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Modulation of Glucose Homeostasis by Nucleotide P2Y2 Receptor and Biological Sex

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MODULATION OF GLUCOSE HOMEOSTASIS BY NUCLEOTIDE P2Y2 RECEPTOR AND BIOLOGICAL SEX

A Master's Thesis

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Master of Science, Cell and Molecular Biology

By

Hailee Anne Marino

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ABSTRACT

Recent insights into the pathological role of Nucleotide $P2Y_2$ receptor suggest $P2Y_2R$ involvement in high fat diet-induced obesity and potentiates insulin resistance. However, these recent insights do not demonstrate how $P2Y_2R$ modulates glucose homeostasis under physiological conditions. Further, it remains unknown how sex biological factors influence $P2Y_2R$ receptor signaling in the regulation of glucose homeostasis. The research objective for the present study is to elucidate the novel roles of $P2Y_2$ in fasting blood glucose and glucose tolerance (basal insulin sensitivity) under resting conditions in males and females. We expected that under physiological conditions $P2Y_2R$ signaling does not contribute to maintaining glucose homeostasis; we did not expect differences in fasting blood glucose and glucose tolerance between the wild type and the $P2Y_2R^{-/-}$ mice. We further hypothesized that sex differences in fasting blood glucose and glucose tolerance would be present in wild type and $P2Y_2R^{-/-}$ mice. We assessed fasting blood glucose (FBG) and glucose tolerance test in C57BL/6 (wild type) and $P2Y_2R^{-/-}$ mice. Mice fasted for 5 hours, and blood was obtained from the tail to assess glucose levels using a glucometer. Mice received 2 g/kg body weight intraperitoneal (IP) injection of 20% dextrose. Glucose levels were measured at time 0 (fasting), then over 90 minutes after IP injection. In the current study, no significant difference was observed in FBG between wild type and $P2Y_2R^{-/-}$ in males and females. However, it was observed that $P2Y_2R^{-/-}$ males had impaired glucose tolerance relative to wild type, while females exhibited no differences. Sex differences in wild type, but not $P2Y_2R^{-/-}$ mice in FBG were observed. However, sex differences in glucose tolerance were observed in WT and $P2Y_2R^{-/-}$ mice, demonstrating females had improved glucose tolerance relative to males. The findings demonstrate $P2Y_2R$ in males contributes to physiological control of glucose homeostasis during a glucose tolerance test. Further, only WT mice demonstrated sex-specific differences in FBG, suggesting a sex-specific role for $P2Y_2R$ under fasting conditions.

KEYWORDS: glucose homeostasis, glucose tolerance, insulin resistance, nucleotide signaling, extracellular nucleotides, inflammation, inflammation-induced insulin resistance, $P2Y_2$ Receptor

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INTRODUCTION

Maintaining whole-body glucose homeostasis (euglycemia) requires coordination and communication between multiple organ systems. The regulatory system consists of central control through neural activity and peripheral control through activities of peripheral organs, including the pancreas, liver, skeletal muscle, and adipose tissue, through the release of hormones. Importantly, peripheral control is key for the insulin-dependent glucose clearance from circulation. If control is disrupted, we become at risk for the development of chronic hyperglycemia and insulin resistance which can lead to disease such as Type 2 Diabetes (T2D). Emerging evidence reveals that proinflammatory mediators interrupt insulin signaling in peripheral tissue, which leads to insulin resistance.

Nucleotide signaling regulates physiological and pathophysiological states, specifically the inflammatory response. Intracellular nucleotides (ATP, ADP, UTP, UDP) released into the extracellular space activate nucleotide signaling by nucleotide receptors. Recently, *in vitro,* and *in vivo* studies have demonstrated that nucleotide receptor $P2Y_2R$ is involved in pathophysiological situations that promote insulin resistance (1, 73, 93, 101, 153). In this study, we set out to better define the physiological role of $P2Y_2R$ in regulating glucose homeostasis by answering the question: What physiological role does $P2Y_2R$ have in fasting blood glucose and whole-body glucose tolerance *in vivo*?

Sex is an important biological variable in maintaining glucose homeostasis through different regulatory mechanisms. Over the past several years, it has been realized the importance of conducting studies that include female and male subjects in both animal and human studies. Animal studies have largely used male animals due to misconceptions of how female

reproductive hormones and the estrus cycle may influence the results or make study design more difficult. However, we know that sexual dimorphisms in health and disease exist and must be accounted for. In the current study we strive to account for sexual dimorphism by using both female and male mice.

Little is known of $P2Y_2R$ signaling and associated regulation of glucose homeostasis between the two sexes. Sex-specific differences in blood glucose tolerance and insulin sensitivity have been established previously in human and animal studies (89, 96, 107, 129, 134). Although sex hormones are an important factor responsible for these differences, genes on X and Y chromosomes and environmental factors also contribute to sex-related differences in cardiovascular, immune, and metabolic system (41, 77, 92). In this study, sex-specific differences in $P2Y_2R$-mediated regulation of glucose homeostasis were assessed.

Our objective for the present study is to elucidate the novel roles of $P2Y_2$ in fasting blood glucose and glucose tolerance (basal insulin sensitivity) under resting conditions in males and females. We expected that under physiological conditions $P2Y_2R$ signaling does not contribute to maintaining glucose homeostasis no differences in fasting blood glucose and glucose tolerance between the wild type and the $P2Y_2R^{-/-}$ mice. We further hypothesized that sex differences in fasting blood glucose and glucose tolerance would be present in wild type and $P2Y_2R^{-/-}$ mice.

Pancreatic Regulation of Blood Glucose

Pancreatic Hormones: Insulin and Glucagon. Pancreatic regulation of glucose homeostasis is mediated by endocrine hormones such as insulin and glucagon. The secretion of these hormones occurs due to the fluctuations in circulating blood glucose concentrations during the absorptive (fed conditions) and postabsorptive (fasting fasting) state (Fig. 1). The endocrine

pancreas releases insulin and “counter-regulatory” hormone glucagon; these hormones are responsible for maintaining glucose homeostasis (Fig. 1) (57, 75, 90, 121). Insulin secretion results from increased blood glucose concentrations (35, 52, 121). Insulin stimulates clearance of glucose, reducing blood glucose. Contrarily, glucagon stimulates endogenous hepatic glucose production, raising blood glucose. When glucose levels decrease between meals glucagon is released from the pancreas to maintain the normal blood glucose level through a negative feedback mechanism (76, 121).The secretion of insulin and activation of insulin receptor signaling are the most important components in maintaining physiological glucose homeostasis.

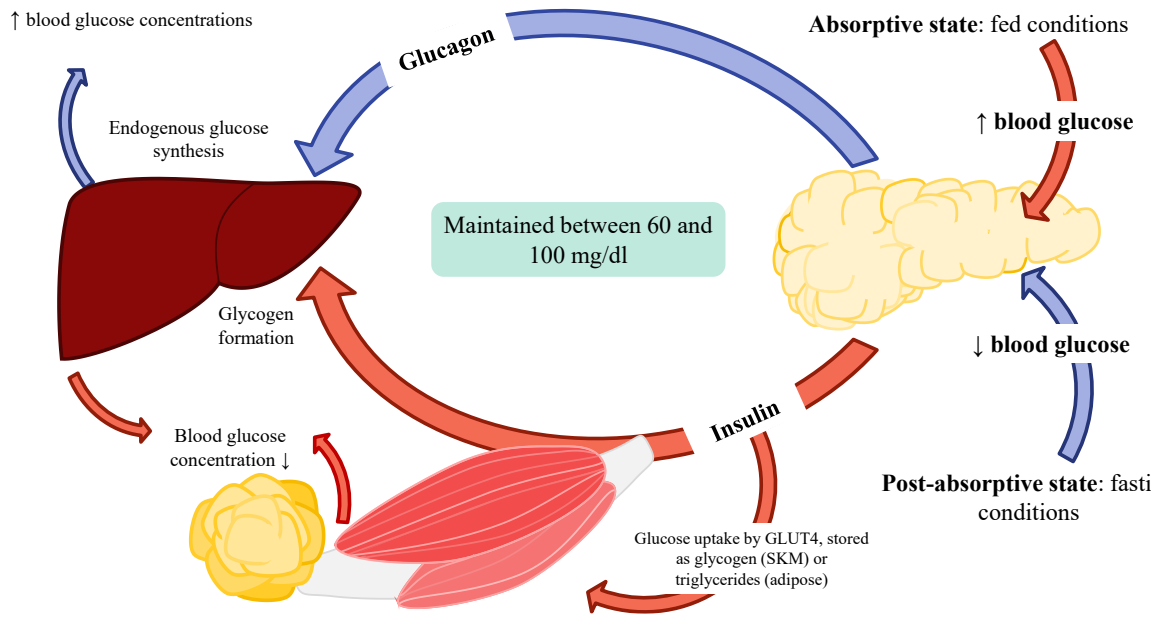

Fig. 1. Maintaining glucose homeostasis is critical for human life to be sustained. The key regulatory system in glucose homeostasis is composed of the pancreas, skeletal muscle, liver, adipose tissue, and pancreatic hormones insulin and glucagon. Regulation occurs under two different states to maintain circulating glucose between 60 and 100 mg/dl. The absorptive state (orange arrows) occurs after eating and is characterized by increase blood glucose levels and the distribution of nutrients. Insulin is secreted in response to increased blood glucose levels to facilitate insulin dependent glucose update into the skeletal muscle and adipose tissue and inhibited endogenous glucose production in the liver. The post-absorptive state (purple arrows) occurs during fasting conditions. Glucose levels start to drop, and the body needs to find a way to provide energy to tissue. Glucagon is released from the pancreas to stimulate endogenous glucose production in the liver, thus provide energy and nutrients to the body between meals.

The endocrine portion of the pancreas is composed of clusters of different cell types referred to as the islet of Langerhans. Islets are composed of α, β, δ, ε, and γ cells (35). Alpha cells release glucagon, β cells release insulin, δ cells release somatostatin, ε cells produce ghrelin, and γ cells produce pancreatic polypeptide (35, 121). The fluctuations of blood glucose concentrations throughout the day are sensed by endocrine cells in the islet of Langerhans (72). As a result, the appropriate hormone is released. The islets are highly vascularized with fenestrated capillaries. These characteristics of the vasculature facilitate glucose delivery and sensing by the islet cells (72).

Glucose-Stimulated Insulin Secretion. Under fed conditions, such as eating a meal, increased circulating glucose triggers insulin secretion from β cells. Glucose is taken up by β cells through glucose transporters, enters glycolysis following phosphorylation by glucokinase, and produces glucose-6 phosphate (G6P). From G6P, pyruvate is produced and enters the TCA cycle leading to an increase in intracellular ATP and raising the ATP/ADP ratio in the β cells (79). Increase in ATP/ADP ratio by high glucose concentration in the β cells leads to closure of the ATP-sensitive K^+ channels (K_{ATP} channels), causing membrane depolarization. As a result, voltage gated Ca^{2+} channels open and allows the influx of calcium. Raised intracellular calcium levels stimulate insulin secretion into the bloodstream (52, 79).

Insulin enters circulation and promotes glucose uptake in peripheral tissues to reduce blood glucose levels, which is a key mechanism to maintain glucose homeostasis. Physiological insulin secretion from β cells is considered biphasic (64). The first phase occurs within two minutes of meal ingestion and sustained for 15-30 minutes, promoting glucose utilization in the periphery. Following this, the second phase of insulin secretion occurs and is sustained until

normal glucose levels are reached. Insulin secretion during the second phase can last for several hours (64).

Insulin in circulation enters the interstitial space and binds to insulin receptors located in the peripheral tissue. The main peripheral tissues responsible for glucose uptake in response to insulin stimulation include skeletal muscle, adipose tissue, and the liver. Insulin receptor activation in the liver also inhibits endogenous glucose production (37). The increase of circulating glucose triggers insulin, the key molecule responsible for the uptake of glucose into the tissue.

Insulin Receptor Signaling. The insulin receptor is a member of the receptor tyrosine kinase family and is the key receptor responsible for glucose uptake in the skeletal muscle and adipose tissue. This well charazitered mechanism is demonstrated in Fig. 2. Insulin binds to the receptor's extracellular domain, causing autophosphorylation of the tyrosine kinase on the intracellular domains. The insulin receptor substrate (IRS-1/2) is recruited to the site of the

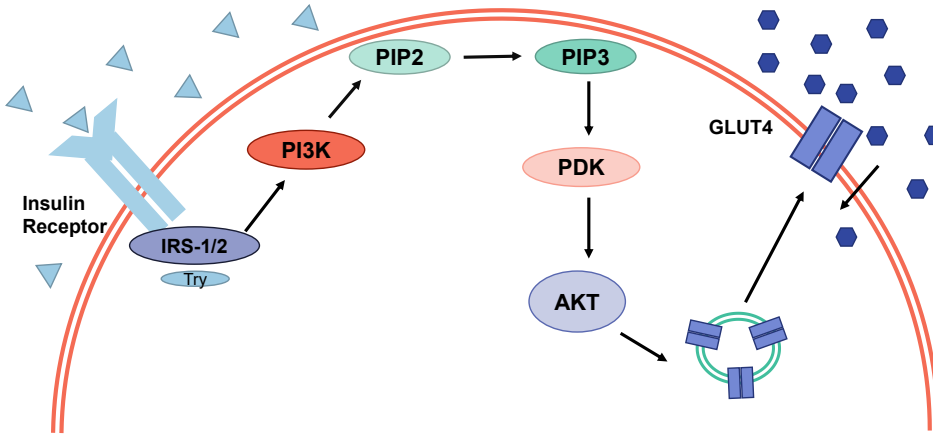

Fig. 2. Insulin receptor signaling transduction during the absorptive state. Insulin binds to the insulin receptor on the skeletal muscle cells or WAT and activates the receptor. This leads to tyrosine phosphorylation of IRS-1/2. IRS-1/2 associates with PI3K. PI3K synthesizes PIP3 from PIP2. PIP3 stimulates the recruitment of PDK and AKT to the plasma membrane. PDK phosphorylates AKT, leading to AKT activation. AKT associates with GLUT4 containing vesicles, stimulating the translocation of GLUT4 to the plasma membrane. GLUT4 facilitates glucose uptake into the cell.

insulin receptor following receptor activation and becomes phosphorylated. Once phosphorylated, IRS isoforms 1 and 2 (IRS-1/2) may activate two different pathways: One pathway is the MAP-K signaling pathway that promotes cell growth and proliferation (54, 108, 136). The other is the phosphoinositide 3-kinases pathway (PI3K) (Fig. 2) which promotes insulin-stimulated glucose uptake into the peripheral tissue that requires insulin for glucose uptake (15) and nitric oxide (NO) production (108, 136).

