gene expression (Emr1, EGF-like module-containing mucin-like hormone receptor-like 1). F4/80 gene expression demonstrated increased expression in the adipose tissue of EP3−/− mice when fed HFD (Figure 19A). In parallel with necrosis, macrophage infiltration of epididymal fat pads increased with increasing body weight. The slope of F4/80 gene expression versus body weight was increased in EP3−/− HFD fed mice compared to HFD fed EP3+/+ mice and the smallest epididymal fat pads from the heaviest EP3−/− HFD fed mice had the greatest amount of both necrosis and F4/80 gene expression. In the obese state, secretion of adipokines is disrupted with increased secretion of pro-inflammatory, insulin resistance promoting cytokines and decreased secretion of anti-inflammatory, insulin-sensitizing cytokines (575). Increased expression of inflammation-associated adipokine genes was found in these epididymal fat pads; expression of TNF-α, Monocyte Chemoattractant Protein-1 (MCP-1), and IL-6 increased in EP3−/− mice when fed HFD (Figure 19B-D). Gene expression of leptin, which is normally increased during obesity, was also increased in epididymal fat pads from EP3−/− mice (Figure 19E). The higher gene expression combined with increased fat mass, were associated with elevated plasma leptin in EP3−/− HFD fed mice (Table 4). Gene expression of adiponectin, which is an insulin-sensitizing cytokine, was not altered in HFD fed EP3+/+ mice (Figure 19F), consistent with what is seen in HFD fed C57BL/6 mice (576-578). In HFD fed EP3−/− mice, which have increased adiposity and inflammation in adipose tissue, gene expression
Figure 18. Vascularization markers are not changed in EP3<sup>−/−</sup> epididymal fat pads
Quantitative real-time RT-PCR analysis of epididymal fat pad gene expression from 20 week old EP3<sup>+/+</sup> and EP3<sup>−/−</sup> mice fed control or HFD. A. Vascular endothelial growth factor A (Vegfa) gene expression was not affected by HFD feeding (P = 0.277) or EP3 genotype (P = 0.184). No significant effect of interaction between dietary fat and EP3 on Vegfa was found (P = 0.0668). Vegfa gene expression was plotted as a function of body weight. The relationships between Vegfa gene expression and body weight are not different in HFD fed mice (P = 0.290). B. Gene expression of the endothelial cell marker, CD31, platelet endothelial cell adhesion molecule-1 (Pecam1), was not affected by HFD feeding (P = 0.302) or EP3 genotype (P = 0.389). Pecam1 gene expression was plotted as a function of body weight. The relationships between Pecam1 gene expression and body weight are not different in HFD fed mice (P = 0.311). N = 8 EP3<sup>+/+</sup> control, 10 EP3<sup>−/−</sup> control, 7 EP3<sup>+/+</sup> HFD, 9 EP3<sup>−/−</sup> HFD. For all figures values are expressed as mean ± SEM.
Figure 19. EP3−/− HFD fed mice have increased expression of inflammation associated adipokines

hormone receptor-like 1, Emr1), was assessed by qPCR. HFD feeding increased F4/80 expression (P < 0.0001). F4/80 gene expression was increased in EP3−/− mice (P = 0.0003). An interaction between dietary fat and EP3 genotype increased F4/80 gene expression (P = 0.0008). F4/80 gene expression was plotted as a function of body weight. The relationship between F4/80 gene expression and body weight were significantly different in EP3−/− mice (P = 0.0003). B. Tumor necrosis factor α (Tnf) gene expression was increased in EP3−/− mice (P < 0.0001). HFD feeding increased Tnf expression (P < 0.0001) and interacted synergistically with EP3 genotype (P = 0.0023). Tnf gene expression was plotted as a function of body weight. The relationship between Tnf gene expression and body weight were not different in HFD fed mice (P = 0.664). C. Monocyte chemotactic protein 1 (MCP-1) gene (Ccl2) expression was measured by qPCR. HFD feeding increased MCP-1 expression (P < 0.0001). F4/80 gene expression was increased in EP3−/− mice (P = 0.0019). An interaction between dietary fat and EP3 genotype increased MCP-1 gene expression (P = 0.0077). MCP-1 gene expression was plotted as a function of body weight. The relationship between MCP-1 gene expression and body weight are not different in HFD fed mice (P = 0.464). D. Interleukin 6 (Il6) gene expression was increased in EP3−/− mice (P = 0.0005). HFD feeding increased Il6 expression (P = 0.0312). EP3 genotype and dietary fat were not found to interact synergistically to affect Il6 expression (P = 0.312). Il6 gene expression was plotted as a function of body weight. The relationship between Il6 gene expression and body weight was not significantly different between EP3−/− and EP3−/− HFD fed mice (P = 0.0559). E. Leptin (Lep) gene expression was increased in EP3−/− mice (P = 0.0212) and by HFD feeding (P < 0.0001). EP3 genotype and dietary fat did not interact synergistically to affect Lep expression (P = 0.688). Lep gene expression was plotted as a function of body weight. The relationships between Lep gene expression and body weight were not different in HFD fed mice (P = 0.103). F. Adiponectin gene (Adipoq) expression was not significantly affected by EP3 genotype (P = 0.0703) or by HFD feeding (P = 0.932). However, an interaction between dietary fat and EP3 genotype decreased Adipoq expression (P = 0.0027). Adipoq gene expression was plotted as a function of body weight. The relationships between Adipoq gene expression and body weight were not different in HFD fed mice (P = 0.294). N = 8 EP3+/+ control, 10 EP3−/− control, 7 EP3+/+ HFD, 9 EP3−/− HFD. For all figures values are expressed as mean ± SEM.
of adiponectin was blunted (Figure 19F). EP3\(^{-/-}\) HFD fed mice have greater amounts of adipose tissue (Figure 12B,D), which in conjunction with the decreased adiponectin gene expression, was manifest by similar plasma levels of adiponectin in the HFD fed EP3\(^{+/+}\) and EP3\(^{-/-}\) mice (Table 4).

**EP3\(^{-/-}\) adipocytes have increased lipolysis**

PGs play an important role in lipolysis (390,401,446), which may also affect adipocyte cell size and epididymal fat pad mass. Adipocytes were isolated from EP3\(^{+/+}\) and EP3\(^{-/-}\) mice and isoproterenol stimulated lipolysis was assessed (Figure 20). Isoproterenol stimulated lipolysis regardless of genotype. PGE\(_2\) inhibited lipolysis in adipocytes isolated from EP3\(^{+/+}\) mice but failed to inhibit lipolysis in adipocytes isolated from EP3\(^{-/-}\) mice or treated with the EP3 antagonist, DG-041. Insulin inhibited lipolysis in both EP3\(^{+/+}\) and EP3\(^{-/-}\) adipocytes indicating that a loss of EP3 does not affect insulin signaling and regulation of lipolysis. We also quantified the PGs in the adipose tissue. We found no significant effect of EP3 genotype or dietary fat on the levels of any of the PGs examined, suggesting that changes in EP3 ligand concentration were not responsible for the observed phenotype (Table 3). Consistent with these data, the expression of the inducible COX-2 gene, Ptgs2, was also unaffected by dietary fat or EP3 genotype (Figure 16B). EP3 receptor expression was also unchanged as the expression of Ptger3 in epididymal fat pads was not affected by HFD feeding (Figure 16A). Taken together, these data are consistent with defects in lipolysis regulation by PGE\(_2\) and lipid storage in EP3\(^{-/-}\) adipocytes by loss of the receptor despite the presence of the cognate ligand.

Because a defect in lipolysis regulation is expected to alter the lipids in the circulatory system, plasma lipids were measured (Table 4). HFD feeding increased plasma triglycerides of
Figure 20. PGE₂ inhibits adipocyte lipolysis through the EP3 receptor
Isoproterenol (ISO) stimulated lipolysis in EP₃⁺/⁺ and EP₃⁻/⁻ adipocytes. PGE₂ inhibited lipolysis in EP₃⁺/⁺ adipocytes but had no effect in EP₃⁻/⁻ adipocytes. In EP₃⁺/⁺ adipocytes treated with the EP3 antagonist, DG-041, PGE₂ was unable to inhibit lipolysis. Insulin inhibited lipolysis in both EP₃⁺/⁺ and EP₃⁻/⁻ adipocytes. All statistics are from unpaired t-tests. † = P < 0.05 vs. EP₃⁺/⁺ + ISO, ‡ = P < 0.05 vs. EP₃⁻/⁻ + ISO. For each group N=3-4. Values are expressed as mean ± SEM.
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Table 3. Prostanoids in epididymal fat pads
Neither dietary fat nor EP3 genotype affected prostaglandin content of epididymal fat pads.
### Table 4. Plasma profiles of EP3⁻/⁻ mice during *ad libitum* feeding

Blood glucose and plasma insulin were obtained from mice via saphenous vein blood draw. C-peptide, leptin, adiponectin, glycerol, alanine aminotransferase (ALT), free fatty acid (FFA), and triglyceride were measured in plasma obtained via cardiac puncture post mortem.
both genotypes when measured during an ad libitum fed state. EP3−/− mice fed HFD displayed a trend toward increased plasma triglycerides and FFA when compared to EP3+/+ mice fed HFD, though neither achieved statistical significance. Plasma free glycerol was unchanged by either dietary fat or EP3 genotype.

**EP3−/− mice have increased ectopic lipid accumulation**

We hypothesized that increased lipolysis and resulting defects in fat pad storage in EP3−/− mice fed HFD, would result in ectopic lipid storage in peripheral tissues. Liver weight was increased in EP3−/− mice when fed HFD, but not control diet (Figure 21A). The increased slope of liver weight versus body weight observed for EP3−/− mice fed HFD as compared to EP3+/+ mice fed HFD (Figure 21B) is consistent with increased ectopic lipid accumulation in HFD fed EP3−/−. Hepatic triglyceride levels were increased in EP3−/− mice fed HFD as compared to EP3+/+ mice fed HFD, with no observed difference between genotypes when fed control diet (Figure 21C). Similarly an increased accumulation of triglycerides was observed in skeletal muscle from EP3−/− mice fed HFD compared to EP3+/+ mice with no difference observed between genotypes fed control diet (Figure 21D). Dietary lipid and EP3 genotype affected the fatty acid composition of hepatic and muscle triglycerides (Tables 5 and 6 respectively). HFD fed EP3−/− mice with smaller epididymal fat pads demonstrated increased hepatic triglycerides indicative of lipid redistribution (Figure 21E). This effect was not seen in EP3+/+ mice. Triglycerides in skeletal muscle did not vary with respect to epididymal fat pad mass in either genotype (Figure 21F).

We expected that lipid redistribution to the liver and subsequent hepatic lipidosis would cause liver damage. We examined the livers histologically using H&E (Figure 21G), and Oil red O (ORO) staining (Figure 21H). Livers from EP3−/− mice fed HFD showed markedly increased levels of steatosis and ORO staining. Steatosis in EP3+/+ mice fed HFD was present but not as
Figure 21. EP3⁻/⁻ mice on HFD develop ectopic lipid accumulation

A. EP3⁻/⁻ mice on HFD had increased liver weight (P = 0.0056). B. Liver weight to body weight ratio was higher in EP3⁻/⁻ mice than EP3⁺/+ (P = 0.0257). C. EP3⁻/⁻ mice on HFD had a higher triglyceride to hepatic tissue ratio, which resulted in increased hepatic triglycerides (P < 0.0001). D. EP3⁻/⁻ mice on HFD had a higher triglyceride to skeletal muscle tissue ratio, which resulted in increased muscle triglycerides (P = 0.0035). E. The relationship between hepatic triglyceride concentration to epididymal fat pad mass differs between EP3⁺/+ and EP3⁻/⁻ mice when fed HFD (P = 0.0059). F. The slope of skeletal muscle triglyceride concentration to epididymal fat pad mass is not different between EP3⁺/+ and EP3⁻/⁻ mice fed HFD (P = 0.612); however, the Y-intercept is changed (P = 0.0297). For A-F, N = 9 EP3⁺/+ control, 10 EP3⁻/⁻ control, 7 EP3⁺/+ HFD, 9 EP3⁻/⁻ HFD. Values are expressed as mean ± SEM. G. H&E staining of livers showed that EP3⁻/⁻ mice fed HFD had increased steatosis. H. Oil Red O staining showed increased lipid staining in livers of EP3⁻/⁻ mice fed HFD. All panels have the same magnification as upper left panel. Images are a representative sample from 3 mice per genotype × diet group, 2 sections per mouse.
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* Bonferroni post-hoc comparison to * in same row P<0.05
** Bonferroni post-hoc comparison to ** in same row P<0.01
*** Bonferroni post-hoc comparison to *** in same row P<0.001

Statistically significant values are highlighted

**Table 5. Fatty acid composition of hepatic triglycerides**

Fatty acid composition of hepatic triglycerides from male EP3\textsuperscript{+/-} and EP3\textsuperscript{-/-} mice, fed either HFD or control diet. N = 9 EP3\textsuperscript{+/-} control, 10 EP3\textsuperscript{-/-} control, 7 EP3\textsuperscript{+/-} HFD, 9 EP3\textsuperscript{-/-} HFD.**
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* Bonferroni post-hoc comparison to * in same row P<0.05  
** Bonferroni post-hoc comparison to ** in same row P<0.01  
*** Bonferroni post-hoc comparison to *** in same row P<0.001  
Statistically significant values are highlighted

**Table 6. Fatty acid composition of skeletal muscle triglycerides**

pronounced as in the EP3\(^{-/-}\) counterparts. When fed control diet, neither genotype displayed hepatic steatosis. Macrophage infiltration of livers was assessed by counting F4/80 positive nuclei (Figure 22A,B) and by qPCR for F4/80 gene expression (Figure 22C). Macrophage infiltration of hepatic tissue was not altered by EP3 genotype or dietary fat. Though EP3\(^{-/-}\) HFD fed livers appear markedly worse than EP3\(^{+/+}\) HFD fed livers, mice from both genotypes showed elevated plasma alanine aminotransferase (Table 4), an enzymatic biomarker of hepatocyte membrane damage, indicating that HFD caused hepatocyte membrane damage in both genotypes though we did not observe fibrosis in the H&E stained sections in either genotype.

