KETONE BODY METABOLISM IN OBESITY AND DIABETES

by

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Abstract

The conservation of ketone body metabolism across various domains of life, including eukaryotes, bacteria, and archaea, underscores its pivotal role in physiological processes. Primarily activated during carbohydrate scarcity, ketone body metabolism provides alternative energy, mainly to the brain, heart, and skeletal muscles. The liver orchestrates ketogenesis, finely tuned by hormones, primarily insulin and glucagon. This thesis delves into the intricate dynamics of ketone body metabolism and its implications on human health, focusing on its role in obesity and potential applications in managing type 2 diabetes (T2D).

Over the past four years, our laboratory has been working to identify innovative targets and approaches aimed at ameliorating hyperglycemia and insulin resistance in conditions such as obesity and T2D. Notably, we've recently uncovered that the catalytic activity of succinyl-CoA:3-ketoacid CoA transferase (SCOT), the pivotal enzyme driving the oxidation of ketone bodies, is heightened in the skeletal muscles of obese mice. SCOT inhibited by drugs or ablated genetically showed improved glycemia in obese and diabetic mice. We hypothesize that administering dietary ketone esters to obese mice, aiming to elevate circulating ketone levels, could potentially exacerbate their glycemic control. Furthermore, we propose that the genetic upregulation of SCOT in skeletal muscles may lead to compromised glycemic regulation in obese mice, thereby heightening their risk of developing T2D.

Chapter 2 of the study investigates the impact of ketone ester (KE) administration on glycemia in obese mice, examining its influence on glucose tolerance, ketone body oxidation, and insulin's role in glycemic control. KE can raise the ketones levels in the circulation effectively to boost a secondary elevation of ketone body oxidation. Contrary to expectations, KE administration

improved glucose tolerance in obese models without relying on ketone body oxidation in skeletal muscles. The islets perifusion with ketone salts suggested that while ketone body oxidation might influence insulin secretion, it's not the primary factor responsible for improved blood glucose levels observed in obese mice. Other metabolic or signaling pathways could play a more substantial role in the observed glycemic improvement. Uncovering these pathways can provide insights into the complex interplay of metabolic factors in obesity and potentially lead to novel therapeutic approaches for managing diabetes and metabolic disorders.

Chapter 3, an ongoing work, focuses on the generation and characterization of SCOT/*Oxct1* (3-oxoacid CoA-transferase 1) transgenic mice. The comprehensive characterization aims to elucidate the physiological shifts and metabolic profile differences due to a chronic upregulation in ketone body oxidation. The results could shed light on SCOT's role in metabolic processes and its potential implications for glycemic management in obese mice, increasing their susceptibility to developing T2D.

The study elucidates the nuanced interplay between ketone body metabolism, insulin secretion, and glucose regulation, particularly under varying nutritional states and obesity. The unexpected outcomes from KE administration and the potential role of βOHB in insulin secretion signal a need for deeper exploration. The differences observed between obese and lean models, the transient nature of insulin secretion, and the varying responses to different degrees of high-fat diet (HFD) present a complex scenario requiring further investigation. The study also emphasizes the need for exploring the long-term effects of ketone ester administration and its potential therapeutic applications, especially in metabolic health and T2D management. The generation of pancreas-specific SCOT knockout models and exploring additional signaling pathways are identified as key

future directions to deepen our understanding of the underlying mechanisms. In conclusion, this study sheds light on the intricate dynamics of ketone body metabolism, its impact on glycemic control in obesity, and its potential therapeutic implications. The findings underscore the complexity of metabolic regulation, highlighting the need for further research to unravel the mechanisms and explore the potential of ketone body metabolism in human health.

Preface

Research Ethics Approval

This thesis is an original work by Kunyan Yang. Ethics approval for the following research was received from the University of Alberta Animal Use and Care Committee, under Animal Use Protocol #00001412 and #00002380.

Collaborative Work

Chapter 2 has been published: Tabatabaei Dakhili SA*, Yang K*, Locatelli CAA, Saed CT, Greenwell AA, Chan JSF, Chahade JJ, Scharff J, Al-Imarah S, Eaton F, Crawford PA, Gopal K, Mulvihill EE, Ussher JR. Ketone ester administration improves glycemia in obese mice. *Am J Physiol Cell Physiol.* 2023 Sep 1;325(3):C750-C757. doi: 10.1152/ajpcell.00300.2023. Epub 2023 Aug 14. PMID: 37575059. *Denotes equal contribution.

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Glossary of Terms

AACS	AcAc CoA synthetase
AcAc	Acetoacetate
AcAc CoA	Acetoacetyl coenyzme A
ACAT	Acetoacetyl coenzyme A thiolase
ACC	Acetyl CoA carboxylase
ACLY	Adenosine triphosphate citrate lyase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ASIC1a	Acid-sensing ion channels 1a
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
bp	Base pair
βOHB	β-hydroxybutyrate
βOHB CoA	β-hydroxybutyryl coenzyme A
BDH1	D-β-hydroxybutyrate dehydrogenase
BDNF	Brain-derived neurotropic factor

BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CoA	Coenzyme A
CPT1	Carnitine palmitoyltransferase I
CS	Citrate synthase
DKA	Diabetic ketoacidosis
DPBP	Diphenylbutylpiperidines
ER	Endoplasmic reticulum
FFA	Free fatty acids
FAO	Fatty acids oxidation
Foxa2	Forkhead box protein A2
FOXO3a	Forkhead box O3a
GABA	Gamma-aminobutyric acid
GPCR	G-protein coupled receptor
GPR40	G-protein coupled receptor 40
GPR41	G-protein coupled receptor 41
GPR109A	G-protein coupled receptor 109A

GTT	Glucose tolerance test
HDACs	Histone deacetylases
HET	Heterozygous
HFD	High-fat diet
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
HMGCS	3-hydroxy-3-methylglutaryl coenzyme A synthase
HMGCL	3-hydroxy-3-methylglutaryl coenzyme A lyase
HMGCR	3-hydroxy-3-methylglutaryl coenzyme A reductase
HSL	Hormone-sensitive lipase
Hsp90	Heat shock protein 90
IL-6	Interleukin 6
IP	Intraperitoneal
K_{ATP} channel	Adenosine triphosphate-sensitive K^+(potassium) channel
KE	Ketone ester
MCT	Monocarboxylate transporter
NAD^+	Nicotinamide adenine dinucleotide
NADH	Hydrogen-reduced nicotinamide adenine dinucleotide

NAFLD	Nonalcoholic fatty liver disease
NF-κB	Nuclear factor-κB
NLRP3	Nucleotide oligomerization domain (NOD)-like receptor protein 3
OAA	Oxaloacetate
Oxct1	3-oxoacid CoA-transferase 1
PC	Pyruvate carboxylase
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PPAR	Peroxisome proliferator-activated receptor
Ppia	Peptidylprolyl isomerase A
PTM	Posttranslational modification
ROS	Reactive oxygen species
RT PCR	Reverse transcription PCR
SCOT	Succinyl-CoA:3-ketoacid CoA transferase
SGLT2	Sodium/glucose co-transporter 2
SNS	Sympathetic nervous system
STZ	Streptozotocin

T1D	Type-1 diabetes
T2D	Type-2 diabetes
TNF-α	Tumor necrosis factor alpha
VLDL	Very low-density lipoprotein
WT	Wildtype

Chapter 1: Introduction

The ketone body metabolism is conserved across various domains of life, including eukaryote [1], bacteria [2], and archaea [3]. Ketone body metabolism is a crucial physiological process that comes into play primarily when the body's carbohydrate stores are low. This typically occurs during periods of fasting, prolonged exercise, or in conditions such as a low-carbohydrate diet or uncontrolled diabetes. The primary goal of ketone body metabolism is to provide an alternative fuel source to glucose for the brain, heart, and skeletal muscles, ensuring the body's energy needs are met under varying nutritional states, which allows the preservation of glucose and protein. A child lacking a key ketogenic enzyme exhibits signs of hypoglycemia [4], while intravenous infusion of ketone bodies in healthy individuals reduces the availability of glucogenic amino acids for gluconeogenesis [5, 6]. Notably, since the brain cannot metabolize fatty acids (FA), ketone bodies can cross the blood-brain barrier and supply approximately two-thirds of its energy needs under hypoglycemic conditions [1].

The liver is the central organ involved in the synthesis of ketone bodies; a process known as ketogenesis. In the state of carbohydrates deficiency, the liver transitions from being an organ primarily engaged in the synthesis of glycogen and fatty acids to one that emphasizes fatty acid oxidation and the production of ketone bodies [7]. Ketone body metabolism is finely regulated by several hormones, primarily insulin and glucagon. Insulin generally suppresses ketogenesis by inhibiting lipolysis (breakdown of fats), while glucagon promotes it by stimulating lipolysis and providing the liver with the necessary fatty acid substrates for ketone body production [7].

The three main ketone bodies produced are acetoacetate (AcAc), beta-hydroxybutyrate (βOHB), and acetone. The synthesis of ketone bodies begins with the breakdown of fatty acids, released

from adipose tissue, into acetyl-CoA molecules within the liver mitochondria. When the concentration of acetyl-CoA exceeds the capacity of the Krebs cycle, these molecules are diverted to form ketone bodies. Once synthesized, ketone bodies are released into the bloodstream and transported to extrahepatic tissues, such as the brain and muscles, where they are converted back to acetyl-CoA and utilized as a source of energy through the Krebs cycle and oxidative phosphorylation. This process is called ketone body oxidation or ketolysis and occurs primarily in the mitochondria of the cells.

Understanding ketone body metabolism is essential as alterations in this metabolic pathway can have significant implications for human health. For example, in conditions such as diabetic ketoacidosis (DKA), the uncontrolled production of ketone bodies can lead to a dangerous drop in blood pH levels [8]. On the other hand, nutritional ketosis, induced by a ketogenic diet, has been explored for potential therapeutic benefits in various health conditions, including epilepsy, obesity, and neurodegenerative diseases. Ketone body also plays a central role in cellular signaling, impacting inflammation, oxidative stress, and regulatory gene expression.

1.1 Introduction to Ketone Body Metabolism

1.1.1 Oxidative Roles of Ketone Body

Ketogenesis

The ketone bodies commonly refer to three molecules, acetoacetate (AcAc), D-β-hydroxybutyrate (D-βOHB) [also known as (R)-(−)-β-hydroxybutyrate] and acetone **(Figure 1.1)**. Ketogenesis occurs primarily in hepatic mitochondria matrix. Specifically, free fatty acids (FFA) in the liver

cytosol are activated to fatty acyl CoAs and enter the mitochondria through carnitine palmitoyltransferase I (CPT1), where they are oxidized to acetyl CoA molecules through β-oxidation. Two molecules of acetyl CoA are condensed by mitochondrial acetoacetyl CoA thiolase (ACAT) to form acetoacetyl CoA (AcAc CoA), freeing one CoA. AcAc CoA is then condensed with a third acetyl CoA by 3-hydroxy-3-methylglutaryl CoA synthase 2 (HMGCS2: mitochondrial isoform) to form 3-hydroxy-3-methylglutaryl CoA (HMG CoA), freeing one CoA. This is the fate-determining step of ketogenesis. Finally, HMG CoA is cleaved by HMG CoA lyase (HMGCL) to form AcAc and acetyl-CoA. AcAc is subsequently converted to D-βOHB by D-βOHB dehydrogenase (BDH1), coupling with the hydrogen-reduced nicotinamide adenine dinucleotide (NADH) generated through β-oxidation. This reversible reaction enables the generation of NAD^+ in a near equilibrium process that predominantly leads to the production of D-βOHB **(Figure 1.2)**. The BDH1 reaction occurs exclusively within mitochondria with strict specificity for D-βOHB, while the D-βOHB/AcAc ratio is reflective of the intramitochondrial NAD^+/NADH ratio, and vice versa [9, 10].

AcAc and D-βOHB, which cannot be metabolized by the liver, are released from hepatocytes into circulation using monocarboxylate transporters (MCT), specifically MCT1 and MCT2, to facilitate their transportation following the concentration gradient in a unidirectional manner [11, 12]. On the other hand, some of the AcAc spontaneously decarboxylated into acetone [13]. Acetone can be eliminated from the body by means of both exhalation and urine, with a portion being metabolized [14, 15]. Several studies have demonstrated the incorporation of radioactively labeled acetone into glucose production in both rats and diabetic humans, although the mechanisms underlying this phenomenon remain unclear [16, 17]. Some amino acids, like leucine, lysine, and tryptophan, serve

as ketogenic precursors. For instance, the catabolism of leucine can result in HMG CoA production, contributing to approximately 2.3% of ketone bodies produced in chronically starved rats [18].

A key distinction between hepatic ketogenesis and extrahepatic ketogenesis lies in the presence of HMGCS2 as it is only expressed in liver and gut [19, 20]. Despite the absence of HMGCS2, fatty acids oxidation (FAO) and glycolysis can generate a large acetyl CoA pool in extrahepatic mitochondria, which shifts the equilibrium of reversible enzymes for ketone body oxidation favoring the production of ketone bodies from acetyl CoA [21, 22]. Exposing rat kidney cortex slices to high levels of fatty acids and AcAc resulted in reduced AcAc consumption and a net ketone bodies production [22]. The capacity of extrahepatic tissues to produce ketone bodies is nullified by their utilization ability, as utilization is the priority in normal physiological conditions. It is unlikely that there would be a net production of ketone bodies within these tissues, as concentrations of ketone bodies in peripheral tissues elevate through the MCT 1/2-faciliated entry of ketone bodies from the circulation [23]. MCT 1/2 transportation is unidirectional and follows the concentration gradient from the plasma to the extrahepatic mitochondria [12].

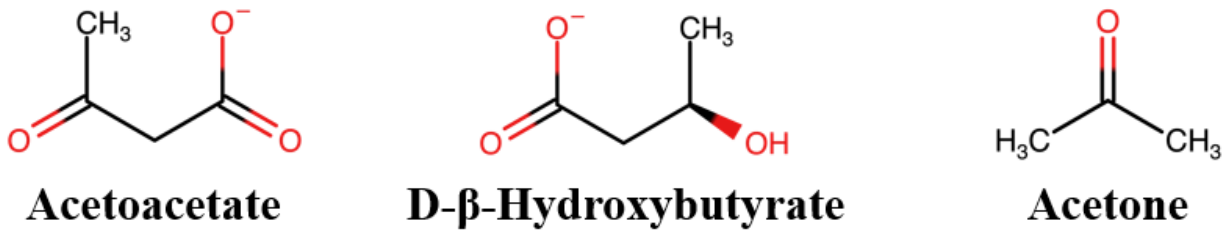

Figure 1.1 *Structures of Ketone Bodies.*

Acetoacetate (AcAc), D-β-Hydroxybutyrate (D-βOHB) and Acetone.

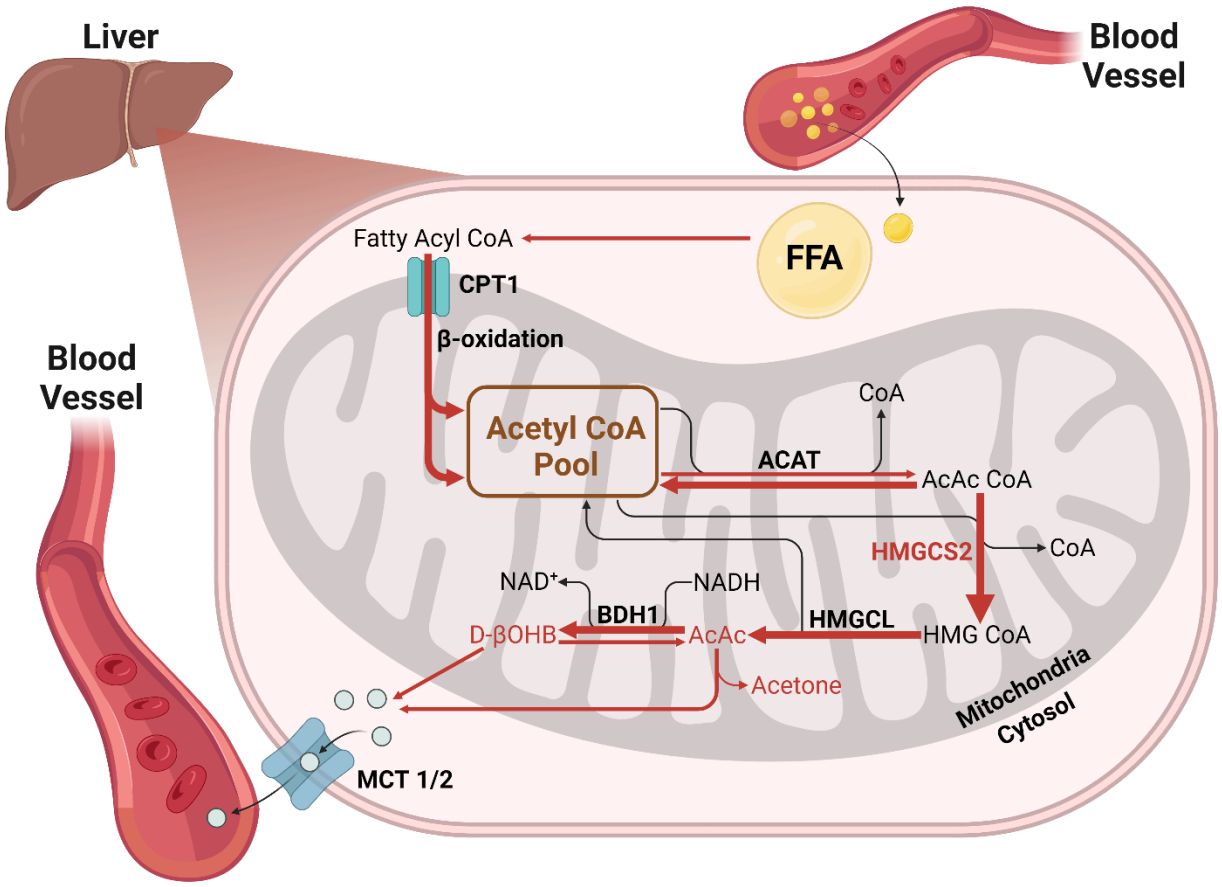

Figure 1.2 *Ketogenic Pathways in Liver.*

Ketogenesis occurs primarily in liver mitochondria matrix and acetyl CoA in the forms of ketone bodies can exit into circulation as alternative sources of energy for extrahepatic tissues. Created with BioRender.com.

Ketolysis

The ketone bodies are primarily produced by the liver and then oxidized in tissues outside of the liver, as hepatocytes lack a key oxidizing enzyme. In the peripheral tissues, AcAc and D-βOHB are converted back into acetyl CoA to enter the Krebs cycle for ATP production **(Figure 1.3)**. The entry of D-βOHB and AcAc from the circulation to extrahepatic tissues is facilitated by MCT 1/2

[12]. The mitochondrial BDH1 then catalyzes the reversable reaction that converts D-βOHB to AcAc coupling the reduction of NAD^+ to NADH [7, 9]. The enzyme succinyl-CoA:3-ketoacid CoA transferase (SCOT) transfers the CoA from succinyl CoA to AcAc, producing AcAc CoA and succinate in a near equilibrium reaction favoring the AcAc production [24]. SCOT is the enzyme required for ketone body oxidation which is not found in the liver. AcAc CoA is further processed through a reversible ACAT reaction, resulting in the production of two acetyl CoA molecules for the subsequent oxidation by the Krebs cycle.