For insulin stimulate glucose uptake, the PI3K pathway is activated (Fig. 2). IRS-1/2 binds with the PI3K and phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2), and generates phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 activates protein 3-phosphoinositide-dependent protein kinase-1 (PDK-1) which subsequently activates AKT, also known as protein kinase B (PKB). AKT binds to vesicles containing glucose transporters, leading to the translocation glucose transporters to the plasma membrane. Through the glucose transporters, glucose is taken into skeletal muscle and adipose tissue, thus decreasing plasma glucose concentrations (15, 37).

Class I Facilitative Glucose Transporters. Glucose transporters (GLUTs) 1-4 are well characterized and classified as Class I Facilitative Glucose Transporters. They are involved in glucose sensing, import and export; and in signal transduction to maintain glucose homeostasis. It should be noted that two additional classes of GLUTs exist: Class II and Class III Facilitative Glucose Transporters. For the context of this thesis, Class I Facilitative Glucose Transporters are highlighted (104). GLUT2 is a sensor of glucose concentrations and an importer of glucose in pancreatic β cells. GLUT2 is also expressed on hepatocytes and facilitates glucose transport and sensing (104, 139, 140). The brain requires constant energy and expresses GLUT3. GLUT3 has a high affinity for glucose and allows for the constant influx of glucose to maintain energy. Like

GLUT3, GLUT1 continuously imports glucose into tissue that requires constant energy, such as erythrocytes. GLUT4 is prominently expressed in skeletal muscle and adipose tissue to take up glucose in response to insulin receptor signaling (65, 140).

Regulation of Blood Glucose by the Liver, Skeletal Muscle, and Adipose Tissue.

Mechanisms of regulating glucose homeostasis by peripheral tissues depends on the metabolic state. Under the absorptive state (fed conditions), when a meal is consumed, glucose is produced from the digestion of complex carbohydrates. Glucose and other monosaccharides are absorbed across the mucosal wall of the small intestines, to enter circulation in the blood. Glucose is cleared in the absorptive state by insulin dependent glucose uptake in the skeletal muscle, liver, and white adipose tissue. Conversely, during the post-absorptive state between meals, while sleeping, and during fasting blood glucose levels fall. Due to the energy demands of the tissue, glucose is produced endogenously to provide energy. The post-absorptive state is governed by glucagon and its actions on the liver. Glucagon acts on the liver to stimulate the breakdown of stored glycogen in the liver or to utilize non-carbohydrate precursor molecules to produce glucose. This is imperative to the maintenance of steady-state glucose supply for energy by utilization of glucose stores, triglycerides, and amino acids (133, 151).

Hepatic Regulation of Blood Glucose. Hepatic regulation of blood glucose occurs during the absorptive state when insulin inhibits endogenous glucose production and promotes glucose storage in the form of glycogen (59). Suppression of endogenous glucose production by insulin prevents hyperglycemia, which can be detrimental to the body and contribute to Type 2 Diabetes. During the absorptive state glucose taken into the liver undergoes the processes of glycolysis and glycogenesis. Under these conditions glycolysis converts glucose to pyruvate to

generate energy. Insulin mediated AKT signaling stimulates glycogenesis in the absorptive state. During glycogenesis, excess glucose molecules are added together to create glycogen as stored energy (121). Between meals glycogen is utilized to provide energy to the body.

In the post-absorptive state, endogenous glucose production in the liver is regulated by glucagon. Glucagon's role in hepatic glucose regulation is to stimulate endogenous glucose production through two pathways, glycogenolysis and gluconeogenesis and inhibiting glycogenesis (75). Glucagon binds to G protein-coupled receptors located on hepatocytes, activating metabolic pathways to produce glucose through adenylyl cyclase/ PKA (4, 9, 59). In post-absorptive state, glycogen is broken down into glucose by Glycogenolysis. Glycogenolysis is activated under short-term fasting condition. Here, glycogen stored from meals is broken down to produce glucose. If glycogen stores are depleted (long-term fasting or starvation), non-carbohydrate precursors are utilized to generate glucose. During starvation, gluconeogenesis is activated so that non-carbohydrates such as amino acids, fatty acids, and glycerol are used to produce glucose.

Skeletal Muscle in Blood Glucose Regulation. The skeletal muscle is considered the predominant insulin-sensitive tissue in the body, contributing 70-80% of insulin-dependent glucose disposal (5, 78). It is well evidenced in the skeletal muscle that exogenous glucose uptake requires three important steps: 1) the delivery of glucose from circulation to the interstitial space; 2) insulin-stimulated translocation of GLUT4; 3) the irreversible phosphorylation of glucose by hexokinase (148). Once glucose is phosphorylated it may enter glycolysis or glycogenesis. Glucose designated for the glycolytic pathway is utilized for ATP synthesis, allowing muscle contraction and movement. Glucose entering glycogenesis are added together to produce glycogen for energy reserves. Unlike stored glycogen in the liver, glycogen

stored in the skeletal muscle cannot be converted back to glucose when blood glucose levels drop (94). When the liver has run out of stored glycogen and hepatic gluconeogenesis starts during starvation, skeletal muscle generates non-carbohydrate precursors that are delivered to the liver and then converted into glucose.

Adipose Tissue in the Regulation of Glucose Homeostasis. White adipose tissue is responsible for maintaining glucose homeostasis through the storage of excess nutrients and secretion of hormones. Excess glucose is taken up by the adipose tissue and converted to glycogen and triglycerides through lipogenesis (122). Lipogenesis is hormonally regulated by insulin, leading to the activation of metabolic pathways that promotes nutrient storage in adipose tissue. Contrarily, when blood glucose levels drop during fasting or starvation, triglycerides are metabolized into free fatty acids (FFAs) and glycerol and released into blood circulation. These two molecules are precursor molecules for endogenous glucose production in the liver when glycogen stores are depleted FFAs and glycerol are released into blood circulation and are brought to the liver (91).

Disruption of Glucose Homeostasis

Insulin resistance occurs when the peripheral tissue no longer effectively responds to insulin, leading to impaired glucose uptake. This leads to hyperinsulinemia and hyperglycemia. Insulin resistance can occur in several pathophysiological conditions, including cardiovascular disease, pre-diabetes, diabetes mellitus, obesity, and metabolic syndrome (103). Even infection and inflammatory diseases share this common trait of insulin resistance.

Development of Type 2 Diabetes. Diabetes is characterized by above-normal blood glucose concentrations. Diabetes can be broken down into three main diseases: Type 2 Diabetes

(T2D), Type 1 Diabetes (T1D), and gestational diabetes. According to the Center of Disease Control (CDC), 34.2 million people across different ages had diabetes as of 2020, encompassing 10.5% of the population of the United States. Of this population, 26.9 million were diagnosed while 7.3 million adults are undiagnosed. Undiagnosed individuals were determined by fasting blood glucose levels and glycated hemoglobin, A1c (29). Additionally, 88 million adults in 2020 were diagnosed with prediabetes, encompassing 34.5% of the population of the United States (29). While prediabetes is a condition preceding diabetes where glucose levels are higher than normal but, in the range to be diagnosed with diabetes.

If glucose homeostasis is disrupted, the development of disease may occur. Prolonged disturbances in glucose homeostasis are associated with T2D. Detection of these disturbances are key, as excessive blood glucose levels are damaging to the body. To assess disturbances two parameters and their diagnostic criteria (Table 1) are used regularly for patients suspected of having prediabetes and T2D: Fasting blood glucose and oral glucose tolerance test (OGTT), a commonly used test for diabetes in humans.

To assess fasting blood glucose (FBG) levels an individual must fast overnight and then come into the clinic to have their blood glucose levels read by having their blood drawn. Fasting blood glucose levels above 100 mg/dl may indicate an abnormality in glucose regulation in the post-absorptive state. Such as abnormal endogenous glucose production, insulin resistance, and impaired insulin secretion. The second measurement is derived from the OGTT. Individuals undergo an overnight fast, go into the clinic to have their FBG read, ingest a drink containing 75 grams of sugar, and have BG levels two hours after. This test is used to evaluate the body's response to exogenous glucose during the absorptive state by comparing the FBG to the blood

glucose levels obtained after the two hours. The increase in blood glucose from "normal" 120 mg/dl after 2 hours suggest a decrease in glucose metabolism such as insulin resistance.

The results derived from both these test aid in the diagnose of prediabetes and T2D (Table 1). For example, prediabetes can be characterized clinically as impaired fasting glucose (IFG) and impaired glucose tolerance (IGT). IFG indicates increase in endogenous glucose production in the liver, potentially due to liver insulin resistance (38, 102). In insulin resistance, the peripheral tissue's sensitivity to insulin is decreased, potentially resulting in IGT. IGT indicates a decrease of glucose uptake in the skeletal muscle and adipose tissue (38, 102).

Table 1. Diagnostic blood glucose values used to identify prediabetes and T2D

	euglycemia (normal glucose tolerance)	prediabetes (impaired glucose tolerance)	type 2 diabetes
fasting glucose	less than 100 mg/dl	100 - 125 mg/dl	above 126 mg/dl
2 Hours Post OGTT Glucose	less than 140 mg/dl	140 – 200 mg/dl	above 200 mg/dl

*Values described are collected from the American Diabetes Association

When insulin resistance develops, the pancreas needs to work "harder" to maintain euglycemia and does this by secreting excess insulin to overcome increasing blood glucose levels (38, 138). This increase in insulin secretion, referred to as hyperinsulinemia, can only combat increasing concentrations of blood glucose and insulin resistance for a given period in most patients. Eventually, β cells may no longer secrete more insulin to match the increasing demands and lead to β-cell dysfunction (Fig. 3). As a result, hyperglycemia appears and insulin secretion decreases. Insulin resistance can be caused by alterations at any step of the signaling

cascade: the insulin receptor, downstream signaling molecules, glucose transporters, and/or intracellular utilization of glucose (38).

Insulin resistance can be caused by alterations at any step of the signaling cascade: the insulin receptor, downstream signaling molecules, glucose transporters, and/or intracellular utilization of glucose (38). Insulin resistance may also be caused by impaired delivery of insulin and glucose to skeletal muscle or adipose tissue from circulation. Inflammation, for example is a cause of insulin resistance by disrupting insulin receptor signaling and impairing cardiovascular delivery of glucose and insulin to the peripheral tissue. The effects of inflammation on glucose homeostasis have been extensively studied and may occur under acute and chronic inflammatory situations.

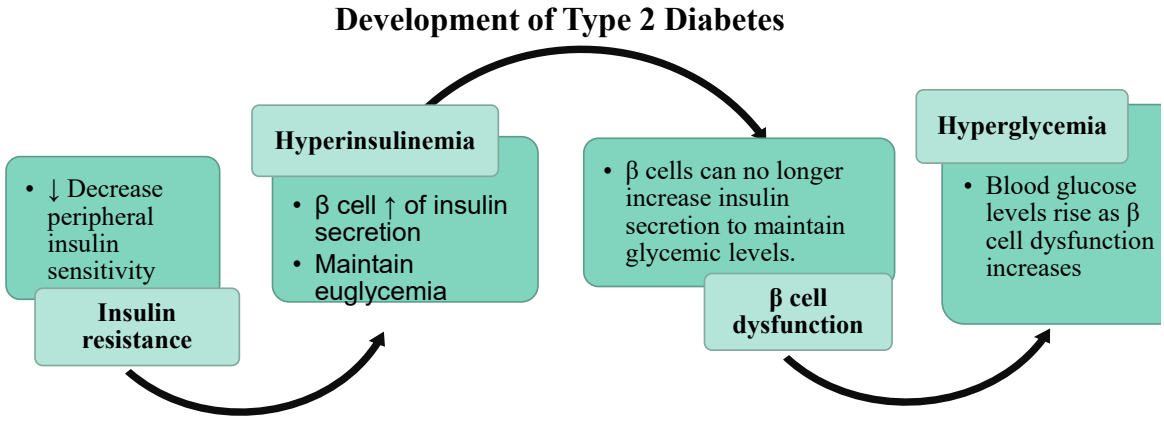

Fig. 3. Development of Type 2 Diabetes. Several disturbances occur leading to T2D. Early in the development of T2D is insulin resistance. Insulin resistance in the peripheral tissue such as skeletal muscle, adipose tissue, and liver occurs due to genetics, aging, obesity, thus the tissue becomes less responsive to insulin in the absorptive state, making it harder for the body to clear glucose from circulation. Due to the decreased insulin sensitivity, the pancreatic β cells pump out more insulin to overcome the insulin insensitivity, defined as hyperinsulinemia. Overtime βcell dysfunction occurs. The overcompensating β are worn out and can no longer secrete excess insulin – this is the defining point in characterizing T2D. Hyperglycemia develops due to the increasing β cell dysfunction.

Chronic Inflammation in the Disruption of Glucose Homeostasis. Chronic, low-grade inflammation is a hallmark of T2D and obesity. Patients with T2D have increased levels of circulating cytokines such as interleukin one beta (Il-1β), tumor necrosis factor alpha (TNF-α), interleukin six (Il-6), and C-reactive protein (CRP) have been considered as therapeutic targets to treat T2D (31, 42, 58, 132). Pro-inflammatory cytokine TNF-alpha was the first inflammatory mediator discovered to potentiate insulin resistance in the adipose tissue. Since then, it is well established that a pro-inflammatory environment evokes local and systemic insulin resistance (31, 50, 58). Increase in production of pro-inflammatory cytokines, chemokines, ATP/UTP, and activation of intracellular signaling molecules such as kinases c-Jun N-terminal kinase (JNK) and IκKB kinase is attributed to the inhibition of insulin effects on glucose uptake. Overnutrition is a suspected cause of the development of inflammation in T2D and obesity.

Overnutrition, namely high-fat diet (HFD), induces stress in the tissue metabolism, stimulating the production of inflammatory mediators (45). Experimental models of high-fat diet-induced obesity in mice have been used to characterize the connection between inflammation and metabolic diseases. HFD feeding alters the immune responses in tissues such as the pancreas, liver, skeletal muscle, and adipose tissue, and enable the development of insulin resistance through the production of pro-inflammatory pathways and cytokines (42). The development of this pro-inflammatory state under HFD feeding is in part, mediated by nucleotide receptor $P2Y_2R$.

Acute Inflammation and the Disruption of Glucose Homeostasis. Glucose homeostasis is disrupted independent of a history of diabetes and pre-diabetes during critical illness, such as severe infection accompanied by the production of pro-inflammatory mediators like chronic inflammation and/or impaired cardiovascular function (6, 27, 42, 55, 87, 130,

141).Clinically, the role of insulin resistance and hyperglycemia in stress, sepsis (LPS-induced inflammation), and severe illness is of great importance. LPS-induced inflammation in experimental models has been used to elucidate the cause-effect relationship between the pro-inflammatory mediators and impaired glucose homeostasis (110). During infection, insulin resistance is facilitated by interruptions in the insulin signaling cascade by pro-inflammatory molecules. Moreover, impaired cardiovascular function disrupts delivery of insulin and glucose from circulation to the peripheral tissue (63, 95, 109).