_Livers from EP3\(^{-/-}\) mice have modest changes in gene expression_

Hepatosteatosis might also be precipitated by an increase in _de novo_ lipid synthesis, decreased lipid secretion in the form of very-low-density lipoproteins (VLDL), and/or reduced \(\beta\)-oxidation of fatty acids. Genes affecting each of these processes were analyzed by qPCR. We found that EP3\(^{-/-}\) mice have reduced carnitine palmitoyltransferase 1A (liver) (Cpt1a) expression regardless of diet, suggesting that a potential, small decrease in \(\beta\)-oxidation (Figure 23A). Fatty acid synthase (Fasn) was also not significantly affected by EP3 genotype (Figure 23B). Expression of genes involved in biosynthesis of VLDL was also investigated. Apolipoprotein B (ApoB) was reduced in EP3\(^{-/-}\) mice but microsomal triglyceride transfer protein (Mttp) was not significantly affected by EP3 genotype (Figures 23C,D).

_EP3\(^{-/-}\) mice are insulin resistant when fed a HFD_

EP3\(^{+/+}\) or EP3\(^{-/-}\) mice had no differences in basal glucose, insulin levels or insulin resistance when fed a control diet (Table 4, Figure 24). When fed a HFD, fasted EP3\(^{-/-}\) mice were hyperglycemic and had glucose levels comparable to what would be expected for EP3\(^{+/+}\) mice of a similar body weight (Figure 24A). However, during the _ad libitum_ fed state HFD fed EP3\(^{-/-}\)
Figure 22. Macrophage infiltration is not changed in the liver of EP3−/− mice
Macrophage infiltration of tissues was assessed by immunohistochemical staining for the macrophage marker F4/80 and by quantitative real-time RT-PCR of its gene EGF-like module-containing mucin-like hormone receptor-like 1 (Emr1). A. Representative images of F4/80 staining in liver tissue. B. Neither HFD feeding nor EP3 genotype affected the percentage of F4/80 positive cells in liver tissue. C. Emr1 gene expression in liver tissue was not affected by either EP3 genotype (P = 0.675) or HFD feeding (P = 0.381). Images are a representative sample from 3 mice per genotype × diet group. Values are expressed as mean ± SEM.
Figure 23. Decreased *Cpt1a* and *Apob* mRNA expression in EP3<sup>−/−</sup> liver
Quantitative real-time RT-PCR analysis of liver gene expression from 20 week old EP3<sup>+/+</sup> and EP3<sup>−/−</sup> mice fed control or HFD. **A.** Carnitine palmitoyltransferase 1A (liver) (*Cpt1a*) gene expression was reduced in EP3<sup>−/−</sup> mice (P = 0.0470). HFD feeding increased *Cpt1a* expression (P = 0.0154) but did not interact synergistically with EP3 genotype (P = 0.633). **B.** Fatty Acid Synthase (*Fasn*) gene expression was reduced by HFD feeding (P = 0.0209). EP3 genotype did not have a significant effect on *Fasn* expression (P = 0.195). **C.** Apolipoprotein B (*Apob*) gene expression was reduced in EP3<sup>−/−</sup> mice (P < 0.0001). HFD feeding increased *Apob* expression (P = 0.0475) but did not interact synergistically with EP3 genotype (P = 0.351). **D.** Microsomal triglyceride transfer protein (*Mttp*) gene expression was reduced by HFD feeding (P = 0.0446). EP3 genotype did not have a significant effect on *Mttp* expression (P = 0.216). For all figures N = 9 EP3<sup>+/+</sup> control, 10 EP3<sup>−/−</sup> control, 7 EP3<sup>+/+</sup> HFD, 9 EP3<sup>−/−</sup> HFD. Values are expressed as mean ± SEM.
Figure 24. EP3⁻/⁻ mice are hyperglycemic, hyperinsulinemic, and insulin resistant when fed a HFD

The effects of HFD and EP3 genotype on insulin resistance were assessed in 20 week old mice. 

**A.** An interaction between dietary fat and EP3 genotype increased fasting blood glucose (P = 0.0002). A similar correlation between body weight and fasting blood glucose was observed between the EP3⁺/⁺ and EP3⁻/⁻ genotypes for mice fed HFD (P = 0.497). 

**B.** HFD feeding increased 16-hour fasted plasma insulin (P < 0.0001). Fasting plasma insulin was increased in EP3⁻/⁻ mice (P = 0.0005). An interaction between dietary fat and EP3 genotype increased fasting plasma insulin (P = 0.0016). The fasting plasma insulin to body weight ratio was greater in EP3⁻/⁻ mice than EP3⁺/⁺ (P = 0.0296). 

**C.** The homeostatic model assessment of insulin resistance (HOMA-IR), a function of fasting blood glucose and plasma insulin, was used to assess insulin resistance. HFD feeding increased the HOMA-IR (P < 0.0001). Insulin resistance was increased in EP3⁻/⁻ mice (P = 0.0010). An interaction between dietary fat and EP3 genotype increased the HOMA-IR score (P = 0.0014). The HOMA-IR to body weight ratio was greater in EP3⁻/⁻ mice than EP3⁺/⁺ (P = 0.0464). For all figures N = 9 EP3⁺/⁺ control, 10 EP3⁻/⁻ control, 7 EP3⁺/⁺ HFD, 9 EP3⁻/⁻ HFD. Values are expressed as mean ± SEM.
mice had lower blood glucose (Table 4). EP3\textsuperscript{\textasciitilde} HFD fed mice were hyperinsulinemic during both fed and fasted conditions (Table 4, Figure 24B); heavier EP3\textsuperscript{\textasciitilde} mice had higher fasting insulin levels than would be expected of a EP3\textsuperscript{\textasciitilde\textasciitilde} mouse of a similar body weight, assuming a linear relationship between body weight and plasma insulin. Calculation of the homeostatic model assessment of insulin resistance (HOMA-IR) indicated that when fed a HFD, EP3\textsuperscript{\textasciitilde} mice are more insulin resistant than EP3\textsuperscript{\textasciitilde\textasciitilde} and that the heaviest EP3\textsuperscript{\textasciitilde} mice were significantly more insulin resistant than would be expected of a EP3\textsuperscript{\textasciitilde\textasciitilde} mouse of a similar body weight, assuming a linear relationship between body weight and HOMA-IR (Figure 24C).

The intraperitoneal ITT is a standard measure of insulin resistance employed in rodents. ITT performed on EP3\textsuperscript{\textasciitilde} HFD fed mice are consistent with insulin resistance in 20 week old animals (Figure 25B). However, these data are difficult to interpret for three reasons: 1) insulin has a half-life of 10 minutes while plasma glucose concentrations during the ITT were measured for 120 minutes 2) the dosing of insulin was based upon body weight, which is greater for EP3\textsuperscript{\textasciitilde}, instead of muscle mass, which was similar 3) fasting glucose concentrations were different in the EP3\textsuperscript{\textasciitilde} HFD fed mice (579).

\textit{EP3\textsuperscript{\textasciitilde} mice do not have capillary rarefaction}

Capillary rarefaction, lessening of the density of capillaries, has been previously shown to cause insulin resistance in mice containing a muscle-specific deletion of the vascular endothelial growth factor (VEGF) (580). EP3 is known to be a positive regulator of VEGF and angiogenesis (571-573,581). Skeletal muscle capillary density was measured to determine if capillary rarefaction was present in EP3\textsuperscript{\textasciitilde} mice potentially contributing to the insulin resistant phenotype of these mice when fed HFD (Figure 26). EP3\textsuperscript{\textasciitilde} mice exhibited no differences in skeletal muscle capillary density regardless of dietary treatment.
Figure 25. Insulin tolerance test of EP3−/− mice
Figure 26. EP3⁻/⁻ mice do not have differences in skeletal muscle capillary density

A. Capillary density in skeletal muscle was assessed by CD31 (PECAM) staining. Neither EP3 genotype (P = 0.675) nor HFD feeding (P = 0.598) affected capillary density. N = 3 mice per group. Values are expressed as mean ± SEM. B. Representative images of CD31 staining for each group.
EP3\(^{-/-}\) mice are not glucose intolerant and do not have increased insulin secretion

An IP-GTT was used to assess glucose handling and in vivo insulin secretion. At eight weeks of age, having been on the control or HFD for four weeks, no difference in IP-GTT was observed between any of the groups (Figure 27A). When assessed at 20 weeks of age, HFD impaired glucose handling, although no effect of EP3 genotype was observed (Figure 27B). All groups of mice exhibited increased plasma insulin in response to glucose challenge during the IP-GTT (Figure 27C). When fed HFD, EP3\(^{-/-}\) mice had elevated insulin levels compared to EP3\(^{+/+}\). In contrast when fed a control diet, plasma insulin levels were unaffected by genotype. For mice fed HFD, the average peak insulin levels were found at 15 minutes post-glucose injection and were significantly higher for the EP3\(^{-/-}\) (0.96±0.29 ng/mL, EP3\(^{+/+}\) vs. 2.61±0.34 ng/mL, EP3\(^{-/-}\)). The change between peak and baseline fasting insulin was not significantly different between any groups. C-peptide levels were also not significantly changed in EP3\(^{-/-}\) HFD fed mice when measured during ad libitum feeding (Table 4).

EP3 blockade reduces glucose stimulated insulin secretion in isles from HFD fed mice

Previous studies have shown that EP3 blockade improves insulin secretion in islets isolated from diabetic mice and humans (74). The effect of EP3 antagonist on GSIS in a separate cohort of EP3\(^{+/+}\) mice fed control or HFD for seven weeks was investigated. At 9.5 weeks of age, mice fed HFD were heavier than mice fed control diet (Figure 28A). Fasted HFD fed mice were both hyperglycemic and hyperinsulinemic (Figure 28B,C). Calculation of the HOMA-IR indicated that mice from both genotypes are more insulin resistant after 7 weeks of HFD feeding (Figure 28D). Pancreatic islets isolated from these mice were assessed for alterations in GSIS by a static incubation insulin secretion assay. EP3 antagonist, DG-041, decreased GSIS in islets
Glucose tolerance was assessed by an intraperitoneal glucose tolerance test (GTT) in male EP3+/+ and EP3-/- mice, fed either HFD or control diet, A. at 8 weeks of age (N = 10 EP3+/+ control, 11 EP3+/- control, 7 EP3+/- HFD, 9 EP3-/- HFD) and B. 20 weeks of age (N = 9 EP3+/+ control, 10 EP3+/- control, 7 EP3+/- HFD, 9 EP3-/- HFD). HFD feeding worsened glucose homeostasis in 20 week old mice (P = 0.0112), but the effect of EP3 genotype was not significant (P = 0.0778). C. Insulin secretion during the GTT in 20 week old mice revealed that EP3-/- HFD fed mice have elevated plasma insulin in response to glucose challenge (P = 0.0002). The EP3 genotype had no effect on plasma insulin levels in mice fed control diets (P = 0.991). Data from B and C at time 0 are the same data that are show in 2A and 2B, respectively. For all figures N = 9 EP3+/+ control, 10 EP3+/- control, 7 EP3+/- HFD, 9 EP3-/- HFD. Values are expressed as mean ± SEM.
Figure 28. EP3 antagonist reduces glucose stimulated insulin secretion in mice fed control diet
A separate cohort of EP3+/− mice was fed control or HFD from 3.5 to 10.5 weeks of age. A. Mice fed HFD showed a trend toward increased body weight throughout the study (P = 0.056) and were significantly heavier at 9.5 weeks of age (P = 0.0053). N = 6 each group. At 10 weeks of age, 6-hour fasting B. blood glucose and C. plasma insulin were found to be elevated in mice fed HFD (P = 0.0118 and P = 0.0141, respectively). D. The HOMA-IR showed that mice fed HFD were insulin resistant (P = 0.0133). For A-C, statistical significance was determined by an unpaired t-test. E. Static incubation insulin secretion from islets from these mice revealed that EP3 antagonist DG-041 reduced GSIS in islets from mice fed control diet (P < 0.05). F. HFD feeding increased glucagon secretion from these islets (P = 0.0182). G. PGE₂ measured in the culture media following the static incubation assay was not found to be different with respect to diet or genotype. For E-G, statistical significance was determined by a series of 2-way ANOVAs comparing diet and [glucose] keeping drug constant or comparing diet and drug keeping [glucose] constant. For B-G, N = 5 mice for each diet. Values are expressed as mean ± SEM.
isolated from control diet fed mice but had no effect on islets isolated from mice fed HFD (Figure 28E). The islets from control diet fed mice without DG-041 treatment demonstrated more GSIS than islets from HFD fed mice. Because these results are in direct contrast with prior studies (74), glucagon and PGE₂ release from these islets was measured. Glucose concentrations and DG-041 treatment did not affect glucagon secretion; however, islets from HFD fed mice had higher glucagon release (Figure 28F). The amount of PGE₂ in the media at the end of the experiment was unaffected by the animals’ diet, glucose concentration, or DG-041 treatment (Figure 28G).