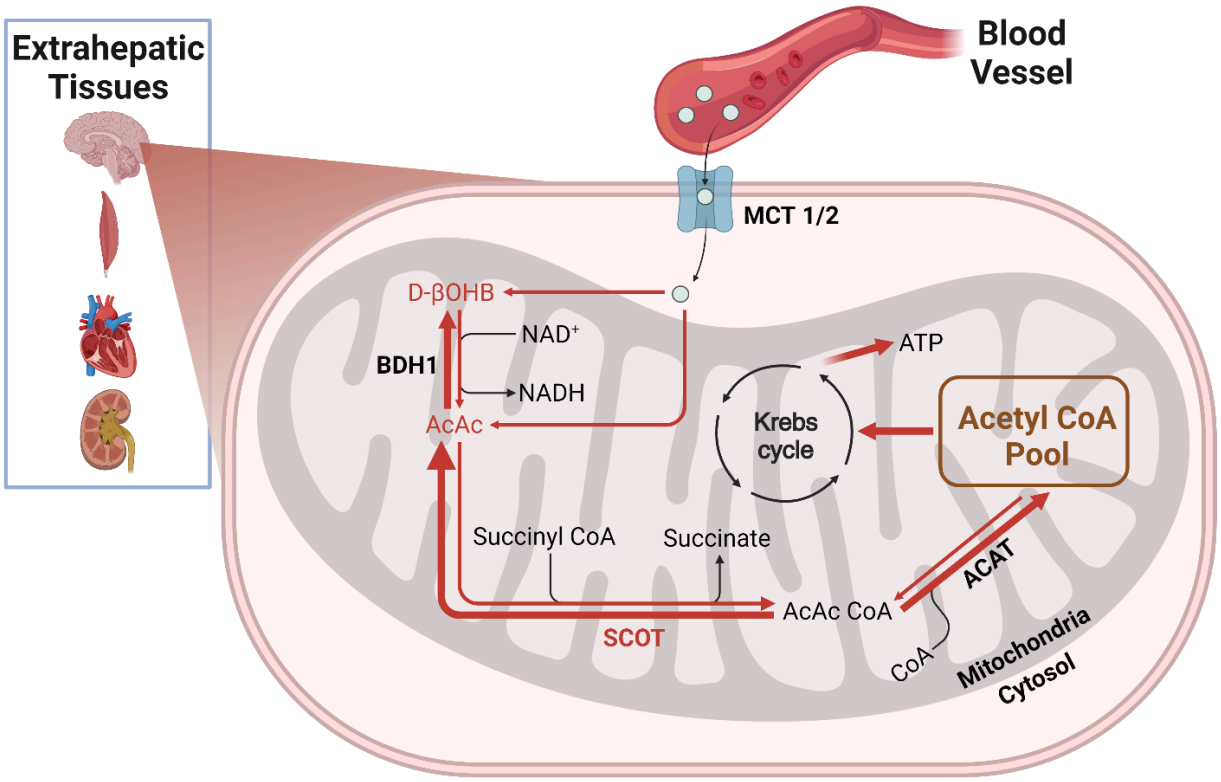

Figure 1.3 *Ketone Body Oxidation Pathways in Extrahepatic Tissues.*

In extrahepatic tissues such as brain, skeletal muscles, heart, and kidneys, circulating ketone bodies are transferred into the cells and converted back to acetyl CoA, which enters the Krebs cycle for ATP production. Created with BioRender.com.

1.1.2 Regulatory Sites of Ketone Body Metabolism

Ketogenic Precursors: Free Fatty Acids (FFA) and Fatty Acids Oxidation (FAO)

The hepatic FAO is required to generate a sufficient acetyl CoA pool for ketogenesis, and depletion of adipocytes can lead to cessation of ketogenesis [25]. Fatty acids used for oxidation in the liver and other tissues primarily come from circulating FFA, as well as from lipolysis of triacylglycerol stored in adipose tissue and plasma very low-density lipoprotein (VLDL) through the action of lipoprotein lipase [26]. The rate of FAO is primarily determined by the levels of intracellular FFA, which is regulated by the availability of circulating FFA and the uptake ability from the circulation [7, 26]. Other factors like hormones, energy levels and carbohydrate metabolism can also regulate FAO [7].

The hormone-sensitive lipase (HSL) is a primary lipolytic enzyme that controls the amount of FFA released from the adipose tissue. The activation of HSL occurs through phosphorylation by cAMP-dependent protein kinase A (PKA). Cyclic adenosine monophosphate (cAMP) can be synthesized from ATP by adenylate cyclase and acts as a second messenger in response to many first messengers, including glucagon, catecholamines, growth hormone and cortisol to stimulate PKA through its production [27-29]. In addition, PKA suppresses acetyl CoA carboxylase (ACC), an enzyme that converts cytosolic acetyl CoA into malonyl CoA. Being a metabolite of glucose, malonyl CoA not only serves as a lipogenic precursor, an opposite process of FAO (see **1.1.4 Nonoxidative Roles of Ketone Bodies**), but also inhibits the CPT1, the enzyme responsible for transporting fatty acyl CoA into the mitochondria, blocking its further oxidation [7]. Meanwhile, cAMP formation as well as the ACC suppression can ensure an amplified fatty acyl CoA transportation across mitochondrial membranes through CPT1 [26]. In the low energy state, AMP-

activated protein kinase (AMPK) in response to high AMP/ATP ratio will also be activated to stimulate HSL and adipose triglyceride lipase (ATGL) that increases FFA release while inhibiting ACC [30].

In contrast, insulin impedes the FAO and hinders the transportation of fatty acyl CoA across mitochondrial membrane by inducing dephosphorylation of HSL and activation of ACC [26, 31]. Insulin has been shown to reduce the affinity of hepatic PKA to cAMP, thus resulting in PKA deactivation [32]. Consequently, the inactivated HSL limits the supply of circulating FFA from adipose tissue and leads to a decline of ketogenesis. Insulin inhibits the FFA release from adipose tissue as seen in rats with insulin injections [33]. Moreover, elevated levels of ketone bodies have an inhibitory effect on the pituitary and thyroid functions, impeding their interaction with PKA and putting a brake on ketogenesis [34].

Table 1.1 *Hormonal Control over FFA and FAO.*

Effects of Glucagon on Lipolysis and FAO	**Effects of Insulin on Lipolysis and FAO**
Increased lipolysis with augmented FFA release from adipose tissue.	Decreased lipolysis with reduced FFA release from adipose tissue.
Amplified fatty acids transportation across mitochondrial membranes, increasing FFA uptake from the circulation for FAO.	Inhibited fatty acids transportation into the mitochondrial matrix, lowing FFA uptake from the circulation for FAO.
Diminished capacity for *de novo* lipogenesis.	Full capacity for *de novo* lipogenesis.
Elevated ketogenesis supported by a large acetyl CoA pool.	Diminished ketogenesis affected by a shrunk acetyl CoA pool.
Increased levels of ketone bodies with a feedback inhibition on the glucagon's effect.	Plummeted levels of ketone bodies from reduced ketogenic rate.

Adapted from Hirsch [27], Holm [28], Rubinstein *et al.* [29], McGarry & Foster [7], Randle [26], Mihaylova & Shaw [30], Horowitz [31], Casal *et al.* [31], and Laeger *et al.* [34].

Ketogenesis: HMGCS2/*Hmgcs2*

HMGCS2 is the key enzyme that is responsible for catalyzing the irreversible step of condensation of AcAc CoA with acetyl CoA to form HMG CoA in hepatic mitochondria. HMGCS2 activity can be regulated by both transcription factors and posttranslational modifications (PTM). The promoter region of *Hmgcs2* gene in rats contains binding regions for nuclear hormone receptor and transcription factors that are sensitive to FFA availability, insulin to glucagon ratio and cAMP production [32]. For example, transcriptional activation of *Hmgcs2* by fatty acids is mediated by the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR) [32, 35]. Forkhead box protein A2 (*Foxa2*) transcription factor also stimulates *Hmgcs2* transcription via glucagon-mediated acetylation but reversed by insulin-mediated phosphorylation [36, 37]. Starvation and high hepatic cAMP levels increase *Hmgcs2* mRNA levels while refeeding and insulin injection produce a quick decrease in expression accompanied by a decline in ketogenesis [32, 38]. Post-translationally speaking, succinyl CoA and succinylation of lysine residues both inhibit enzymatic activity of HMGCS2 while fatty acids and glucagon cause de-succinylation and HMGCS2 activation [39, 40]. Fasting-induced phosphorylation of a serine residue also significantly promotes HMGCS2 activity [41].

The dual regulations from transcription factors and PTM affect both the activity and the abundance of the enzyme as changes in mRNA level of *Hmgcs2* are reflected on the HMGCS2 protein level [42]. However, the same conclusion cannot be made in human physiology. Regulation through PTMs offers a means of promptly modifying enzymes in response to metabolic shifts. In contrast, alterations in the overall levels of *Hmgcs2* transcription offer a more sustained form of control against nutritional fluctuations.

Ketolysis: SCOT

SCOT is an essential enzyme involved in ketone body utilization found exclusively in extrahepatic mitochondria, which serves as an important marker for assessing ketone body utilization. In rats, muscles perfused with ketone bodies significantly increased the activity of SCOT compared to muscles perfused without ketone bodies [43]. Increased SCOT activity has been observed in skeletal muscles from obese mice as a possible maladaptation [44]. By using a class of drugs namely diphenylbutylpiperidines (DPBP) to inhibit SCOT, there is an improvement in glucose intolerance of obese mice [45]. In obese mice, the skeletal muscle-specific removal of SCOT yielded results comparable to the suppression of DPBP [44]. In diabetes, SCOT activity is diminished by chronic DKA and administering insulin to diabetic rats only leads to a partial restoration of enzymatic activity [46]. Moreover, AcAc has shown a pattern of substrate inhibition to SCOT when the activity curve rise to a maximum and then descend as the levels of AcAc increases, which could be a contributing factor to DKA [47]. PTMs like nitration, and oxidative alterations of SCOT are proposed to be harmful facets of diabetes. Tyrosine-nitrated SCOT has been identified as a potential factor contributing to the impaired activity of SCOT in diabetic conditions [48]. On the contrary, nitrohydroxylation of tryptophan boosts SCOT catalytic activity in the rat heart [49].

1.1.3 The Integration of Ketone Body Metabolism and Energy Homeostasis

Hepatic Adaptation

The ketogenic environment is affected by fluctuations in dietary status and alterations in demands for circulating carbohydrates. Elevation in ketogenesis is observed under physiological conditions (fasting, prolonged exercise and fat feeding) as well as in the pathological condition of diabetes while refeeding after fasting and insulin injections decrease ketogenic rates [7, 20, 40, 50]. The liver is essential in regulating glucose, lipid balance, and ketogenesis in animals, mainly by reacting to shifts in nutritional conditions through changes in circulating insulin and glucagon levels. In the absorptive phase (high insulin to glucagon ratio), increased glucose uptake and the esterification of fatty acids contribute to augmented storage of glycogens and triacylglycerols [51]. Insulin curbs FFA release by directly suppressing lipolysis, FAO **(Table 1.1)** and the supply of α-glycerophosphate essential for the re-esterification of FFA [52]. In the post-absorptive state, characterized by a low insulin-to-glucagon ratio, there's an uptick in FFA release, fueling ketogenesis to satisfy the body's energy requirements. This surge in ketone production can simultaneously dampen glucose uptake, possibly predisposing the body to insulin insensitivity [51]. Glucagon elevates ketogenesis without influencing the liver's uptake of FFA in perfused liver of fed rats [33]. Moreover, the initiation of FFA mobilization from adipose tissue is more significantly influenced by the relative or absolute absence of insulin than by the presence of other hormones [53].

While the enhanced delivery of FFA to the liver is a crucial factor, it alone is not enough to trigger ketosis; the rate of β-oxidation plays a determining role in how much of the FFA are diverted towards lipogenesis [53]. Elevated levels of FFA *in vivo* does not necessarily lead to an increase

in ketone production [54], and ketosis can be reversed even when concentrations of free fatty acids in the plasma are maintained at high levels [55]. While it's well established that the liver's lipogenic ability decreases during fasting, healthy individuals subjected to a 36-hour fast often show a varying increase in hepatic lipid content [56]. The acetyl CoA derived from hepatic FAO could be directed towards fatty acids synthesis, ketogenesis and the Krebs cycle [7] **(Figure 1.4)**. The increased influx of FFA produces an accelerated expansion of acetyl CoA pool in mitochondria, which can overflow to the cytosol for *de novo* lipogenesis (see **1.1.4 Nonoxidative Roles of Ketone Body**). Meanwhile, around 40% of FFA are utilized for ketogenesis in the liver [1, 33].

While some believe that a lack of oxaloacetate (OAA) can hinder the Krebs cycle and thus promote ketogenesis, the mechanisms named anaplerosis and cataplerosis act to maintain the balance of the Krebs cycle. Anaplerosis and cataplerosis are metabolic processes where, respectively, intermediates are replenished in the Krebs cycle through the synthesis of cycle intermediates, and intermediates are removed from the cycle for biosynthesis or gluconeogenesis, maintaining the cycle's balance and functionality [57]. OAA serves as a precursor for the synthesis of citrate, with both molecules acting as vital intermediates in the Krebs cycle. Because of the heightened carbohydrate demand and reduced glucose availability during fasting, OAA is shuttled from the mitochondria to the cytosol, where it aids in gluconeogenesis [58, 59]. Nevertheless, OAA can be generated from pyruvate through the reaction mediated by pyruvate carboxylase (PC), serving as an archetypical anaplerotic enzyme [57]. When acetyl CoA levels rise from FAO during ketogenic states, acetyl CoA activates PC to ensure a consistent replenishment of OAA [57, 60]. The enzyme pyruvate dehydrogenase (PDH), which oxidizes pyruvate to acetyl CoA, is inhibited both by a heightened acetyl CoA to CoA ratio resulting from FAO and by phosphorylation driven by acetyl CoA via PDH kinase [61], thereby leaving pyruvate available for the production of OAA. Although

there is some evidence of citrate synthase suppression by fatty acyl CoA, it is concluded that the Krebs cycle inhibition is shown to have a relatively minor impact on ketogenesis [7, 62]. During fasting, the marked reduction in lipogenesis is the main factor that directs the increased flow of acetyl CoA towards ketogenesis, resulting in the mild ketosis observed [53, 63]. However, in severe DKA, the situation arises when the delivery rate of fatty acids surpasses the capacity for esterification, leading to the production of acetyl CoA at rates that greatly exceed the tissue's capability to process it through oxidative and lipogenic routes.

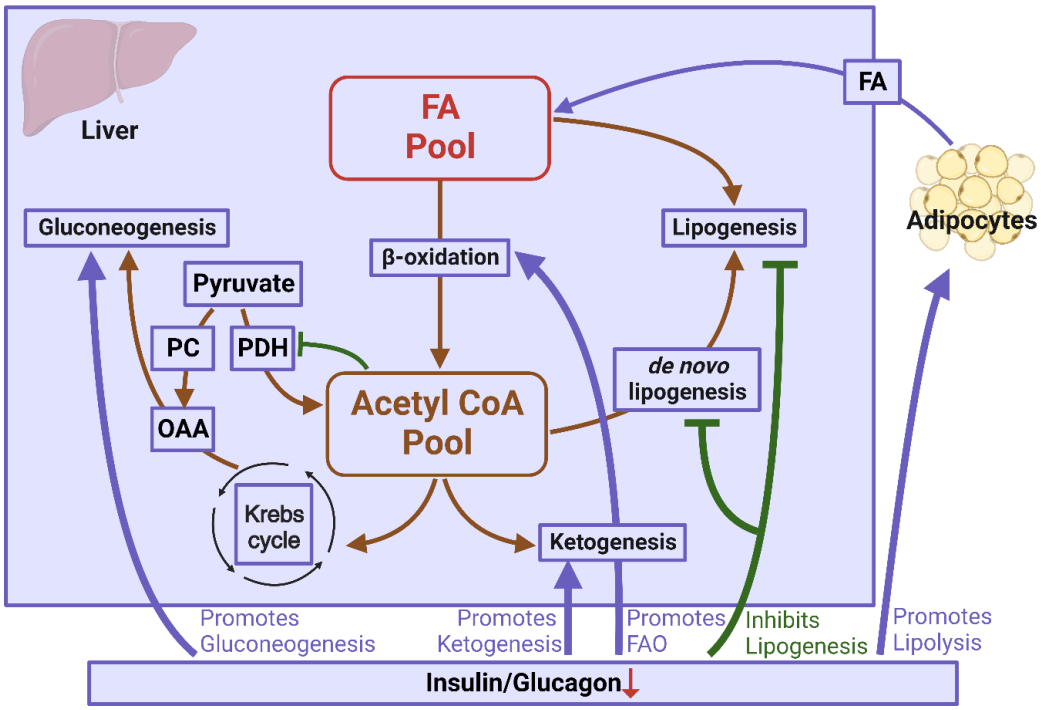

Figure 1.4 *Glucose, Lipid Balance, and Ketogenesis in Liver.*

In situations like fasting, which is characterized by a low insulin/glucagon ratio, there is a surge in FFA supply, primarily amplifying β-oxidation with an inhibited fatty acids esterification and subsequently enriching the liver's acetyl CoA reserves. The liver processes FAO-derived acetyl CoA majorly through three ways: lipogenesis, ketogenesis, and the Krebs cycle. Since the Krebs cycle has a minimal impact on ketogenesis, it is primarily the downregulation of lipogenesis that contributes to the initiation of ketosis. Created with BioRender.com.

Ketone Body Utilization in Different Tissues

The capacity of extrahepatic mitochondria for ketone body utilization is somewhat proportional to the levels of circulating ketone body in the bloodstream and will reach a plateau when the levels of ketone bodies continue to elevate [64]. Ketosis occurs when there is an imbalance between the production and removal rates of ketone bodies. This discrepancy has been suggested as the cause of ketosis in fasting rats, rabbits, sheep, dogs as well as in fasting obese humans [56]. Nowadays, the ketone sensor with 10 μL of blood can gives us an accurate concentration of βOHB within 30s [65]. The βOHB in the blood is converted to AcAc by the BDH1 embedded in the sensor, turning NAD^+ into NADH in the process. When NADH is converted back to NAD^+ by a redox mediator, it produces a current that's directly linked to the βOHB levels. In humans, the usual concentration of ketone bodies in postprandial serum is approximately 0.05 mM, which rises to around 0.3 mM after an overnight fast and reach levels of 4-7mM with prolonged starvation **(Table 1.2)** [1, 20]. For our C57 mice obtained from the Jackson Laboratory, ketone body levels are typically below 0.5 mM in the fed state. However, in the fasted state, these levels increase. After an overnight fast, they range between 0.5-1 mM, and rise above 1-2 mM following a prolonged fast of 48 hours or more.

Some tissues exhibit unique adaptations for ketone body metabolism. Notably, rat cardiac muscles, exercised diaphragms, and skeletal muscles demonstrate a pronounced preference for ketone body [10, 66, 67]. Moreover, ketone body utilization accelerates with an uptick in metabolic rate, as seen during muscle exertion [68]. The role of insulin in the uptake and utilization of ketone body provides some insights on how different tissues react to ketone bodies in different states. Recent *in vivo* studies have revealed that insulin induces a tissue-specific response in ketone body

utilization. For instance, diaphragm and skeletal muscle fibers have a reduced uptake of AcAc in diabetic rats compared to their non-diabetic counterparts. When both insulin and glucose are given together, striated muscle fibers from both diabetic and control rats exhibit an enhanced uptake of AcAc, an outcome not attainable with either glucose or insulin individually [59, 69]. Glucose, potentiated by insulin, boosts the uptake of ketone bodies into adipose tissue, leading to reduced oxidation and increased conversion into fatty acids [70]. Conversely, the heart responds differently to insulin. In rat hearts perfused with insulin, ketogenic factors (such as diabetes, starvation, fatty acids, ketones, and pyruvate) hinder glucose uptake [71]. Glucose by itself doesn't influence the utilization or clearance of AcAc, even when insulin is present. Administering insulin without glucose leads to decreased AcAc removal and reduced D-βOHB formation in the hearts of fed rats. Furthermore, regardless of metabolic conditions of fasting or being well-fed, insulin decreases the oxidation rate of AcAc significantly by 22-35% [66]. Recent research employing isolated working hearts has demonstrated that in murine models, insulin does not affect the rates of cardiac ketone oxidation under various physiological conditions [72-74].

Considering that skeletal muscle mass makes up 35%-40% of total body weight, the primary influence on ketone body metabolism in response to insulin is attributed to skeletal muscles. Scow and Chernick observed that the removal of ketone body from the bloodstream was impaired in pancreatectomized rats but was restored with insulin administration [25]. Balasse and Havel discovered that diabetic dogs exhibited reduced ketone utilization compared to their control counterparts; however, introducing glucose and insulin enhanced the clearance and oxidation of the introduced ketones [68]. A different research team discovered that elevating the infusion rate of glucose, either by itself or combined with insulin, led to enhanced tissue absorption of infused AcAc in fasted, starved, or alloxan-diabetic rats [75].

Table 1.2 *Different States of Circulating Ketone Bodies Levels in Human and Mouse.*

States	Ketone Bodies Levels in Human	Ketone Bodies Levels in Mouse
Postprandial	0.05~0.1 mM	<0.5 mM
Overnight Fast	Up to 0.3mM	0.5~2 mM
Prolonged Fast (2 Weeks)	4~7 mM	
Prolonged Exercise	1~2 mM	1~1.5 mM
Ketogenic Diet	0.6~1.5 mM	0.5~2 mM
Hyperketonemia	1~3 mM	
Ketoacidosis	>3 mM	
Diabetes	Up to 25mM	

Adapted from Cahill [1], Robinson & Williamson [20], Veech [76], Laffel [77], Greenwell *et al.* [78], Holcomb *et al.* [79] and *in vivo* observation.