Bacterial infections have the potential to become severe and illicit a strong pro-inflammatory response, septic shock, and organ failure (40, 125). Gram-negative bacteria contain an endotoxin on their outer membrane called lipopolysaccharides (LPS). When LPS is released from the bacterial wall it binds to toll-like receptor 4 (TLR4), on the plasma membrane of immune cells to facilitate the activation of the innate immune system (111). Activation of TLR4 leads to the transcription of inflammatory cytokines by inducing NF-κB translocation to the nucleus. The inflammatory cytokines and molecules include TNF-α, Il-6, Il-1β, Il-10, type 1 interferons, nitric oxide (NO), reactive oxygen species (ROS), and leads to the release of extracellular nucleotides from the immune cells (55, 90, 132, 150). The ramifications of the pro-inflammatory response and increase in pro-inflammatory mediators can lead to insulin resistance in the peripheral tissue by impeding insulin receptor signaling and glucose uptake.

Activation of cytokine receptors, TLR4, and nucleotide receptors by extracellular nucleotides interrupts insulin signaling by inhibiting activation of the PI3K-AKT pathway. During normal insulin signaling, insulin binds to the insulin receptor leading to the phosphorylation of a tyrosine on IRS-1/2 and leads to AKT activation and GLUT4 translocation to the membrane to bring glucose into the cell. During the pro-inflammatory state, a serine on the

IRS-1/2 molecule is phosphorylated instead of tyrosine, or phosphorylation of AKT is inhibited. This impedes GLUT4 translocation to the cell membrane (115, 116).

Hyperinsulinemic-euglycemic clamp studies demonstrated impaired muscle glucose uptake when treated with high doses of LPS. In this study, alterations to the insulin signaling pathway were not observed, but impaired cardiovascular function was proposed to be responsible for the insulin resistance. It is suspected that insulin and glucose delivery from circulation to the interstitial space might be disrupted under these conditions (99). In another study, skeletal muscle microvascular alterations were observed using intra-vital microscopy using insulin with a fluorescent tag, showing alterations in microvascular flow and decrease of insulin accumulation in the interstitial space in mice challenged with LPS (95). One reason for cardiovascular challenges might be due to overproduction of NO through iNOS during the immune response as part of the host defense (28, 30, 84). Previous literature demonstrates increased amounts of NO lead to impaired cardiac function, including decreases in mean arterial pressure and blood pressure (63). In one study, a hyperinsulinemic clamp revealed impaired glucose uptake/disposal in wild type mice treated with sodium nitroprusside (SNP), an NO donor relative, suggesting that alterations in the cardiovascular system effect the delivery of glucose to the skeletal muscle in the presence of NO. Further, iNOS KO mice were rescued from impaired glucose uptake when treated with LPS relative to wild type mice, suggesting that the overproduction of NO from iNOS hinders delivery of glucose from blood circulation to the peripheral tissue by impairing cardiovascular function, thus leading to insulin resistance (63).

Extracellular Nucleotide Signaling

ATP was initially recognized as an extracellular signaling molecule when ATP was identified as a neurotransmitter in the 1970s (20). In the decades following, the role of nucleotides and nucleotide receptors has gone beyond neurotransmission. Nucleotide receptor subtypes are expressed in most cell types. Nucleotide receptor activation has a wide variety of effects and gained interest as therapeutic targets for inflammatory and cardiovascular disease.

Physiological Release and Function of Extracellular Nucleotides. ATP is predominantly synthesized in the mitochondria and maintained at a micromolar intracellular concentration (14). Intracellularly, ATP is used for energy to maintain cellular metabolism, fueling anabolic reactions. Physiological release of nucleotides into the extracellular space occurs in a controlled manner via secretory vesicles, ATP-transporters, hemichannels such as pannexin and connexins, and channel proteins (Fig. 4) (85). Physiological extracellular concentration of nucleotides is maintained at nanomolar concentrations. To maintain this concentration and prevent over activation, the release and degradation of extracellular nucleotides must remain in balance (80, 81). Extracellular triphosphates (ATP) can be converted to diphosphates (ADP), into monophosphates (AMP), and to adenosine by ectoenzymes that are expressed on the plasma membrane, such as NTPDase1, ecto-ATPDase, CD39, apyrase, and CD37 (Fig. 4) (120). Extracellular nucleotides influence neurotransmission, hormone secretion, and vascular homeostasis, immunity, and blood glucose level under physiological conditions (13, 17, 18, 21).

Pathophysiological Release and Function of Extracellular Nucleotides. During pathophysiological events, extracellular ATP concentrations rise to micromolar concentrations and become uncontrollable (Fig. 4) (22, 43, 68). The uncontrolled release occurs in cell injury, cell death, necrosis, and apoptosis of the affected cells and tissue (85, 146). The regulated release

of extracellular nucleotides occurs under pathological conditions, such as infection. Pathogens such as bacteria or viruses contain pathogen-associated molecular patterns (PAMPs) (protein, carbohydrates, DNA, or RNA) that are recognized by the innate immune system and elicit an innate immune response. For example, LPS from bacteria, is a PAMP that elicits the controlled release of ATP from immune cells via connexins and exocytosis (43).

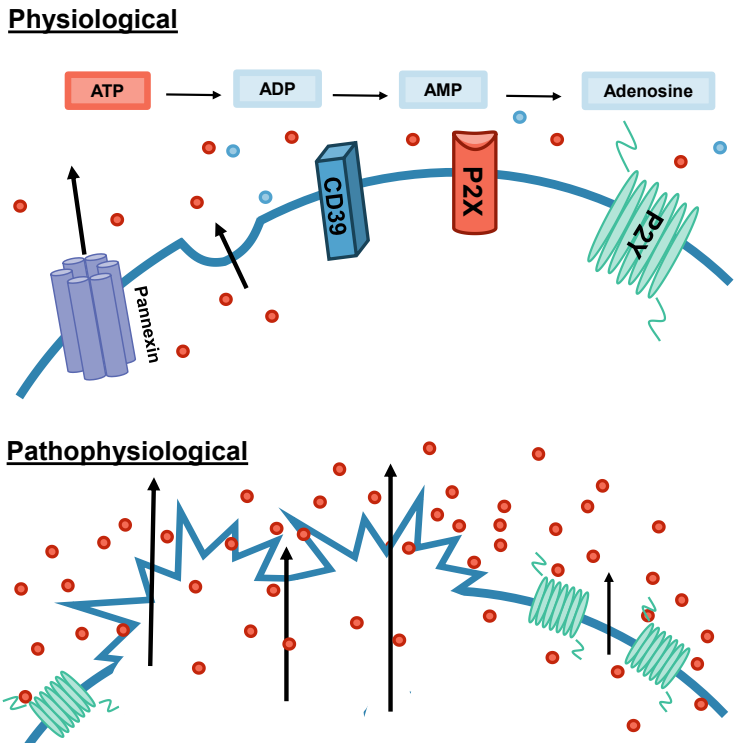

Fig. 4. Nucleotide signaling under physiological and pathophysiological conditions**.** Extracellular nucleotides (ATP/UTP) and metabolites are signaling molecules and ligands for nucleotide receptors (orange, green). Top: physiological conditions nanomolar concentrations of ATP are tightly regulated n the extracellular space by controlled release through channels (purple) and exocytosis, then conversion into metabolites by ectonucleotidases (blue). Top: pathophysiological conditions lead an increase to micromolar concentrations of extracellular nucleotides, increasing the ligand for nucleotide receptors, thus increasing expression and activity. Under these conditions, excess nucleotides from damaged, dying, or infected cells to act as danger and find me signals for immune cells during infection and inflammation.

Nucleotides released to extracellular space from damaged or dying cells serve as danger-associated molecular patterns (DAMPs) to activate immune cells, referred to as a "danger signal". Extracellular nucleotides participate in the innate immune response to activate and induce immune cell mobilization by acting as a "find-me" signal to draw them to damaged or infected tissue (23, 68, 146) and inducing pro-inflammatory cytokine release (33) at the site of infection to promote host defense. For example, extracellular nucleotides exert action on nucleotide receptors to enable immune cells such as neutrophils and macrophages to migrate to and subsequent phagocytosis of affected cells (85, 146). Additionally, extracellular nucleotide are involved in the production of pro-inflammatory cytokines by exerting action on $P2X_7R$ and $P2Y_2R$ on macrophages participates in the activation of NLRP3 inflammasome and leading to Il-1β production, further activating the pro-inflammatory response (12, 36).

Purinergic Receptor Signaling: Nucleotide Receptors. The action of nucleotides and their metabolites are mediated by purinergic receptors which are classified as two families: P1 and P2 receptors (Fig. 5). Purinergic receptors are broadly expressed in animal and mammalian tissue on the plasma membrane (114). P1, adenosine receptors (A1, A2A, A2B, and A3), are G protein-coupled receptors activated by adenosine (21, 70). P2 receptors are categorized into P2X and P2Y subtypes. P2X receptors (P2X1-7) are ionotropic, ligand-gated ion channels activated by ATP. Nucleotide P2Y receptors are classified as metabotropic G protein-coupled receptors (GPCRs) and couple to G proteins Gi, Gq/11, and Gs. Mammalian cells express 8 subtypes of the P2Y family ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors). P2Y receptors are activated by a variety of nucleotides such as ATP, UTP, and their metabolites, ADP and UDP. ADP activates $P2Y_1$, $P2Y_{12}$, $P2Y_{13}$; ATP activates $P2Y_2$, $P2Y_{11}$; UDP activates $P2Y_6$;

UDP-glucose activates $P2Y_{14}$; UTP activates $P2Y_2R$ and $P2Y_4R$ (44, 48, 71, 85). The receptor of our interest, $P2Y_2R$, is equipotently activated by UTP and ATP.

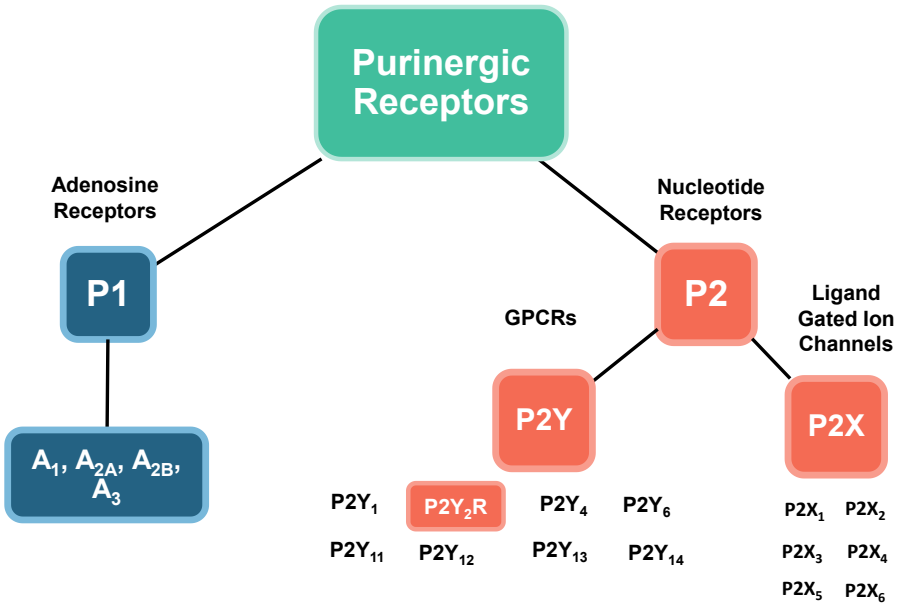

Fig. 5. Purinergic receptor subtypes. Purinergic receptors are divided into adenosine receptors, P1 (blue) and nucleotide receptors, P2 (orange). Nucleotide receptors are further broken down further by receptor type. P2X receptors are ligand gated ion channels activated by ATP. P2Y receptors are G protein-coupled receptors that upon activation by various nucleotides (ATP, ADP, UTP, UDP, and UDP-glucose) associate with intracellular G proteins. The receptor of interest for this research is the $P2Y_2$ receptor. This receptor is equipotently activated by ATP and UTP and expressed throughout the body, exerting a wide variety of functions physiologically and pathophysiological.

$P2Y_2$ Receptor

The gene for $P2Y_2R$ is located on chromosome 11 in *Homo sapiens* and chromosome 7 in *Mus musculus* (97, 105). Expression of $P2Y_2R$ is observed in most tissue types. For example: bone, skeletal muscle, liver, pancreas, adipose tissue, tissue composing the urinary system, cardiovascular system, and immune system, but not limited to these tissues (114). $P2Y_2R$ is a GPCR, forming 7-transmembrane spanning helixes connected by intra- and extracellular loops, extracellular N-terminus, and an intracellular C-terminus. The first extracellular loop contains an

arginine-glycine-aspartate (RGD), allowing for the recognition of integrins. The C terminus contains an Src homology domain (SH3) and associates with tyrosine kinases (48). $P2Y_2R$ activation induces a conformational change that allows for the interaction with G proteins on the intracellular side of the membrane. $P2Y_2R$ associates with G alpha subunits $G_{q/11}$, $G_{i/o}$, $G_{12/13}$. $P2Y_2R$ coupling with $G_{q/11}$ and $G_{i/o}$ induces pathway PLCβ activation producing IP3 and DAG, leading to intracellular calcium signaling, mobilization, and release. Integrins, a_v, bound to the RGD domain in the first extracellular loop of the $P2Y_2$ receptor is required for $P2Y_2R$ to couple with $G_{i/o}$ and $G_{12/13}$, leading to activation of GTPases, Rho, and Rac, respectively (48). The interaction of the RGD domain with integrin αVβ3/β5 promotes nucleotide-induced chemotaxis which is recognized as a step in the inflammatory response, cell migration, and proliferation (48).

The role of $P2Y_2R$ in the cardiovascular and immune systems has been well established. Activated $P2Y_2R$ under pro-inflammatory states plays important role in tissue damage, chronic inflammation, or the progression of diseases. For example, substantial research implies that $P2Y_2R$ potentiates disease states, including inflammatory diseases, such as atherosclerosis, high-fat diet-induced obesity, asthma, fatty liver disease, and sepsis (11, 49, 93, 98, 127). Studies focused on $P2Y_2R$ in vascular inflammation and immune cells have been key in characterizing the role of $P2Y_2R$ in the intra- and extravascular inflammatory response.