*EP₃⁻/⁻ mice have lower blood pressure*

Blood pressure in EP₃⁻/⁻ mice fed control and HFD was measured because it is a component of metabolic syndrome (582). EP₃⁻/⁻ mice were found to have lower systolic blood pressure regardless of dietary treatment (Figure 29).

**Discussion**

In the present studies, we found that EP₃⁻/⁻ mice when faced with a nutritional challenge are prone to obesity and dysregulation of adipocyte function. These findings show a novel interaction between EP3 genotype and dietary fat content. The effects of EP3 gene deletion are pleiotropic with changes observed in activity, body mass, inflammation and adipocyte function. We demonstrate here that the obesity phenotype is not penetrant when EP₃⁻/⁻ mice are maintained on either normal chow diet or on control diet; however, EP₃⁻/⁻ mice fed a HFD become more obese. These findings are consistent with previous studies that EP₃⁻/⁻ mice are obese, hyperleptinemic, insulin resistant and have impaired glucose homeostasis when fed breeder chow (102). EP₃⁻/⁻ mice were not hyperphagic but did show a significant reduction in activity, which may contribute to the increased body mass of these mice. This is significantly
Figure 29. Decreased blood pressure in EP3⁻/⁻ mice
Blood pressure was measured in 20 week old mice after 16 weeks of control or HFD feeding. EP3⁻/⁻ mice had lower blood pressure (effect of genotype: $P = 0.0400$). $N = 3$ EP3⁺/+ control, 4 EP3⁺/+ HFD, 4 EP3⁻/⁻ HFD. Values are expressed as mean ± SEM.
different from previous studies, which showed a very modest increase in food intake and an increase in activity (102). Moreover those studies showed an increase in activity in the light cycle, whereas we observed no changes in activity in the light cycle, but a decrease in activity in the dark cycle. These observed differences may be due to differences in experimental conditions such as the diet (breeder chow vs. HFD) or the methodology used to collect the activity data (surgical radiotelemetry implantation vs. non-invasive “beambreak”). In any event, we do not observe an increase in food consumption to account for the obesity, and we do observe decreases in activity consistent with the obesity phenotype observed.

The EP3<sup>−/−</sup> mice demonstrate ectopic lipid accumulation and insulin resistance similar to the phenotype of AdPLA<sup>−/−</sup> mice, which lack PGE<sub>2</sub> – EP3 signaling in adipose tissue (101). Importantly, the phenotype of EP3<sup>−/−</sup> mice differs from that of AdPLA<sup>−/−</sup> mice in that EP3<sup>−/−</sup> mice are obese and have more adipose tissue whereas AdPLA<sup>−/−</sup> mice are lean. This suggests that the increased obesity and overall adiposity in EP3<sup>−/−</sup> HFD fed mice are due to a lack of EP3 signaling in non-adipose sites of action (e.g. central nervous system), in contrast to AdPLA<sup>−/−</sup> mice, which presumably have reduced EP3 signaling only in adipose tissue. It is our hypothesis that deletion of the EP3 receptor in a number of tissues contributes to the overall increased obesity, fat mass, and adipocyte size; changes in the adipose tissue of EP3<sup>−/−</sup> mice, including increased inflammation, cell death, and lipolysis, contribute to the proportional decrease of epididymal fat pad mass and adipocyte size and the increased ectopic lipid accumulation. PGs have been shown to play an important role in adipocyte function (390). Norepinephrine signaling in adipose tissue increases intracellular cAMP and thereby activates PKA, which phosphorylates and activates HSL, the rate-limiting step in lipolysis (401,403-405). PGE<sub>2</sub> inhibits norepinephrine-stimulated lipolysis (92,393,411,412,417-419). Adipocytes from EP3<sup>−/−</sup> mice had a loss of PGE<sub>2</sub>-evoked
inhibition of lipolysis similar to that observed in AdPLA<sup>−/−</sup> mice. Insulin is also a potent suppressor of lipolysis in adipose tissue. We did not observe a statistically significant difference in either plasma triglyceride or free fatty acid levels, even though HFD fed EP3<sup>−/−</sup> mice had elevated plasma insulin. The failure of the elevated insulin to suppress free fatty acid levels indicates that the adipose tissue from in HFD fed EP3<sup>−/−</sup> mice is insulin resistant. In addition, significant accumulation of triglycerides in liver and skeletal muscle were observed in these mice and are further indications of persistent, chronic dyslipidemia and adipocyte dysfunction. It is of interest that while we observed an increase in macrophage infiltration and inflammatory cytokine levels in adipose tissue of the EP3<sup>−/−</sup> mice, these changes were not observed in the fat pads of AdPLA<sup>−/−</sup> mice (101), suggesting that unrestrained lipolysis alone is sufficient to contribute to ectopic lipid accumulation.

Increased necrosis was observed in parallel with increased macrophage infiltration in epididymal fat pads of EP3<sup>−/−</sup> HFD fed mice. Adipocyte cell death and macrophage infiltration may also result in the release of lipids from adipocytes and may exacerbate ectopic triglyceride accumulation (583-585). Previous studies demonstrated that when wild type C57BL/6 mice are fed a 60% HFD adipocyte death in epididymal fat pads results in decreased adipocyte size, lighter epididymal fat pads, increased macrophage infiltration, increased liver mass and steatosis, and insulin resistance (584). This study noted a similar correlation to what we have observed; epididymal fat pad weight negatively correlated with body weight in the heaviest mice in the group. In addition, Strissel, et al. found that the mice with the lightest epididymal fat pads had increased liver weight suggesting lipid redistribution from the epididymal fat pads to ectopic tissues, which likely contributed to insulin resistance (584). Data presented here are consistent with this phenotype and suggest that the deletion of the EP3 receptor may exacerbate or
accelerate this adipose tissue remodeling and resultant ectopic lipid accumulation. Thus increased lipolysis coupled with increased adipocyte cell death in the heaviest mice, as evidenced by the disproportionately increased macrophage infiltration, accounts for the reduced epididymal fat pad mass and the severe increase in hepatic triglycerides in the heaviest EP3\(^{-/-}\) mice.

Although we saw an increase in circulating insulin levels in the obese, insulin resistant EP3\(^{-/-}\) mice, we did not observe a corresponding increase in GSIS in isolated pancreatic islets. This difference may be partly attributed to normalization of the GSIS to insulin content in perifusion studies, implying loss of EP3 did not alter intrinsic islet function. Because basal insulin and glucose levels were increased in obese EP3\(^{-/-}\) mice the hyperglycemia combined with other factors such as increased \(\beta\)-cell mass and/or altered autonomic tone to the islet may have sustained insulin secretion. Alternatively, increased insulin resistance results in increased plasma insulin due to reduced insulin clearance by the liver. Obese EP3\(^{-/-}\) mice were not glucose intolerant, thus the underlying hyperinsulinemia was critical in maintaining normal glucose tolerance despite concomitant insulin resistance.

It has long been appreciated that \(\text{PGE}_2\) modulates GSIS and has been suggested that blockade of the EP3 receptor would improve insulin secretion (197). Studies have indicated that pharmacological blockade of the EP3 receptor in pancreatic islets improves GSIS (74,233). In our studies we found no evidence suggesting improved GSIS in either chow fed (Chapter II) or control and HFD fed animals. It should be noted that previous studies showing GSIS differences in human islets only observed differences in EP3 mediated GSIS in islets isolated from diabetic individuals; no differences in GSIS were observed upon antagonist treatment of islets from non-diabetic individuals (74).
In summary, the EP3−/− mice are sensitive to caloric overload and display increased obesity, lipolysis, and adipocyte cell death, which is accompanied by reallocation of lipid storage from fat to ectopic tissues, leading to hepatic lipidosis and insulin resistance. These phenotypes are not penetrant when mice are fed control or chow diets, which contain less dietary fat. These studies demonstrate that EP3 is an important player in adipose tissue physiology.
CHAPTER IV
THE PGE\textsubscript{2} EP3 RECEPTOR REGULATES DIET-INDUCED ADIPOSITY IN 36 WEEK OLD MALE MICE

Introduction

The prevalence of diabetes increases significantly with age and affects approximately 18-30\% of the elderly population in the United States (586-590). Type 2 diabetes is the most prevalent form of diabetes and has the highest economic burden of $159.5 billion per year (9). Type 2 diabetes results from a reduction in both insulin sensitivity and $\beta$-cell function. Aging populations are characterized by both declining $\beta$-cell function and insulin sensitivity (591,592). This includes a decreased responsiveness of $\beta$-cells to changes in glucose concentrations (592). Understanding the mechanisms of insulin secretion by $\beta$-cells will provide therapeutic targets to pharmacologically regulate these, improving $\beta$-cell function and ameliorating Type 2 diabetes.

Treating diabetes in the elderly is challenging for a number of reasons. Oftentimes elderly patients suffer multiple conditions, hence polypharmacy is common (593). In addition, elderly patients frequently suffer from cognitive or physical disorders that limit the ability to manage their disease (594,595). Furthermore, clinical presentation and symptoms of diabetes can be altered by age making it difficult to diagnose diabetes in the elderly (594). Drugs which boost a patient’s own glucose stimulated insulin secretion (GSIS), such as exendin-4, a glucagon like peptide-1 (GLP-1) receptor agonist, are particularly useful because they augment a patient’s insulin secretion in response to blood glucose removing the need to tightly regulate meals and injections (594). Drugs with similar mechanisms of action would also benefit elderly diabetic patients, especially if they can be taken orally.

Many nonsteroidal anti-inflammatory drugs (NSAIDS), such as aspirin, have long been known to increase GSIS (50,57,58,105,106). Therapeutically beneficial concentrations cause
adverse side effects and investigations into these drugs as therapies for diabetes were abandoned (80). NSAIDS act by inhibiting cyclooxygenase (COX) enzymes. COX converts arachadonic acid to PGH$_2$, which is converted into five primary bioactive prostanoids: PGE$_2$, PGF$_{2\alpha}$, PGD$_2$, PGI$_2$ (prostacyclin), and TXA$_2$ (thromboxane). The local action of PGs depends on activation of a family of specific GPCRs designated EP for E-prostanoid receptors, FP, DP, IP and TP receptors, for the other PGs, respectively (43,107). PGD$_2$, PGE$_2$, and PGF$_{2\alpha}$ are produced in pancreatic islets in response to glucose and may function as a way to fine-tune GSIS (74,169,172,174,175,177,178,181). PGD$_2$ and PGF$_{2\alpha}$ are typically associated with increasing insulin release, while PGE$_2$ has been published to inhibit insulin release (71,74,177,183-197). COX inhibition is hypothesized to increase GSIS by reducing PGE$_2$ levels (50,105,172).

Expression of EP receptors has been observed in the mouse (181,235) and human pancreas (236), human pancreatic stellate cells (237), in human (221), mouse (74,181), rat (196,197,238), and guinea pig islets (71), mouse β-cells (239), in HIT and Min6 β-cell lines (71,74,227,240), 832/13 rat insulinoma cell line (238), and in the α-cell line, αTC1 (74). These aforementioned gene expression studies suggest that EP3, the G$_{\alpha_i}$ coupled receptor, has the highest expression of the four EP receptors in islets. Expression of all three EP3 splice variants and PGE$_2$ production from islets are increased in diabetes (74). EP3, the G$_{\alpha_i}$ coupled EP receptor, has been hypothesized to be responsible for mediating PGE$_2$’s inhibition of GSIS as prior studies have shown that EP3 agonists decrease GSIS which can be ameliorated by an EP3 antagonist (71,74,194,197,233). However, EP3 blockade and gene knockout were not found to affect GSIS in my hands (Chapter II, Chapter III). PGE$_2$ is also an important inflammatory mediatory with the EP3 receptor evoked response mediating interleukin-1β (IL-1β), and lipopolysaccharide (LPS) induced febrile response (310). Inflammatory cytokines increase with age and a low level
state of inflammation is associated with diabetes (24,25). Blockade of PGE₂-EP₃ signaling would be anti-inflammatory and may have salutary effects on diabetes further augmenting GSIS by lessening the auto-inhibitory response of islet-derived PGs in response to glucose.

I hypothesized that EP₃⁻/⁻ mice have enhanced GSIS that led to the improvement in glucose homeostasis. I have shown that EP₃⁻/⁻ mice have improved glucose homeostasis at 40 weeks of age when they are fed a standard chow diet (Chapter II). This effect is not seen at 20 weeks of age when both genotypes show a similar response to an IP-GTT. Only the 40 week old EP₃⁻/⁻ mice manifested an improvement in glucose homeostasis because improvements in β-cell function may not significantly affect glucose homeostasis in younger animals that already have proper glycemic control. I hypothesized that in a setting of diet induced type 2 diabetes, that aged EP₃⁻/⁻ mice would similarly show an improvement in glycemic control.