1.1.4 Nonoxidative Roles of Ketone Body

L-βOHB Metabolism

The synthesis of L-β-hydroxybutyrate (L-βOHB) [also known as (S)-(+)-β-hydroxybutyrate] can occur as a by-product from the β-oxidation of FAO **(Figure 1.5)**, but no free L-βOHB could be detected in mammalian tissues except for exogenous L-βOHB administration [80]. Theoretically, L-βOHB is generated from L-β-hydroxybutyryl CoA (L-βOHB CoA) catalyzed by L-βOHB CoA deacylase. L-βOHB CoA is one of the β-oxidation-derived intermediates and can be converted to AcAc CoA through a reversible reaction facilitated by the mitochondrial L-βOHB CoA dehydrogenase [81]. AcAc CoA then breaks down to acetyl CoA molecules by ACAT. L-βOHB is most likely the product of a congested Krebs cycle that stalls the progression of the β-oxidation. Key enzymes for L-βOHB metabolism are found in the liver, heart, brain, and skeletal muscles

[81]. However, Scofield *et al.* demonstrated that only the D-βOHB was produced from β-oxidation in the liver from fasted rats [82]. L-βOHB, therefore, is more likely to be produced outside of liver.

The metabolic fate of L-βOHB for oxidation is limited by the stereospecificity of mitochondrial BDH1 towards D-βOHB. During exercise, when radioactively labeled D, L-βOHB mixture is infused into the human body, muscular uptake of L-βOHB was significantly lower than that of D-βOHB, suggesting a limited impact of L-βOHB on respiration [10]. In rats, D-βOHB is the more favored substrate for oxidation, but L-βOHB is found to be used extensively for synthesis of fatty acids and sterols in the spinal cords, brains and kidney from suckling rats [83]. L-βOHB can be converted to L-βOHB CoA with the assistance of L-βOHB CoA ligase to be further transformed into AcAc and acetyl CoA for sterol biosynthesis or *de novo* lipogenesis [84]. L-βOHB CoA ligase exhibits its highest activity in the brain, which highlights the role of L-βOHB in complex lipid synthesis in the nervous system [81]. Moreover, L-βOHB utilization is mediated via AcAc or AcAc CoA as the metabolism of [^{14}C]-labelled ketones and [^{3}H]-labelled ketones show a 90% loss of tritium in the products [83].

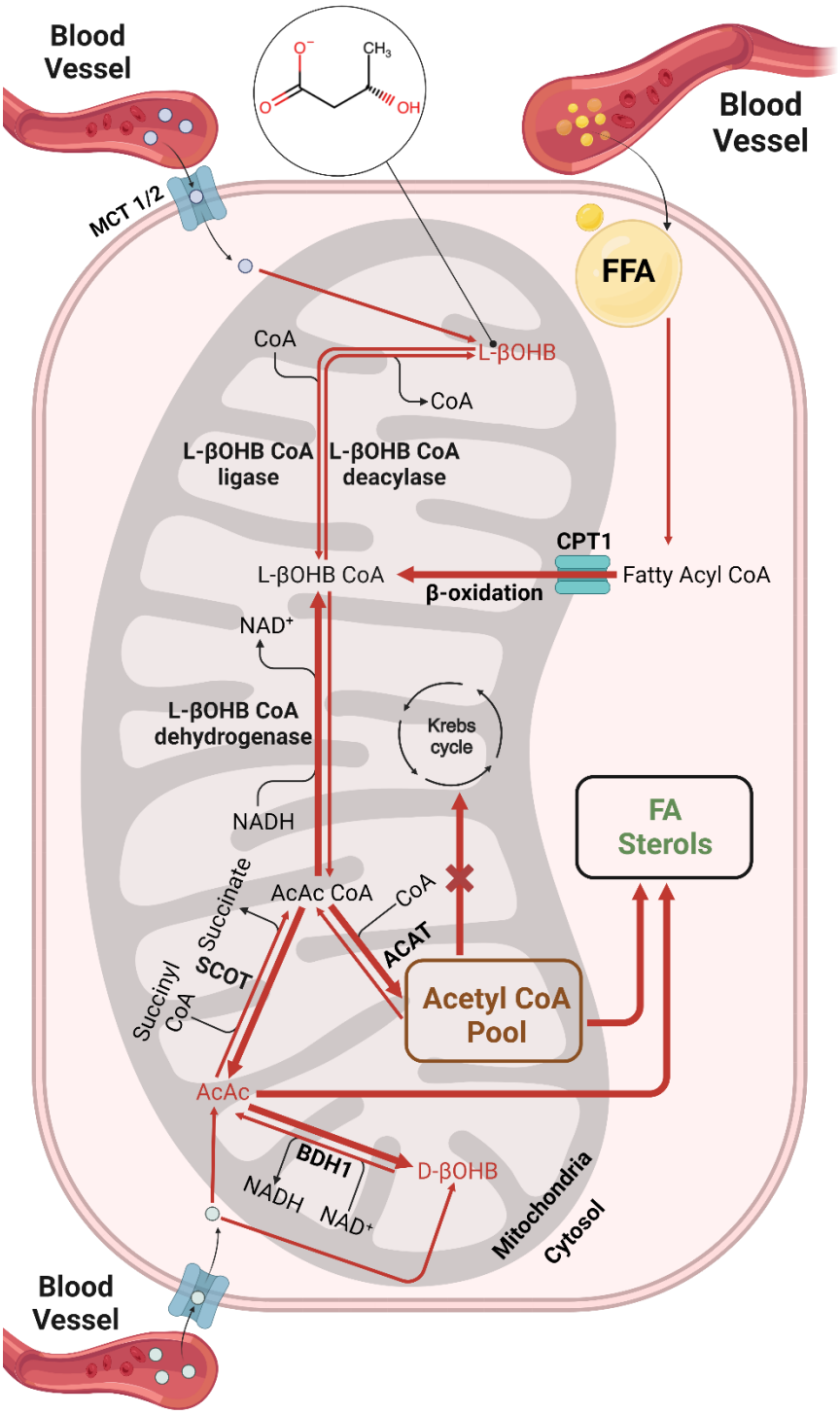

Figure 1.5 *Structure of L-β-Hydroxybutyrate and Its Proposed Mitochondrial Metabolic Pathways.*

When the Krebs cycle is strained by an expanding acetyl CoA pool, exogenous L-βOHB follows the proposed conversion route from L-βOHB to acetyl CoA and AcAc, which could be sources for fatty acids synthesis or sterol biosynthesis in the cytosol. Adapted from Lincoln *et al.* [80], Reed & Ozand [81], Scofield *et al.* [82], and Webber & Edmond [83]. Created with BioRender.com.

Lipogenesis and Cholesterol Synthesis

Beyond their terminal oxidation in the Krebs cycle to produce ATP and their excretion through urine and breath, ketone bodies can contribute to anabolism through *de novo* lipogenesis and cholesterol biosynthesis **(Figure 1.6)**. It is generally accepted that triacylglycerols are synthesized in the cytosol and then transported to lipid droplets for storage in the adipose tissues [85]. Ketone bodies are also efficient substrates for complex lipid synthesis in various lipogenic tissues such as the brain [83], liver [86], adipose tissue [87], and lactating mammary gland [88].

In mitochondria, AcAc is exported to cytosol where it is converted to AcAc CoA by AcAc CoA synthetase (AACS)-catalyzed ATP-dependent reaction. In the cytoplasm, AcAc CoA can be either converted to acetyl CoA via a reversible reaction catalyzed by cytosolic thiolases (cThiolases) or channeled by the cytosolic isoform of HMG CoA synthase 1 (HMGCS1) to produce HMG CoA [89]. Cytosolic acetyl CoA can then be transformed into malonyl CoA by ACC, paving the way for *de novo* lipogenesis. HMG CoA, on the other hand, is directed to the rate-limiting step of sterol biosynthesis catalyzed by HMG CoA reductase (HMGCR) [90]. AACS is a high-affinity enzyme for AcAc, as AcAc concentration in the fed state is sufficient for *de novo* lipogenesis [86]. Ketone bodies are significantly more efficient than glucose as a substrate for de novo lipid synthesis, thus sparing glucose for lipogenesis. Radioactively labelled ketones bodies, especially the AcAc, are preferred for sterols incorporation and lipogenesis rather than glucose in the brain [83]. During the suckling period, AACS activity increases, correlating with heightened AcAc integration into lipids. Furthermore, ketone bodies are favored for cholesterogenesis over other lipogenic precursors [89].

Ketone bodies are especially vital in the brain development during the neonatal stage when they aid in the synthesis of lipid, cholesterol and amino acid in addition to the oxidative importance of

ketone bodies in the nervous system [91]. However, SCOT-dependent oxidation is still the primary fate of ketone bodies, as excessive hyperketonemia occurs when primary oxidative function is impaired [92].

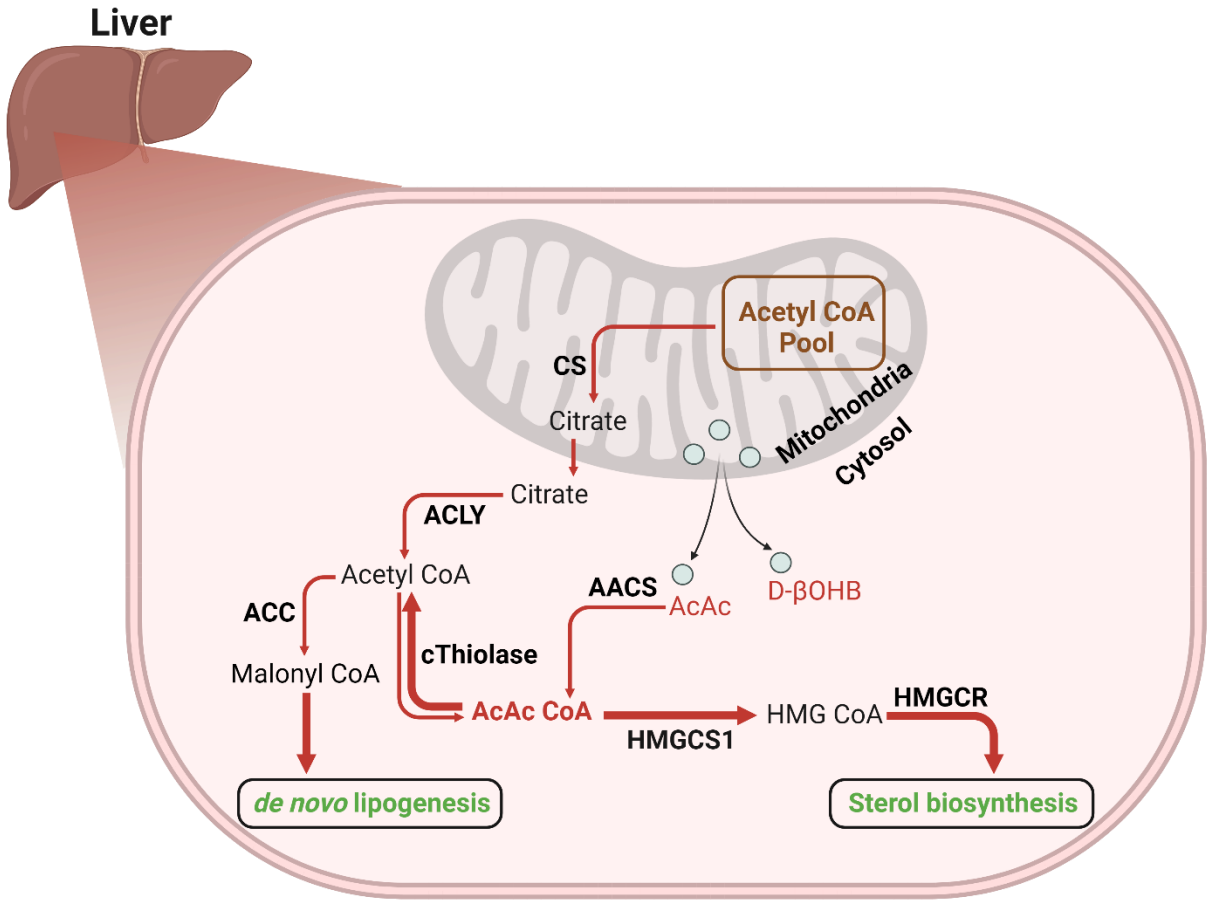

Figure 1.6 *Nonoxidative Metabolism of Ketone Body in Liver.*

Cytosolic AcAc-CoA can be directly utilized for sterol biosynthesis through HMG CoA or contribute to the cytosolic acetyl CoA pool through catalyzation of cytosolic thiolases (cThiolases). Another source of cytosolic acetyl CoA could be mitochondrial acetyl CoA. In the mitochondrial, citrate synthase (CS) converts acetyl CoA into citrate to be exported to the cytoplasm and then is converted back to acetyl CoA by ATP citrate lyase (ACLY). Acetyl CoA is subsequently transformed into malonyl CoA by ACC, initiating *de novo* lipogenesis. Created with Biorender.com.

1.2 Physiological Roles of Ketone Body

1.2.1 Ketone Body as a Mediator of Oxidative Stress and Inflammation through Signaling and Posttranslational Modification (PTM)

The ketone bodies, beyond being energy sources, have distinct signaling functions in cellular equilibrium. When the levels of circulating ketone bodies exceed the energy needs of oxidation, signaling activities commence [24]. As previously discussed, D-βOHB is preferentially utilized in extrahepatic oxidation due to the stereospecificity of mitochondrial BDH1. However, it is possible for both D- and L-isomers of βOHB to be involved in the signaling.

Oxidative stress arises from the imbalance between the generation of reactive oxygen species (ROS) and the biological system's capacity to neutralize these reactive elements. This situation is implicated in a variety of pathological conditions and often leads to inflammatory responses. AcAc and βOHB play opposing roles in cellular signaling. AcAc tends to promote oxidative stress, leading to heightened inflammation [93], whereas βOHB typically reduces both oxidative stress and inflammation [94]. Hence, the physiological effects of ketone bodies depend on the ratio of AcAc to βOHB. While extended periods of nutrient deprivation diminish inflammation due to the βOHB-mediated effect [95], the persistent ketosis seen in type 1 diabetes (T1D) promotes inflammatory conditions that can be attributed to AcAc [96]. Fasting-induced ketosis results in a two to four-fold increase in βOHB compared to AcAc. Meanwhile, the chronic ketoacidosis observed in diabetes is associated with elevated levels of AcAc, as they are not cleared from the circulation as rapidly as βOHB [97]. The benefits of ketogenic diets are partly attributed to βOHB's capacity to reduce inflammation by suppressing the NOD-like receptor protein 3 (NLRP3) inflammasome [98]. There is evidence showing that AcAc and βOHB regulate the diabetes-

associated bone loss bidirectionally. βOHB suppresses mineralization that would worsen the bone loss while AcAc enhances it [99].

βOHB

During situations like fasting, exercise, or diabetes, βOHB has been observed to inhibit Class I histone deacetylases (HDACs), leading to the enhanced expression of genes such as forkhead box O3a (*FOXO3a*) that combat oxidative stress [100]. βOHB enhances mitochondrial respiration in cortical neurons through the activation of histone acetyltransferase, serving as an antioxidant strategy to counteract ROS production [101]. Histone lysine residues undergo β-hydroxybutyration from direct interaction with βOHB, leading to enhanced capacity of oxidative phosphorylation via epigenetic alterations [102]. Mice treated with βOHB have shown enhanced resistance to oxidative stress [100]. Endoplasmic reticulum (ER) stress is linked to harmful ROS and protein misfolding. Starvation in rats or βOHB treatment led to higher βOHB levels accompanied by reduced ER stress and increased production of antioxidant enzymes in both animals and cells [103]. Fasting can induce a profound quiescent state in muscle stem cells, marked by a postponement in cell-cycle entry and increased stress resilience. βOHB, acting as an HDAC inhibitor, plays a pivotal role in this process by activating the p53 pathway, preserving the stem cell characteristics and guarding against cellular harm [104].

The inflammation decreases as it adjusts to limited glucose during fasting or rigorous exercise [103]. However, the precise function of βOHB in regulating the innate immune response requires further elucidation. βOHB can interact with several G-protein coupled receptors (GPCR), particularly G-protein coupled receptor 109A (GPR109A), to influence inflammatory responses.

βOHB but not AcAc activates GRR109A to suppress adenylyl cyclase-mediated cAMP production, thus inhibiting the lipolytic action in adipose tissue [105]. This creates a feedback loop where ketosis can limit the extent of ketogenesis by reducing the FFA release. However, the protective effects of βOHB through GPR109A have not been confirmed in live studies. GPR109A, apart from being expressed in adipocytes, is also present on various immune cells like macrophages and neutrophils. βOHB among other GPR109A ligands have shown anti-inflammatory effects across various conditions like atherosclerosis, obesity, and neurological diseases [106]. The GPR109A-regulated responses include the inhibition of nuclear factor-κB (NF-κB) pathway [94], which is absent when GPR109A is not present [107]. NF-κB is an inducible transcription factor and its activation involves the release of pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) [108]. The anti-inflammatory effect of βOHB is enhanced with increased expression of GPR109A associated with diabetes and neurodegeneration [94, 107]. βOHB treatment also reduces inflammation markers in human immune cells as well as the mouse by suppressing the activation of NLRP3 inflammasome [95, 103].

AcAc

When AcAc levels increase, either independently or in conjunction with high glucose, they can intensify harm to endothelial cells via oxidative stress and ROS buildup, which is believed to be a major factor in the heightened cellular damage observed in T1D [93, 96]. AcAc promotes inflammation through increased secretion of TNF-α in human monocytes, a trend observed in diabetic patients with elevated ketone concentrations [93]. In diabetes, inflammation arises not

solely from DKA but also from hyperglycemia, which increases oxidative stress and consequently leads to the production of inflammatory factors [109].

1.2.2 Therapeutic Roles of Ketogenic Diet

Neuronal Function

The high-fat and very low-carbohydrate based ketogenic diet have shown therapeutic effect against stroke [110], neurological disorders [76] and neurodegenerative diseases [111, 112]. Ketogenic diet has been used as a treatment for refractory epilepsy since the early 20^{th} century. For many with epilepsy, anticonvulsant drugs may be ineffective or cause severe side effects, making the ketogenic diet a beneficial alternative for improved seizure control [76]. The exact mechanism behind the beneficial effects on the nervous system is yet to be fully understood. One hypothesis suggests that ketone bodies reduce the need for carbohydrates. By conserving glucogenic amino acids like glutamate, they can be converted into gamma-aminobutyric acid (GABA), a key inhibitory neurotransmitter with recognized antiseizure properties [113]. Moreover, decreased brain glycolysis promotes ketogenesis in epilepsy management, leading to increased ATP availability and fewer depolarization events, thereby enhancing neuronal stability [114, 115]. In addition to its antioxidant properties, βOHB boosts the levels of brain-derived neurotropic factor (BDNF), which fosters neurogenesis, encourages neurite outgrowth and synaptogenesis, and aids in preventing apoptosis [101, 116]. Ketone bodies may also participate in seizure termination by activating acid-sensing ion channels 1a (ASIC1a) by their pH-lowering effect in the blood [117-119].

Ketone bodies may decrease free radical production and inflammation in the brain, given the observed reduction in toxic hydroxyl radical levels and elevated GPR109A levels in the brains and microglial cells of individuals with Parkinson's disease and in pro-inflammatory settings [76, 120]. βOHB exhibits complex interactions with the G-protein coupled receptor 41 (GPR41). In one study, researchers identified that βOHB functions as a partial antagonist, decreasing the activity of the sympathetic nervous system (SNS). This in turn results in reduced energy expenditure and a decreased heart rate, primarily by blocking short chain fatty acid signaling via GPR41 [121]. Contradictorily, another research group discovered that the same concentration of βOHB could act as an agonist, stimulating GPR41 and consequently heightening SNS activity [122]. This dual behavior highlights the multifaceted nature of βOHB's interaction with GPR41.

Heart Failure

The heart is the most energy-demanding organ in humans yet has limited energy-storing capacity. 90% of the ATP used by the heart comes from glucose and fatty acids, while amino acids and ketone bodies make up the rest [123]. Several studies have reported an association of heart failure in humans [124, 125] and mice [126] with increased ketone body uptake and utilization accompanied by elevated levels of circulating ketones [127, 128]. Ketogenic diets as well as ketone body infusion seem to exert cardioprotective effects [129-131]. It has been suggested that ketone bodies are more energetically efficient, providing more energy in the form of heat for ATP synthesis compared to glucose and fatty acids [76, 132]. However, Karwi and Lopaschuk contend that glucose generates more ATP per oxygen consumed (exhibiting a higher P/O ratio) compared to ketone bodies, while fatty acids release more energy per 2-carbon units than ketones [133].