$P2Y_2R$ During the Intravascular Inflammatory Response. $P2Y_2R$ plays important roles in the intravascular and extravascular events during the pro-inflammatory response. Intravascularly, $P2Y_2R$ influences leukocyte motion via regulating adhesion and junctional molecules. Endothelial cell $P2Y_2R$ activation leads to the expression of cell adhesion molecules VCAM-1, ICAM-1, PE-CAM, and VE-cadherin. These molecules are important molecules for

the regulation of vascular permeability and recruitment and extravasation of leukocytes (83, 126, 128, 147). $P2Y_2R$ plays a role in vascular permeability, a hallmark of inflammation. This is evidenced by *in vivo* activation of $P2Y_2R$ by UTP in the cremaster muscle, leading to increased venular permeability to albumin in wild type while there was no change in $P2Y_2R^{-/-}$ mice (61). *In vitro* studies demonstrate the cellular mechanism, activated $P2Y_2R$ is recruited to the endothelial cell-cell junctions and co-localized with VE-Cadherin (83), a junctional cell adhesion molecule important for regulating the integrity of the endothelial barrier.

To be recruited to a site of damaged or inflamed tissue, leukocytes must increase interaction with endothelium lining the inner surface of vasculature and subsequently extravasate. This process is referred to as the leukocyte adhesion cascade, a multi-step process leading to transmigration and is key in the intravascular response to inflammation (82). Models of the disease show that $P2Y_2R$ is important for the expression of VCAM-1, which is required for leukocyte-endothelial interaction and extravasation. $P2Y_2R$ induces VCAM-1 expression facilitating the adhesion of monocytes to the endothelial cells, eosinophils in lung inflammation, and monocyte infiltration into the adipose tissue during high-fat diet-induced obesity (2, 93, 143). Studies using the $P2Y_2R$ global knock-out model have shown that the loss of $P2Y_2R$-mediated VCAM-1 expression is protective during inflammatory diseases and reduced immune cell infiltration, demonstrating that $P2Y_2R$ expression can exacerbate the pro-inflammatory response (2, 93, 128, 143). Furthermore, recent data collected in our lab demonstrate $P2Y_2R$'s role in endothelial-leukocyte interaction *in vivo* in the cremaster muscle under stimulated conditions (UTP) and basal conditions using the $P2Y_2R$ knock out model. Data from this study demonstrates that $P2Y_2R$ modulates basal leukocyte rolling and adhesion under physiological

conditions and is necessary for leukocyte rolling when challenged under stimulated conditions with UTP.

P2Y$_2$R in Extravascular Inflammation. P2Y$_2$R facilitates extravascular pro-inflammatory response through the activation of pro-inflammatory and stress associated signaling pathways, expression of pro-inflammatory cytokines and mediators, and activation and augmentation of immune cells. ATP/UTP is released from damaged, inflamed, and infected tissue and acts as danger signals. When cells are assaulted, ATP/UTP is released to the extracellular space acting as a danger signal to recruit immune cells to the site of damage. Extracellular ATP/UTP activates P2Y$_2$R on the plasma membrane of neutrophils and augment neutrophil chemotaxis (32, 69). The release of ATP from the leading edge of neutrophils bind to P2Y$_2$R in an autocrine fashion to amplify chemotactic signals (32). Further, apoptotic and damaged cells release ATP, activating macrophages via P2Y$_2$R, allowing phagocytosis of dying cells (47).

Activation of P2Y$_2$R leads to increase in the transcription of pro-inflammatory mediators from immune cells and the peripheral tissue, potentiating the inflammatory responses. P2Y$_2$R accomplishes transcriptional activation by increasing NF-κB phosphorylation and translocation to the nucleus, leading to the production of pro-inflammatory cytokines and mediators (39, 48). Like TLR4 activation, P2Y$_2$R activation leads to the production of cytokines such as Il-6, TNF-alpha, Il-1β, and other pro-inflammatory mediators like nitric oxide via iNOS. As discussed previously, these contributors to the development of insulin resistance (1, 49, 62). Mice with a P2Y$_2$R-deficiency on myeloid cells show a decrease in plasma Il-6 and TNF-α and decreased expression of iNOS when challenged with LPS; showing that P2Y$_2$R in cells derived from myeloid cells promotes acute inflammation and the production of pro-inflammatory mediators.

In vitro studies in macrophages show $P2Y_2R$-mediated Il-1β production through activation of the NLRP3 inflammasome (12, 36). Additionally, activation of the NLRP3 inflammasome is shown to contribute to insulin resistance in obesity (142).Effects of $P2Y_2R$ on blood glucose regulation are linked to the diverse role of $P2Y_2R$ in the pro-inflammatory response. Together, $P2Y_2R$ involvement in pro-inflammatory responses may negatively impact glucose homeostasis during these situations.

Current Knowledge of $P2Y_2R$ in Glucose Homeostasis

The role of $P2Y_2R$ as a regulator of glucose homeostasis has been emerging over the past several years. Early studies suggested the role of extracellular nucleotides in insulin secretion and glucose uptake in the periphery by exerting action on the P2 receptor, however, these studies did not look specifically into $P2Y_2R$'s role (24, 25). Recent studies show $P2Y_2R$ has a negative impact on glucose homeostasis and insulin sensitivity (24, 25). These studies show $P2Y_2R$ impacts insulin sensitivity *in vitro* in skeletal muscle cells and liver cells and *in vivo* during high fat diet-induced obesity in mice. Here, we focus on the analysis of the current literature describing $P2Y_2R$'s role in glucose homeostasis and possible ways the receptor may be involved.

$P2Y_2R$ Potentiates Insulin Resistance by Altering the Insulin Signaling Pathway *In Vitro*. An increase in extracellular nucleotide concentration and hyperglycemic conditions activate $P2Y_2R$ and lead to insulin resistance in, as demonstrated by two recent studies in human skeletal muscle cells and human HepG2 hepatocyte cells (73, 101). In both studies, $P2Y_2R$ expression and function was confirmed by RT-PCR and measurement of intracellular calcium mobilization after ATP stimulation. Incubating respective cells in high concentrations of glucose lead to the release of extracellular ATP, as previously observed in earlier studies in HUVEC and smooth muscle cells (SMC) (106, 112). $P2Y_2R$ activation under hyperglycemic conditions

resulted in attenuated insulin induced AKT activation, the pathway required for glucose uptake, suggesting that $P2Y_2R$ activation contributes to the development of insulin resistance. In both cell types stimulating $P2Y_2R$ activation promoted the ERK1/2 signaling, a mitogenic pathway involved in insulin resistance and inflammation. Together, these studies suggest ATP released under hyperglycemic conditions activates $P2Y_2R$, which leads to defective insulin-stimulated glucose uptake by favoring activation of mitogenic and inflammatory pathways associated with insulin resistance (11, 50).

$P2Y_2R$ Potentiates Obesity and Insulin Resistance *in vivo*. $P2Y_2R$ plays a role in the pathogenesis of obesity and insulin resistance. Recent data demonstrated $P2Y_2R$ potentiates HFD induced obesity (93, 153). Merz et al. tested insulin sensitivity in $P2Y_2R^{-/-}$ and WT (C57BL/6 background) mice following HFD (15 weeks). In brief, insulin sensitivity was assessed by using an insulin tolerance test and quantifying plasma C-peptide levels, an indicator of plasma insulin concentration (100). $P2Y_2R^{-/-}$ mice had increased insulin sensitivity following HFD, further illustrated by lower C-peptide. Zhang et al showed $P2Y_2R$ KO mice had lower plasma insulin levels following HFD. They further demonstrated that key molecules responsible for insulin stimulated glucose uptake such as the insulin receptor, IRS-1 (insulin receptor substrate 1), and GLUT4 mRNA expression was increased in the KO mice relative to WT mice. This suggest that $P2Y_2R$ under HFD conditions inhibits the expression of molecules responsible for insulin stimulated glucose uptake and attenuates insulin receptor signaling. Collectively, these studies implicate the putative role of $P2Y_2R$ in attenuating insulin signaling and potentiating dysglycemia in HFD.

Adamson et. al assessed if $P2Y_2R$ on immune cells facilitate the development of HFD induced-obesity and insulin resistance in chimeric myeloid-specific $P2Y_2R$ mice. In brief, WT or

$P2Y_2R^{-/-}$ bone marrow was transplanted into WT mice, creating chimeric knockout mice WT-transplant or KO-transplant. Following either regular diet or HFD (15-16 weeks), glucose tolerance and plasma insulin were then assessed. Whole-body glucose tolerance and insulin sensitivity were unchanged between KO-transplant mice and WT-transplant on an HFD group or between mice on a regular diet. This indicates that the myeloid-$P2Y_2$ receptor does not contribute to impaired glucose tolerance and insulin resistance under HFD-induced obesity.

Contrarily, Merz et al assessed the function of $P2Y_2R$-induced VCAM-1 expression of the endothelium and its contributions to HFD-induced obesity and insulin resistance. Wild type mice had increased immune cell infiltration into the adipose tissue relative to $P2Y_2R^{-/-}$ mice. Endothelial cells were isolated from the stromal vascular fraction and tagged to quantify VCAM-1 expression using flow cytometry. This showed wild type mice on the HFD had increased VCAM-1 expression relative to WT mice on a chow diet and $P2Y_2R^{-/-}$ mice on an HFD. These data suggested that $P2Y_2R$ activity is increased on an HFD, leading to increased VCAM-1 expression to facilitate immune cell infiltration. This pro-inflammatory environment mediated by $P2Y_2R$ may contribute to the insulin resistance discovered in the wild type mice, but absent the $P2Y_2R^{-/-}$ mice. Collectively, these data suggest that endothelial $P2Y_2R$ (and subsequent VCAM-1 expression) and not Myeloid-$P2Y_2R$ facilitates immune cell infiltration, accumulation, and inflammation in the adipose tissue leading to the development of insulin resistance.

$P2Y_2R$-mediated Nitric Oxide Production and Glucose Homeostasis. $P2Y_2R$ is involved in modulating blood flow through the production of nitric oxide. Additionally, nitric oxide is important in regulating blood glucose homeostasis by participating in the delivery of insulin and glucose to the peripheral tissue from the vasculature. $P2Y_2R$ is constitutively expressed in the vascular endothelial cells and its agonists, ATP and UTP, maintain

endothelium-dependent vasodilation under rest conditions (19, 22, 26). This is evidenced by the fact that endothelial-specific $P2Y_2$ knock-out mice exhibit hypertension due to attenuated endothelial nitric oxide (147). This may provide a role for $P2Y_2R$ in the delivery of insulin from the vasculature to the interstitial space through the expression of eNOS and NO production.

Delivery of insulin from the vasculature to interstitial space is an important step in glucose uptake. The production of nitric oxide allows for increase perfusion, capillary recruitment, vasodilation to peripheral tissue, and trans-endothelial insulin transport to facilitate this step. In diseases such as obesity and insulin resistance, NO production is reduced (124). Further, insulin resistance has been shown to develop in eNOS knockout mice (46). The production of NO is partially mediated by insulin acting on endothelial insulin receptors to activate eNOS in the vasculature through the PI3K-AKT pathway (144). Delivery of insulin from the vasculature to interstitial space is an important step in glucose uptake. The production of nitric oxide allows for increase perfusion, capillary recruitment, vasodilation to peripheral tissue, and trans-endothelial insulin transport to facilitate this step and in diseases such as obesity and insulin resistance, NO production is reduced (124).

It is unclear if $P2Y_2R$-mediated eNOS signaling participates in the delivery of insulin to the peripheral tissue. Given that $P2Y_2R$ is an important regulator of endothelium-dependent vasodilation, this suggests a potential role of $P2Y_2R$ in facilitating changes in the vasculature that positively influence insulin delivery and subsequent glucose uptake in the peripheral tissue. This is supported by a study performed in diabetic and healthy humans, where the objective was to assess the vasodilatory effect of $P2Y_2R$ agonist ATP and UTP in the vasculature of the skeletal in diabetic patients (137). In this study, patients were infused with ATP and UTP through the femoral artery, both previously being defined as vasodilators. Diabetic patients' vasodilatory

response to ATP and UTP are decreased along with leg blood flow relative to healthy patients, suggesting attenuated nucleotide signaling in diabetic patients. The attenuated nucleotide signaling, potentially, through $P2Y_2R$ may lead to decreases in NO bioavailability and contribute to insulin resistance seen in patients with T2D. This may suggest endothelial $P2Y_2R$ is important to maintain euglycemia via the delivery of insulin to the peripheral tissue and requires further investigation.

Contrarily, under acute inflammatory conditions, $P2Y_2R$-mediates iNOS expression may suggest a negative impact for $P2Y_2R$ on maintaining euglycemia. The production of NO through iNOS during the immune response is part of the host defense. $P2Y_2R$-mediated iNOS expression is evidenced by *in vitro* studies in macrophages. LPS challenge *in vitro* macrophages increases $P2Y_2R$ mRNA expression. Further, iNOS protein expression is increased when challenged with LPS plus ATP or UTP with a correlating increase in NO production (49). Supporting this, $P2Y_2R$-deficiency on myeloid cells in mice leads to a decrease in *NOS2* (iNOS) gene expression when challenged with an acute dose of LPS compared to control mice suggesting partial protection from acute inflammation (1).

The pro-inflammatory environment and cardiovascular impairments created by iNOS lead to impaired glucose uptake and insulin (30). NO production by iNOS occurs in excess and has negatively affected cardiovascular function (decrease in cardiac output, blood flow, and perfusion) leading to impaired insulin-stimulated glucose uptake in the muscle due to impaired glucose delivery. This is evidenced in two studies where endotoxemia in mice decreased glucose uptake in skeletal muscle due to a decrease in glucose delivery to the tissue due to impaired cardiac function (63, 99). In this way, it is hypothesized that $P2Y_2R$-mediated iNOS expression and overproduction of NO lead to impaired muscle glucose uptake. Inhibiting $P2Y_2R$ activation

under these conditions may attenuate the cardiac impairments that facilitate impaired glucose uptake. The pro-inflammatory environment and cardiovascular impairments created by iNOS lead to impaired glucose uptake and insulin (30). NO production by iNOS occurs in excess and has negatively affected cardiovascular function (decrease in cardiac output, blood flow, and perfusion) leading to impaired insulin-stimulated glucose uptake in the muscle due to impaired glucose delivery. This is evidenced in two studies where endotoxemia in mice decreased glucose uptake in skeletal muscle due to a decrease in glucose delivery to the tissue due to impaired cardiac function (63, 99). In this way, it is hypothesized that $P2Y_2R$-mediated iNOS expression and overproduction NO, leading to impaired muscle glucose uptake. Inhibiting $P2Y_2R$ activation under these conditions may attenuate the cardiac impairments that facilitate impaired glucose uptake.