Young EP₃⁻/⁻ mice become obese and insulin resistant when fed a HFD but maintained similar body weights as compared to EP₃⁺/⁺ when fed a lower fat control diet (Chapter III). EP₃⁻/⁻ mice on HFD began to diverge from EP₃⁺/⁺ at approximately 12 weeks of age when they were shown to have an increased percentage of body fat as measured by NMR. The body weight of these HFD fed mice began to diverge soon after. In order to assess the effects of HFD in older mice, EP₃⁺/⁺ and EP₃⁻/⁻ were fed the same control (10% calories from fat) and HFD (45% calories from fat) for 16 weeks diets beginning at 20 weeks of age.

Methods

Animal procedures & high fat diet feeding

Mice utilized for these experiments were maintained as previously described (Chapter II). Beginning at age 20 weeks, male C57BL/6 EP₃⁺/⁺ or EP₃⁻/⁻ mice were fed a control diet (10% calories from fat; 4.3% fat by weight, D12450Bi, Research Diets, New Brunswick, NJ) or high
fat diet (HFD) (45% calories from fat; 24% fat by weight, D12451i, Research Diets) for 16 weeks. All procedures were approved by the Institutional Animal Care and Use Committee at Vanderbilt University.

*Energy balance*

A separate cohort of mice utilized for energy balance studies were fed HFD beginning at ~17 weeks of age for 10.5 weeks until ~28 weeks of age. Energy balance studies were performed in a Promethion system (Sable Systems International, North Las Vegas, NV) for five days by the Vanderbilt Mouse Metabolic Phenotyping Center.

*Tissue fatty acid composition*

Triglycerides were extracted from ~100 mg flash frozen liver by the Mouse Metabolic Phenotyping Center Lipid Lab as previously described (Chapter III).

*Histology*

Livers from 3 mice per genotype × diet treatment were fixed in 10% formaldehyde overnight at 4°C and subsequently stored in 70% ethanol at 4°C prior to paraffin embedding and hematoxylin and eosin (H&E) staining. Liver samples from 3 mice per genotype × diet treatment for were fixed by freezing in OCT for Oil Red O (ORO) staining. All slides were imaged with a 20× objective using an Olympus BX51 microscope. Histology was performed by the Vanderbilt Translational Pathology Shared Resource.

*Intraperitoneal glucose tolerance tests*

IP-GTT was performed on male C57BL/6 EP3+/+ or EP3−/− mice as previously described (Chapter II).
Intraperitoneal insulin tolerance tests

Male EP3\(^{+/+}\) or EP3\(^{-/-}\) mice were fasted for 6 hours during the light cycle and fasting blood glucose level were measured with the Accu-Check Aviva blood glucose meter using blood obtained from the tail. Following intraperitoneal injection of 34.1 µg/kg insulin (human recombinant insulin, Sigma-I9278), for 24 week old mice, or 0.75 U/kg insulin (human recombinant insulin, Novolin R, Novo Nordisk, Princeton, NJ), for 36 week old mice, in 0.9% normal saline, blood glucose levels were measured at 15, 30, 60, 90, and 120 minutes post-injection.

Mouse islet perifusion

Pancreatic islets were isolated from male C57BL/6 EP3\(^{+/+}\) or EP3\(^{-/-}\) mice and perifusion assays were performed on fresh islets at the Vanderbilt Islet Procurement & Analysis Core as previously described (Chapter II). Mice utilized for islet perifusion experiment were 37 to 39.5 weeks old and had been fed HFD beginning around 17 weeks of age for 20.5 to 21.5 weeks.

Total Pancreatic Insulin

Insulin was extracted from whole pancreata. Excised pancreata were placed in 5 ml of acid-ethanol (1.8% 10N HCl in 70% ethanol) and frozen overnight at -20°C. Tissues were homogenized with a Tissue Master 240 (OMNI International, Kennesaw, GA) and frozen overnight at -20°C. Samples were centrifuged for 30 minutes at 2000 RPM in an Allegra 21 (Beckman Coulter, Brea, CA) and supernatant was transferred to a new tube. Supernatant was frozen overnight at -20°C and homogenization and centrifugation were repeated. Supernatants were normalized to 15 ml per sample with acid-ethanol. Insulin content was analyzed in duplicate by radioimmunoassay by Vanderbilt University Hormone Assay & Analytical Services Core.
Statistics

Statistics were performed as previously described (Chapter III).

Results

Body composition and energy balance of EP3<sup>−/−</sup> mice

To test the hypothesis that glucose handling in EP3<sup>−/−</sup> mice is more sensitive to diet than observed for EP3<sup>+/+</sup> mice, EP3<sup>+/+</sup> or EP3<sup>−/−</sup> males were fed either HFD (45% calories from fat) or a micronutrient matched control diet (10% calories from fat). Body weight in both EP3<sup>+/+</sup> and EP3<sup>−/−</sup> HFD fed mice increased at a greater rate than in animals fed control diet, with a divergence in body weight becoming apparent by 4 weeks of matched diet feeding (P = 0.0007; Figure 30A). Moreover, by 8 weeks after beginning the HFD, EP3<sup>−/−</sup> mice were heavier than EP3<sup>+/+</sup> animals (P = 0.0296; adjusted P-value Bonferroni post hoc test). Body composition analyses throughout the experimental time period revealed that lean body mass was affected by the EP3 genotype and age when fed a HFD (P = 0.0487, effect of interaction between genotype and age for HFD fed mice only; Figure 30B). Increased weight gain in EP3<sup>−/−</sup> mice on HFD coincided with increased fat mass, though this was not statistically significant (2-way ANOVA P = 0.0904; Figure 30C). Post-mortem analysis of epididymal fat pads confirmed that HFD feeding increased fat mass (P = 0.0008) but was not significantly altered by EP3 genotype (P = 0.746; Figure 30D). This test was underpowered and therefore unable to detect an effect of EP3 genotype on epididymal fat pad mass (power ≈ 0.05). Energy balance studies of HFD fed mice showed that EP3<sup>−/−</sup> mice do not have differences in food consumption (Figure 31B). EP3<sup>−/−</sup> mice did not have a significant change in movement (Figure 31C), but spent significantly more time inactive during the dark cycle (Figure 31D).
Figure 30. EP3−/− mice are obese when fed a HFD
A. Male EP3+/+ and EP3−/− mice fed control diet or HFD were weighed weekly from age 20 weeks to age 36 weeks. No difference in body weight was observed between genotypes in the control fed animals. EP3−/− mice fed HFD were significantly heavier than EP3+/+ animals beginning at 28 weeks of age. Body composition of B. lean and C. fat were assessed at four week intervals by pulsed NMR. For A-C N = 3 EP3+/+ control, 9 EP3−/− control, 7 EP3+/+ HFD, 7 EP3−/− HFD, values are expressed as mean ± SEM. D. Epididymal fat pads were weighed post mortem at the conclusion of the study. N = 3 EP3+/+ control, 4 EP3−/− control, 2 EP3+/+ HFD, 7 EP3−/− HFD. Values are expressed as mean ± StDev.
Figure 31. EP3$^{-/-}$ mice spend more time still during the dark cycle

A. Male EP3$^{+/+}$ and EP3$^{-/-}$ mice were fed HFD for 10.5 weeks between the ages of 16.5 weeks of age and 29 weeks of age. B. Total food intake was measured in these mice. Mice consumed more food during the dark cycle ($P = 0.0055$), but no significant difference between EP3 genotypes ($P = 0.952$) or interaction between light cycle and genotype ($P = 0.426$) were observed. C. Total movement was measured in the same mice. All mice were found to move more during the dark cycle ($P = 0.0029$). D. Mice were still for a greater amount of their time in the light cycle ($P = 0.0223$). EP3$^{-/-}$ mice were found to have a greater proportion of still time than EP3$^{+/+}$ mice during the dark cycle. For all figures N = 3 EP3$^{+/+}$ HFD, 3 EP3$^{-/-}$ HFD. All values are expressed as mean ± SEM.
Hepatic lipidosis in EP3\textsuperscript{−−} mice

Liver weight was increased in EP3\textsuperscript{−−} mice (P = 0.0145, effect of genotype) and by HFD feeding (P = 0.0164, effect of diet; Figure 32A). Hepatic triglyceride levels were also increased by HFD feeding (P = 0.0344, effect of diet) with hepatic triglycerides in EP3\textsuperscript{−−} mice increased when fed HFD (P = 0.0497, effect of interaction; Figure 32B). Dietary lipid and EP3 genotype affected the fatty acid composition of the hepatic triglycerides (Table 7). We observed steatosis in the livers histologically using H&E (Figure 32C), and Oil red O (ORO) staining (Figure 32D). Livers from EP3\textsuperscript{−−} mice fed HFD showed markedly increased levels of steatosis and ORO staining. Steatosis in EP3\textsuperscript{+/−} mice fed HFD was present but not as pronounced as in the EP3\textsuperscript{−−} counterparts. When fed control diet, neither genotype displayed significant hepatic steatosis.

Glycemic control in EP3\textsuperscript{−−} mice

Glucose handling was assessed by IP-GTT. At 24 weeks of age, having been on the control or HFD for four weeks, no difference in IP-GTT was observed between any of the groups (Figure 33A). When assessed at 36 weeks of age, animals fed HFD demonstrated impaired glucose handling (Figure 33B, P = 0.0171), although an effect on genotype was not observed (P = 0.0706). EP3\textsuperscript{−−} HFD fed mice had elevated blood glucose at the start of the IP-GTT (P = 0.0228) that reduced the ability to detect changes in AUC. ITT performed on animals fed HFD showed that they were insulin resistant at 36 weeks of age independent of genotype. EP3\textsuperscript{−−} mice appear to have decreased insulin sensitivity (Figure 33D), although these data are difficult to interpret due to the short half-life of insulin injected (10 minutes) compared to the long time period analyzed for the ITT (579). Interpretation of the response of EP3\textsuperscript{−−} mice to exogenous insulin administration is further complicated because EP3\textsuperscript{−−} HFD fed mice showed elevated 6-hour fasting insulin (P < 0.0001). Exogenous insulin administration to EP3\textsuperscript{−−} HFD fed mice
Figure 32. EP3−/− mice fed HFD develop hepatic steatosis
A. EP3−/− mice on HFD had increased liver weight. B. EP3−/− mice on HFD had increased hepatic triglycerides. C. H&E staining of livers showed that EP3−/− mice fed HFD had increased steatosis. D. Oil Red O staining showed increased lipid staining in livers of EP3−/− mice fed HFD.

2-3 mice per genotype × diet group, 2 sections per mouse. For all figures N = 3 EP3+/+ control, 4 EP3−/− control, 2 EP3+/+ HFD, 7 EP3−/− HFD. All values are expressed as mean ± StDev.
### Table 7. Fatty acid composition of hepatic triglycerides


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* Bonferroni post-hoc comparison to * in same row P<0.05

** Bonferroni post-hoc comparison to ** in same row P<0.01

*** Bonferroni post-hoc comparison to *** in same row P<0.001

Statistically significant values are highlighted
Figure 33. EP3<sup>−/−</sup> mice have elevated blood glucose
Glucose tolerance was determined in male EP3<sup>+/+</sup> and EP3<sup>−/−</sup> mice, fed either HFD or control diet. A. At 24 weeks of age neither HFD (P = 0.904) nor EP3 genotype (P = 0.342) affected glucose homeostasis. B. At 36 weeks of age HFD impaired the response to glucose challenge (P = 0.0171). Neither EP3 genotype (P = 0.0706) nor an interaction between EP3 genotype and dietary fat (P = 0.8148) affected response to glucose challenge. Insulin tolerance was determined in male EP3<sup>+/+</sup> and EP3<sup>−/−</sup> mice, fed either HFD or control diet, C. (C') at 24 weeks of age and D. (D') 36 weeks of age. At both ages EP3<sup>−/−</sup> HFD fed mice had elevated blood glucose, as compared to EP3<sup>+/+</sup>, after a 6-hour fast at the beginning of the ITT (P = 0.0031 and 0.0001, respectively). For all figures N = 3 EP3<sup>+/+</sup> control, 9 EP3<sup>−/−</sup> control, 7 EP3<sup>+/+</sup> HFD, 7 EP3<sup>−/−</sup> HFD. All values are expressed as mean ± SEM.
caused a 102 mg/dl drop in blood glucose in the first 30 minutes which is similar to the response observed in EP3+/+ and control diet fed mice. Elevated fasting glucose is an indication of insulin resistance and was found in EP3−/− HFD fed mice after both the 16- and 6-hour fasts.

Pancreatic islet insulin secretion and content in EP3−/− mice

EP3 blockade has been reported to improve GSIS in islets from diabetic mice (74). Pancreatic islets isolated from EP3+/+ and EP3−/− mice fed HFD were assessed for alterations in GSIS by islet perfusion. EP3−/− islets showed impaired GSIS but were unaffected by PGE2 (Figure 34A).

One physiologic response to increasing insulin resistance is an increase in pancreatic β-cell proliferation (596). Moreover, Gaα inhibits β-cell proliferation, possibly through interactions with EP3 (134), thus we examined islet and total pancreatic insulin as an indicator of β-cell mass. Insulin content of islets used for the perfusion experiment was not significantly different (Figure 34B). In addition, no statistically significant effect of EP3 genotype (P = 0.541) or HFD feeding (P = 0.346) on total pancreatic insulin content was found in 36 week old mice (Figure 34C). These tests were underpowered and therefore unable to detect differences in total pancreatic insulin (power ≈ 0.05).