Increasing ketones oxidation in the heart doesn't seem to improve cardiac efficiency [74], which is further confirmed by clinical observation of ketone body infusion in patients with heart failure with reduced ejection fraction [131]. The cardioprotective effect likely arises from the augmentation of ketone substrates availability for ATP synthesis [133]. Recent findings have illuminated the connection between sodium/glucose co-transporter 2 (SGLT2) inhibitors, which exhibit significant protective effects in patients with diabetic cardiomyopathy [134, 135], and the observed elevation in βOHB levels in both humans [136, 137] and mice [138], associated with increased ketogenesis [136]. However, the therapeutic role of ketone bodies in heart failure remains highly debated.

Weight Management

Ketogenic diet is also proven effective in promoting weight loss [139] and reducing hepatic triacylglycerol content in subjects with NAFLD [140]. A 2016 study revealed that a short-term ketogenic diet with a nearly carbohydrate-free regimen effectively lowered body weight, waist size, blood pressure, and insulin resistance in severely obese adults [141]. Also, meta-analysis of clinical studies regarding ketogenic diets highlights the appetite-suppressing effects of such diets [142]. Extended periods of starvation are frequently characterized by a loss of appetite [143, 144], while the energy produced through the oxidation of visceral βOHB dictates the length of satiety intervals and the initiation of subsequent food intake [145]. This suggests that ketone bodies may enhance satiety through mechanisms involving both oxidation and signaling [34]. Nevertheless, the underlying molecular mechanisms involved remain unresolved. Recent research has revealed that a low-fat diet rich in complex carbohydrates can improve glycemia and induce fat loss in obese

mice, while a cocoa butter-based ketogenic diet does not yield the same results [78]. The efficacy of the ketogenic diet in weight loss is thus questionable. The application of the ketogenic diet is linked to multiple vascular complications. These include elevated blood cholesterol levels due to high-fat intake [76] and dilated cardiomyopathy related to elevated fatty acids in the blood [146]. Such adverse effects constrain the diet's applicability, particularly among older individuals. The outcomes from different dietary components of ketogenic diets can vary significantly; for instance, a vegetarian diet is associated with a 21% reduced risk of NAFLD [147]. Therefore, it is essential for anyone considering a ketogenic diet for weight loss to discuss it with a healthcare professional or registered dietitian to ensure it is appropriate and to receive guidance on balanced nutrition.

1.2.3 Roles of Exogenous Ketone Body in Energy Conservation

Aside from ketogenic diet, another way to stimulate ketosis is through administration of exogenous ketone bodies including ketone salts or ketone esters (KE). Consuming exogenous ketone bodies can achieve nutritional ketosis even under non-ketogenic conditions. In this state, while blood glucose and insulin levels remain stable, peripheral glucose oxidation is reduced due to the inhibitory impact of ketone bodies on glycolysis [148, 149]. Ketone salts are AcAc or βOHB that is bound to salt ions, typically sodium. Ingesting or infusing sodium ketone salts can elevate βOHB levels to around 5mM within 2 hours in rodents with weight-lowering effect [34]; however, this could come with an excessive sodium consumption [150]. The KE, on the other hand, provides a safe and tolerable way to induce nutritional ketosis efficiently [151]. The proposed pathway **(Figure 1.7)** of Cox *et al.* explains how D-βOHB is released from (R)-3-hydroxybutyl (R)-3-hydroxybutyrate KE after ingestion. The enhancement in performance seen with KE

supplementation in professional cycling has garnered significant attention [152], especially given that the muscles of trained athletes exhibit an elevated capacity for the uptake and oxidation of ketone bodies [153]. Recently, KE has been demonstrated to enhance athletic performance during exercise, potentially due to the promotion of intramuscular FAO. This is accompanied by preserved glycogen due to reduced glycolysis and decreased lactate accumulation in skeletal muscle that improve the exercise endurance [154]. KE-induced ketosis may not be advantageous for sports such as sprinting, which primarily depend on intense glycolytic activity for energy. Additionally, muscle loss is cut down, potentially facilitating a quicker recovery from exercise [155].

The preservation of energy, especially carbohydrates, is crucial for the brain during starvation due to its inability to utilize fatty acids. The introduction of exogenous ketone bodies can shift the priority of energy substrates, impacting both glucose oxidation and FAO across various species. Administration of ketone bodies to man [156], dogs [157-159] and rats [160] decreases blood glucose as well as plasma FFA. Exogenous ketone bodies help conserve carbohydrates by reducing glycolysis, sparing the glucose for glycogen synthesis. In perfused rat hearts, AcAc reduced glucose uptake by half, while the synthesis rates of glycogen remained unchanged [66]. When ketone bodies are administered in conjunction with insulin, reduction in glucose uptake is observed with increased intracellular glucose levels, and an enhanced rate of glycogen synthesis [71]. Insulin speeds up the transport of glucose into cells but doesn't necessarily enhance its phosphorylation, indicating that ketone bodies might reverse the impact of insulin on glucose transport within the heart [51]. In eviscerated-nephrectomized rabbits [161] and healthy dogs [158], ketone bodies infusion is found to decrease glycolysis with unaffected glucose uptake. Despite the suppression of glycolysis, the level of cytosolic ATP increases because of the mitochondrial oxidation of the

ketone bodies [76]. Ketone bodies in rat muscles are observed to favorably enter the Krebs cycle to produce energy while simultaneously inhibiting glycolysis and boosting glycogen storage [162].

The effect of exogenous ketone body on lowering blood glucose levels and circulating FFA is postulated as the results of insulin secretion, but the literatures show distinct results depending on the species and the magnitude of ketosis induced. Ketone bodies are shown to be insulinogenic in dogs [157] and rats [160]. Transient increments in plasma insulin have been observed with acute infusions of AcAc in man [163, 164]. Yet, other studies have reported that sustained ketone body infusions lead to hypoglycemia without any noticeable rise in plasma insulin levels in the peripheral blood [156, 165, 166]. This is probably because all endogenously produced insulin first passes through the liver before entering systemic circulation, where it becomes accessible to peripheral tissues [167]. Thus, the impact of insulin on blood glucose levels mainly results from a reduced hepatic release of glucose, achieved by inhibiting gluconeogenesis and glycogenolysis, leading subsequently to a net hepatic absorption of glucose [168, 169].

The levels of insulin-induced hypoglycemia correlate with the elevation of ketone levels brought about by exogenous ketones. Madison's team found a sustained pancreatic response with higher doses of ketones [157], in contrast to Balasse *et al.* of a brief insulin reaction with lower ketone doses [158]. Pi-Sunyer *et al.* established that insulin response is proportional to the concentrations of ketones administered [170]. Insulin levels in the peripheral arterial circulation significantly increase when ketone concentrations reach 1.5 to 2 mM, a range observed in short-term fasting but below that seen in DKA [170].

Reduced FFA levels appear to correlate with the administered ketone concentrations. Additionally, the release of FFA from adipose tissue is more responsive to insulin than glucose uptake in

peripheral tissues [170]. Sodium D, L-βOHB infusion in dogs can extinguish the norepinephrine induced FFA and directly inhibit FFA outflow from adipose tissue [171]. Insulin also promotes a reduction in FFA production by adipose tissue and increases FFA intake by the liver [158]. It is possible that exogenous ketones inhibit release of FFA from adipose tissue directly independent of insulin since plasma FFA decreases in sodium AcAc-infused diabetic rats [160]. *In vitro* work indicates that ketone bodies affect the lipase activation by hindering the cAMP production [52, 172]. The reduction of FFA in the bloodstream may result from the combined actions of ketones and insulin, potentially serving as a protective measure against the onset of ketoacidosis.

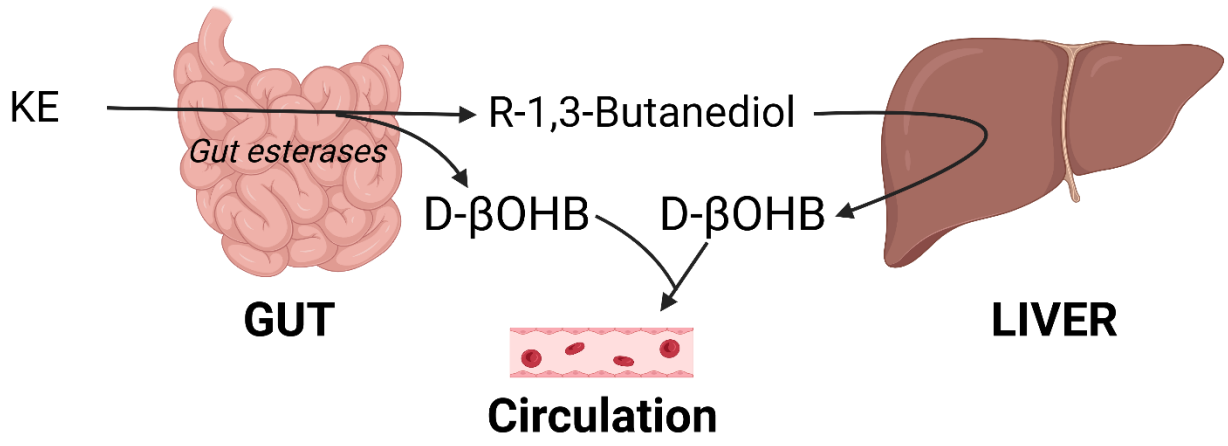

Figure 1.7 *Proposed Pathway of (R)-3-Hydroxybutyl (R)-3-Hydroxybutyrate KE Metabolism.*

One molecule of KE, when ingested, can release one molecule of D-βOHB in the gut and then into the blood. The other metabolite R-1,3-butanediol undergoes further enzymatic reaction in the liver to release another molecule of D-βOHB into the circulation. The combined D-βOHB production constitutes an efficient and prolonged ketosis. Adapted from Cox *et al.* [154]. Created with BioRender.com.

1.3 Potential Therapeutic Approaches Targeting Ketone Body Metabolism for Obesity and Diabetes

1.3.1 Roles of Ketone Body in Obesity and Diabetes

Obesity and diabetes represent two of the most prevalent and challenging public health issues globally. Obesity is characterized by an excess accumulation of body fat, usually resulting from an imbalance between caloric intake and energy expenditure. Diabetes, on the other hand, is a chronic metabolic disorder marked by elevated levels of blood glucose, or blood sugar, which can lead to serious damage to the heart, blood vessels, eyes, kidneys, and nerves over time [173]. Obesity and diabetes are closely interconnected, with obesity serving as a significant risk factor for the onset of type 2 diabetes(T2D) [173].

Obesity is associated with an increased β-cell responsiveness, which is attributed to excessive food intake and lipid accumulation, leading to β-cell hyperplasia independent of insulin resistance [174, 175]. Nevertheless, insulin resistance is tightly associated with ectopic lipid accumulation in cases of obesity [176], whereas the hypersensitivity could culminate in β-cell fatigue and subsequent failure, signaling the emergence of T2D [177]. Additionally, in β-cells, alterations in glucose metabolism and lipid signaling are influenced by an increased supply of fatty acids from adipose tissues [178], as long-term Intralipid infusion has been shown to impair insulin sensitivity to glucose and glycolytic capacity both *in vivo* and in isolated perfused pancreas studies [179, 180]. The collective impact of these factors contributes to the predominant metabolic alterations observed in T2D, namely hyperglycemia and intensified lipolysis [181, 182].

Both obesity and diabetes are characterized by increased concentrations of FFA from lipolysis due to elevated cAMP levels [183], and in cases of diabetes where insulin levels are low from β-cell failure, this results in enhanced mobilization of FFA and suppressed fatty acid synthesis, thereby boosting FAO, and expanding the acetyl CoA pool for ketogenesis. Studies have reported that individuals experiencing severe DKA exhibited splanchnic production rates of 195 and 385 g per day, a notable increase compared to the 150 g per day observed in obese individuals [56, 184]. A diabetic patient weighing 75 kg has the potential to utilize around 180 g of ketone bodies daily, compared to the 143 g per day utilization observed in individuals with obesity [56, 185]. DKA arises from the overproduction of ketone bodies that exceeds the physiological needs of the body, coupled with impaired uptake and oxidation of these ketone bodies due to an insulin-deficient state [59, 68, 186, 187]. Prolonged exposure to ketone bodies further reduces the ability of the islets to produce insulin [180], thereby perpetuating a detrimental cycle. However, it is important to clarify that the harm caused by DKA arises from acidosis due to the excessive production of ketone bodies rather than the presence of ketone bodies themselves [8].

Increased activity of CPT1 and HMGCS2 also contribute to the augmented ketogenesis in diabetes and insulin injection to diabetic rats greatly reduces ketonuria and *Hmgcs2* expression [32, 38, 42]. The steady state of OAA differentiates pathological ketosis from physiological ketosis. In liver and kidney cortex where gluconeogenesis takes place, the rate of gluconeogenesis is especially high when the demand for carbohydrate synthesis is great as in diabetes. At the same time, the supply of OAA from PC remains unchanged even though the removal of OAA by gluconeogenesis is greatly increased, which leads to an elevated ketogenesis [58, 188]. Excessive demand for carbohydrates in diabetes puts extra strains on the hepatic Krebs cycle, which causes the accumulation of ketone bodies. Ketone bodies are significant alternative metabolites to glucose in

diabetic conditions. It is suggested that the conversion of AcAc to acetone may serve as an additional mechanism to counteract DKA, as acetone plays a role in regulating pH [58, 181, 189]. During periods of fasting in humans, a significant amount (up to 37%) of AcAc can be converted into acetone, resulting in elevated levels of acetone in the bloodstream, if not promptly oxidized, they can have detrimental effects on the central nervous system, causing narcosis [15, 190]. Some diabetic patients suffer from severe acetonemia. Sulway and Malins reported that the concentration of plasma acetone in some diabetic patients was equal to or greater than that of plasma AcAc and its slow elimination due to its hydrophilic and lipophilic properties causes long-lasting elevated acetone levels even after blood glucose, AcAc and D-βOHB levels returned to normal [189].

The interplay between obesity and diabetes has multifactorial roots, encompassing genetic, environmental, behavioral, and lifestyle factors. Lifestyle modifications, such as a balanced diet and regular physical activity, remain fundamental in managing and preventing both conditions. Understanding the intricate relationship between obesity and diabetes is essential for developing effective strategies for prevention, intervention, and treatment, aiming to curb the growing prevalence of these interconnected metabolic disorders and improve the quality of life for affected individuals.

1.3.2 Targeting Ketone Body Oxidation to Improve Glycemic Regulation in Obesity and Diabetes

Currently, it is estimated that over 400 million people globally are afflicted with diabetes, with T2D accounting for approximately 90% of these cases, largely attributable to prevailing obesity [173]. Pre-T2D manifests through insulin resistance; however, pronounced hyperglycemia can be

counteracted by augmented insulin secretion from islet β-cells. As the pathology characterized by insulin resistance and hyperinsulinemia advances, the continual demand for insulin secretion places heightened stress on islet β-cells, ultimately leading to β-cell dysfunction and the emergence of clinical T2D. Metformin is a first-line medication for the treatment of Type 2 Diabetes (T2D); however, it cannot be prescribed to patients with a low glomerular filtration rate [191]. Additionally, more newly developed medications aimed at reducing hyperglycemia in T2D by enhancing insulin secretion, such as glucagon-like peptide-1 receptor agonists, find inducing gastrointestinal issues such as nausea, vomiting, diarrhea and constipation [191]. Consequently, there is a persisting clinical need for the identification and development of novel therapies for T2D.

Our laboratory's work is centered on exploring the function of ketone body metabolism in addressing metabolic disorders associated with obesity and diabetes [44, 45, 78]. In our prior work, we found that obese mice exhibit increased SCOT activity in their skeletal muscles. As previously mentioned, SCOT is an essential enzyme of ketone body oxidation in extrahepatic mitochondria and can be upregulated by the abundance of circulating ketones. Moreover, a group of DPBP drugs, such as pimozide, penfluridol, and fluspirilene, can inhibit SCOT, enhancing the glucose tolerance of both obese and T2D mice with the participation of pyruvate dehydrogenase (PDH) but independent of weight loss, lipid accumulation, or brain SCOT activity. Pimozide and its drug class DPBP were first developed in 1963 as the treatment of schizophrenia and Tourette syndrome with its primary target being the dopamine D_2 receptor subfamily [192, 193], as well as a potent inhibitor of the voltage-dependent Ca^{2+} channel [194]. Similar improvement in glycemic profile is achieved in obese mice with muscle specific SCOT knockout ($SCOT^{Muscle-/-}$), thereby further supporting a beneficial role for skeletal muscle SCOT inhibition in the alleviation of obesity/T2D-induced glucose intolerance. It is suggested that inhibiting ketone body oxidation could enhance

glycolytic flow through PDH to maintain energy homeostasis in obese mice. This, in turn, could alleviate the glucose intolerance associated with obesity and diabetes.

Additionally, the cocoa-butter based ketogenic diet does not enhance glucose tolerance in obese mice, further suggesting that the elevation of ketone body oxidation is a maladaptive response resulting from the metabolic disorder associated with obesity and diabetes. Although the mechanistic link between ketone body metabolism and glucose homoeostasis requires further investigation, manipulation of ketone body oxidation may be a novel approach for developing treatment for hyperglycemia in individuals with obesity and T2D.

1.4 Hypotheses and Aims

In fasting mice, obesity has been linked to lower blood ketones and elevated ketone body oxidation in the skeletal muscles [44]. SCOT inhibition or SCOT deletion in skeletal muscles improves glucose intolerance in obese mice [44, 45]. During fasting state, it is possible that rise in ketone body oxidation is a maladaptive perturbation to the enhanced ketogenesis associated with obesity. Since increases in ketone supply can boost ketone body oxidation rates thereby increasing the SCOT activity [195], we hypothesize that providing obese mice with dietary ketone esters to increase circulating ketone levels may worsen their glycemic control. Additionally, we theorize that the genetic overexpression of SCOT in skeletal muscles could result in impaired glycemia in obese mice, making them more susceptible to uncontrolled hyperglycemia and β-cell failure.

The aims of each study, which addressed specific portions of the overarching hypotheses, are listed below by chapter:

Chapter 2: Ketone ester administration improves glycemia in obese mice.

1. To examine whether KE-induced nutritional ketosis would impact glucose tolerance.
2. To examine whether ketone body oxidation in muscle is required for glycemic control in obese mice by administering KE in $SCOT^{Muscle-/-}$ mice.
3. To assess the role of insulin in the effect of KE on glycemic control.

Chapter 3: Generation and Characterization of SCOT/*Oxct1* Transgenic Mice (Ongoing work)

1. To generate homozygous 3-Oxoacid CoA-Transferase 1 (*Oxct1*) transgenic mice.
2. To characterize glycemic profile of obese homozygous *Oxct1* transgenic mice.

Chapter 2: Ketone Ester Administration Improves Glycemia in Obese Male Mice

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*Denotes equal contribution.

2.1 Abstract

During periods of prolonged fasting/starvation, the liver generates ketones [i.e., β-hydroxybutyrate (βOHB)] that primarily serve as alternative substrates for ATP production. Previous studies have demonstrated that elevations in skeletal muscle ketone oxidation contribute to obesity-related hyperglycemia, whereas inhibition of succinyl CoA:3-ketoacid CoA transferase (SCOT), the rate-limiting enzyme of ketone oxidation, can alleviate obesity-related hyperglycemia. As circulating ketone levels are a key determinant of ketone oxidation rates, we tested the hypothesis that increases in circulating ketone levels would worsen glucose homeostasis secondary to increases in muscle ketone oxidation. Accordingly, male C57BL/6J mice were subjected to high-fat diet-induced obesity, whereas their lean counterparts received a standard chow diet. Lean and obese mice were orally administered either a ketone ester (KE) or placebo, followed by a glucose tolerance test. In tandem, we conducted isolated islet perifusion experiments to quantify insulin secretion in response to ketones. We observed that exogenous KE administration robustly increases circulating βOHB levels, which was associated with an improvement in glucose tolerance only in obese mice. These observations were independent of muscle ketone oxidation, as they were replicated in mice with a skeletal muscle-specific SCOT deficiency. Furthermore, the R-isomer of βOHB produced greater increases in perifusion insulin levels versus the S-isomer in isolated islets from obese mice. Taken together, acute elevations in circulating ketones promote glucose-lowering in obesity. Given that only the R-isomer of βOHB is oxidized, further studies are warranted to delineate the precise role of β-cell ketone oxidation in regulating insulin secretion.