The previous studies discussed provide evidence that under pathophysiological conditions, $P2Y_2R$ activation is increased and negatively impacts glucose homeostasis by potentiating insulin resistance. The presence of high glucose concentrations leads to ATP and UTP release that increase the expression and activation of $P2Y_2R$. However, there is a lack of information on how $P2Y_2R$ modulates glucose homeostasis under physiological conditions. It is unknown if $P2Y_2R$ activity contributes to glucose homeostasis in the postabsorptive and absorptive state, or if these conditions contribute to increased or decreased receptor activity. In the current study we set out to define the role of $P2Y_2R$ in glucose homeostasis under physiological conditions. We utilized the global $P2Y_2R$ knock out mouse model to evaluate if the absence of the receptor altered fasting glucose values and the response to glucose tolerance test relative to wild type mice, allowing us to elucidate the role of $P2Y_2R$ in glucose homeostasis. In this study, we found that $P2Y_2R$ may contribute to physiological glucose homeostasis in the

absorptive state specifically in male mice; further we found that sex-specific differences in fasting, or the post-absorptive state may be mediated by $P2Y_2R$.

Sex-Specific Regulation of Glucose Homeostasis

In biomedical research, male biased studies were the norm to circumvent any perceived interference from the female reproductive system or the assumption that what was discovered in males applied to females. However, in recent years, it has been realized that both female and male research subjects are necessary to conduct research that accounts for the sexual dimorphisms we see in health and disease and are important in considering symptoms of certain disease and efficacy of certain drugs. The combination of genetic and hormonal sex differences alters a variety of physiological parameters between the sexes.

Reproductive (sex) hormones such as estrogen partially contribute to sexual dimorphisms between men and women. There are several key sexual dimorphisms in glucose homeostasis that are evidenced to be influenced by sex hormones. Sex differences are observed in fasting blood glucose, glucose tolerance, and insulin sensitivity, and in the development of metabolic diseases such as T2D (34, 60). For example, it is reported that increased insulin sensitivity in women relative to men is correlated to the differing concentrations of estrogen between the sexes (88, 129). This is exemplified in age-related studies by observing the changes of estrogen concentrations through the life span in women and the effect on insulin sensitivity. For instance, women early in their reproductive years are more insulin sensitive relative to woman who have gone through menopause, where there is a significant decrease in estrogen. When comparing young adult women and age-matched men, women are less prone to the development of insulin resistance. In-post-menopausal women the incidences of insulin resistance began to match men,

suggesting that estrogen has a protective role against insulin resistance (89). This highlights the importance of reproductive hormones in the regulation of the glucose homeostasis and how they contribute to sexual dimorphisms.

Further, female humans and mice have lower fasting blood glucose levels relative to males and females, in addition to being more insulin sensitive. However, blood glucose levels remain higher relative to men 2 hours post-oral glucose tolerance test OGTT in women (89). This suggest that women have decreased glucose tolerance relative to men, despite documented increased insulin sensitivity. However, when an OGTT is performed in humans, the bolus of glucose ingested contains a standardized concentration of glucose for all patients. This may indicate that the rate of glucose uptake in women is decreased in women due to body fat-content and height differences. Whereas in mice the bolus of glucose ingested or administered by injection is based on body weight, which may lead to a more accurate reflection of glucose tolerance. During intraperitoneal GTT (IPGTT) in C57BL/6 mice, females have better glucose tolerance and insulin sensitivity compared with males (86). When an OGTT is performed in humans, the bolus of glucose ingested contains a standardized concentration of glucose for all patients whereas in mice the bolus of glucose ingested or administered by injection is based on body weight, which may lead to a more accurate reflection of glucose tolerance.

Sex differences contribute to body fat and skeletal muscle distribution and abundance, which further contribute to sex-specific differences in glucose homeostasis. For instance, white adipose tissue distribution may influence differences in metabolic parameters among women. For example, fat is distributed in the subcutaneous space primarily in women, however, accumulates in the visceral organs in men. The accumulation of visceral fat is tightly correlated with insulin resistance and T2D. Accordingly, men are more likely to develop T2D and cardiovascular

disease. Similarly, it has been reported skeletal muscle differs between men and women in energy metabolism, fiber composition, and contractile speed (56). Supporting this, to a study from 1990 showed that men and women exhibit differences in glucose uptake. In this study, human subjects were given a glucose challenge and after 3 hours, women had higher glucose uptake relative to men (113).

Genetic sexual dimorphisms in glucose homeostasis are attributed to the X and Y chromosomes, gene expression, and epigenetics. Sex-specific differences gene expression in key molecules in various tissues involved in glucose homeostasis. For instance, it has been shown expression of GLUT4 mRNA in female mice is higher than that in males. When these mice were challenged with exogenous insulin, females have significantly higher phosphorylated AKT relative to males (86). The increase in gene expression in females may be attributed to estrogen's ability to regulate transcription. Estrogen binds to nuclear estrogen receptors and become transcription factors, leading to the recruitment of transcriptional coactivators to promoter and enhancer regions to activate gene expression (67). Given that women have higher levels of estrogen, this may enhance transcription of relevant receptors, transporters, and signaling molecules involved in glucose homeostasis in women, leading to better insulin sensitivity.

Regulation of genes not directly involved in metabolic pathways may have sex-specific influence on glucose homeostasis. For instance, in $Casp2^{-/-}$ mice females are protected from impaired glucose tolerance in older adults. In contrast, knocking out this gene promotes impaired glucose tolerance in older males (152). Further, a study evaluating G-protein coupled receptor kinase 2 (GRK2) in sex- and age- related differences in glucose homeostasis under high fat diet feeding show young female mice have better insulin sensitivity correlating with lower GRK2 expression compared to age-matched male and middle-aged female animals This demonstrates

expressional differences in genes and proteins may be influenced by sex hormones and contribute to sex-specific differences in development of impaired glucose tolerance (8, 152).

Collectively, sex-specific differences in glucose homeostasis are complex and are regulated by a variety of biological factors. In the studies discussed here, we see evidence that sex differences in glucose homeostasis are mitigated by sex hormones and sexual dimorphisms in gene expression. How these sex biological factors influence $P2Y_2$ receptor signaling in the regulation of glucose homeostasis remains unknown and requires investigation. In the current study we set out to illuminate the novel physiological function of $P2Y_2R$ in glucose homeostasis in both male and female mice and to evaluate potential sexual dimorphism in $P2Y_2R$ activation in glucose homeostasis. In the current study we observed different response to glucose tolerance test in male $P2Y_2R^{-/-}$ mice relative to wild type littermates, but not in female wild type and knock-out littermates. We observed sex specific differences in fasting blood glucose and glucose tolerance test in wild type males and females and $P2Y_2R^{-/-}$. The discovery of sex differences in fasting blood glucose in wild type mice, but not in $P2Y_2R^{-/-}$ may suggest involved of $P2Y_2R$.

METHODS

Animals

The Institutional Animal Care & Use Committee (IACUC) of Missouri State University approved all procedures of the study designated as protocol 18-006.0 (Appendix). Animals were purchased from Jackson Laboratory (Stock #: 009132 Bar Harbor, Maine). The $P2Y_2R^{+/-}$ (C57BL/6J background) mice were originally established by the lab of Dr. Beverley Koller (149). All mice were fed a standard chow diet (Laboratory Rodent Diet 5001, Purina, cat # 0001319 St. Louis, Missouri). Mice were housed in the Professional Building vivarium in filtered cages. The mice were on a twelve-hour light-dark cycle (6 am-6 pm dark, 6 pm-6 am light) at 72 °F.

Both female and male $P2Y_2R^{-/-}$ and C57BL/6 (wild type) littermates were utilized during this study (Fig. 6). Each experiment was performed on a pair of same-sex $P2Y_2R^{-/-}$ and wild type littermates. Mice were between the ages of 8 and 12 weeks old for all experiments performed. Female mice weighed between 17.8 and 25.3 grams; male mice weighed between 22.5 and 28.9 grams. A total of 38 mice were used for the study while the data generated from 36 mice were included in the thesis. Nine wild type and nine $P2Y_2R^{-/-}$ male mice were used in the experiments for this thesis. Ten wild type and 10 $P2Y_2R^{-/-}$ female mice were used in the experiments for this thesis. One wild type and one $P2Y_2R^{-/-}$ female mice were not included in data analysis. These mice had missing ear tags at the time of the experiment. After completing the experiment, the two mice were mixed and placed into the wrong cage and could not be re-genotyped to confirm the genotype associated with the experiment; the data from these two experiments were not included in the data analysis (Fig. 6).

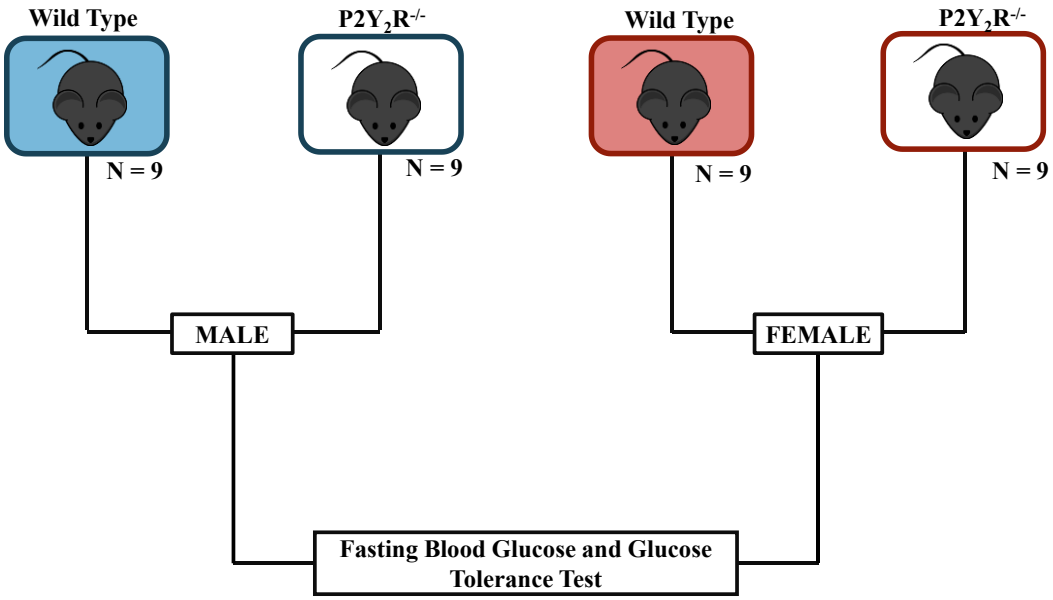

Fig. 6. Experimental groups of mice**.** Female and male $P2Y_2R^{-/-}$ mice and wild type littermate control mice were utilized during this study. Experiments were performed on a pair of same sex littermates.

Mouse Colony Breeding and Genotyping

Female and male heterozygous $P2Y_2^{+/-}$ were bred at Missouri State University, producing 50% heterozygous, 25% wild type (WT), and 25% $P2Y_2R^{-/-}$ mice. Mice were weaned at 28 days old. One week before weaning ears were tagged for identification, 0.5-2mm of their tails were snipped using sterile razors and placed into 1.5 ml tubes for genotyping. Male and female WT and $P2Y_2R^{-/-}$ offspring were utilized for this study. Heterozygous offspring were utilized as breeders to produce $P2Y_2R^{-/-}$ and wild type mice.

Three primers were used to amplify DNA for genotyping. One antisense primer and 2 sense primers, due to mutant *P2ry2* allele (Table 2) in the knockout mice. These primers were purchased through IDT DNA. The $P2Y_2R$ knock out model was created by inserting a neomycin cassette into the *P2ry2* cDNA at base pairs 552 to 1149, thus disrupting gene function (149). For our genotyping, one sense primer was created to amplify part of the neomycin gene, while the

other sense primer bound to the intact WT *P2y2* gene. The same antisense primer paired with the sense primers generated two distinct bands to for wild type and knock out alleles: 418 bp for wild type and 598 bp for knockout. Fig. 7 shows the gel electrophoresis results for wild type, knockout, and heterozygous mice with the primers designed for genotyping.

Genotyping was accomplished in 3 steps: DNA extraction, PCR, and gel electrophoresis. DNA was extracted from mouse tail snips using components of Thermo Scientific Phire Animal

Table 2. Primers used for genotyping the $P2Y_2R$ mouse colony.

	Primers	**DNA Sequence**
oRU23	anti-sense	5’ -AGCCACCCGGCGGGCATAAC- 3’
oRU24	sense	5’ -GAGGGGGACGAACTGGGATAC- 3’
oRU25	sense	5’ -AAATGCCTGCTCTTTACTGAAGG- 3’

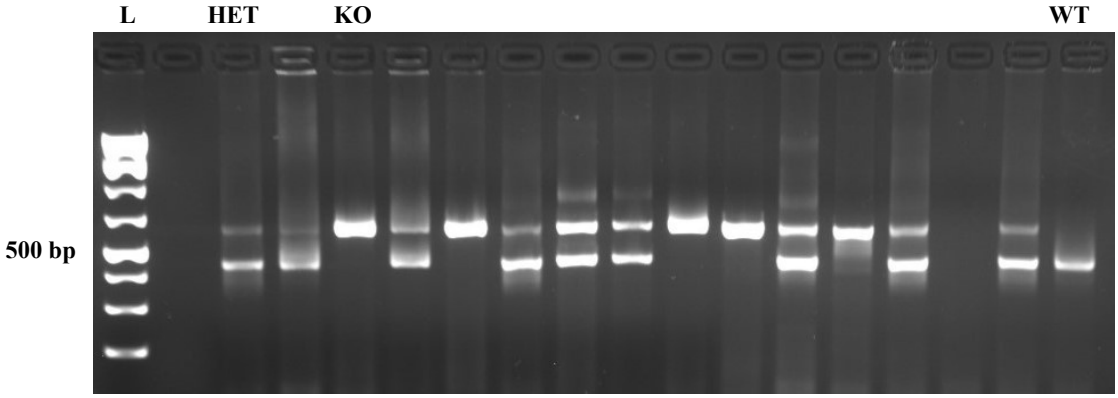

Fig. 7. Genotyping results for the $P2Y_2R$ breeding colony. Wild type and the knockout $P2Y_2R$ gene were amplified, and the products electrophoresed on a 1% agarose gel in 1 X sodium borate (SB) buffer. Left, 100 base pair (bp) ladder (L). Products from single heterozygous (HET), $P2Y_2R^{-/-}$ (KO), and wild type (WT) mice are indicated above the lane. Expected band sizes are: 598 bp KO, 414 bp WT; HET mice will contain both bands.

Tissue Direct PCR Kit: Dilution Buffer (Thermo Scientific Cat # F-132-5ML, Waltham, Massachusetts) and DNA Release Additive (Thermo Scientific, Cat # FERB93, Waltham, Massachusetts). Twenty microliters of dilution buffer and 1 µl of DNA release were added directly to the tail snips. The centrifuge tubes were spun down and then vortexed to make sure tail snips were in solution. The tail snips incubated at room temperature for 5 minutes, then at 98 ℃ for 2 minutes. The centrifuge tubes were vortexed again with the liquid containing the DNA extract and tail snip left in the tube. Following this, DNA extract was used for PCR or frozen at -20 ℃.