Discussion

Blockade of PGE2 action at the inhibitory EP3 receptor has been hypothesized to be a therapeutic target to improve GSIS, which would be beneficial for the treatment of diabetes. I have previously shown that 40 week old EP3−/− mice have improved glucose homeostasis when fed a standard chow diet (Chapter II). Here, I investigated the effect of HFD feeding in older mice. EP3−/− mice are more prone to developing obesity when fed HFD regardless of whether HFD feeding begins in young (4 weeks) or older (20 weeks) mice. The effects of HFD feeding
Figure 34. Islets from EP3<sup>−/−</sup> HFD fed mice have reduced GSIS
A. Perifusion of islets from HFD fed EP3<sup>+/+</sup> and EP3<sup>−/−</sup> mice showed reduced GSIS in EP3<sup>−/−</sup> HFD fed islets (P = 0.0337). GSIS was unaffected by PGE<sub>2</sub> (P = 0.295). B. Insulin content of islets used for perifusion was not different (P = 0.302). For A&B, N = 3 EP3<sup>+/+</sup> HFD, 3 EP3<sup>−/−</sup> HFD. Values are expressed as ± SEM. C. Measurement of total pancreatic insulin content reveals no differences with respect to either dietary fat or EP3 genotype. For C, N = 2 EP3<sup>+/+</sup> control, 3 EP3<sup>−/−</sup> control, 2 EP3<sup>+/+</sup> HFD, 7 EP3<sup>−/−</sup> HFD. Values are expressed as mean ± StDev.
were apparent more readily after transition to the HFD in older animals. This may be due to the animals being at a mature body weight when the diet treatment began. Dietary fat did not significantly affect body weight in younger mice until approximately 8 weeks of age, when they were near their mature body weight. EP3$^{-/-}$ mice become more obese than EP3$^{+/+}$ only when fed a HFD and then only after they have reached mature body weight.

EP3$^{-/-}$ mice appear to be more insulin resistant than EP3$^{+/+}$ mice when fed HFD. EP3$^{-/-}$ HFD fed mice had elevated glucose throughout the ITT due to an elevated baseline 6-hour fasting glucose. We previously reported that EP3$^{-/-}$ mice become insulin resistant when fed a HFD due to lipid redistribution to ectopic tissues (Chapter III). We observed a similar increase in liver weight and hepatic triglycerides in EP3$^{-/-}$ mice only when fed HFD.

We were not able to detect significant changes in glucose tolerance in part because EP3$^{-/-}$ HFD fed mice had elevated fasting glucose and the Accu-Check Aviva blood glucose meter has a maximum reading of 600 mg/dl, hence higher values were not able to be measured. It is not surprising that HFD fed EP3$^{-/-}$ mice have high glucose during an IP-GTT given that we found decreased GSIS in islets isolated from HFD fed EP3$^{-/-}$ mice. Decreased GSIS from EP3$^{-/-}$ islets is in contrast to a prior report that has shown that EP3 antagonist, L-798,106, raises GSIS in diabetic islets (74). We previously reported that EP3 antagonist, DG-041, lowers GSIS in islets from control diet fed animals (Chapter III). However, our previous study found no effect of DG-041 on GSIS in islets from HFD fed mice (Chapter III). It is possible that off target effects of the EP3 antagonists have lead to some of these discrepancies.

Our results are consistent with an earlier report which showed that EP3$^{-/-}$ mice fed a breeder chow become more glucose intolerant and insulin resistant as they age (102). These results are also consistent with our previous report of glucose handling in younger EP3$^{-/-}$ HFD
fed mice (Chapter III). In both studies, diet affected glucose homeostasis, but the effect of the EP3 genotype was not statistically significant.

These data show that obesity and related phenotypes become worse with age in EP3−/− mice when fed a HFD. The phenotypes are remarkably similar to what we have reported in younger mice of the same genotypes fed the same diets (Chapter III). Older EP3−/− mice fed HFD tended to have more obesity, worse insulin sensitivity, and poorer glucose homeostasis. Furthermore, we observed no improvement in glucose homeostasis in control diet fed animals which is in contrast to our prior report of improved glucose homeostasis in EP3−/− mice fed a standard chow diet.
CHAPTER V
THE EFFECT OF THE EP3 ANTAGONIST DG-041 ON MALE MICE WITH DIET-INDUCED OBESITY

Introduction

EP3$^{-/-}$ mice have been shown to be more susceptible to diet induced obesity (Chapter III, IV). The increased obesity in EP3$^{-/-}$ mice is the result of an interaction between the EP3 genotype and dietary fat, with EP3$^{-/-}$ mice becoming obese only on a HFD but having normal body weights and phenotypes on control or chow diets. In addition to becoming more obese, EP3$^{-/-}$ mice have increased lipolysis and adipocyte necrosis causing a redistribution of lipid to ectopic tissues resulting in insulin resistance (Chapter III).

These effects of EP3 on the metabolic phenotype were noted in a model of chronic EP3 disruption, utilizing mice that had a global EP3 gene disruption since birth. EP3 is expressed during gestation (597); hence, it is possible that some of the observed phenotypes may be in part due to the lack of EP3 during gestation and the early stages of growth. Maternal obesity and over-nutrition predispose offspring to obesity (598); hence, it is possible that EP3 loss in dams may contribute to the predisposition of obesity observed in their pups. Furthermore, PGE$_2$ is known to play an inhibitory role in adipocyte development inhibiting adipogenesis through the EP4 receptor (324,335,352,364).

In order to examine the effects of acute inhibition of EP3 on metabolic phenotype, EP3 antagonist DG-041 (556) was administered to wild-type mice for one week and changes in body weight, insulin sensitivity, and ectopic lipid distribution were assessed. DG-041 administration caused a reduction in skeletal muscle triglyceride content while showing a trend toward increased hepatic triglycerides which is constant with inhibition of hepatic lipolysis.
Materials & Methods

Mice

All mice were obtained from Jackson Labs. For pharmacokinetics studies, approximately 16 week old, 28 gram male C57BL/6 were utilized. Mice utilized for diet induced obesity studies were male F1 progeny of male C57BL/6 crossed with female Balb/c mice (Jackson Labs #100007). For diet studies mice were housed at Vanderbilt for three days on a standard chow diet (Laboratory Rodent Diet 5001, LabDiet) after which time they were fed either control (D12450Bi, Research Diets), HFD (D12451i, Research Diets), or very high fat diet (VHFD) (60% calories from fat; 34.9% fat by weight, D12492i, Research Diets). After 17 weeks of defined diet feeding, mice were injected with 20 mg/kg DG-041 or vehicle twice daily for one week. Mice were weighed weekly and body composition was assessed every four weeks by pulsed NMR (Bruker Intraments, The Woodlands, TX) at the Vanderbilt Mouse Metabolic Phenotyping Center. At the termination of the experiment, mice were euthanized by isoflurane overdose and liver, epididymal fat pads, and skeletal muscle were dissected.

In vivo pharmacokinetics

DG-041 was dissolved in DMSO, and was then diluted to one part DMSO+DG-041, six parts PEG400, and 3 parts 0.9% saline. Vehicle made of one part DMSO, six parts PEG400, and 3 parts 0.9% saline was used as the control. 20 mg/kg DG-041 was administered by subcutaneous injection. Plasma was collected by sapheneous vein blood draw into EDTA coated tubes (Sarstedt, Nümbrecht, Germany) with the final blood draw being obtained by cardiac puncture in euthanized mice. Plasma was stored at -80 °C until DG-041 was measured by HPLC/MS/MS analysis as previously described (599).
Assessment of insulin resistance

Mice were fasted for 6 hours during the light cycle. EDTA plasma was collected by sapheneous vein blood draw and insulin was quantified by RIA at the Vanderbilt Hormone Assay Core. While collecting blood for plasma, blood glucose was measured in excess sapheneous vein blood with an Accu-Check Aviva glucometer and glucose test strips (Roche Diagnostics). HOMA-IR was calculated as the fasting insulin level (µU/mL) × blood glucose level (mg/dL) / 405.

Histology

Livers from all mice were fixed, sectioned, and imaged as previously described (Chapter IV).

Tissue fatty acid composition

Triglycerides were extracted from ~100 mg flash frozen liver by the Mouse Metabolic Phenotyping Center Lipid Lab as previously described (Chapter III).

Plasma chemistry

Plasma triglycerides and FFAs were quantified from EDTA plasma as previously described (Chapter III).

Results

In vivo pharmacokinetics of DG-041

DG-041 achieved a maximum plasma concentration of 1385 nM (± 716) at 1 hours following subcutaneous administration and displayed an average AUC of 331.4 nM·h over the 26 hours (Figure 35). DG-041 was also found in the brain indicating that it crosses the blood-brain barrier. DG-041 was administered subcutaneously at 20 mg/kg twice daily in subsequent
Figure 35. Plasma concentration-time profile of DG-041 following subcutaneous administration

20 mg/kg DG-041 was administered to mice by subcutaneous injection. Plasma DG-041 concentrations were measured from sapheneous vein blood at the indicated times. N = 4-6. Brain DG-041 concentrations were measured from mice euthanized at the indicated time. N = 1 each. Values are expressed as mean ± StDev.
experiments because DG-041 has an IC50 of 11 nM in [3H]Ligand Displacent Binding Assay for the mouse EP3 receptor and the plasma concentrations of DG-041 at this dose will provide sufficient coverage antagonize endogeneous EP3 receptors (600).

_C57BL/6xBalb/c mice are obese when fed a HFD_

Wild-type C57BL/6xBalb/c male mice were fed either HFD (45% calories from fat), VHFD (60% calories from fat) or a micronutrient matched control diet (10% calories from fat). Body weight increased in each of the three groups over the course of the study (Figure 36A). Body weight in both HFD and VHFD fed mice increased at a greater rate than in animals fed control diet, with a divergence in body weight becoming apparent between six and eleven weeks of age. Body composition analyses over the course of the study revealed that fat mass also increased with age and dietary fat (Figure 36B). Lean mass was also increased by dietary fat (Figure 36C).

_C57BL/6xBalb/c mice are insulin resistant when fed a HFD_

Blood glucose and plasma insulin were measured in C57BL/6xBalb/c mice that had been fed control diet, HFD, or VHFD for 17 weeks. Regardless of dietary fat, mice in the fasted state had no differences in basal glucose levels (Figure 37A). Increasing dietary fat increased fasting insulin levels (Figure 37B). Calculation of the homeostatic model assessment of insulin resistance (HOMA-IR) indicated that increasing dietary fat increases insulin resistance (Figure 37C).

_EP3 antagonist does not affect body composition_

20 week old C57BL/6xBalb/c mice that had been fed control diet, HFD, or VHFD were administered EP3 antagonist, DG-041, or vehicle for one week by twice-daily subcutaneous injections. Body weight decreased in HFD and VHFD fed mice following administration of
Figure 36. C57BL/6×Balb/c mice are obese when fed a HFD

A. Male mice fed control diet, HFD, or VHFD were weighed between 4 weeks of age and 20 weeks of age. At 3 weeks of VHFD feeding, mice were significantly heavier than mice fed control diet (P = 0.0243). At 4 weeks of VHFD feeding, mice were significantly heavier than mice fed HFD (P = 0.0341). At 8 weeks of HFD feeding, mice were significantly heavier than mice fed control diet (P = 0.0219).

B. Body composition of fat was assessed by pulsed NMR. Fat mass in mice was increased by dietary fat and age (P < 0.0001).

C. Body composition of lean mass was assessed by pulsed NMR. Lean mass in mice was increased by dietary fat and age (P < 0.0001).

For all figures N = 8 control, 8 HFD, 9 VHFD. Values are expressed as mean ± SEM.
Figure 37. C57BL/6×Balb/c mice become hyperinsulinemic and insulin resistant when fed a HFD

The effects of dietary fat on insulin resistance was assessed in 20 week old C57BL/6×Balb/c mice. **A.** Dietary fat had no effect on blood glucose after a 6-hour fast ($P = 0.365$). **B.** Increasing dietary fat increased fasting plasma insulin ($P = 0.0001$). **C.** The homeostatic model assessment of insulin resistance (HOMA-IR), a function of fasting blood glucose and plasma insulin, revealed that increasing dietary fat increased insulin resistance ($P = 0.0008$). For all figures $N = 8$ control, 8 HFD, 9 VHFD. Values are expressed as mean ± SEM.
Figure 38. EP3 antagonist does not affect body composition
20 week old C57BL/6×Balb/c mice were administered DG-041 or vehicle by subcutaneous injection for one week (20.5 to 21.5 weeks of age). A. Administration of vehicle with or without DG-041 decreased body mass in mice fed HFD or VHFD. B. Body composition of fat was assessed by pulsed NMR. Injection of vehicle with or without DG-041 tended to decrease fat mass in mice fed HFD or VHFD. C. Body composition of lean mass was assessed by pulsed NMR. Lean mass was not significantly affected by DG-041 or vehicle. For all figures N = 4 control + vehicle (circle + solid line), 3 control + DG-041 (circle + DG-041), 4 HFD + vehicle (square + solid line), 4 HFD + DG-041 (square + dotted line), 4 VHFD + vehicle (triangle + solid line), 5 VHFD + DG-041 (triangle + dotted line). Differences between pre-DG-041 (week 20.5) and post-DG-041 (week 21.5) treatment were determined by Bonferroni post-hoc comparison and are indicated on figure. Values are expressed as mean ± SEM.
vehicle with or without DG-041 (Figure 38A). This indicates that the decreased body weight was due to the vehicle and/or the stress of twice-daily handlings and injections. A concomitant decrease in fat mass was also observed in these mice (Figure 38B). Lean mass was not significantly altered by DG-041 or vehicle (Figure 38C). Post-mortem analysis of liver weight and epididymal fat pad weight found that dietary fat increased liver and epididymal fat pad mass (Figure 39). In HFD and VHFD fed mice, DG-041 did not affect either liver or epididymal fat pad mass.