2.2 Introduction

During prolonged periods of fasting/starvation, the liver produces ketone bodies such as β-hydroxybutyrate (βOHB), acetoacetate (AcAc), and to a lesser extent acetone (collectively referred to herein as ketones; [24]). Owing to their water solubility, ketones can be easily transported via the circulatory system. They can cross the blood-brain barrier, making them a vital alternative energy source for the brain during insufficient glucose availability, since neurons have a limited capacity to store glucose and support fatty acids oxidation [1].

The significance of ketone metabolism in maintaining physiological homeostasis during fasting has been recognized for several decades. Although the brain is a significant consumer of ketones, other tissues, such as the heart and skeletal muscle, are also reliant on ketones as an essential energy source. Ketone metabolism appears to become even more important of a fuel source in various pathologies, as it has been demonstrated that the failing heart has an increased reliance on ketones as an oxidative fuel source, which is likely an adaptive response to declines in fatty acids oxidation [24, 124]. Furthermore, it has also been suggested that ketones may serve as a super fuel that provide additional energy, which is based on observations that exogenous ketone ester (KE) administration can augment exercise performance in humans [154].

Recent studies have also observed that expression and activity of the rate-limiting enzyme of ketone oxidation, succinyl CoA:3-ketoacid CoA transferase (SCOT), is elevated in the skeletal muscles of obese mice. Furthermore, both pharmacological and genetic inhibition of SCOT activity in mice alleviated obesity-induced hyperglycemia [44, 45]. Therefore, it appears that increases in muscle ketone oxidation may contribute to the pathology of type 2 diabetes. As increases in circulating ketone supply are a critical determinant of ketone oxidation rates [196],

we hypothesized that elevating circulating ketones would compromise blood glucose control in lean mice, while potentially exacerbating dysglycemia in obese mice.

Potential strategies to address our hypothesis involve elevating endogenous ketones via extended fasting or adhering to a ketogenic diet. Although these are robust approaches to increase ketones, they induce many other physiological changes (i.e., increases in circulating fatty acids and subsequent fatty acids oxidation rates) that could potentially compromise our ability to assess the effect of increased circulating ketone levels on glycemia [78]. Conversely, the emergence of exogenous KE beverages represents a more viable alternative to increase circulating ketones without impacting other key physiological parameters secondary to extended fasting or adhering to a ketogenic diet. Commercially available KE beverages are able to robustly elevate circulating ketone levels in a dose-dependent manner, owing to their unique metabolic activation process. This mechanism involves the cleavage of the KE molecule by gut carboxylesterases, resulting in the release of D-βOHB and 1,3 butanediol **(Figure 2.1A)**. Once the latter molecule reaches the liver it undergoes further metabolism, yielding another D-βOHB [154]. As such, we aimed to investigate the ability of orally administered KEs to increase circulating ketones in both lean and obese mice, while assessing glucose tolerance outcomes.

2.3 Methods

2.3.1 Animal Care

All animal procedures were approved by the University of Alberta Health Sciences Animal Welfare Committee and all animals received care in accordance with the guidelines approved by the Canadian Council on Animal Care. Animals were housed at 22°C (temperature-controlled) in a 12 h light and 12 h dark cycle (lights on from 6 am to 6 pm), with all mice receiving standard environmental enrichment (chip bedding, a plastic tube, 8 ounces of crinkle paper, and Nestlet square bedding comprised of short fiber cotton) and ad libitum access to food and water. 8-week-old male mice were either fed a standard chow; or a high-fat diet (45%kcal from lard, Research Diets D12451 or 60%kcal from lard, Research Diets D12492) for 8 weeks to induce experimental obesity. After 8 weeks on dietary protocols, one group of male mice were sacrificed by isoflurane inhalation followed by cervical dislocation and proceeded to islet isolation. Another group of male mice underwent 3-hour fast before being randomly assigned to receive either placebo drink or ketone ester (1719 mg/kg, HVMN) by oral gavage. All animals were subjected to intraperitoneal (IP) glucose tolerance tests (IPGTTs) at 2-h post-treatment. At study completion, mice were treated with either placebo drink or ketone ester through oral gavage following a 3-h fast prior to euthanization. Mice were euthanized 2-h post-gavage with an IP injection of sodium pentobarbital (12 mg), following which tissues were extracted and immediately snap-frozen in liquid N_2 using liquid N_2-cooled Wollenberger tongs.

2.3.2 Assessment of Glucose Tolerance

Mice were transferred to clean static cages with all cage enrichments and fasted for 3-h with free access to drinking water before treated with placebo drink or ketone ester by oral gavage. Whole blood samples were collected from tails at 0-, 5-, 10-, 15-, 30-, 60-, 90-, and 120-minute post-gavage for circulating β-hydroxybutyrate levels measurement using the FreeStyle Precision Neo blood ketone monitoring system (Abbott) and for circulating glucose levels measurement using Contour Next blood glucose monitoring system (Bayer). IPGTTs were performed at 2-h post-gavage. Mice were administered glucose (2 g/kg) via IP injection with whole blood samples collected from tail at 0-, 5-, 10-, 15-, 30-, 60-, 90-, and 120-minute post-glucose administration using Contour Next blood glucose monitoring system (Bayer) to measure blood glucose levels.

2.3.3 Measurement of Circulating Glucose, βOHB, and Insulin Levels

Following our 8 wk dietary protocol, mice were transferred to clean static cages with all cage enrichments and fasted for 3 h with free access to drinking water before randomization to oral administration of either a placebo [provided by Health Via Modern Nutrition (HVMN)] or KE (1,719 mg/kg, HVMN). Mice were randomly assigned for sampling to measure circulating glucose, βOHB, and insulin Levels. Mouse tail whole blood samples were collected at 0, 5, 10, 15, 30, 60, 90, and 120 min post-gavage for measurement of circulating βOHB levels using the FreeStyle Precision Neo blood ketone monitoring system (Abbott). The βOHB in the blood is converted to AcAc by the BDH1 embedded in the sensor, turning NAD^+ into NADH in the process. When NADH is converted back to NAD^+ by a redox mediator, it produces a current that's directly linked

to the βOHB levels. Circulating glucose levels were obtained at the same time points using the Contour Next blood glucose monitoring system (Bayer).

Whole blood samples were collected from mouse tail (~10 μL) during the IPGTT immediately prior to injecting glucose (0-min) and at 15-min post-glucose administration. Plasma insulin was measured using a commercially available enzyme-linked immunosorbent assay kit according to the manufacturer provided protocols (ALPCO Diagnostics). In brief, 5 μL of isolated plasma was added in a microplate with 75 μL of enzyme conjugate, following which the plate was incubated in an orbital microplate shaker at 750 rpm for 2-h at room temperature. After the incubation period, the microplate was washed six times with a provided wash buffer and subsequent reaction was initiated by adding 100 μL of the provided tetramethyl benzidine to each well followed by room-temperature incubation in the orbital microplate shaker at 750 rpm. The reaction was terminated after a 30-min incubation by adding 100 μL of a provided stop solution. The plasma insulin levels were determined by measuring optical density of each sample at 450 nm using Synergy H1 Hybrid Reader (BioTek).

2.3.4 Islet Isolation and Perifusion

Mice were sacrificed by isoflurane inhalation followed by cervical dislocation. The common bile duct was clamped at the duodenum and collagenase XI (7.5 mg/mL; Sigma C7657) in HBSS (5 mM glucose, 1 mM MgCl2) was injected into the common bile duct until the pancreas was fully inflated. After removal, excised pancreases were digested at 37°C for 12 minutes and washed with HBSS (5 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$). The islets were isolated with Histopaque

gradient (Sigma, 10771), handpicked in complete RPMI (10% FBS, 1% penicillin/streptomycin), and rested overnight at 37°C, 5% O 2.

80 islets per mouse were perifused in a Biorep perifusion system (Biorep technologies). Perifusion solutions were made in KRBH buffer (115 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 24 mM $NaHCO_3$, 10 mM HEPES, and 1% BSA; pH = 7.4). The islets were equilibrated at 37°C and 2.8 mM glucose in the perifusion machine for 42 minutes before the addition of other solutions. Flow rate was consistent at 100 µL/min and treatments are indicated at the top of figures (10 mM glucose ± 1 or 10 mM (S)-3-hydroxybutanoic acid sodium (HY-W050031), 1 or 10 mM (R)-3-hydroxybutanoic acid sodium (HY-W015851), 10 mM ketone ester (HVMN), placebo, and 30 mM KCl or 16.7 mM glucose). Perifusion effluents were collected every 2 minutes, held at 4-8°C, and stored at -80°C until insulin was determined by Mouse Ultrasensitive or High Range Insulin ELISA (ALPCO, 80-INSMSU-E01 or 80-INSMSH).

2.3.5 Statistical Analysis

All values are presented as means ± standard error of the mean (SEM). An unpaired, two-tailed Student's t test was used to assess statistical significance between two groups. Multiple time points were compared using a repeated-measures two-way ANOVA followed by a Bonferroni post hoc analysis. The above data analysis was completed using GraphPad Prism 9 software. Differences were considered significant when $P < 0.05$.

2.4 Results

2.4.1 KE Administration Increases Circulating Ketone Levels without Impacting Glucose Tolerance in Lean Male Mice

Acute treatment of lean male C57BL/6J mice fed a standard laboratory chow diet with a KE (1,719 mg/kg) via oral gavage led to robust increases in circulating βOHB levels that were sustained for at least 2 h and peaked within the first 15–30 min **(Figure 2.1B)**. Circulating glucose concentrations during this 2-h period remained similar between both groups **(Figure 2.1C)**. At 2 h post-KE or placebo administration, all mice were subjected to intraperitoneal glucose tolerance testing, which demonstrated no change in glucose tolerance in mice with acute increases in their circulating ketones **(Figure 2.1D&E)**.

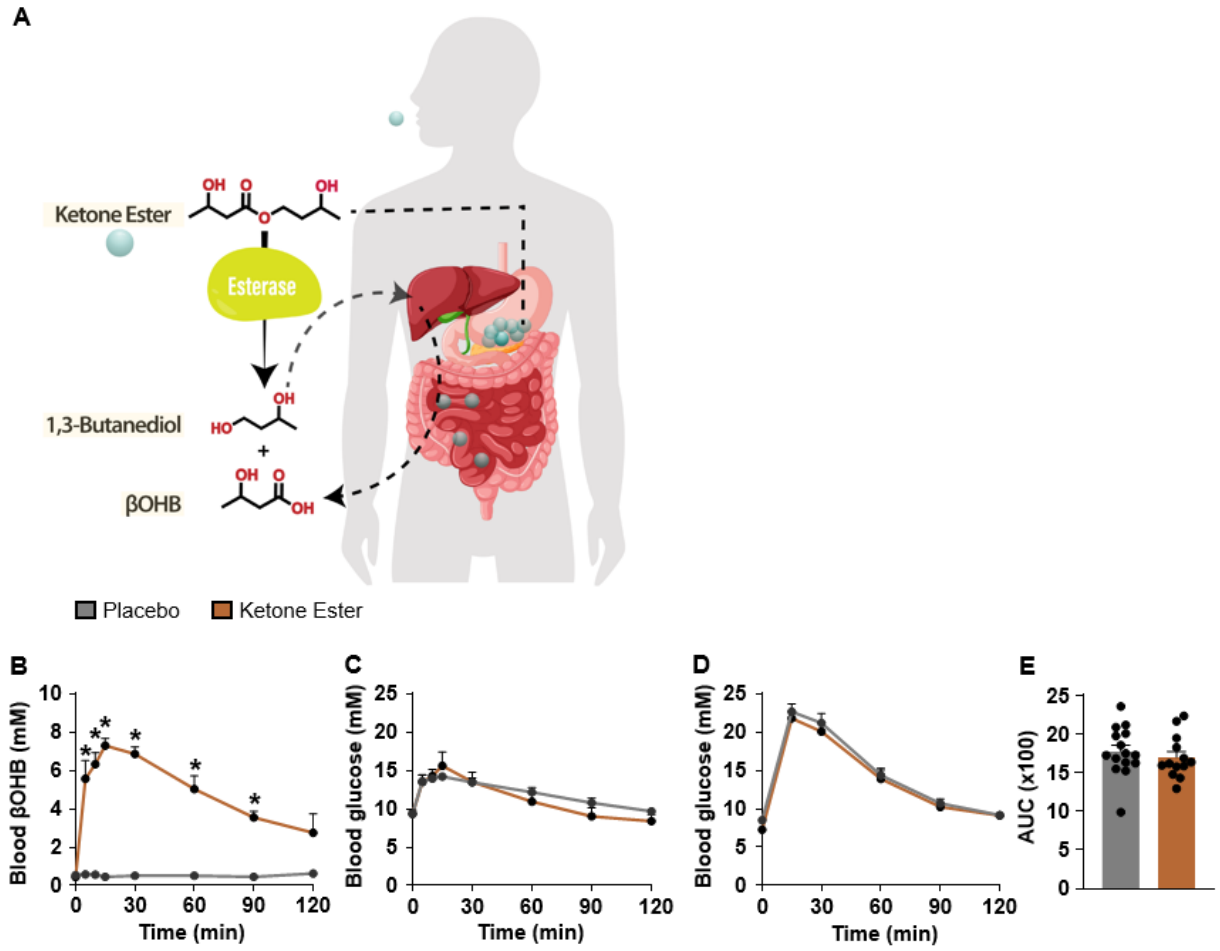

Figure 2.1 *Exogenous KE administration increases circulating ketone levels without impacting glucose tolerance in lean male mice.*

A: Schematic representation of ketone ester metabolism and conversion following oral administration. Circulating βOHB levels (B) and circulating glucose levels (C) in lean C57BL/6J mice that were treated with either placebo or KE (n = 8 or 9). Glucose tolerance (performed 2 h following oral gavage; D) and the associated AUC (n = 13–15; E). Values represent means ± SE. Differences were determined using either an unpaired Student's t test or a repeated measures two-way ANOVA, followed by a Bonferroni post hoc analysis. *P < 0.05 vs. placebo-treated counterparts. Figure 2.1A was designed by Freepik. AUC, area under the curve, βOHB, β-hydroxybutyrate; KE, ketone ester.

2.4.2 KE Administration Increases Circulating Ketone Levels and Improves Glucose Tolerance in Obese Male Mice

In our next set of studies, male C57BL/6J mice were subjected to experimental obesity via 8 wk of supplementation with an HFD containing 45% kcal from lard, following which all mice were orally administered either a KE (1,719 mg/kg) or placebo. We once again observed robust increases in circulating βOHB levels that were sustained for at least 2 h and peaked within the first 15–30 min **(Figure 2.2A)**. Circulating glucose concentrations remained similar between KE- and placebo-treated mice during this time frame, but we did observe mild nonsignificant increases in circulating insulin levels **(Figure 2.2B&C)**. Moreover, circulating glucose levels were significantly lower in the KE-treated obese mice immediately before starting the intraperitoneal glucose tolerance test **(Figure 2.2D)**. In contrast to our observations in lean mice, the obese mice that were administered the KE now displayed a robust improvement in glucose tolerance that was associated with a trend toward increases in circulating insulin before glucose administration **(Figure 2.2E-G)**. Similar results were observed in male C57BL/6J mice that were obese and fed an HFD containing 60% kcal from lard for 8 wk **(Figure 2.2H-N)**. However, KE administration produced a more robust glucose-lowering before the assessment of glucose tolerance in obese mice fed the 60% kcal HFD **(Figure 2.2I)**.

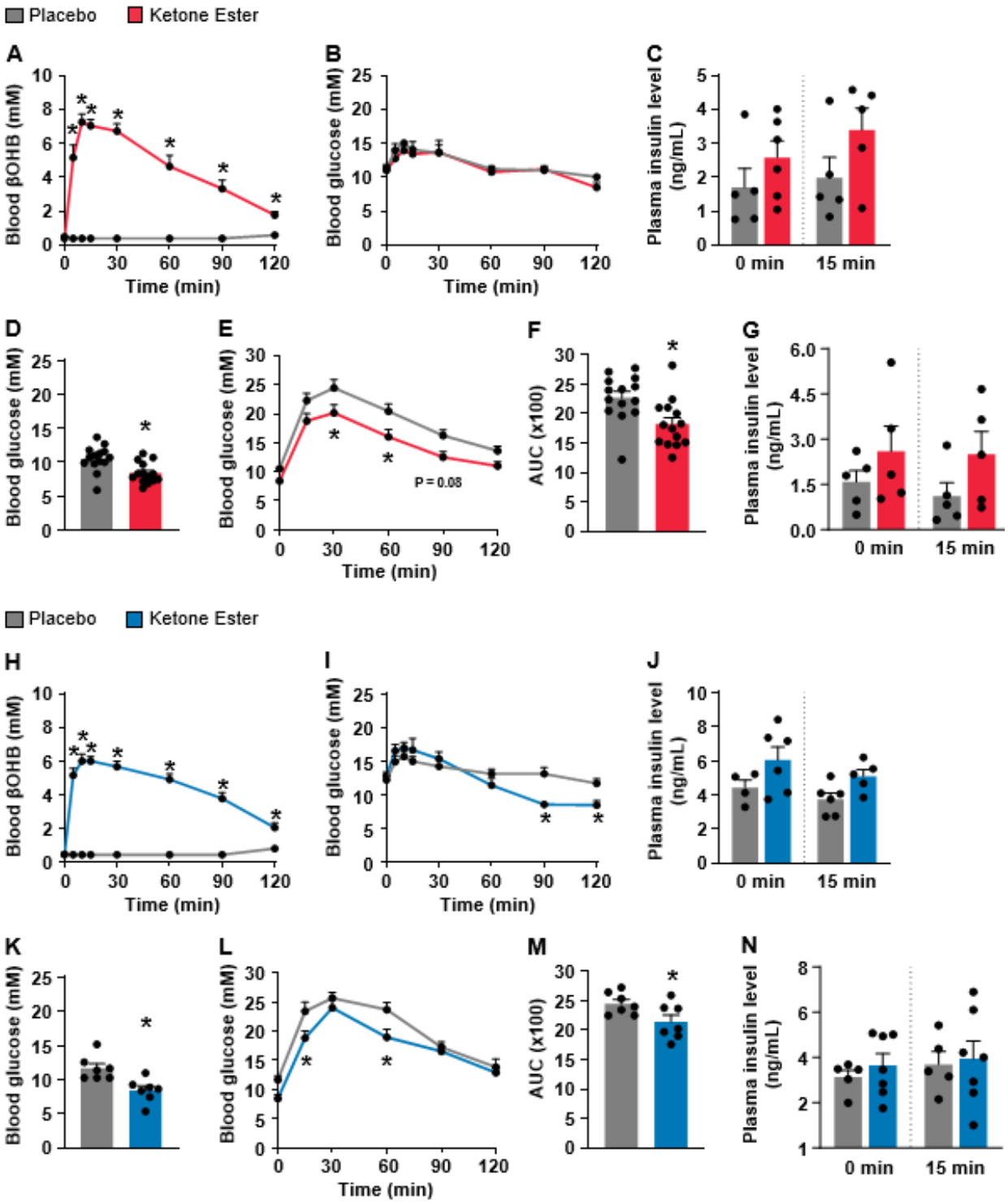

Figure 2.2 *Exogenous KE administration increases circulating ketone levels and improves glucose tolerance in obese male mice.*

Circulating βOHB levels (A), circulating glucose levels (B), and corresponding insulin levels (C) in C57BL/6J mice fed a 45% HFD and treated with either placebo or KE (n = 5 or 6). Starting glucose levels before glucose tolerance (D), glucose tolerance (performed 2 h following oral gavage; E), the associated AUC (n = 14; F), and corresponding insulin levels (n = 5 or 6; G). Circulating βOHB levels (H), glucose levels (I), and corresponding insulin levels (J) in C57BL/6J mice fed a 60% HFD and treated with either placebo or KE (n = 4–7). Starting glucose levels before glucose tolerance (K), glucose tolerance (performed 2 h following the gavage; L), the associated AUC (n = 7; M), and corresponding insulin levels (n = 5–7; N). Values represent means ± SE. Differences were determined using either an unpaired Student's t test or a repeated measures two-way ANOVA, followed by a Bonferroni post hoc analysis. *P < 0.05 vs. placebo-treated counterparts. AUC, area under the curve; βOHB, β-hydroxybutyrate; HFD, high-fat diet; KE, ketone ester.