Extracted DNA was amplified by PCR using DreamTaq master mix (Thermo Scientific, Cat # K1071, Waltham, Massachusetts), and primers oRU23, oRU24, oRU25. For the PCR, a master mix was created based upon the number of tail snips and controls. In Table 3 an example of how the master mix was set up is provided. Each PCR reaction contained 12.5 µl of Dream Taq Master Mix, 0.25 µl of oRU23 (100 µM), oRU24 (100 µM), oRU25 (100 µM), 10.0 ul of H_2O. After the master mix was aliquoted to the PCR tubes, 1.75 µl of DNA was added to each

Table 3. Example for creating a 25 µl Master mix for 7 samples, controls, and1 µl extra

25 µl PCR reaction	per reaction	master mix (7X)
Dream Taq Master Mix	12.5 µl	87.5 µl
oRU23 (100 µM)	0.25 µl	1.75 µl
oRU24 (100 µM)	0.25 µl	1.75 µl
oRU25 (100 µM)	0.25 µl	1.75 µl
H_2O	10.00 µl	70 µl
Total:	23.25 µl	—
template DNA	1.75 µl added to each tube after the master mix	

tube for each mouse. The thermocycler was set according to the parameters depicted in Table 4.

Following PCR, gel electrophoresis was performed. PCR reactions were removed from the thermocycler and vortexed. For Dream Taq containing loading dye, reactions were loaded into the gel as is. For PCR reactions where Dream Taq did not include loading dye, 5 µl of 6X loading dye was added to each reaction. The PCR product was run on 1% to 1.5% agarose gel containing Ethidium Bromide (EtBr) at the following volumes, depending on the size of the gel: 2 ul of EtBr was added to 50 ml gel, 10 ul of EtBr was added to 100 ml and 125 ml gels. Each agarose gel was run at 230V for 30 to 35 minutes in 1X sodium borate (SB) buffer. Fig. 7 demonstrates the gel electrophoresis results for wild type, knockout, and heterozygous mice.

Table 4. Thermocycler settings for mouse colony genotyping

	initial denaturation	denaturation	annealing	extension	repeat	final extension	hold
Temp	95 ℃	95 ℃	60 ℃	72 ℃	Repeat	72 ℃	4 ℃
Time	2 min	30 sec	30 sec	30 sec	2-4 36X	5 min	∞

Intraperitoneal Glucose Tolerance Testing

To evaluate the glucose response, an intraperitoneal glucose tolerance test (IPGTT) was performed (Fig. 8) (7, 10, 11, 16, 145). Mice were placed into separate cages for fasting for 5 hours before IPGTT. These cages were clean, free of food, but access to water ad libitum. A stock volume of 50% dextrose (Patterson Veterinary Supply, #78008986, Kansas City, Missouri) was diluted to 20% (w/v) dextrose in saline for intraperitoneal (IP) injection at 2g/kg body weight. Ten to fifteen minutes before the GTT mice were removed from their cage and place in a raised, uncovered container for easy access, then lidocaine was applied to the tail for local anesthesia. Lidocaine was applied by gently taking the mouse by the tail and wiping it onto the

tail. After 10 to 15 minutes, the lidocaine was wiped off and 70% EtOH was used to sanitize the tail. One to two millimeters of the tail tips were snipped with surgical scissors. The tail was gently massaged to promote blood flow to the tip to measure blood glucose levels using blood glucose test strips coupled with a glucometer (ReliOn, Walmart, Bentonville Arkansas). The fasting (t = 0) blood glucose reading was assessed immediately before the IP injection of dextrose. Fig. 8 shows a schematic timeline of each important time point in the experiment.

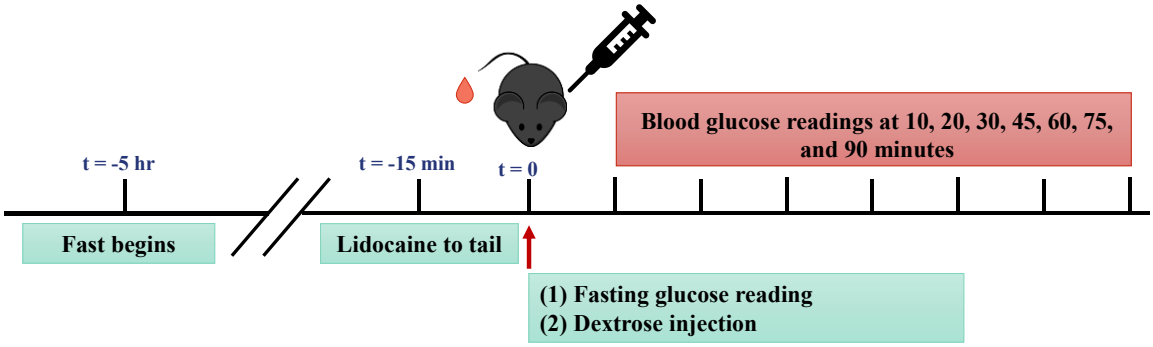

Fig. 8. Schematic timeline of intraperitoneal glucose tolerance test. Mice were removed from vivarium and placed into the cages with water but no food to initiate the 5 hours fast (t = -5 hr). Ten to fifteen minutes (t = -15 min) prior to the glucose tolerance test lidocaine was applied to the tail. Fasting blood glucose was read at the end of the 5 hours fast (t = 0) and immediately after IP injection of 20% dextrose was administered. Following blood glucose levels were read at the indicated time points over 90 minutes.

After the fasting blood glucose reading the IP injections were performed. For the IP injections mice were physically restrained by experimenter. The mice were placed on the bench top with the dominant hand gently holding the mouse just below the base of the tail. With the non-dominant hand, the mice were picked up by the scruff of the neck with the thumb and index finger. The tail was then placed between the palm and the pinky finger. From this point the abdomen was exposed for the IP injection. The IP injection was administered in the lower right or left quadrant of the abdomen at a 45°C angle. After the IP injection blood glucose reading were taken at 10, 20, 30, 45, 60, 75, and 90 minutes after IP injection. After the final glucose

reading, the tail was anesthetized again using lidocaine. Ten minutes after the second application of lidocaine, the tail blood samples were collected in SARSTEDT Microvette CB 300 K2E centrifuge tubes (Thermo Scientific, Cat# NC9141704, State) for an assessment of insulin level. To obtain plasma, 100 to 150 µl of blood was centrifuged at 10 g for 10 minutes at 4 °C (Eppendorf Centrifuge 5415 R, Cat#E216059, Germany). The plasma was aliquoted into a 1.5 ml Eppendorf tube and stored at -80 °C for future quantification of insulin levels. After each experiment, mice were anesthetized using isoflurane followed by euthanasia by cervical dislocation.

Statistical Analyses

All values are presented as Mean ± SEM. Prism GraphPad and College of Health and Human Services R-STATs department were used to evaluate significant differences between groups: female WT vs KO, male WT vs KO, WT male vs female, and KO male vs female. Glucose tolerance test data were analyzed using two-way ANOVA with repeated measures. This is to evaluate the effect of the genotype on blood glucose response, which consists of two (two independent variables, wild type, and $P2Y_2R^{-/-}$ mouse strains) by eight repeated measures at indicated time points. The analysis for the effect of sex on glucose response also used two-way ANOVA with repeated measures. Sisak's multiple comparison test was utilized to determine if a significant difference in glucose concentration at each time point between genotypes or sexes. Fasting blood glucose levels between the four groups to evaluate the effect of genotype and sex were analyzed with a 2-way ANOVA, and then two-tailed unpaired t-test to evaluate effect of genotype on fasting blood glucose and the effect of sex on fasting blood glucose. Statistical significance was reported as p-value less than 0.05 ($p < 0.05$).

The results Tables generated in Prism using a 2-way ANOVA with repeated measures for IPGTT data and a 2-way ANOVA for fasting are in the results (Tables 5,8,9, 10, 11). Results tables generated from 2-tailed t-test for sex differences in fasting are in the results (Tables 6,7)

RESULTS

Influence of $P2Y_2$ Receptor and Sex under Fasting Conditions

Clinically, fasting blood glucose (FBG) is utilized to screen for pre-diabetes and diabetes. In animal research, fasting blood glucose levels may allow investigators to evaluate blood glucose homeostasis under various conditions. We used fasting blood glucose values to assess $P2Y_2R$ and sex in glucose homeostasis in the post-absorptive state.

$P2Y_2$ Receptor Activity Under Fasting Conditions. To assess fasting blood glucose in $P2Y_2R^{-/-}$ and wild type mice, blood glucose levels at *t = 0*, fasting without food for 5 hours in the same-sex littermates were measured (Fig. 9, Table 5). Fasting blood glucose is not expected to differ between wild type and knock out mice under physiological conditions. No significant difference in fasting blood glucose levels due to genotype in male wild type (106.6 ± 4.6 mg/dl; $n = 9$) and $P2Y_2R^{-/-}$ (100.4 ± 4.7 mg/dl; $n = 9$) and, in female wild type (88.9 ± 5.7 mg/dl; $n = 9$) and $P2Y_2R^{-/-}$ (95.1 ± 4.5 mg/dl; $n = 9$) mice. These data (Fig. 9, Table 5) suggest $P2Y_2R$ does not affect fasting blood glucose in sex- and littermate-matched mice**.**

Sex Differences Under Fasting Conditions. Sex differences in FBG were assessed between all four groups. It is expected that sex specific differences in fasting blood glucose are present in both wild type and $P2Y_2R^{-/-}$ mice. Sex differences were significant when all four groups were analyzed with a 2-way ANOVA (Fig. 9, Table 5). To assess the effect of sex on FBG within wild type mice and $P2Y_2R^{-/-}$ mice using a 2-tailed t test between wild type male and female (Table 6) and $P2Y_2R^{-/-}$ males and females (Table 7). In wild type mice, the fasting glucose levels were significantly lower in females relative to males (Fig. 9, Table 6). However,

$P2Y_2R^{-/-}$ females and males did not differ significantly in fasting glucose levels (Fig. 9, Table 7). The results suggest $P2Y_2R$ may contribute to sex differences affecting FBG

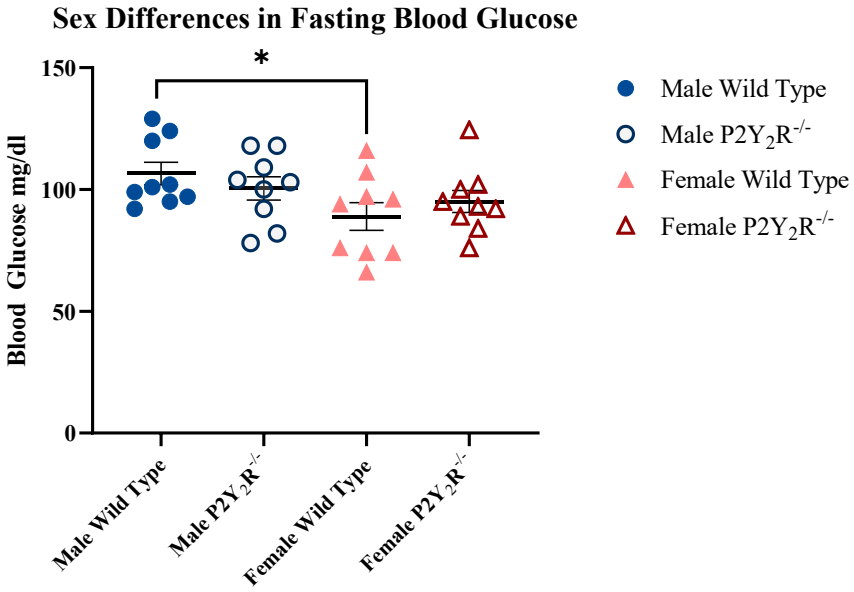

Fig. 9. Sex differences in fasting blood glucose levels. $P2Y_2R$ did not alter fasting blood glucose in males. Fasting blood glucose levels (at $t = 0$) were measured following 5 hours of fasting using a glucometer. FBG value were analyzed between the 4 groups using a 2-way ANOVA, showing sex, not genotype has a significant effect on FBG ($p < 0.05$). FBG was then analyzed for sex differences using 2-tailed t-test to evaluate the effect of sex in wild type and knock out mice. Data are presented as mean ± SEM ($n = 9$) and * denotes statistical significance ($p < 0.05$).

Table 5. 2-way ANOVA for fasting blood glucose between all 4 groups results.

Source of Variation	% Of total variation	P value	P value summary	Significant?	
Interaction	3.987	0.2212	ns	No	
Sex	14.06	0.0255	*	Yes	
Genotype	8.162e-005	0.9955	ns	No	
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	339.2	1	339.2	F (1, 32) = 1.557	P=0.2212
Sex	1196	1	1196	F (1, 32) = 5.489	P=0.0255
Genotype	0.006944	1	0.006944	F (1, 32) = 3.187e-005	P=0.9955
Residual	6973	32	217.9		

* Source of variation (top, bold) and the ANOVA table (bottom, bold).

Table 6. 2-tailed unpaired t-test for WT sex differences in fasting glucose

Unpaired t test	
P value	0.0284
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.409, df=16

Table 7. 2-tailed unpaired t-test for $P2Y_2R^{-/-}$ sex differences in fasting glucose

Unpaired t test	
P value	0.4235
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.8213, df=16

Influence of $P2Y_2$ Receptor and Sex on Glucose Tolerance

To assess the effects of $P2Y_2R$ and sex on glucose homeostasis in the absorptive state (fed conditions), IPGTT was performed by measuring blood glucose concentration over a 90-minute time-course after intraperitoneal injection (IP) of glucose (Fig. 10).

$P2Y_2$ Receptor Activity in Glucose Tolerance, Fed Conditions. To determine if $P2Y_2R$ regulates glucoses in the absoprtive state, the time course response to IPGTT in $P2Y_2R^{-/-}$ mice was compared with sex- and littermate-matched wild type mice (Fig. 10, Table 8, Table 9). The results for the glucose tolerance test are expected to remain the same between male wild type and $P2Y_2R^{-/-}$ mice. Male $P2Y_2R^{-/-}$ mice displayed higher blood glucose levels over 90 min in response to glucose challenge relative to wild type males, shown in Fig. 10*A* and Table 8 ($p < 0.05$). These data suggest $P2Y_2R$ is involved in maintaining physiological glucose tolerance.

Unlike male mice, females did not have significant interaction between time and genotype when data were analyzed with two-way ANOVA with repeated measures.Although, the time course response to IPGTT in female wild type mice showed a trend of lower blood glucose levels compared with female $P2Y_2R^{-/-}$ mice, there was no significant difference, as shown in Fig. 10*B* and Table 9 ($p > 0.05$). These data may suggest $P2Y_2R$ is not involved in matianing phsyilogical glucose tolerance in females, but plays a male-specific role in glucose tolerance. Fig. 10*C* shows all four groups plotted onto one graph.