**EP3 antagonist does not affect insulin sensitivity**

Blood glucose and plasma insulin were measured in 6-hour fasted C57BL/6xBalb/c mice that had been fed control diet, HFD, or VHFD and were administered either vehicle or EP3 antagonist, DG-041, for one week by twice daily subcutaneous injections. Blood glucose and plasma insulin were not significantly affected by DG-041 treatment, though mice fed VHFD had reduced blood glucose when treated with vehicle but not DG-041 (Figure 40A,B). Consequently, HOMA-IR was also unaffected by DG-041 (Figure 40C). Treatment of the mice with vehicle improved insulin sensitivity; this may be due to the decreased body weight of HFD and VHFD fed mice.

**Circulating plasma lipids are not affected by EP3 antagonist**

Plasma FFAs and triglycerides were measured in ad libitum fed C57BL/6xBalb/c mice that had been fed control diet, HFD, or VHFD and were administered either vehicle or EP3 antagonist, DG-041, for one week by twice-daily subcutaneous injections (Figure 41). Dietary fat did not affect either plasma FFA or triglyceride concentrations. DG-041 also did not affect either plasma FFA or triglyceride concentrations.
Figure 39. Liver and epididymal fat pad mass in mice treated with EP3 antagonist
The effect of EP3 antagonist, DG-041, on tissue weight was measured in 21 week old C57BL/6×Balb/c mice fed control, HFD, or VHFD. A. Increasing amounts of dietary fat increased liver mass in vehicle treated mice (One-way ANOVA test for linear trend, P = 0.0094). DG-041 did have a significant effect on the weight of the liver (Two-way ANOVA effect of drug, P = 0.0984), except in mice fed control diet. N = 4 control + vehicle, 3 control + DG-041, 4 HFD + vehicle, 4 HFD + DG-041, 4 VHFD + vehicle, 4 VHFD + DG-041. B. Dietary fat affected epididymal fat pad mass (Two-way ANOVA effect of diet, P = 0.0142). DG-041 did not significantly affect the weight of the epididymal fat pads (Two-way ANOVA effect of drug, P = 0.647). N = 4 control + vehicle, 3 control + DG-041, 4 HFD + vehicle, 4 HFD + DG-041, 4 VHFD + vehicle, 5 VHFD + DG-041. Values are expressed as mean ± SEM.
Figure 40. EP3 antagonist does not affect insulin sensitivity
The effect of EP3 antagonist, DG-041, on insulin resistance was assessed in 20 week old C57BL/6×Balb/c mice fed control, HFD, or VHFD. "A. Treatment of the mice with vehicle significantly reduced fasting blood glucose (Two-way ANOVA comparing vehicle and diet, effect of vehicle: \( P = 0.0401 \)), though this trend was not observed in DG-041 treated mice (Two-way ANOVA comparing DG-041 and diet, effect of DG-041: \( P = 0.358 \)). B. Treatment of the mice with vehicle or DG-041 did not have a significant effect on plasma insulin (Two-way ANOVA comparing vehicle/DG-041 and diet, effect of vehicle: \( P = 0.0539 \) and effect of DG-041: \( P = 0.244 \)). C. The homeostatic model assessment of insulin resistance (HOMA-IR), a function of fasting blood glucose and plasma insulin, showed that treatment of the mice with vehicle significantly reduced HOMA-IR ((Two-way ANOVA comparing vehicle and diet, effect of vehicle: \( P = 0.0235 \)), though this trend was not observed in DG-041 treated mice (Two-way ANOVA comparing DG-041 and diet, effect of DG-041: \( P = 0.0701 \)). For all figures \( N = 4 \) control + vehicle, 3 control + DG-041, 4 HFD + vehicle, 4 HFD + DG-041, 4 VHFD + vehicle, 5 VHFD + DG-041. Differences between pre-DG-041 (week 20.5) and post-DG-041 (week 21.5) treatment were determined by Bonferroni post-hoc comparison and are indicated by asterisk on figure. Values are expressed as mean ± SEM.
Figure 41. EP3 antagonist does not affect plasma lipids

The effect of EP3 antagonist, DG-041, on plasma lipids was measured in 21 week old ad libitum fed mice. **A.** Increasing amounts of dietary fat did not affect the amount of plasma free fatty acid (One-way ANOVA test for linear trend, \( P = 0.522 \)). DG-041 also did not significantly affect the amount of plasma free fatty acids (Two-way ANOVA effect of drug, \( P = 0.839 \)). **B.** Increasing amounts of dietary fat did not affect the amount of plasma triglycerides (One-way ANOVA test for linear trend, \( P = 0.176 \)). DG-041 also did not significantly affect the amount of plasma triglycerides (Two-way ANOVA effect of drug, \( P = 0.164 \)). For all figures \( N = 4 \) control + vehicle, 3 control + DG-041, 4 HFD + vehicle, 4 HFD + DG-041, 4 VHFD + vehicle, 5 VHFD + DG-041. Values are expressed as mean ± SEM.
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† (14:0+16:0+17:0+18:0) / (16:1+18:1ω9+18:1ω7+18:2+18:3ω6+18:3ω3+20:3ω6+20:4+20:5+22:4ω6+22:5ω6+22:5ω3+22:6)

* Bonferroni post-hoc comparison to * in same row P<0.05
** Bonferroni post-hoc comparison to ** in same row P<0.01
*** Bonferroni post-hoc comparison to *** in same row P<0.001

Statistically significant values are highlighted

**Table 8. Fatty acid composition of hepatic triglycerides**
Fatty acid composition of hepatic triglycerides from male EP3+/+ and EP3−/− mice, fed either HFD or control diet. N = 4 control + vehicle, 3 control + DG-041, 4 HFD + vehicle, 4 HFD + DG-041, 4 VHFD + vehicle, 5 VHFD + DG-041.

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<td>mean ± SEM</td>
<td>mean ± SEM</td>
<td>mean ± SEM</td>
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†(14:0+16:0+17:0+18:0) / (16:1+18:1ω9+18:1ω7+18:2+18:3ω6+18:3ω3+20:3ω6+20:4+20:5+22:4ω6+22:5ω6+22:5ω3+22:6)

* Bonferroni post-hoc comparison to * in same row P<0.05
** Bonferroni post-hoc comparison to ** in same row P<0.01
*** Bonferroni post-hoc comparison to *** in same row P<0.001

Statistically significant values are highlighted

**Table 9. Fatty acid composition of skeletal muscle triglycerides**

Fatty acid composition of skeletal muscle triglycerides from male EP3+/+ and EP3−/− mice, fed either HFD or control diet. N = 4 control + vehicle, 3 control + DG-041, 4 HFD + vehicle, 4 HFD + DG-041, 4 VHFD + vehicle, 5 VHFD + DG-041.
**EP3 antagonist decreases skeletal muscle triglycerides**

Because EP3<sup>−/−</sup> mice have been shown to have increased ectopic lipid accumulation when fed a HFD (Chapter III), ectopic lipid accumulation was measured in DG-041 treated mice that had been fed control diet, HFD, or VHFD. Triglycerides in both liver and skeletal muscle were increased with increasing dietary fat. DG-041 did not have a significant effect on liver triglycerides (Figure 42A). Hepatic steatosis was observed in livers of mice fed HFD and VHFD, but did not appear to be affected by DG-041 (Figure 43). In skeletal muscle, DG-041 caused a marked reduction in the amount of triglyceride, especially in the mice fed VHFD (Figure 42B). Dietary fat and DG-041 treatment affected the fatty acid composition of hepatic and muscle triglycerides (Tables 1 and 2, respectively).

**Discussion**

Inbred strains of mice are known to have inherent genetic differences that affect their metabolic phenotype (601-609). C57BL/6×Balb/c F1 hybrid mice were investigated for their susceptibility to diet induced obesity and diabetes because C57BL/6 and Balb/c mice are known to differ in their susceptibility to HFD. C57BL/6 have been reported to be more prone to obesity than Balb/c mice (601), while Balb/c mice have more severe HFD-induced hepatic lipid accumulation (604-606). We chose to investigate C57BL/6×Balb/c F1 mice because they would have maximal heterozygosity while still being genetically and phenotypically uniform. These mice were found to be susceptible to diet induced obesity and insulin resistance. In fact, they consistently weighed more than C57BL/6 mice in previous studies (Chapter III). This is likely due to hybrid vigor of the C57BL/6×Balb/c F1 mice, although the differences due to maternal effects may also influence the phenotype of C57BL/6×Balb/c F1 mice (610-612).
Figure 42. EP3 antagonist decreases skeletal muscle triglycerides

The effect of EP3 antagonist, DG-041, on triglyceride accumulation in ectopic tissues was measured in 21 week old C57BL/6×Balb/c mice fed control, HFD, or VHFD. A. Increasing amounts of dietary fat increased the amount of hepatic triglyceride (One-way ANOVA test for linear trend, $P = 0.0234$). DG-041 did not significantly affect the amount of triglycerides in the liver (Two-way ANOVA effect of drug, $P = 0.132$). B. Dietary fat affected the amount of triglycerides found in the skeletal muscle (One-way ANOVA test for linear trend, $P = 0.0005$). DG-041 significantly reduced the amount of triglycerides found in the skeletal muscle (Two-way ANOVA effect of drug, $P = 0.0004$). For all figures $N = 4$ control + vehicle, $3$ control + DG-041, 4 HFD + vehicle, 4 HFD + DG-041, 4 VHFD + vehicle, 5 VHFD + DG-041. Values are expressed as mean ± SEM.
Figure 43. Hepatic steatosis in mice treated with EP3 antagonist
H&E staining of livers showed that dietary fat increased steatosis. No noticeable effect of DG-041 treatment on steatosis was observed. Images are a representative sample of sections from 4 control + vehicle, 3 control + DG-041, 3 HFD + vehicle, 4 HFD + DG-041, 4 VHFD + vehicle, 5 VHFD + DG-041.
In some studies, EP3 antagonists have been shown to increase GSIS in islets from diabetic individuals (74). Although my studies have not found an effect of DG-041 on isolated islets from HFD fed mice, EP3−/− mice fed HFD had increased plasma insulin suggesting decreased insulin clearance (Chapter III). The increased plasma insulin in EP3−/− mice fed HFD is associated with insulin resistance, which can be attributed to an increased ectopic lipid accumulation in these mice (Chapter III). In order to assess the effects of EP3 loss in a setting of diet induced obesity without the concomitant ectopic lipid accumulation and insulin insensitivity, EP3 antagonist, DG-041, was administered to C57BL/6xBalb/c mice fed control, HFD, or VHFD. Plasma insulin, blood glucose, and insulin sensitivity were not significantly affected by DG-041 treatment. These results may have been somewhat confounded by the administration of this drug, because treatment with the vehicle alone cause a reduction in body mass and fat mass in HFD and VHFD fed animals. This reduction in body mass and fat mass may have contributed toward a trend of improved insulin sensitivity in these mice.

Ectopic and plasma lipids were measured in these mice after treatment with EP3 antagonist, DG-041, for one week to determine if acute EP3 inhibition could affect lipid distribution. Mice treated with DG-041 were shown to have slightly increased hepatic triglycerides in VHFD fed animals, but surprisingly showed significantly decreased skeletal muscle triglycerides. These results are not consistent with increased lipolysis from adipocytes due to EP3 inhibition (101,284,Chapter III), which would result in increased triglycerides in both liver and skeletal muscle. These results are, however, consistent with decreased hepatic lipolysis. PGE2 has been shown to decrease very-low-density lipoproteins (VLDL) secretion from hepatocytes (506,527). Decreased triglycerides during the rapid weight loss is not likely because both vehicle and DG-041 treated mice lost weight, but only DG-041 mice demonstrated
decreased skeletal muscle triglycerides. PGE$_2$ has also been shown to decrease the expression of genes involved in the packaging of lipoproteins: apolipoprotein B (ApoB) and microsomal triglyceride transfer protein (MTTP) (454,478). In EP3$^{+/}$ mice Apob expression was decreased while the decrease in Mttp expression was not statistically significant (Chapter III). Decreased hepatic lipolysis is consistent with reduced triglyceride accumulation in most ectopic tissues, such as skeletal muscle, with increased hepatic triglycerides, a trend toward which was observed in mice fed VHFD and treated with DG-041. This effect of EP3 on hepatic lipolysis was likely not seen in EP3$^{+/}$ mice fed HFD because the chronic EP3 inhibition and increased adipocyte lipolysis ultimately caused skeletal muscle triglyceride accumulation despite fewer triglycerides being released from the liver.

These studies suggest that EP3 may have a previously unrecognized role in metabolic physiology, being a regulator of hepatic lipolysis. Furthermore, they also establish that C57BL/6×Balb/c F1 mice are susceptible to diet induced obesity and insulin resistance.
The EP3 receptor affects obesity, insulin resistance, and glycemic control

The present studies highlight the importance of the EP3 receptor in obesity, insulin resistance, and glycemic control. Prior to beginning these studies, it was known that EP3\(^{-/-}\) mice are obese, hyperglycemic, hyperinsulinemic, and insulin resistant when fed a breeder chow diet (102). There was also good evidence suggesting that PGE\(_2\) signals through the EP3 receptor to inhibit GSIS (74,198-200,233). In addition, there was also ample evidence showing that PGE\(_2\) inhibits lipolysis in adipocytes, also occurring in an EP3 dependent manner (101,284,390). In order to further elucidate the role of EP3 in metabolism, physiological studies were performed on global EP3\(^{-/-}\) mice. These studies show that EP3 is a key regulator of multiple tissues and organs that are central to metabolic physiology.