2.4.3 Increases in Muscle Ketone Oxidation Are Not Required for the Glucose Lowering Actions in Response to KE Administration in Obese Male Mice

Because improvements in glucose tolerance following increases in circulating ketone levels were in direct contrast to our hypothesis, we repeated our studies in $SCOT^{MuscleKO}$ mice. These mice are incapable of oxidizing ketones within their muscle due to skeletal muscle-specific deletion of SCOT, the rate-limiting enzyme of ketone oxidation[44]. Male $SCOT^{MuscleKO}$ mice were subjected to experimental obesity via the provision of an HFD containing 45% kcal from lard for 8 wk. While the peak increases in circulating ketones we observed in C57BL/6J mice were recapitulated in obese $SCOT^{MuscleKO}$ mice, the increase was more sustained in the latter, consistent with their inability to oxidize ketones in their muscle **(Figure 2.3A)**. We also noted a consistent decrease in blood glucose levels in $SCOT^{MuscleKO}$ mice during KE administration **(Figure 2.3B)**, which may be attributed to a mild but nonsignificant increase in insulin levels at 15 min post-KE

administration **(Figure 2.3C)**. This decrease in circulating glucose levels remained immediately before administering intraperitoneal glucose for the assessment of glucose tolerance **(Figure 2.3D)**. Similar to what we observed in obese male C57BL/6J mice, KE administration improved glucose tolerance in obese SCOT^{MuscleKO} mice **(Figure 2.3E&F)**, which was once more associated with a marginal but nonsignificant increase in insulin levels at the start of the glucose tolerance test **(Figure 2.3G)**.

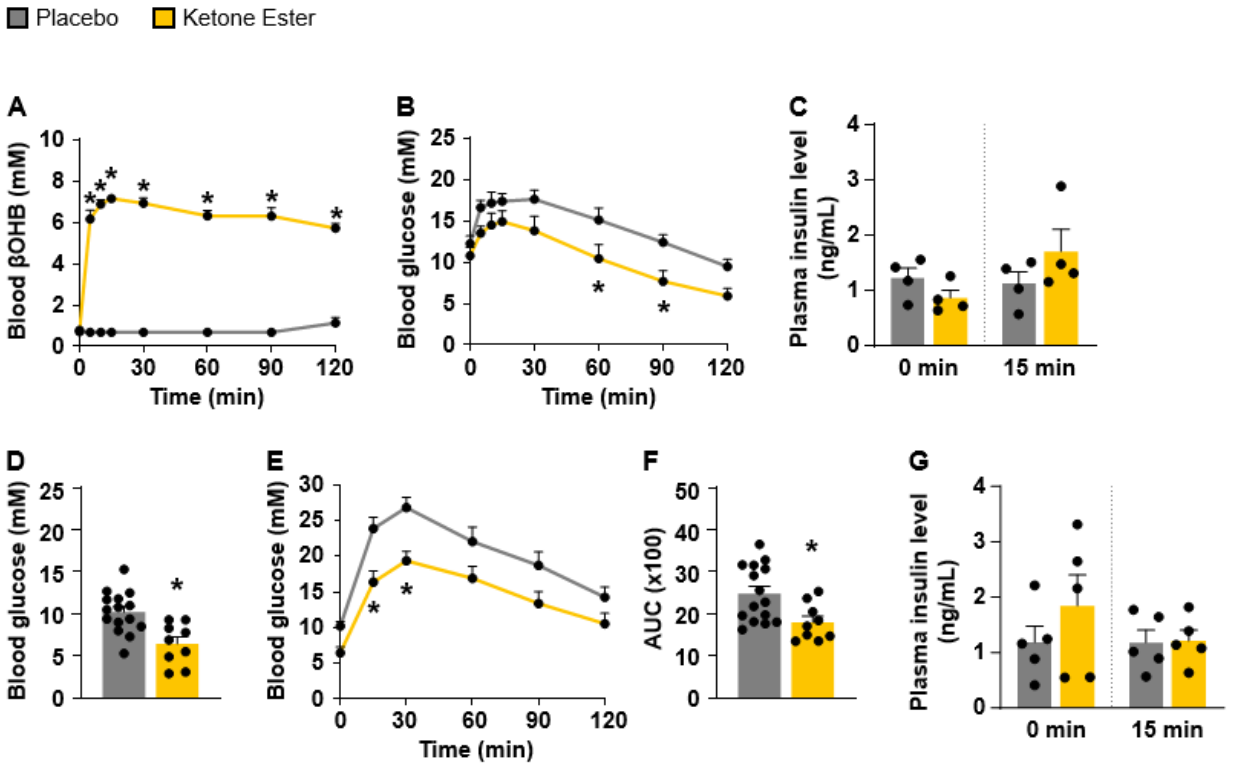

Figure 2.3 *Ketone oxidation in muscle is not required for the glucose lowering actions in response to exogenous KE administration in obese male mice.*

Circulating βOHB levels (n = 7–10; A), circulating glucose levels (n = 7–10; B), and corresponding insulin levels (n = 4; C) in obese SCOT^{MuscleKO} mice fed a 45% HFD treated with either placebo or KE. Starting glucose levels before glucose tolerance (D), glucose tolerance (performed 2 h following oral gavage; E), the associated AUC (n = 7–15; F), and corresponding insulin levels (n = 5 or 6; G). Values represent means ± SE. Differences were determined using either an unpaired

Student's t test or a repeated measures two-way ANOVA, followed by Bonferroni post hoc analysis. *P < 0.05 vs. placebo-treated counterparts. AUC, area under the curve; βOHB, β-hydroxybutyrate; HFD, high-fat diet; KE, ketone ester.

2.4.4 The R-Isomer of βOHB Demonstrates Increases in Insulin Secretion Versus the L-Isomer in Perifused Islets of Obese Mice

Although insulin levels were not significantly elevated by KE treatment in any of our previous studies, the values we observed were consistently higher with KE administration. We posited a more sensitive and dynamic approach to measuring insulin may prove to be more informative to evaluate the relationship between elevations in ketones and insulin secretion. As such, we assessed the impact of increased ketones on insulin secretion via islet perifusion experiments. Islets were isolated from standard chow-fed lean and HFD-fed obese male C57BL/6J mice. Direct treatment of perifused islets with the KE produced no effect on insulin secretion **(Figure 2.4A-C)**. consistent with the KE not being hydrolyzed into βOHB in the absence of gut carboxylesterase activity. In contrast, islets from lean and obese mice increased insulin release in response to 10 mM R- and S-βOHB, but the R-isomer provoked a greater insulin response that was statistically significant only in obese mice **(Figure 2.4D-G)**.

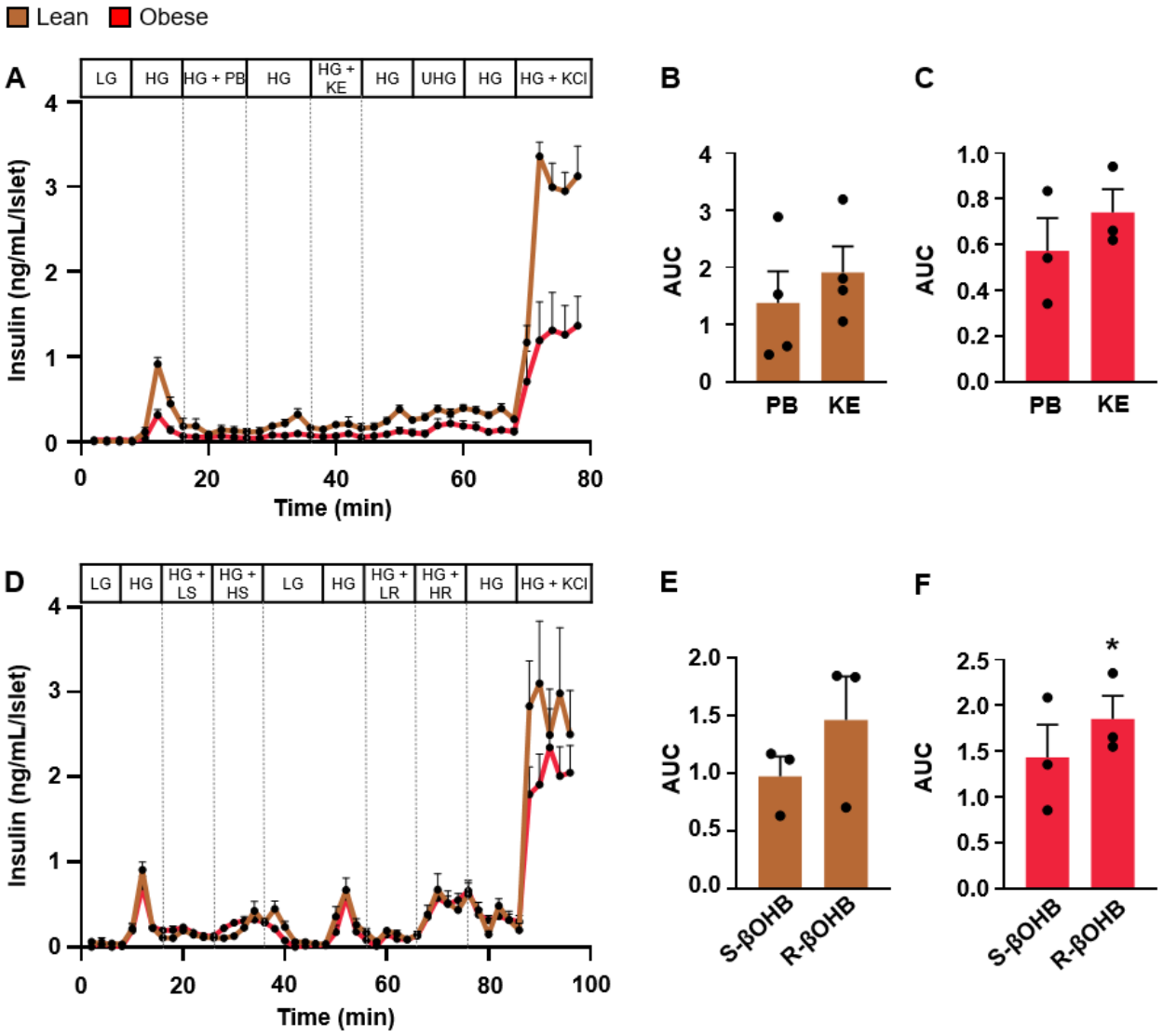

Figure 2.4 *R- and S-isomers of βOHB stimulate insulin secretion in perifused islets of obese mice.*

Insulin levels following treatment of perifused islets from lean and obese C57BL/6J mice with placebo or KE (n = 3 or 4; A), and the associated AUCs from the lean (B) or obese (C) mice. Insulin levels following treatment of perifused islets from lean and obese C57BL/6J mice with R-βOHB or S-βOHB or KE (n = 3; D), and the associated AUCs from the lean (E) or obese (F) mice. Values represent means ± SE. Differences were determined using an unpaired Student's t test. *P < 0.05. AUC, area under the curve; βOHB, β-hydroxybutyrate; KE, ketone ester.

2.5 Discussion

In this study, we observed that acute increases in circulating ketone levels improved glucose tolerance in obese but not lean mice. Our findings presented evidence against the hypothesis that elevation of circulating ketones would provide excess substrate and subsequent enhancement of ketone oxidation in skeletal muscle, which in turn could potentially worsen hyperglycemia. Nonetheless, these findings do not contradict that inhibiting SCOT activity to decrease skeletal muscle ketone oxidation may be a promising strategy to improve glucose homeostasis, as previously demonstrated [44]. In fact, our results suggest that muscle ketone oxidation is dispensable for the observed phenotypes, since obese $SCOT^{MuscleKO}$ mice that are incapable of oxidizing ketones in their muscle exhibited comparable glucose-lowering effects following KE administration.

Because circulating insulin levels were consistently higher with KE administration despite not being significant, we performed dynamic islet perifusion studies to more accurately assess the impact of ketones on insulin secretion. R-βOHB produced mild but significant increases in insulin secretion when compared with S-βOHB in the islets of obese male mice, though a trend to an increase was also observed from islets of lean male mice. Since only R-βOHB can be oxidized by βOHB dehydrogenase in the mitochondria to generate ATP, our results suggest that the glucose-lowering effects we observed after acute elevations of circulating ketones may be partly attributed to ketone oxidation within islet β-cells. Importantly, several studies have been conducted to explore the potential influence of ketones on insulin and glucagon secretion [156, 157, 165, 166, 197, 198]. In one study, the effects of βOHB and AcAc on insulin and glucagon secretion were explored using a perfused rat pancreas model, whereby both βOHB and AcAc exhibited a dose-dependent effect

on stimulating insulin and glucagon secretion (largest responses observed at 10 mM). Consistent with our findings, it has been observed that ketones at physiological levels (below 1.0 mM) do not stimulate insulin secretion [199]. Ketones could thus serve a protective role during diabetic ketoacidosis and limit their self-inflicted harm by stimulating insulin secretion, which would decrease peripheral lipolysis and further increases in hepatic ketogenesis. In another study, the infusion of βOHB or AcAc in alloxan-induced diabetic dogs decreased blood glucose levels with a concurrent increase in insulin secretion. Conversely, no such effects were observed in dogs that had undergone pancreatectomy, indicating the essential role of the pancreas (and likely its associated hormones) in mediating this phenotype [157].

Our observations are in agreement with several previous studies demonstrating that ketones have a direct and stimulatory effect on the activity of islet β cells. However, it remains unknown how increases in circulating ketones promote islet β-cell insulin secretion, though islet ketone oxidation may be involved since perifusion insulin levels were greater following treatment with R-βOHB versus S-βOHB. Prior studies have shown that ketones can increase the sensitivity of the ATP-sensitive potassium (K_{ATP}) channel. The augmented K_{ATP} channel sensitivity triggered by ketone oxidation generated ATP may result in amplified effects on membrane depolarization of the β cell, leading to an influx of calcium ions and eventual insulin secretion. It has been also shown that activation of the G protein-coupled receptor 41, which can be stimulated by ketones or short-chain fatty acids such as propionate, results in increases in insulin secretion [200]. Similarly, ketones have been demonstrated to serve as activating ligands for the G-protein coupled receptor 43 [201], and agonism of this receptor has also been demonstrated to enhance insulin secretion in isolated islets [202].

In general, the intricate molecular and cellular mechanisms underlying the insulin secretory effects of ketones remain largely unexplored and are likely to involve a multitude of complex and interconnected pathways. It will be important for future studies to explore how ketone oxidation specifically drives insulin secretion through characterization of islet-specific mouse models incapable of oxidizing ketones. Nonetheless, it is evident that the exogenous administration of ketones also exerts acute beneficial effects on glycemic control, and it will be important to determine whether such observations persist with chronic administration. It is also unclear why KE administration only improved glucose tolerance in obese mice, but it is possible that since lean mice already have well-controlled glucose homeostasis, their glucose tolerance could not be improved further.

It should be noted that KE administration produced a more rapid glucose-lowering in obese mice fed 60% kcal HFD versus those fed a 45% kcal HFD. It remains unknown what accounts for these differences, and if ketones have contrasting actions on islets from obese mice fed the two different HFDs, as our islet perifusion studies were performed in obese mice fed a 45% kcal HFD. It is also noteworthy that the immediate glucose-lowering actions post-KE administration appeared to be strongest in the obese $SCOT^{MuscleKO}$ mice. Reasons for this are unclear, but the rise in circulating ketones was more prolonged in these animals where values were >6 mM throughout the entire 2-h period. It will be of interest to determine whether there are specific thresholds regarding circulating ketone levels and the downstream actions on glucose homeostasis.

A key limitation of our study is that our experiments were performed solely in male mice, and thus it will be important to determine whether our observations translate to females. Preliminary studies in our laboratory suggest that acute increases in circulating ketones, secondary to oral KE

administration, also improve glucose tolerance in obese but not lean female C57BL/6J mice **(Figure 2.5)**. Another limitation of our studies is that we did not perform oral glucose tolerance tests, which produce more potent actions on insulin secretion than intraperitoneal glucose tolerance tests due to stimulating incretin hormone (i.e., glucagon-like peptide-1) secretion. Our preliminary evidence suggests that KE administration does not improve oral glucose tolerance **(Figure 2.6)**, though future studies will need to probe into these discrepancies and assess whether the glucose-lowering actions of KEs are negligible in the presence of incretins.

Taken together, our study adds to the growing body of evidence demonstrating the multifaceted role of ketone metabolism in the regulation of whole-body glucose homeostasis. Although adherence to a ketogenic dietary pattern has also been proposed to improve glycemia in obesity [203, 204], the low-carbohydrate nature of ketogenic diets is associated with decreases in circulating insulin. Moreover, adherence to oral consumption of commercially available KE beverages would likely be more feasible than ketogenic dieting over the long term.

2.6 Grants

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2.7 Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

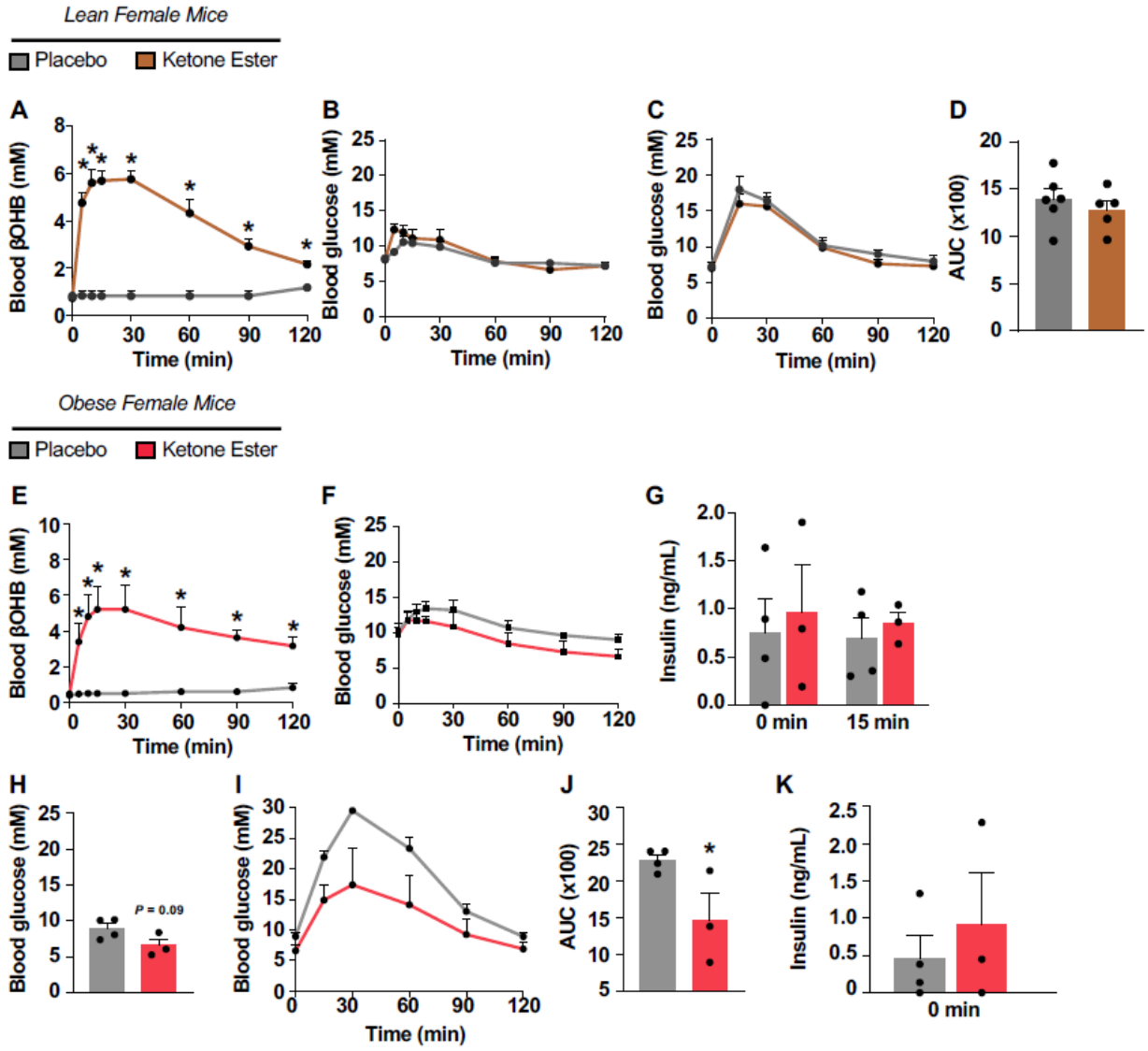

Figure 2.5 *Exogenous KE Administration in Female Mice Recapitulates the Observations in Male Mice.*

(A) Circulating βOHB levels, (B) circulating glucose levels, (C) glucose tolerance (performed 2 h following oral gavage), and (D) the associated AUC of the glucose tolerance test in female C57BL/6J mice fed a standard chow diet and treated with either placebo or KE (n=5-6). (E)

Circulating βOHB levels, (F) circulating glucose levels, and (G) corresponding insulin levels in female C57BL/6J mice fed a 45% high-fat diet and treated with either placebo or KE (n=3-4). (H) Starting glucose levels prior to glucose tolerance, (I) glucose tolerance (performed 2 h following oral gavage), (J) the associated AUC of the glucose tolerance test, and (F) corresponding insulin levels (n=3-4). Values are mean ± SEM. Differences were determined using either an unpaired Student's t test or a repeated-measures two-way ANOVA, followed by Bonferroni post-hoc analysis. *P < 0.05 vs. placebo-treated counterparts. AUC – area under the curve.