Sex-Specific Differences in Glucose Tolerance in Wild Type Controls and $P2Y_2R^{-/-}$ Mice. The effect of sex on glucose homeostasis in the absorptive state was analyzed by comparing glucose tolerance test between sexes in wild type and $P2Y_2R^{-/-}$ mice (Fig. 11, Table 10, 11). Sex differences between male and female mice during a glucose tolerance are expected. Female mice are expected to have lower blood glucose levels over time relative to males. The time course response to IPGTT in female wild type mice was lower than male wild type mice ($p < 0.05$) (Fig. 11*A* Table 10). The female $P2Y_2R^{-/-}$ mice displayed lower blood glucose levels over the time relative to males $P2Y_2R^{-/-}$ mice ($p < 0.05$), shown in Fig. 11*B* and Table 11. Consistent with previous literature and data reported, blood glucose levels over time are lower in female wild type mice, indicating improved insulin sensitivity and glucose clearance relative to wild type males. In $P2Y_2R^{-/-}$ mice, we see these sex differences as well. However, these data suggest the sex differences in $P2Y_2R^{-/-}$ mice may be exacerbated due to the statistically significant increase in blood glucose levels in $P2Y_2R^{-/-}$ males relative to wild type and requires further investigation.

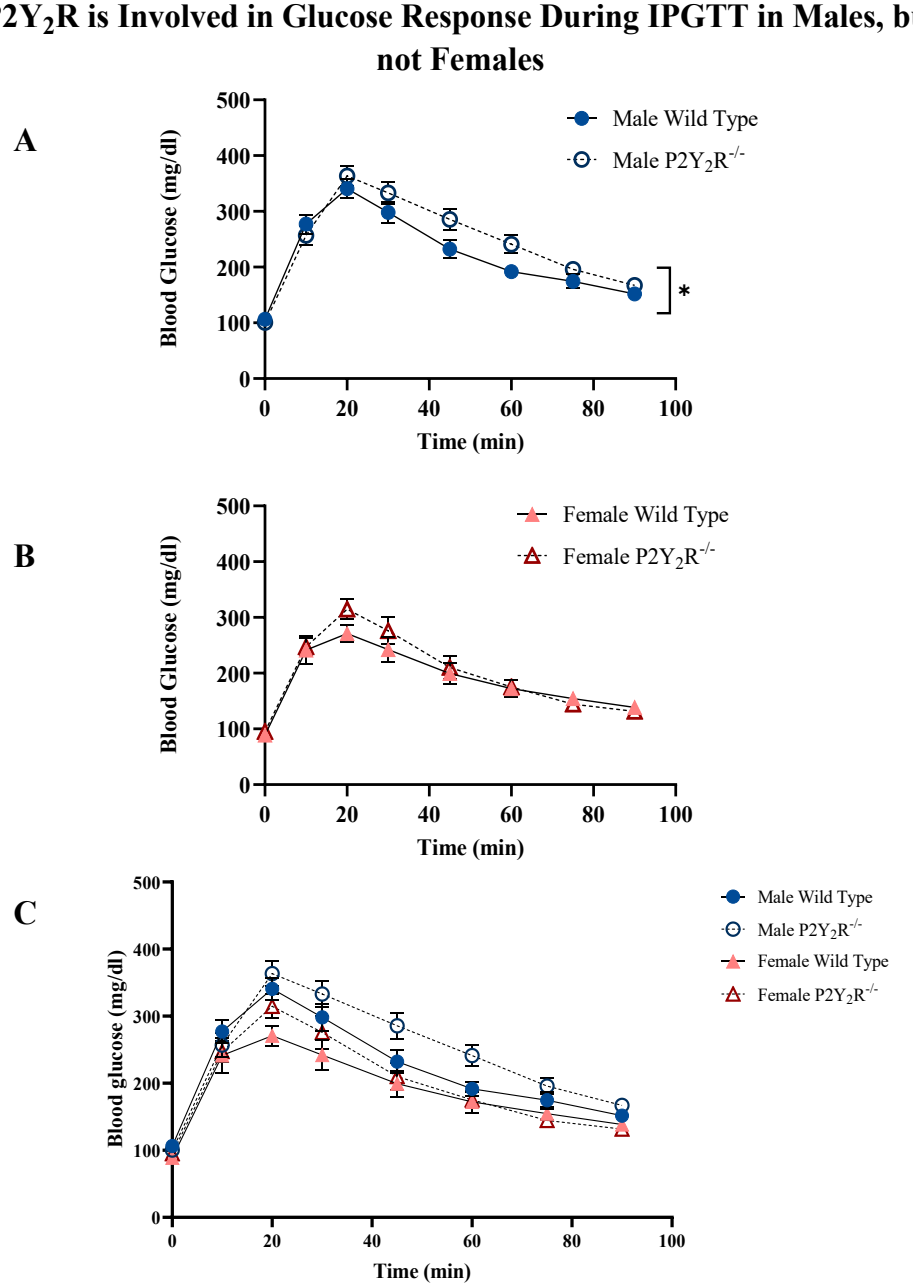

Fig. 10. $P2Y_2R$ is involved in glucose response during IPGTT in males, but not females. Intraperitoneal glucose tolerance test (IPGTT) was used to determine the role of $P2Y_2R$ in glucose homeostasis. Mice fasted for 5 hours before *Ip* administration of 20% (v/v) dextrose at 2 g/kg body weight. A: Blood glucose responses to IPGTT over 90 minutes in male wild type and $P2Y_2R^{-/-}$. B: Blood glucose responses to IPGTT over 90 minutes in female wild type and $P2Y_2R^{-/-}$ mice. C: All four group's blood glucose response plotted onto one graph. Data are presented as mean ± SEM and * denotes statistical significance ($p < 0.05$).

Table 8. WT and $P2Y_2R^{-/-}$ male mice 2-way ANOVA repeated measures results.

Source of Variation	% Of total variation	P value	P value summary	Significant?	Geisser-Greenhouse's epsilon
Time x Genotype	1.776	0.0151	*	Yes	
Time	74.50	<0.0001	****	Yes	0.3766
Genotype	1.430	0.1770	ns	No	
Subject	11.47	<0.0001	****	Yes	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Time x Genotype	20402	7	2915	F (7, 112) = 2.625	P=0.0151
Time	855907	7	122272	F (2.636, 42.17) = 110.1	P<0.0001
Genotype	16427	1	16427	F (1, 16) = 1.995	P=0.1770
Subject	131755	16	8235	F (16, 112) = 7.417	P<0.0001
Residual	124352	112	1110		

* Source of variation (top) and the ANOVA table (bottom)

Table 9. WT and $P2Y_2R^{-/-}$ female mice 2-way ANOVA repeated measures results.

Source of Variation	% Of total variation	P value	P value summary	Significant?	Geisser-Greenhouse's epsilon
Time x Genotype	1.230	0.4294	ns	No	
Time	65.41	<0.0001	****	Yes	0.3237
Genotype	0.4615	0.4683	ns	No	
Subject	13.38	<0.0001	****	Yes	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Time x Genotype	11144	7	1592	F (7, 112) = 1.008	P=0.4294
Time	592631	7	84662	F (2.266, 36.25) = 53.61	P<0.0001
Genotype	4182	1	4182	F (1, 16) = 0.5519	P=0.4683
Subject	121242	16	7578	F (16, 112) = 4.799	P<0.0001
Residual	176866	112	1579		

*Source of variation (top) and the ANOVA table (bottom)

Sex Influence Glucose Response During IPGTT in Both Wild Type and $P2Y_2R^{-/-}$ Mice

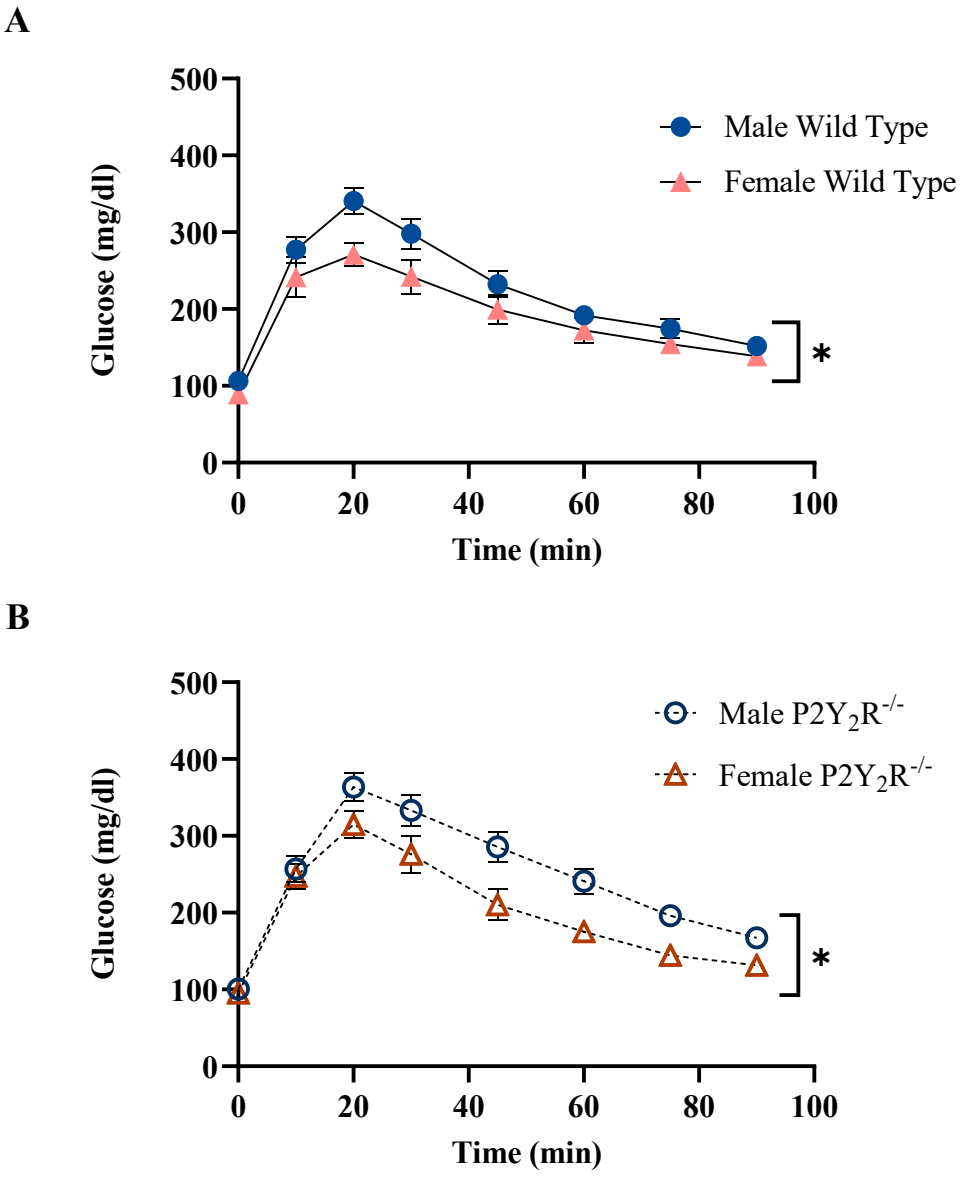

Fig. 11. Sex influences glucose response during IPGTT in both wild type and $P2Y_2R$-/- mice. The effect of sex on glucose homeostasis was assessed using age-matched male (n = 9) and female (n = 9) wild type (A) and P2Y2R-/- mice (B). Mice have fasted for 5 hours before *ip* administration of 20% (v/v) dextrose at 2 g/kg body weight. Blood glucose was measured at the indicated time points. Data are presented as mean ± SEM and the symbol * denotes statistical significance ($p < 0.05$)

Table 10. Sex differences in wild type mice 2-way ANOVA repeated measures results.

Source of Variation	% Of total variation	P value	P value summary	Significant?	Geisser-Greenhouse's epsilon
Time x Sex	1.343	0.2882	ns	No	
Time	65.26	<0.0001	****	Yes	0.3136
Sex	4.110	0.0321	*	Yes	
Subject	11.93	<0.0001	****	Yes	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Time x Sex	12861	7	1837	F (7, 112) = 1.238	P=0.2882
Time	624938	7	89277	F (2.195, 35.12) = 60.15	P<0.0001
Sex	39353	1	39353	F (1, 16) = 5.513	P=0.0321
Subject	114212	16	7138	F (16, 112) = 4.810	P<0.0001
Residual	166229	112	1484		

* Source of variation (top) and the ANOVA table (bottom)

Table 11. Sex differences in $P2Y_2R^{-/-}$ mice 2-way ANOVA repeated measures results.

Source of Variation	% Of total variation	P value	P value summary	Significant?	Geisser Greenhou epsilor
Time x Sex	1.343	0.2882	ns	No	
Time	65.26	<0.0001	****	Yes	0.3136
Sex	4.110	0.0321	*	Yes	
Subject	11.93	<0.0001	****	Yes	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Time x Sex	12861	7	1837	F (7, 112) = 1.238	P=0.288
Time	624938	7	89277	F (2.195, 35.12) = 60.15	P<0.000
Sex	39353	1	39353	F (1, 16) = 5.513	P=0.032
Subject	114212	16	7138	F (16, 112) = 4.810	P<0.000
Residual	166229	112	1484		

*Source of variation (top) and the ANOVA table (bottom)

DISCUSSION AND CONCLUSION

Currently, there are no published data demonstrating $P2Y_2$ receptor activation in the physiological regulation of glucose homeostasis. In this current study we set out to define the novel role of the $P2Y_2$ receptor in physiological glucose regulation in both male and female mice. Previous studies demonstrate that $P2Y_2R$ signaling is increased under pathophysiological conditions, such as obesity and insulin resistance demonstrating $P2Y_2R$ is a key player. However, it is unknown if $P2Y_2R$ signaling is involved in glucose homeostasis under physiological conditions. Further, it is known that sex differences in gene expression and reproductive hormones contribute to the physiological regulation of glucose homeostasis. In the previous studies discussed, experiments were conducted in solely male or female mice and did not account for sex-specific differences in glucose homeostasis.

In the current study we set out to define the novel role of sex differences in $P2Y_2R$ signaling. The objective of this study is to answer the questions (1) what physiological role does $P2Y_2R$ have *in vivo* in fasting blood glucose and glucose tolerance and (2) do sex-differences in $P2Y_2R$ signaling change these parameters between female and male mice? Using four groups of mice, 2 sexes, 2 mouse strains (WT and $P2Y_2R^{-/-}$ mice), we found the absence of $P2Y_2R$ impaired glucose tolerance, thus glucose homeostasis in the absorptive state, in males, but not in female mice. Further, sex-difference in fasting blood glucose in wild type mice, but not in and $P2Y_2R^{-/-}$ mice. Moreover, sex difference in GTT in both wild type and $P2Y_2R^{-/-}$ mice were found, however, $P2Y_2R$-mediated modulation during GTT was only observed in males, but not females.