**Body Weight**

These studies demonstrate that EP3\(^{-/-}\) mice become obese only when they are fed a HFD. EP3\(^{-/-}\) mice begin to become more obese than EP3\(^{+/+}\) on a HFD after 14 weeks of age. When placed on a HFD after 14 weeks of age, EP3\(^{-/-}\) mouse weights begin to diverge immediately suggesting that this phenotype is only observed in mature mice that have finished growing. An earlier study has shown that EP3\(^{-/-}\) mice are obese (102). However, that study was the first report of obesity in EP3\(^{-/-}\) mice, despite being published nine years after the first description of EP3\(^{-/-}\) mice (310,555). The present studies show that there is a diet × genotype interaction with the EP3 genotype; EP3\(^{-/-}\) mice only become obese on a HFD. The first report of obesity in EP3\(^{-/-}\) mice utilized a breeder chow diet for all mice, which has a higher fat content than most standard chow
diets (102). It is possible that the obesity phenotype in EP3−/− mice was not observed for so long because most researchers maintained their mice on standard chow diets.

EP3−/− mice fed breeder chow were reported to be more obese due to increased food consumption (102). They also reported that EP3−/− mice were more active during the light cycle and that these mice spent their extra time awake eating. This increase in motor activity and food intake was not found throughout the day, but rather at a limited number of specific time points. These data are in contrast to what we have observed. We found that EP3−/− mice fed HFD have no differences in food consumption, but rather had decreased movement in the dark cycle. We hypothesize that the decreased activity is contributing to the observed increase in obesity in EP3−/− HFD fed mice.

Pancreas and islets

PGE₂ is typically thought to inhibit GSIS through the EP3 receptor (74,198-200,233). The current studies did not find that PGE₂-EP3 signaling inhibited GSIS. In fact, EP3 inhibition more frequently reduced GSIS contrary to our expected results. These results are in contrast to two prior studies which demonstrated that the EP3 antagonist L-798,106 improved GSIS (74,233). It should be noted that these studies found no effect of L-798,106 on islets at concentrations below 10 μM, while L-798,106 has an Kᵢ for the human EP3 receptor of 300 pM (613). In Ins-1 (832/3) cells, L-798,106 only began to block the actions of 50 nM PGE₂ and 10 nM sulprostone at 100 nM and did not fully restore GSIS until 10 μM (74). Given that 100 nM is ~300 fold greater than Kᵢ, more than a marginal response of L-798,106 is expected. The lack of effect of L-798,106 on GSIS at low concentrations is consistent with off target effects causing the observed responses at concentrations greater than 10 μM; especially off target effects at the EP4 receptor for which L-798,106 has a Kᵢ of 916 nM (613).
IP-GTT studies also did not show an improvement in plasma insulin or glucose clearance in EP3\(^{-/-}\) mice. Despite islets from EP3\(^{-/-}\) mice not having improved GSIS, HFD fed EP3\(^{-/-}\) mice were consistently hyperinsulinemic. These EP3\(^{-/-}\) mice still exhibited increased plasma insulin in response to glucose challenge indicating that they maintained functional \(\beta\)-cells.

The elevated plasma insulin and glucose levels in EP3\(^{-/-}\) HFD fed mice demonstrate that these mice are insulin resistant. Because insulin secretion does not appear to be increased by EP3 gene knockout, it is possible that the higher plasma insulin concentrations are due to reduced insulin clearance. EP3\(^{-/-}\) HFD fed mice had lower blood glucose than did EP3\(^{+/+}\) HFD fed mice when measured during ad libitum feeding. This indicates that these mice may be able to secrete enough insulin to overcome the insulin resistance and maintain normal glucose levels.

**Adipose Tissue**

PGE\(_2\) has long been known to play an important role in obesity, having a role in both adipogenesis and adipocyte function, specifically lipolysis (390). C/EBP\(\beta\) is transiently expressed during the early stages of adipogenesis and is critical for inducing the expression of PPAR\(\gamma\) and C/EBP\(\alpha\), which are necessary for the final stages of adipogenesis (349,350). \(Cebpb\) was expressed at significantly higher levels in EP3\(^{-/-}\) HFD fed mice indicating that adipogenesis might be increased. C/EBP\(\beta\) is also a transcription factor for COX2 (346-348). Despite \(Cebpb\) up regulation, \(Ptgs2\) and PG levels were not different in the epididymal fat pads.

Numerous studies have shown that PGE\(_2\) inhibits lipolysis in adipocytes (90-92,101,296,297,321,392-398,400). The main pathway leading to lipolysis from GPCRs is through the PKA pathway. Norepinephrine signaling in adipose tissue increases intracellular cAMP and thereby activates PKA, which phosphorylates and activates HSL, the rate-limiting step in lipolysis (401,403-405). PGE\(_2\) inhibits norepinephrine stimulated lipolysis (92,393,417-
Several studies using EP receptor selective agonists suggest that PGE₂ inhibits lipolysis through the EP3 receptor (396,421,427). Recently two studies confirmed these results using EP3 selective antagonists, showing that their EP3 antagonists block PGE₂ mediated inhibition of cAMP accumulation (284) and FFA release (101). The present studies also confirm these prior results showing that a different EP3 antagonist and EP3 gene knockout block PGE₂ mediated inhibition of glycerol release. These three different mechanisms of measuring PGE₂ mediated inhibition of lipolysis each tell us something different. Measuring cAMP shows that canonical drivers of lipolysis are affected by EP3 (284). Glycerol is only released during the breakdown of triglycerides confirming that lipolysis is occurring. FFAs are the main lipids that are released into the circulatory system during lipolysis (101).

The cAMP-PKA signaling pathway that regulates lipolysis in adipocytes also regulates Ucp1 expression (319). PGE₂ has been shown to increase Ucp1 expression through the EP4 receptor, which classically opposes the actions of EP3 (282,286,324). In addition, increased Ucp1 expression was found in the epididymal fat pads of AdPLA⁻/⁻ mice (101). Ucp1 expression was greatly increased in the epididymal fat pads of HFD fed EP3⁻/⁻ mice, which is consistent with increased lipolysis in these tissues. Increased Ucp1 expression is expected to increase energy expenditure. Consistent with this, EP3⁻/⁻ HFD fed mice were found to have increased CO₂ production.

We found that EP3⁻/⁻ mice fed a HFD had a relative decrease in epididymal fat pad mass and adipocyte cell size in the heaviest EP3⁻/⁻ HFD fed mice. In addition to having a loss of PGE₂-evoked inhibition of lipolysis, necrosis was increased in these same fat pads, both of which are likely to contribute to the loss of mass in the epididymal fat pads of the EP3⁻/⁻ HFD fed mice.
The *in vivo* consequences of these defects were to cause a reallocation of lipid from adipose tissue to ectopic tissues in EP3\(^{-/-}\) HFD fed mice.

Necrosis and macrophage infiltration were increased in the epididymal fat pads of EP3\(^{-/-}\) HFD fed mice. Adipocyte cell death and macrophage infiltration also results in the release of lipids from adipocytes contributing to ectopic triglyceride accumulation (583-585). When C57BL/6 mice are fed a 60\% very HFD adipocyte death in epididymal fat pads peaks at 16 weeks of very HFD feeding resulting in decreased adipocyte size, lighter epididymal fat pads, increased macrophage infiltration, increased liver mass and steatosis, and insulin resistance (584). This study noted a similar correlation to what we have observed; epididymal fat pad weight is positively correlated with body weight until \(\sim\)35 grams but negatively correlated in mice \(\sim\)40 grams and greater (584). In addition, they found that the mice with the lightest epididymal fat pads had increased liver weight suggesting lipid redistribution from the epididymal fat pads to ectopic tissues, which likely contributed to insulin resistance (584). We were not able to find a significant correlation between epididymal fat pad PGE\(_2\) content or Ptger3 gene expression with either body weight or epididymal fat pad mass, suggesting that lipolysis alone does not account for the striking relationship between body weight and epididymal fat pad mass found in EP3\(^{-/-}\) mice. We hypothesize that increased adipocyte cell death in the heaviest mice, as evidenced by the disproportionately increased macrophage infiltration, accounts for the reduced epididymal fat pad mass and the severe increase in hepatic triglycerides in the heaviest EP3\(^{-/-}\) mice.

An earlier study has shown that loss of PGE\(_2\)-EP3 mediated inhibition of lipolysis in AdPLA\(^{-/-}\) mice causes ectopic lipid accumulation and insulin resistance due to FFAs leaving adipocytes and then being stored in ectopic tissues (101). My results provide confirmation of this
phenotype, with EP3\textsuperscript{−/−} mice having both ectopic lipid accumulation and insulin resistance when fed a HFD. However, the phenotype of EP3\textsuperscript{−/−} mice differs from that of AdPLA\textsuperscript{−/−} mice in that EP3\textsuperscript{−/−} mice are obese and have more adipose tissue whereas AdPLA\textsuperscript{−/−} mice are lean. In addition, adipose tissue from EP3\textsuperscript{−/−} HFD fed mice had increased expression of inflammatory genes, F4/80, TNF-\(\alpha\), IL-6, and MCP-1, which were unchanged in AdPLA\textsuperscript{−/−} mice. This suggests that the lipolysis phenotype alone is sufficient to cause the increased ectopic lipid accumulation and insulin resistance. We hypothesize that the increased obesity and adiposity in EP3\textsuperscript{−/−} HFD fed mice are due to decreased movement caused by a lack of EP3 signaling in the nervous system, while AdPLA\textsuperscript{−/−} mice only have reduced EP3 signaling in adipose tissue and therefore do not have changes in behavior. It is also possible that the presence/lack of EP3 in the immune system contributes to the divergent phenotypes of EP3\textsuperscript{−/−} and AdPLA\textsuperscript{−/−} mice. It is our hypothesis that changes in the non-adipose tissues of EP3\textsuperscript{−/−} mice contribute to the overall increased obesity, fat mass, and adipocyte size; whereas changes in the adipose tissue of EP3\textsuperscript{−/−} mice, namely lipolysis and cell death, contribute to the proportional decrease of epididymal fat pad mass and adipocyte size and the increased ectopic lipid accumulation.

**Immune cells and inflammation**

PGE\textsubscript{2} is well known to be an important regulator of inflammation (41,44). Macrophage infiltration of epididymal fat pads and liver tissue were measured. F4/80, a marker of macrophage, gene expression was elevated in the epididymal fat pads of EP3\textsuperscript{−/−} HFD fed mice indicating increased macrophage infiltration. Gene expression of proinflammatory cytokines, TNF-\(\alpha\), MCP-1, and IL-6, were also increased in the epididymal fat pads of EP3\textsuperscript{−/−} HFD fed mice. No change in macrophage infiltration was observed in the liver. This indicates that the inflammation observed in the adipose tissue is likely due to a local change in the adipocytes and
not due to a global change in the macrophage themselves. Necrosis and increased lipolysis were observed these epididymal fat pads, both of which could contribute to macrophage recruitment in this tissue (574,614).

Liver

Obesity and other models of liver damage increase hepatic PGE$_2$ production and expression of PGE$_2$ generating enzymes (454,458,465). The current studies show that the livers of EP3$^{-/-}$ mice develop steatosis and acquire excess triglycerides when the mice are fed a HFD. Increased liver weight was also observed in EP3$^{-/-}$ mice fed a breeder chow diet, though progression of fatty liver disease was not evaluated in that study (102). It is likely that the excess triglyceride accumulation in the livers of EP3$^{-/-}$ HFD fed mice is due to increased lipolysis from the adipose tissue, with the liver simply acting as a secondary storage site.

Though our hypothesis is that in our model the steatotic livers are due to defects in adipose tissue, PGE$_2$ has been reported to increase triglyceride content in hepatocytes by suppressing very low-density lipoprotein synthesis and triglyceride transport (454,506,527). In the current studies, mice treated with an EP3 antagonist have decreased muscle triglycerides, which is consistent with decreased VLDL transport out of the liver. In addition, EP3$^{-/-}$ mice have decreased Apob expression, but Mttp expression was unchanged. It should be noted that over 50% of ApoB protein is degraded and not used for VLDL synthesis, so the decrease in Apob expression itself does not indicate changes in hepatic lipolysis. Studies examining PGE$_2$ mediated inhibition of hepatic lipolysis suggests that PGE$_2$ mediates these effects via an EP2/4 mediated signaling pathway (465). Because the signaling pathway of EP3 classically opposes that of EP2/4, it is possible that EP3 plays a protective role in the liver mitigating EP2/4 induced hepatic lipid accumulation.
Future Directions

The present studies show a severe phenotype of metabolic dysfunction in mice with a global EP3 gene deletion in a setting of diet induced obesity that is likely due to the important functions of the EP3 receptor in multiple organ systems. In addition to characterizing the phenotypes of global EP3\(^{-/-}\) mice, I have also investigated the effects of EP3 in isolated islets and adipocytes in an ex vivo setting. Further studies utilizing isolated tissues and cells from EP3\(^{-/-}\) mice, as well as the use of temporal and tissue specific EP3 gene deletion models in a setting of diet induced obesity, will greatly aid in the understanding of how the EP3 receptor functions in the various tissues to affect metabolic physiology. In addition to PGE\(_2\)-EP3 signaling other PG receptors also play a role in these metabolically important tissues and the roles of many of these other receptors have not been well characterized in a setting of metabolic stress.