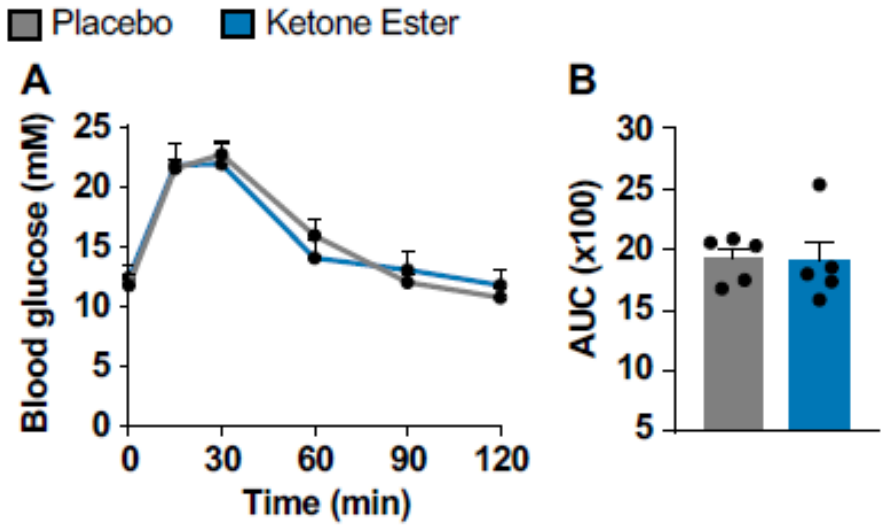

Figure 2.6 *Exogenous KE Administration Does Not Improve Oral Glucose Tolerance in Male Mice.*

(A) glucose tolerance (performed 2 h following oral gavage), and (B) the associated AUC of the glucose tolerance test in male C57BL/6J mice fed a 60% high-fat diet for 3-weeks and treated with either placebo or KE (n=5). Values are mean ± SEM. Differences were determined using either an unpaired Student's t test or a repeated measures two-way ANOVA, followed by Bonferroni post-hoc analysis. AUC – area under the curve.

Chapter 3: Generation and Characterization of SCOT/*Oxct1* Transgenic Mouse (Ongoing work)

3.1 Abstract

Emerging evidence underscores the potential role of skeletal muscle ketone oxidation in mediating glycemic responses and insulin resistance associated with obesity and Type 2 Diabetes T2D. To delve deeper into these observations, a transgenic mouse model overexpressing SCOT was developed by transfecting mouse embryos with an adenoviral vector bearing the myosin heavy chain kinase 8 (MHCK8) promoter - exclusive to muscle fibers - and the mouse *Oxct1*. When crossed with a Cre-expressing mouse, the loxP-flanked stop codon preceding mOxct1 is excised, leading to heightened SCOT protein expression. Semi-quantitative RT PCR data indicates that tamoxifen-induced HSA^{Cre} effectively cleaved the loxP-flanked stop codon. However, no significant upregulation of SCOT was found at mRNA levels and western blotting. Challenges with the SCOT transgenic model might stem from the random vector insertion, making homozygote determination through conventional PCR challenging. There's also the possibility that homozygous animals are yet to be generated or that a single transgene copy doesn't suffice to discern differences at mRNA and protein stages. Addressing the genotype ambiguity, strategies like whole genome sequencing could be employed to pinpoint transgene transfection sites, facilitating specific genotyping protocols. Alternatively, breeding between a wildtype (WT) mouse and offspring from heterozygous (HET) parents might help in discerning the presence of homozygous transgenes, based on the resultant pup genotypes.

3.2 Introduction

Previous studies have established that there's an increase in the mRNA/protein expression and enzyme activity of SCOT in the skeletal muscles of HFD-induced obese male mice [44]. While SCOT an essential enzyme responsible for ketone body oxidation, treatment of SCOT inhibitors improved glycemia in obese male mice [44, 45]. Likewise, when SCOT was removed from the skeletal muscles of mice, there was a significant improvement in blood sugar levels and glucose tolerance during experimental obesity or T2D [44]. In humans, a study comparing lean and age-matched obese women found that the rectus abdominus muscle samples from obese participants exhibited reduced βOHB oxidation rates [205], indicating abnormalities in muscle ketone metabolism related to obesity. There's increasing evidence suggests that changes in skeletal muscle ketone oxidation might play a role in exacerbating glycemia and potentially the insulin resistance seen in obesity and T2D; thus, creating a transgenic mouse that overexpresses SCOT could provide valuable insights into its effects on the *in vivo* glycemic profile.

The term "vector" refers to a piece of DNA containing foreign DNA that can replicate within an organism, facilitating the transfer or propagation of the inserted DNA. Since viruses are efficient in delivering their genome into cells, viral genomes as foreign DNA vectors are utilized in generating transgenic animals [206]. An adenovirus carrying a vector **(Figure 3.1)** with the myosin heavy chain kinase 8 (MHCK8) promoter, followed by a loxP-flanked stop codon and the mouse *Oxct1*, is utilized for transfecting mouse embryos. MHCK8 is exclusively expressed in muscle fibers, including skeletal muscles and cardiac muscles [207]. Successful vector transfer is selected by ampicillin, and the adenoviruses carrying the vector insert their genomes at random locations. The transfected embryos are then placed back to female mice for further breeding of transgenic

mice, which is done at the Texas A&M Institute for Genomic Medicine. The Cre-loxP system is a widely used technology for manipulating gene expression in a cell-specific manner [208]. This method allows researchers to selectively activate, inactivate, or modify genes in particular cell types, providing a powerful tool for studying gene function and creating animal models for various diseases. When the transgenic mouse is crossed with another mouse carrying Cre, Cre can excise the DNA at loxP sites, removing the stop codon in between. Without the stop codon, m*Oxct1* can be transcribed and expressed as additional SCOT protein. Further genotyping and metabolic analysis are done to confirm the existence of the transgene, the occurrence of the excision, and the corresponding phenotypes.

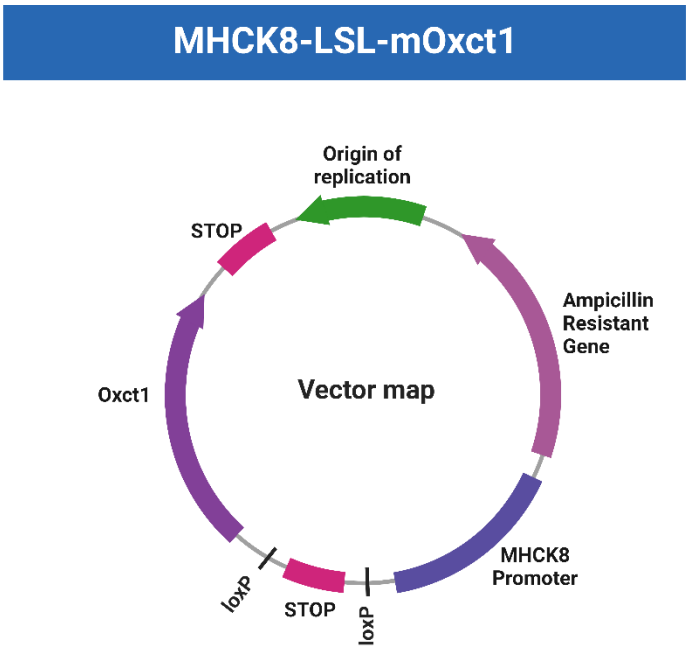

Figure 3.1 *Vector map of MHCK8-LSL-mOxct1 for adenoviral transfection.*

The vector map is provided by the Texas A&M Institute for Genomic Medicine. Created with BioRender.com.

3.3 Methods

3.3.1 Animal Care

All animal procedures were approved by the University of Alberta Health Sciences Animal Welfare Committee and all animals received care in accordance with the guidelines approved by the Canadian Council on Animal Care. Animals were housed at 22°C (temperature-controlled) in a 12 h light and 12 h dark cycle (lights on from 6 am to 6 pm), with all mice receiving standard environmental enrichment (chip bedding, a plastic tube, 8 ounces of crinkle paper, and Nestlet square bedding comprised of short fiber cotton) and ad libitum access to food (chow diet) and water. The mouse carrying the transgene was generated by Texas A&M Institute for Genomic Medicine and transferred to the University of Alberta. Then, the transgenic mouse is crossed with mouse with a tamoxifen-inducible human α-skeletal actin-Cre (HSA^{Cre}) which is specifically expressed in the skeletal muscle [209] to generate pups expressing both the transgene and HSA^{Cre} **(Figure 3.2)**. 6-week-old mice received daily IP injections of tamoxifen (100mg/kg) suspended in corn oil for 5 consecutive days and followed by a one-week washout post-tamoxifen. At washout completion, mice were fasted 2-h prior to euthanization. Mice were euthanized with an IP injection of sodium pentobarbital (12 mg), following which tissues were extracted and immediately snap-frozen in liquid N_2 using liquid N_2-cooled Wollenberger tongs. At the same time, the transgenic mouse, originally created in the C57BL/6N background, is backcrossed with the C57BL/6J strain, which is commonly used in our studies, allowing for consistent research conditions.

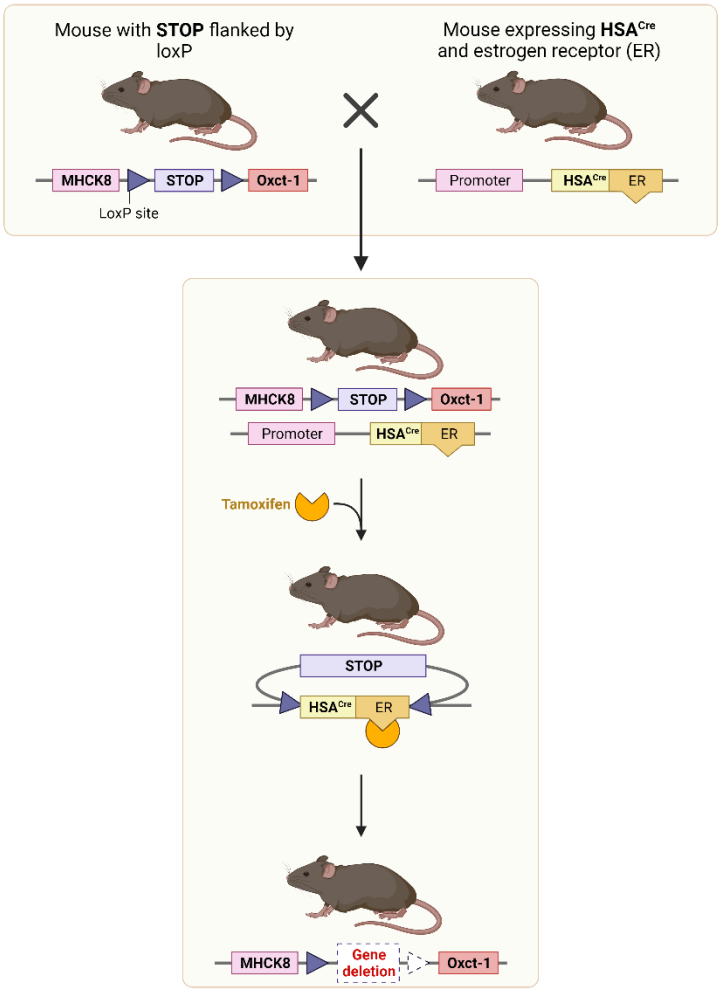

Figure 3.2 *Generation of transgenic mice carrying a tamoxifen inducible HSA^{Cre}.*

The transgenic mouse is crossed with another mouse carrying tamoxifen inducible HSA^{Cre} to produce the transgenic mice carrying tamoxifen inducible HSA^{Cre}. Once tamoxifen is injected and binds to estrogen receptor (ER), Cre is activated to cut at loxP sites, removing the stop codon in between. Created with BioRender.com.

3.3.2 Genotyping with Polymerase Chain Reaction (PCR)

Mouse tails were used to extract DNA for genotyping. After collected, tails were incubated in the tail lysis buffer at 55°C, preferably overnight. Next, equivalent volume of the phenol-chloroform mixture combining 20 mL of phenol, 20 mL of chloroform, and 800 µL of isoamyl alcohol was added and shaken vigorously. After centrifuging, transfer the top aqueous layer into a new tube with double the volume of absolute ethanol. After spinning, carefully decant the supernatant, wash the resultant DNA pellet with 70% ethanol, and resuspend the pellet in 10 mM Tris. Extracted DNA from mouse tails is combined with *Oxct1* primers and Taq (AdvanTech) to perform polymerase chain reaction (PCR) with the annealing temperature at 63°C. The products of PCR can identify the presence of the *Oxct1* transgene at 208 base pair (bp) in the Midori-green stained 2.5% agarose gel, with an internal control band at 324 bp to determine the quality of the DNA. The detection of Cre has a slightly different protocol, with an annealing temperature at 63°C, Cre at 100 bp with a control band at 324 bp.

3.3.3 Semi-quantitative Reverse Transcription PCR (RT PCR)

Semi-quantitative RT PCR involves the generation of complementary DNA (cDNA) from RNA and a conventional PCR through the Midori-green stained 2.5% agarose gel. Total RNA was isolated from gastrocnemius muscles (20 mg) using TRIzol reagent (15596018; Thermo Fisher Scientific). In brief, samples were homogenized in TRIzol, following which chloroform was added to the mixture and centrifuged at 14,000 x g for 10 minutes at 4°C. Isopropanol was added to the resulting aqueous layer then centrifuged at 16,000 x g for 10 minutes at 4°C. The pellet was washed with 1 mL of 75% ethanol to remove any remaining impurities and left to air-dry for 30 minutes,

then subsequently resuspended in 30 µL of RNAase-free water. RNA concentration was quantified using a NanoDrop 2000 spectrophotometer. cDNA was synthesized from total RNA with the aid of the SuperScript III synthesis system (Invitrogen). Lastly, PCR was run with the primer MHCK8-m*Oxct1* which detects the excision of the loxP-flanked stop codon.

3.3.4 Real-Time Quantitative PCR (qPCR)

The real-time PCR was executed using the CFX connect Real-time PCR machine (Bio-Rad Laboratories Inc.) with SYBR Green (Kapa Biosystems). The quantification of relative mRNA transcription levels was accomplished using the $2^{-\Delta\Delta Ct}$ method, as detailed by Livak and Schmittgen [210]. Peptidylprolyl isomerase A (*Ppia*) served as the housekeeping gene for normalization.

3.3.4 Western Blotting

Gastrocnemius muscle tissues (20 mg) were homogenized in lysis buffer containing 50 mM Tris HCl (pH 8 at 4°C), 1 mM Ethylenediaminetetraacetic acid (EDTA), 10% glycerol (w/v) and 0.02% Brij-35 (w/v), 1 mM dithiothreitol (DTT), 0.5 mM of sodium orthovanadate (Na_3VO_4), 2.5 mM of sodium fluoride (NaF) and protease and phosphatase inhibitors (Sigma Aldrich). The protein samples were quantified using the Bradford Assay and subjected to western blotting protocols. OXCT1 (12175-1-AP; Proteintech) and HSP90 (610418; BD Transduction Laboratories) antibodies were prepared in a 1:1000 dilution in 3% bovine serum albumin (BSA). Anti-mouse (7076S; Cell Signaling Technology) or anti-rabbit (7074S; Cell Signaling Technology) secondary

antibodies were prepared in a 1:2000 dilution in 5% milk. Afterwards, the membranes were incubated with secondary antibodies for 1.5 hours and fluoresced using Clarity Western Enhanced Chemiluminescence Substrate (Bio-Rad). Western blot images were processed using the ChemiDoc imaging systems (Bio-Rad) and densitometry was performed using Image Lab software (Bio-Rad).

3.4 Results

3.4.1 Random Insertion of Transgene Limits the Genotyping Results

The DNA was extracted from the tails of the transgenic mice to determine their genotypes. The PCR was used to determine the genotypes of transgenic mice using the *Oxct-1* and Cre primers. However, it is unable to determine the homozygosity of the transgene due to the nature of random insertion, the resulting genotypes from the PCR are wildtype (WT: without transgene) and heterozygous (HET: with transgene). In the PCR result with *Oxct-1* transgene as the primer, the higher band at 324 bp is the quality control band while the lower band at 208bp is *Oxct-1* **(Figure 3.3A)**. Therefore, the lane with two bands is HET indicating the presence of transgene, while the lane with one band is WT. On the other hand, in the PCR result with Cre as the primer, the higher band at 324 bp is the quality control band while the lower band at 100 bp is Cre **(Figure 3.3B)**. The plus (+: with Cre) and minus (-: without Cre) are used to indicate the presence of HSA^{Cre}. Therefore, the lane with two bands has Cre (+), and the lane with one band does not have Cre (-). The complete genotypes are determined by combining the PCR results of *Oxct-1* and Cre.

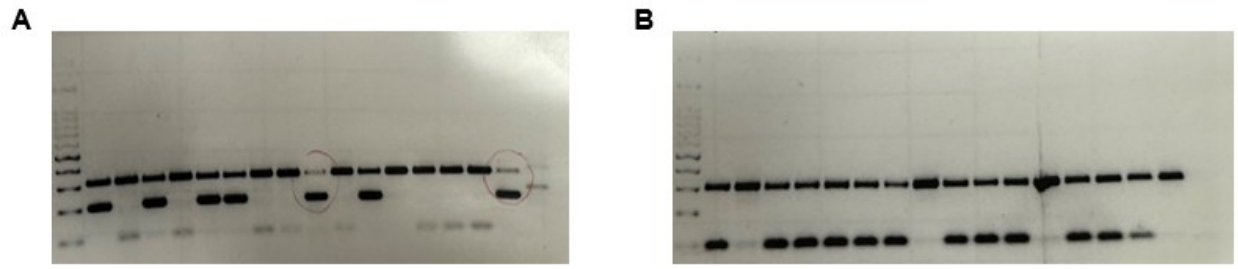

Figure 3.3 *Random Insertion of Transgene Limits the Genotyping Results to WT and HET.*

The PCR results with *Oxct-1* transgene primer (A) display the higher band as the quality control band (324 bp) while the lower band as *Oxct-1* (208bp). The PCR results with Cre primer (B) display the higher band as the quality control band (324 bp) while the lower band as Cre (100 bp).

3.4.2 Semi-quantitative RT PCR Confirms Excision of Stop Codon with No Difference at mRNA and Protein Levels

The gastrocnemius muscle from the same cohort of transgenic mice were used for all the following analysis. The semi-quantitative RT PCR analysis with the *MHCK8-mOxct1* primer reveals stronger signals associated with the HET+ transgenic mice **(Figure 3.4A)**. The lanes containing samples from left to right: DNA electrophoresis ladder, HET+, HET+, HET+, HET+, WT+, WT+, WT+, negative control (without cDNA samples). The lighter signals from WT+ indicates the presence of unspecific bands. The qPCR employing the *Oxct1* primer exhibits almost up to a 2-fold change with no significance at the mRNA level when comparing samples from HET+ to ones from WT+ **(Figure 3.4B)**. The western blotting displays similar results of SCOT with no significant difference found between samples from WT+ and ones from HET+ **(Figure 3.4C&D)**.