Assessment of Fasting Blood Glucose

Fasting blood glucose is used to evaluate blood glucose regulation under postabsorptive condition. Fasting blood glucose levels are primarily regulated by the liver via hepatic endogenous glucose production under the metabolic state with refrainment from eating for a given period. The impaired fasting glucose in humans is defined as FBG > 100 mg/dl. In the current study, mice underwent a morning fast for 5 hours starting at 7:30 am. The duration of fasting is an important aspect of assessing metabolic abnormalities. Overnight fasting (12-18 hours) is reported to provide a steady-state FBG concentration, however, due to the high metabolic rate of mice fasting for this long induces metabolic stress and significant weight loss in the animals (10, 16). Five hours fast, on the other hand, reduce metabolic stress and is equivalent to human physiology (3, 10). Overnight fasting has been suggested for evaluating FBG in high-fat diet models to reduce variability. However, in the current study mice were kept on a regular chow diet to evaluate physiological blood glucose regulation.

In an article from the Jackson laboratory (51) it has been reported that the normal range of fasting blood glucose levels in mice is between 80 and 100 mg/dl after a 4 to 6 hour fast. However, a variety of factors can influence the level of FBG baseline in each set of experiments. FBG values that have been reported has a big range: 151.35±12.61 mg/dl in 18-week old C57BL/6J mice after 4-hour fast (66), 96.2±10.2 mg/dl 12-hour daytime fast and 85.1±22.5 mg/dl 12 hour night time fast in male C57BL/6J (135), 141.98±4.32 mg/dl in 18- to 19-week old male mice after a 6 hour fast (7), 124.33±7.2 mg/dl in 16-week old male C57BL/6 mice with an unknown fasting time (53), 106.31±12.61 mg/dl in 12- to 16- week old 657BL/6 male mice after a 4 hour fast (74). Given this information, fasting blood glucose levels may vary between studies due to the mice (age, strain, littermate), fasting period, researchers (animal handling skills), and experimental environment where the test is performed.

In the current study, a pair of littermates, one wild type and one $P2Y_2R^{-/-}$ mouse with the same sex, were used for an experiment to evaluate FBG and IPGTT and minimize genetic variance. The WT mice had 106±4.6 mg/dl FBG in males and 88.9±5.7 mg/dl in females (Fig. 9, Table 5, Table 6). These data were consistently collected by one person in a consistent environment with efforts to eliminate any stress by placing mice in a quiet area for fasting. Further, the person handling the mice for each experiment and performing the experiments was kept consistent in the current study, reducing stressors from different handlers between experiments. In future studies, if multiple persons are performing experiments, it is necessary to randomize experimental conditions, such alternating who is performing experiments on certain cohorts of mice (123). In comparison to the FBG values reported from previous literature for wild type mice, our fasting blood glucose levels in both females and males are consistent with the values from literature reported above, despite differences in age, fasting time, and sex of the mice. These data demonstrate that the data collected in the current study are consistent with previously published literature and show the importance of maintaining consistency and reducing stressors in metabolic studies (Fig. 9, Table 5, Table 6). Further, fasting blood glucose values for $P2Y_2R^{-/-}$ mice under physiological conditions have not been reported in literature yet. $P2Y_2R^{-/-}$ mice had 100.4±4.7 mg/dl in males and 95.1±4.5 mg/dl in females (Fig. 9, Table 5, Table 7). We did not observe differences in FBG between wild type and $P2Y_2R^{-/-}$ pairs in both males and females, suggesting that $P2Y_2R$ does not alter FBG for either males or females.

Sex-specific differences in FBG have been observed in WT mice, but not found in $P2Y_2R^{-/-}$ mice. A significant difference was revealed in wild type mice with females displaying lower FBG relative to males. As reported previously, women have lower FBG relative to male counterparts. Contrarily, there is no sex-specific differences in FBG in $P2Y_2R^{-/-}$ mice (Fig. 9,

Table 7). The findings demonstrate that $P2Y_2R$ modulates sex-difference in FBG, suggesting that $P2Y_2R$ regulates FBG in different manners between males and females. Given that FBG level is primarily regulated by hepatic endogenous glucose production, our findings suggest that contribution of $P2Y_2R$ to sex-specific differences in FBG under physiological conditions may be through different responses of the liver to the fasting status between males and females. It is worthy investigating hepatic glucose metabolism modulated by $P2Y_2R$ in males and females with respect to its interaction with sex hormones regulating endogenous glucose production.

Assessment of $P2Y_2$ Receptor Role During Glucose Tolerance Test

Glucose tolerance test are useful tools to monitor and detect changes in blood glucose in response to an exogenous glucose load and allow for detection euglycemia or dysglycemia (impaired glucose tolerance). In human, oral glucose tolerance test (OGTT) is used clinically as a diagnostic tool for pre-diabetes, T2D, and gestational diabetes. In animal models, glucose tolerance test allows researchers to characterize glucose metabolic phenotype in various disease models under different conditions, such as sex, environment, treatments, and genetic modifications. Exogeneous glucose can be administered through different routes, including oral (OGTT), intravascular (IVGTT), or intraperitoneal (IPGTT) method, which subsequently influences the levels of blood glucose because of different regulatory mechanisms. GTT results may vary in how a glucose challenge is administered. OGTT is used in both humans and animals, however, intravenous GTT (IVGTT) and intraperitoneal GTT (IPGTT) are often used in animals.

GTT Assessment for Wild Type and $P2Y_2R^{-/-}$ Male Littermates. Current literature reported an important role of $P2Y_2R$ in high-fat diet induced obesity. The data collected from

those studies show that $P2Y_2R$ contributes to insulin resistance and obesity under pathophysiological conditions (93, 153) . In the current study under physiological conditions, we observed higher blood glucose levels during glucose tolerance test in male $P2Y_2R^{-/-}$ knockout mice compared with WT mice, suggesting a novel protective effect of $P2Y_2R$ on maintaining glucose tolerance in male mice (Fig. 10*A*, Table 8). Interestingly, in Zhang et al's study using regular diet, the male $P2Y_2R^{-/-}$ mice had lower blood glucose levels over time of GTT relative to wild type mice (153). However, they did not report any significant differences in GTT between WT and $P2Y_2R^{-/-}$ mice with normal diet. In addition, the genetic background for their mice is B6D2 which is different from our mice (C57BL/6NJ), and the age of mice used for their study is older than mice used in our study. We observed that $P2Y_2R^{-/-}$ male mice increased blood glucose levels starting at $t = 20$ relative to wild type mice (Fig. 10*A*). It is possible that aging-induced decrease in insulin sensitivity could mask the effect of $P2Y_2R$ on the regulation of GTT. It is also possible that differences in the genetic background of the mice could influence these differences (107, 117, 131). In similar HFD experiments performed by Merz et al, mice with C57BL/6 background were used, the same background as our mice (93). However, data was only reported for the HFD diet fed mice for GTT and ITT experiments. In conclusion our findings demonstrate $P2Y_2R$ modulates physiological glucose tolerance in male mice with a C57BL/6 background with age between 8 and 12 weeks old.

Impaired glucose tolerance and insulin resistance can result from dysfunction of cardiovascular system (95, 99). Substantial data has shown $P2Y_2R$ regulates the homeostasis of the cardiovascular system. For instance, hypertension is observed in both global $P2Y_2R^{-/-}$ mice and endothelial-specific knockout of $P2Y_2R$ mice (26, 118, 119, 147), implicating vasodilatory effect of $P2Y_2R$ in endothelial cells. In our lab, we recently observed a similar phenomenon in

$P2Y_2R$'s role in leukocyte-endothelial interaction. Data shows that under physiological conditions *in vivo*, $P2Y_2R^{-/-}$ mice had increased leukocyte rolling and adhesion, hallmarks of inflammation, relative to wild type controls, thus suggesting $P2Y_2R$ is important in maintaining physiological leukocyte-endothelial interactions. Collectively with the current study, it is suggested that $P2Y_2R$ is important in multiple organ systems in maintaining physiological functions. It remains to study if $P2Y_2R$-dependent glucose homeostasis is regulated through the cardiovascular system.

GTT Assessment for Wild Type and $P2Y_2R^{-/-}$ Female Littermates. Interestingly, differences in glucose tolerance were not observed between female $P2Y_2R^{-/-}$ and WT littermates (Fig. 10*B*, Table 9). We do observe a trend, however, between $t = 10$ and $t = 30$ of increased glucose concentration on the glucose response curve (Fig. 10*B*). This suggests several possibilities. $P2Y_2R$ may not be necessary for regulating glucose tolerance in females. Differences in insulin secretion from the pancreas may be occurring in compensatory response to high blood glucose levels. Finally, increasing sample size in the female groups may allow us to either reach a statistically significant difference or corroborate current findings.

Assessment of Sex-specific Difference During Glucose Tolerance Test

Sex differences in glucose homeostasis are well documented. Based on previous literature in mice, we expected female mice to have lower blood glucose levels for GTT relative to male mice. In the current study, sex differences in our wild type mice were as expected. We show that female wild type mice have lower blood glucose levels over the course of the glucose tolerance test, suggesting female WT mice have better glucose clearance than WT males (Fig. 11*A*, Table 10). This may be reflective of increased insulin sensitivity reported in both female mice and

humans. Currently, sex-specific differences in the $P2Y_2R^{-/-}$ animal model are not reported in literature. Here, we characterized sex-specific differences in glucose tolerance in the $P2Y_2R^{-/-}$ model. Female $P2Y_2R^{-/-}$ mice have lower blood glucose levels in GTT time course curve relative to males were (Fig. 11*B*, Table 11). These data demonstrate that $P2Y_2R$ does not alter sex-differences in glucose tolerance between female and male mice.

In mice, females can tolerate a glucose challenge better compared to male counterparts, consistent with the data in both wild type and $P2Y_2R^{-/-}$ mice in the current study. As discussed earlier, this is not reflected in humans between men and women. During an oral glucose tolerance test, women have higher blood glucose concentrations 2 hours after glucose challenge compared with men. There are a few possibilities responsible for the discrepancy in GTT between human and mice. As described previously, exogenous glucose is given to patients via oral route vs. mice via intraperitoneal injection. Regulatory mechanisms of blood glucose between oral and intraperitoneal routes differ, including decrease in blood glucose modulated by incretins that are produced from the small intestine in response to ingested glucose. When performing an OGTT with patients each patient is administered a standard amount of glucose, 75 g. However, in mice, the concentration of glucose was administered based upon body weight, standardizing it for each experimental subject. Those factors may account for the inconsistency in GTT sex difference between human and mice.

Study Limitations and Future Directions

It is imperative to assess insulin action to make more-solid conclusions from the data collected in the current study. Due to the limitation of time and resources, this was not completed in this thesis research. The mechanisms of $P2Y_2R$ modulating GTT in males and females

remains to be investigated. In general, three possible ways to potentiate glucose tolerance by $P2Y_2R$ in males include increase in: (1) blood insulin level; (2) insulin receptor sensitivity; (3) insulin diffusion rate from blood to skeletal muscle or adipose tissue. Likewise, mechanisms of sex differences in $P2Y_2R$-mediated sex difference in FBG for WT mice as well as sexual dimorphism in glucose tolerance for both wild type mice $P2Y_2R^{-/-}$ remain to be studied. We predict that sex difference in the regulation of blood glucose level by the liver is responsible for distinctive FBG between males and females under fasting condition. Furthermore, the roles of sex hormones, genes encoded on sex chromosomes, and epigenetic factors in the different regulatory mechanisms between males and females are unclear. Further study on identifying genes located on X or Y chromosome involved in sex-specific differences in regulation of blood glucose level under various conditions is needed.

Standard protocol for working with the $P2Y_2$ receptor is to challenge the receptor with agonist UTP in comparison to basal activation. UTP challenge can be used to further elucidate if receptor activation modulates glucose tolerance. This is a way to see if direct receptor activation influences the conditions being evaluated. It is shown in hepatocyte and cultured skeletal muscle cells that UTP activation stimulates $P2Y_2R$ and favors insulin resistance under hyperglycemic conditions. *In vivo* activation of $P2Y_2R$ using UTP versus basal conditions during glucose tolerance test in wild type mice in a combination with the findings from the current study may allow us to comprehensively evaluate the role of $P2Y_2R$ in the regulation of blood glucose. While UTP challenge in $P2Y_2R$ knock out mice also allows us to tease out the activation of other P2Y receptors that are activated by UTP.

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APPENDIX

From: Institutional Animal Care and Use Committee <IACUC@MissouriState.edu>
Sent: Tuesday, April 27, 2021 12:13 PM
To: Goerndt, Angela M <AGoerndt@MissouriState.edu>; Marino, Hailee A <Hailee909@live.missouristate.edu>
Subject: RE: IACUC Protocols

I am so sorry. I just now saw this email. I just didn't see it before.

Yes. The approval date is 10/12/17 and because Cayuse is gone, I have no record of any email that would have been sent stating that. I can write a letter or whatever might be acceptable to the graduate college? I will talk with Dr. Masterson to see if that is acceptable.

Dr. Wang is listed on the protocol that was pulled from Cayuse which is the only "original" we have so I think you should be fine there.

Janene

From: Goerndt, Angela M <AGoerndt@MissouriState.edu>
Sent: Tuesday, April 20, 2021 1:52 PM
To: Marino, Hailee A <Hailee909@live.missouristate.edu>
Cc: Institutional Animal Care and Use Committee <IACUC@MissouriState.edu>
Subject: Re: IACUC Protocols

Hi Hailee,

This is the earliest version of 18-006.0 that I have. I hope it's what you need. I did notice it does not have an official approval date on it. From looking at the annual reviews, it appears the approval date was 10/12/2017. I'm copying our IACUC administrator. Maybe she can help us track down the approval notification that was sent to Dr. Morris. I can't find an amendment that removes Dr. Morris and makes Dr. Wang the official PI. Maybe Janene can help us with that too. 🙂

Kind regards,
Angie

IACUC verification

Proctor, Janene A <JaneneProctor@MissouriState.edu>
Fri 4/30/2021 12:21 PM
To: Marino, Hailee A <Hailee909@live.missouristate.edu>
Cc: Goerndt, Angela M <AGoerndt@MissouriState.edu>

1 attachments (107 KB)
Marino grad letter IACUC.pdf;

Hello Hailee,

I talked with Dr. Masterson in the Grad College. She told me to write a letter and instruct you to upload the letter into BlackBoard rather than the protocol.

I have attached the letter.

You're good to go on the IACUC front. 🙂

Congrats on your graduation.

Janene Proctor

Research Administration Specialist &
IACUC Administrator
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