Further elucidation of the obesity and insulin resistance phenotypes

The present studies show a striking diet \(\times\) genotype interaction where EP3\(^{-/-}\) mice are obese when fed a HFD but not when they are fed a control or standard chow diet. One of the main unanswered questions is: why are EP3\(^{-/-}\) mice are only more obese on the HFD, but maintain similar body weights on control and standard chow diet? The present studies suggest that EP3\(^{-/-}\) HFD fed mice are less active than EP3\(^{+/+}\), but do not investigate control diet fed mice. An earlier study has suggested that EP3\(^{-/-}\) mice become more obese due to hyperphagia and night eating (102). If EP3\(^{-/-}\) mice are consistently hyperphagic or less active, then why do they not also gain more weight when they are fed diets with a lower fat content? The observation that the EP3\(^{-/-}\) mice only display the obesity phenotype on the HFD suggests that there is some mechanism that is causing them to either have increase food consumption or decreased activity only when they are faced with a HFD challenge. Measuring food consumption and energy
expenditure in mice fed both control and HFD will be necessary to determine if diet affects food
consumption or activity in EP3/ mice. It would also be beneficial if paired mice could be
obtained from the same litter because maternal effects may influence behavior and obesity
susceptibility.

Further studies characterizing insulin resistance in EP3/ mice may be warranted. The
HOMA-IR, used in the present studies, suggests that EP3/ HFD fed mice are insulin insensitive,
but this test has only been validated in humans. The ITTs performed here also suggest that these
mice are insulin resistant, but they are confounded by the increased body weight of EP3/ HFD
fed mice, the higher fasting glucose levels fed mice, and the short half-live of insulin of EP3/ HFD
(579). The hyperinsulinemic-euglycemic clamp is the gold standard for determining insulin
resistance. Furthermore, this test can be coupled with a radiolabeled glucose tracer to show
which tissues are insulin resistant. From my studies, we can hypothesize that liver and skeletal
muscle from EP3/ HFD fed mice are insulin insensitive, but this has not been shown. It would
also be interesting to determine if adipose tissue is insulin resistant in vivo, as I have only shown
that the adipocytes from chow fed mice are insulin sensitive ex vivo. PGE2 has been show to
increase glucose metabolism in adipocytes (409), hence the effect of EP3 on adipocyte insulin
sensitivity may be of interest. The use of hyperinsulinemic-euglycemic clamps would be
especially interesting in pair fed mice, such a study would be useful in determining whether
EP3/ HFD mice are insulin resistant in the absence of hyperphagia.

It is possible that EP3/ mice have a defect in their reward circuitry where they will
become more easily “addicted” to the HFD and not to lower fat diets. If this were the case, then
these mice would be expected to only consume more food when fed the HFD. In order to totally
eliminate the overeating and/or eating at the wrong time component of these studies, the current
studies presented could be repeated in pair fed mice. If EP3\(^{+/+}\) and EP3\(^{-/-}\) mice were fed the same amount of control or HFD each morning and evening, then the effect of EP3 on glucose homeostasis and insulin sensitivity could be determined in the absence of an overeating/nighteating phenotype. Though caloric intake would be controlled, EP3\(^{-/-}\) mice may still be more obese than EP3\(^{+/+}\) due to decreased energy expenditure.

*Characterizing the role of EP3 in energy expenditure and thermogenesis*

It is possible that EP3 plays a role in thermogenesis and energy expenditure. EP3 is already well known to be necessary for pyrogen-induced fever (309-311). Hence, it is also possible that EP3 may play a role in normal thermogenesis. Through its role in fever, EP3 regulates sympathetic nervous signaling to adipocytes which increases lipolysis and uncoupled thermogenesis. In addition to potentially regulating norepinephrine signaling to adipose tissue, EP3 is also expressed on adipocytes and mediates PGE\(_2\)’s inhibition of lipolysis which is linked to thermogenesis through similar signaling mechanisms.

EP3\(^{-/-}\) HFD fed mice have increased *Ucp1* expression in epididymal fat pads, but it is not clear if this leads to a significant increased uncoupled thermogenesis, though we did find an increase in CO\(_2\) production from EP3\(^{-/-}\) HFD fed mice. In addition, epididymal adipose tissue is not the main site of adipocyte thermogenesis, so it would be interesting to determine if *Ucp1* expression is increased in other white adipose tissue in EP3\(^{-/-}\) HFD fed mice. Thermogenic capacity could be tested in EP3\(^{-/-}\) mice by examining oxygen consumption, cold tolerance, and changes in body temperature in response to a \(\beta_3\) adrenergic receptor specific agonist. Furthermore, if global EP3 deletion is found to affect thermogenesis, it would be of interest to determine the contribution of EP3 in both adipocytes and the preoptic nucleus.
Further characterization of the role of EP3 in adipose tissue

The role of EP3 in these other adipose depots should also be examined because epididymal fat pads are not functionally representative of all fat depots in mice. EP3<sup>−/−</sup> mice are more obese than EP3<sup>+/+</sup> mice when fed HFD; but in the largest EP3<sup>−/−</sup> mice, the epididymal fat pads were smaller. We attributed this to decreased adipocyte size due to necrosis and lipolysis, but this does not explain where the extra weight is being stored. Some extra weight is being stored in the liver and other ectopic tissues, but it is has not been determined whether other fat depots have altered lipid storage in EP3<sup>−/−</sup> mice.

EP3<sup>−/−</sup> mice tended to have larger epididymal fat pads than EP3<sup>+/+</sup> when fed a HFD. Adipocyte size was increased in EP3<sup>−/−</sup> mice accounting for some of the increased mass. It is also possible that adipogenesis was increased in EP3<sup>−/−</sup> HFD fed mice and that a relative decrease in adipogenesis in the heaviest EP3<sup>−/−</sup> HFD fed mice could account for some of the relatively decreased fat pad mass. Expression of Cebpb was increased in EP3<sup>−/−</sup> HFD fed epididymal fat pads possibly indicating increased adipogenesis. Further studies examining the expression of C/EBPβ target genes and late stage adipogenesis transcription factors, Pparg and Cebpa, will be useful for determining whether adipogenesis is altered in EP3<sup>−/−</sup> mice. PGC-1α is a cofactor that modulates PPARγ’s activity. Transcription of the gene encoding Pgc1a can be induced by p38α MAPK phosphorylation of the ATF-2 transcription factor (319). p38α MAPK activation in adipocytes can be mediated by the same cAMP-PKA signaling that also activates lipolysis. Because of this, we can predict that future studies will find Pgc1a expression increased in the epididymal fat pads of EP3<sup>−/−</sup> HFD fed mice which may play a role in adipocyte proliferation and differentiation in these tissues. In addition to measuring the expression of adipogenic genes, future studies examining the role of EP3 in adipogenesis should also examine more direct
measurements of proliferation. Proliferation and differentiation of EP3\(^{-/-}\) mouse embryonic fibroblasts may be examined to determine the EP3’s role in adipogenesis in vitro.

Studies examining the phenotype of an adipocyte specific EP3 knockout mouse would be of interest to determine which of the phenotypes I have observed in the global EP3\(^{-/-}\) model are caused by the lack of EP3 in adipose tissue. I have hypothesized that the lipid redistribution and insulin resistance phenotypes are due to increased adipocyte lipolysis and cell death. However, it is not certain that all of these phenotypes are completely due to defects in the adipocytes.

**Further characterization of the role of EP3 in liver tissue**

I have hypothesized that the lipid redistribution to liver and insulin resistance phenotypes observed in EP3\(^{-/-}\) mice are due to changes in the adipose tissue. It is possible that the lack of EP3 in the liver itself contributes to the increased hepatic lipid accumulation in global EP3\(^{-/-}\) HFD fed mice. I have already shown that EP3\(^{-/-}\) livers have decreased Cpt1a expression, which is consistent with reduced β-oxidation. Further experiments, such as measuring hepatocyte cellular metabolism, are needed to determine if hepatic β-oxidation is reduced in EP3\(^{-/-}\) mice. Also, EP3\(^{-/-}\) mice may also have reduced hepatic lipolysis. Again further experiments are needed to determine if this occurs. Measuring plasma triglyceride levels following the administration of a VLDL uptake inhibitor could be used to assess hepatic lipolysis in vivo.

PGE\(_2\) has also been implicated in hepatic gluconeogenesis (75,76,482,494,505-516). The lack of EP3 in the liver may be contributing to the elevated glucose levels in EP3\(^{-/-}\) HFD fed mice. Glycogen content from the livers can be measured to determine if there are defects in glycogen storage and more direct measurements of gluconeogenesis may be obtained utilizing cultured hepatocytes.
Further characterization of the role of EP3 in the pancreas and islets

The current studies have shown that EP3 inhibition, by either gene knockout or receptor blockade, does not improve GSIS in isolated islets or in vivo. Assessment of glucose homeostasis by IP-GTT showed no significant difference between EP3\(^{+/+}\) and EP3\(^{-/-}\) regardless of diet. Future studies should investigate the effect of global EP3 gene knockout on insulin secretion and glucose homeostasis in an oral glucose tolerance test (O-GTT). The lack of EP3 in islets may be beneficial in an O-GTT because incretins, which are secreted by the intestines, are expected to raise β-cell cAMP above baseline levels. Increasing intracellular cAMP above basal levels has been postulated to be necessary to observe the effects of PGE\(_2\) in islets.

I did not find a significant change in the amount of total pancreatic insulin. However, our collaborators, Maureen Gannon and her lab, have found that the mice utilized for these studies have increased β-cell replication, with replication occurring after 16 weeks of HFD feeding. This is consistent with prior studies that have shown that PGE\(_2\) inhibits β-cell proliferation (188,195) and that G\(_{\alpha_z}\) knockout mice have increased β-cell replication and mass (134). Further investigation into the mechanism behind EP3 inhibition of β-cell proliferation is warranted. Though EP3 antagonists may not be useful for treating diabetes, blockade of similar receptors may be useful for improving β-cell proliferation and mass.

In addition to affecting insulin secretion, EP3 may also play a role in other cell types in the pancreas. Several studies have suggested that PGE\(_2\) increases glucagon secretion in vivo (183,189,208-210,217,218,234). When glucagon secretion was measured from islets treated with an EP3 antagonist in our studies, no clear result was obtained. Examining glucagon secretion from EP3\(^{-/-}\) islets will be useful to determine the role of EP3 in α-cells. Furthermore, in vivo glucagon levels should also be measured as circulating glucagon plays a major role in glucose
homeostasis. In addition to having an effect on α-cells, my ligand binding studies suggest that EP3 is also be expressed in the exocrine tissue of the pancreas. Exocrine tissue in the pancreas secretes digestive enzymes and hence EP3 deletion from these tissues may affect digestion. This could lead to changes in food absorption that could affect energy intake, obesity, and diabetes.

Further characterization of the role of EP3 in immune cells

PGE$_2$ and EP3 are important regulators of immune function (41,44). Inflammation has been shown to be an important contributor to the pathogenesis of obesity and insulin resistance (23-25). The current studies show increased inflammation in the epididymal fat pads but not the liver of EP3$^{-/-}$ HFD fed mice. This suggests that these changes in inflammation are due to localized changes in these tissues and not a change in immune cell function. However, it has yet to be established whether EP3 genotype in the immune cells affects inflammation or the adiposity phenotype. This could be investigated by utilizing models of bone marrow and/or T cell transplant in EP3$^{+/+}$ and EP3$^{-/-}$ mice.

The role of prostaglandins in diabetic complications

Aside from the aforementioned tissues and organ systems, PGs play an important role in many other physiological systems. The involvement of PGs in these other physiological processes may implicate PGs in other diabetic complications such as nephropathy, retinopathy, peripheral neuropathy, and vascular complications (615). EP3 is also involved in adrenocorticotropic hormone release in response to endotoxin implicating EP3 in regulation of the hypothalamic-pituitary-adrenal (HPA) axis (616). By regulating the HPA axis, EP3 may also exert some of its effects on obesity and glucose homeostasis.
Summary

In closing, the present studies have answered several important questions about the role of the EP3 receptor in metabolic physiology. Studies by Sanchez-Alavez, et al. have shown obesity in EP3<sup>−/−</sup> mice, which was in disagreement with prior studies that found no differences in body weight (102,310,555). My studies have reconciled these opposing results by showing that EP3<sup>−/−</sup> mice are only obese when fed a diet containing high dietary fat. In addition, the studies by Sanchez-Alavez, et al. reported that EP3<sup>−/−</sup> mice are insulin resistant (102). My studies have demonstrated that EP3<sup>−/−</sup> mice are likely insulin resistant due to increased ectopic lipid accumulation. Furthermore, my studies are the first studies, to my knowledge, to utilize islets and adipocytes isolated from EP3<sup>−/−</sup> animals to investigate the effects of EP3 on GSIS and lipolysis, respectively. Despite my extensive phenotypic characterization of EP3<sup>−/−</sup> mice during HFD feeding, the present studies have only served to create more questions. Further studies will provide tissue specific information about the role of the EP3 receptor in diet induced obesity and diabetes.
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