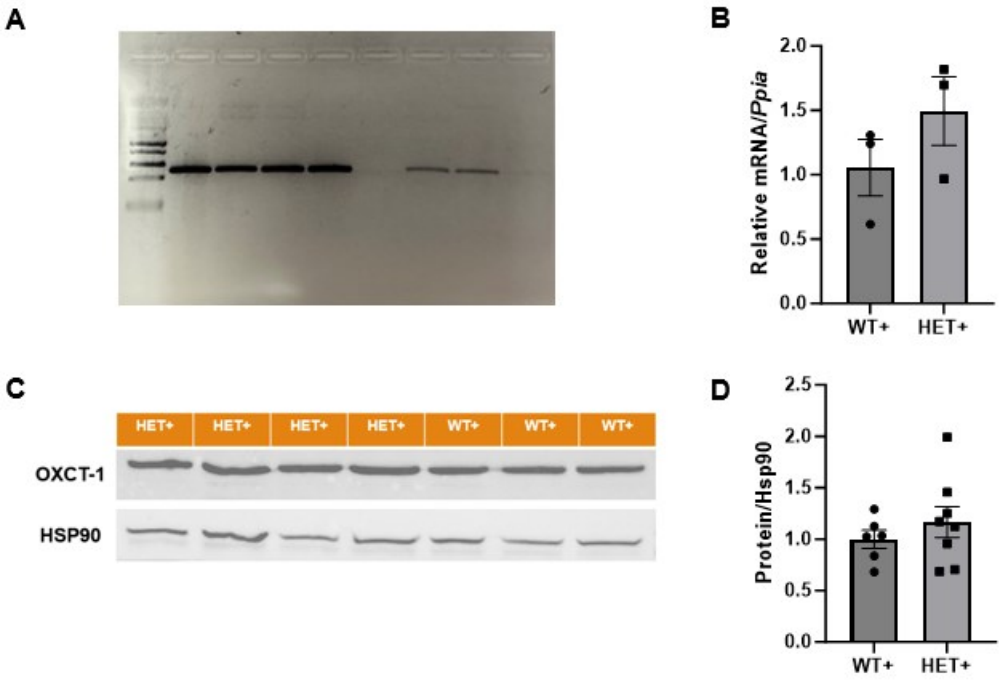

Figure 3.4 *Excision of Stop Codon in Transgenic Mice Shows No Difference at mRNA and Protein Levels.*

Imaging results of semi-quantitative RT PCR (A) regarding *MHCK8-mOxct1* in gastrocnemius muscles from WT+ and HET+ mice. Relative mRNA levels (B) of *Oxct1* in gastrocnemius muscles from WT+ and HET+ mice (n=3). Immunoblot (C) and its quantification (D) demonstrating OXCT1 level in gastrocnemius muscles from WT+ and HET+ mice (n= 6-8). Values represent means ± SE. Differences were determined using either an unpaired Student's t test. Figure 3.4D quantification is performed by Bio-rad Image Lab. *Ppia*, peptidylprolyl isomerase A. Hsp90, heat shock protein 90.

3.5 Discussion

Although this is still a work in progress, there are some conclusions that could be drawn from the current SCOT transgenic mouse models. Based on the results of semi-quantitative RT PCR, tamoxifen-induced HSA^{Cre} has successfully cleaved the loxP-flanked stop codon as the signals from the HET+ were stronger than the ones from WT+. Despite the observation, the elevation of SCOT at mRNA levels is not significant when comparing HET+ with WT+, and the results from the western blotting reveal no substantial difference in the SCOT protein levels between HET+ and WT+. Several possibilities could contribute to the issues of the current SCOT transgenic model. First, as the insertion of the vector is random, it is difficult to determine the homozygotes by genotyping through conventional PCR. Therefore, it is possible that the phenotypes of homozygote are not associated with the accurate genotype. Also, it is also possible that homozygous animals have not been generated yet, and one copy of transgene is not potent enough to show difference at mRNA and protein levels. In skeletal muscles, research indicates that two copies of a similar promoter, MHCK7, are required to exhibit approximately a 2-fold difference in transcriptional activity, and MHCK8 necessitates three copies to achieve around a 4-fold difference in transcriptional activity [207].

One way to determine the presence of homozygous transgene is through whole genome sequencing. By applying this technique, the transfection sites of the transgene will be discovered to develop site-specific genotyping protocols. The other way is using the WT mouse to set up a breeding pair with the pups with unconfirmed genotypes coming from two HET parents. If all the pups coming from this new breeding pair are HET, the unconfirmed genotype in the breeding pair would be a homozygote.

Chapter 4: General Discussion

Our experimental results demonstrated that acute KE administration improved the glucose tolerance in obese mice while the lean littermates remained unaffected. The improved glycemia is independent of the ketone body utilization in skeletal muscles, while the role of insulin appears to be minimal, as suggested by a transient surge in insulin both *in vivo* and *ex vivo*. D-βOHB (R-βOHB) rather than L-βOHB (S-βOHB) perifusion induced a more robust insulin secretion in isolated islets from obese mice, indicating that insulin stimulation is more likely to originate from ketone body oxidation instead of ketone body signaling pathways.

Our theory posits that the elevation of plasma ketones stimulates the β-cells to secrete a small amount of insulin, which primarily targets the liver. This results in decreased glucose output and increased FFA uptake at the liver [211], without influencing peripheral glucose usage. Previous research indicates that exogenous ketone infusion in dogs elevates pancreatic-venous insulin levels [149] while also causing hypoglycemia and a reduction in FFA [157, 170]. Ketone bodies can also enhance the K_{ATP} channel's sensitivity to ATP levels [212], potentially intensifying membrane depolarization and increased Ca^{2+} influx in β-cells, prompting enhanced insulin secretion driven by elevated ATP from ketone body oxidation **(Figure 4.1)**. Insulin secretion may as well be influenced by the signaling properties of ketones. βOHB signaling through G-protein coupled receptor 40 (GPR40) releases a positive influence on ketone body production within β-cells and results in insulin secretion [213]. Similar effects can be achieved through binding the G-protein coupled receptor 43 [201].

Insulin secretion is posited to be initiated by acute exposure to exogenous ketones when circulating levels exceed 2 mM [170]; notably, our study observed a peak at 8 mM, suggesting potential insulin

secretion. However, it is hard to detect insulin fluctuations in the peripheral blood when the insulin secretion is transient [163]. The peak insulin levels are observed 5 min into continuous infusion and 2.5 min following an acute dose, then fall gradually back to the baseline [170] probably due to the feedback inhibition of hypoglycemia. The suppressive effect of insulin on hepatic glucose output could be overcome by the stimulating effect of peripheral hypoglycemia [214, 215]. In addition, peripheral insulin levels may not provide an accurate representation of insulin secretion patterns of β-cells. The maximal insulin values might have been missed in our tail-blood samples since they were collected 15 minutes after KE administration. It's plausible that minor quantities of secreted insulin could be filtered out by the liver from the portal circulation, reducing hepatic glucose release without showing in peripheral blood measurements. Nonetheless, we noticed a slight, non-significant rise in insulin levels in the 45% HFD-induced obese models, but no such increase was evident in the 60% HFD-induced obese models. This observation downplays the role of insulin in our *in vivo* findings, and it is plausible that the increase in circulating ketones from KE consumption might ameliorate the glycemia in obese models through other mechanisms.

As previously reported, acute injections of exogenous ketones can result in a decrease in blood FFA levels [162]. In addition to insulin's inhibitory effect on lipolysis, ketone bodies independently impede lipase activation by suppressing cAMP production [52, 172] and inhibit lipolysis through the activation of GPR109A [144]. The FFA availability can also be decreased by an upregulation of FAO from KE ingestion [154]. The reduction in circulating FFA can potentially enhance insulin sensitivity, as seen when fat injection in normal rabbits led to almost complete insulin insensitivity [51]; therefore, KE might act as a restorative agent in counteracting the elevated FFA levels and associated insulin resistance observed in obesity. In obese mice, the GTT could potentially trigger

an improved insulin response compared to their lean counterparts where the insulin sensitivity remains unaffected.

The lowering in circulating glucose levels may result from the combined effects of insulin and ketone bodies at the hepatic level to reduce glucose output. Obesity is often accompanied by β-cell hyperresponsiveness to numerous secretagogues which could lead to insulin hypersecretion when stimulated [163]. A potential contributing factor is the increased cAMP levels seen in obesity, as activation of cAMP-dependent PKA can enhance Ca^{2+} channel function, leading to accelerated insulin exocytosis [216]. In obese mice, the observed hyperinsulinemia indicates persistently elevated insulin secretion. The reduction in plasma FFA concentration and improved insulin sensitivity can lead to a diminished gluconeogenesis and a subsequent decrease in hepatic glucose production [217]. Recent discovery indicates that KE drink can lower the availability of L-alanine for gluconeogenesis [218], underscoring the potential role of ketone bodies in inducing hypoglycemia and enhancing protein conservation. Should exogenous ketones have a similar effect on the liver as in adipose tissue, decreased cAMP levels might directly suppress the phosphorylase activity that drives glycogenolysis [219]. The effect is manifested by the lower glucose levels prior to GTT in the KE-treated obese group compared to the placebo-treated group.

Additionally, obesity is associated with chronic, low-grade systemic inflammation, marked by elevated levels of pro-inflammatory cytokines such as TNF-α and IL-6, primarily from adipose tissue, which can disrupt insulin signaling and potentially result in insulin resistance [220]. βOHB through binding the GPR109A exhibits anti-inflammatory effects in obesity [106]. These effects involve inhibiting the release of pro-inflammatory cytokines like IL-6 and TNF-α [94]. Considering the substantial release of D-βOHB following acute KE consumption, it's conceivable

that the anti-inflammatory properties of D-βOHB could decrease the levels of circulating cytokines in obese mice, subsequently bolstering the insulin signaling pathway.

In our 60% HFD-induced diabetic mouse models treated with STZ, the absence of glycemia improvement likely indicates a reliance on unimpaired β-cells in mice, as STZ is known to induce β-cell death (**Appendix 1**). The glucose-lowering effect of exogenous ketones has been observed in diabetic patients who have a compromised insulin secretory capacity [221], pointing to the presence of alternative mechanisms in humans for lowering glucose levels other than the effect of insulin. The glucose-lowering effect of exogenous ketones may be contingent on the induced ketone levels, as sodium D-βOHB intake at a dosage equivalent to KE only raised circulating ketone levels to around 2 mM, unlike the 8 mM achieved with KE, resulting in no improvement in glycemia of the obese mice (**Appendix 2**). Plus, minor elevation of ketones levels from sodium D, L-βOHB infusion has been shown not to affect insulin sensitivity in humans [222].

The conclusions from **chapter 2** are different from our expectation, which could stem from several aspects. First and foremost, the improvement of glucose intolerance in diet-induced obesity observed in previous studies can be attributed to augmented peripheral glycolysis that compensates for the energy deficit resulting from reduced ketone body oxidation because of the loss of SCOT function. In contrast, the enhanced glucose tolerance seen in this study may originate from the exogenous KE-induced hypoglycemia that is potentiated by obesity as well as the positive influence of the ketone bodies on paving the roads for insulin actions upon glucose challenges. Despite the variations in nutritional states from prior research, acute KE administration invariably has a positive effect on glycemia in fasted conditions, as demonstrated by improved oral GTT responses in fasted humans with impaired glucose tolerance relative to a placebo group [223]. Also,

the acute elevation of circulating ketones is more effective in inducing insulin secretion compared to long-term treatment [163, 164], which could probably explain why the ketogenic diet fails to enhance glycemia in obese mice [157]. Despite tamoxifen induced HSA^{Cre} successfully activated the stop codon excision in **Chapter 3**, no significant difference was observed in mRNA and protein levels of SCOT/*Oxct1* between HET+ and WT+. The challenges with determining the homozygosity of the transgene due to random vector insertion and the potential insufficiency of a single transgene copy to show differences contribute to the issues of the model.

Recent research has noted an elevation in plasma insulin after the oral intake of exogenous ketones, a response not seen with intravenous infusion, even though both methods resulted in comparable increases in blood β-OHB concentrations and decreases in blood glucose [224]. These observations imply that the method of administration and/or hormones derived from the gut might contribute to shaping the response to exogenous ketones. This strongly indicates the likelihood of the existence of additional mechanisms, independent of insulin, that mediate the glucoregulatory effects of ketones.

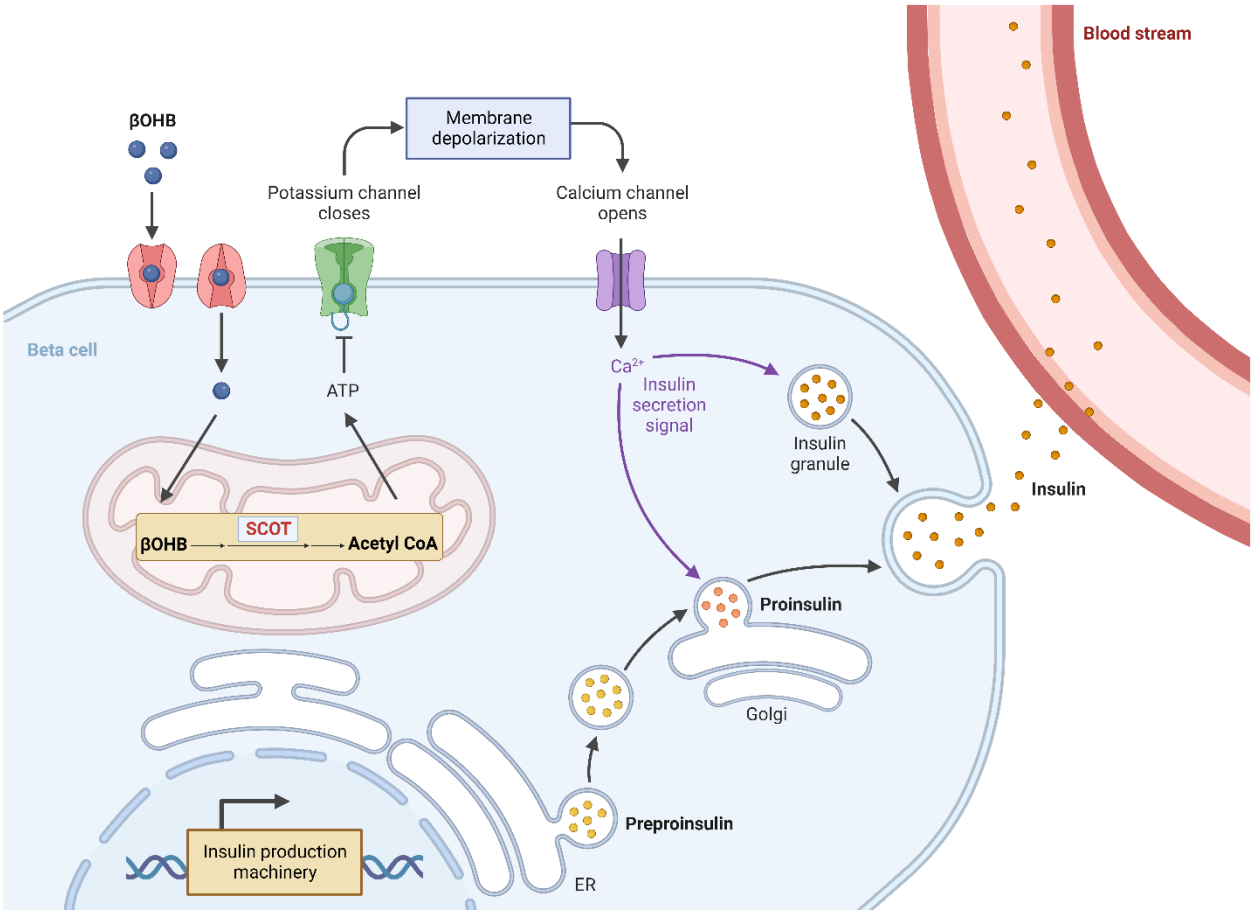

Figure 4.1 *Proposed pathway of insulin secretion induced by ketone body oxidation.*

The proposed model suggests that ketones are transported into the islets' beta cells for oxidation. This oxidation produces ATP, which then prompts the closure of the ATP-sensitive potassium channel, causing membrane depolarization. Subsequently, calcium channels open, allowing calcium ions to enter and offset the extramembrane potassium. This influx of calcium also triggers the release of insulin through exocytosis. Created with BioRender.com.

Chapter 5: Future Directions

While our study provides valuable insights into the role of ketone body metabolism in regulating obesity-induced glucose intolerance, several questions remain unanswered. First, the generation of pancreas-specific SCOT knockout models stands as a crucial next step. This approach aims to elucidate if the release of insulin can be directly attributed to ATP produced from pancreatic ketone oxidation. Coupled with this, it's vital to explore other potential signaling pathways that might facilitate Ca^{2+} release in the pancreas, deepening our grasp of the intricate processes that govern insulin secretion. Although the exact interplay between ketone body signaling and insulin signaling pathways remains ambiguous, it is imperative to explore if the anti-inflammatory properties of ketone bodies contribute to enhanced glucose tolerance in obese mice. Nevertheless, molecular studies can be undertaken to investigate the roles of insulin in the liver and peripheral tissues following the ingestion of KE.

While the acute effects of KE are becoming clearer, a thorough investigation into the long-term ramifications of ketone ester administration remains paramount. Injecting exogenous ketones to alloxan-diabetic rats subcutaneously twice daily for a three-day period notably enhanced both glucose and fat metabolism. This improvement might be attributed to the effects of insulin, which boosted glycogen storage, as well as accelerated glycolysis and lipogenesis processes [162]. There is also a recent free-living single-arm pilot study that reported enhanced glycemic control in individuals with T2D who consumed a KE three times daily for 28 days [225]. This study would pave the way for assessing the broader implications of ketone esters, especially in the realms of metabolic health and potential therapeutic applications in T2D management.

To finish where I left off in **Chapter 3**, a comprehensive characterization of SCOT transgenic mice would provide insights into the physiological shifts caused by chronic upregulation of muscular ketone body oxidation, offering a comparative metabolic profile against wild-type controls. The priority is to determine the location of transgene insertion through whole-body genotyping, as it would facilitate the development of site-based primers for homozygote identification and associated phenotypes. The chronic elevation of ketone body oxidation might disrupt the energy homeostasis by affecting glycolysis and FAO which could lead to metabolic disorders in obese models. Moreover, it is plausible that there may be no discernible difference in the basal level of SCOT, particularly as the euthanization of the mice was performed without inducing a fasting state. To fully maximize SCOT expression in transgenic mice, it may be beneficial to euthanize the mice under fasting conditions or after administering exogenous KE, since the SCOT activity is upregulated by the concentrations of circulating ketones. Overexpression of SCOT *in vitro* also disrupts aminotransferase activity of amino acids [226]. It would also be interesting to see the effect of SCOT expression on the muscle function as muscles of trained athletes exhibit an elevated capacity for the uptake and oxidation of ketone bodies [153].

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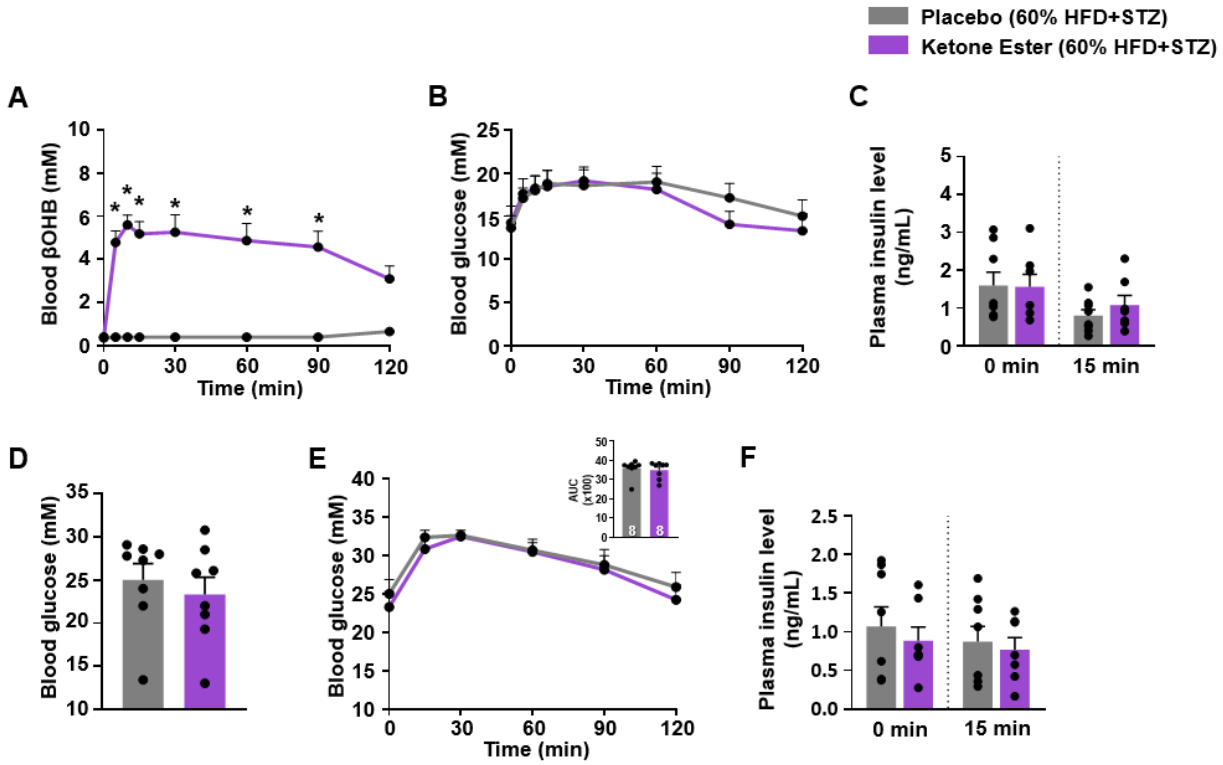
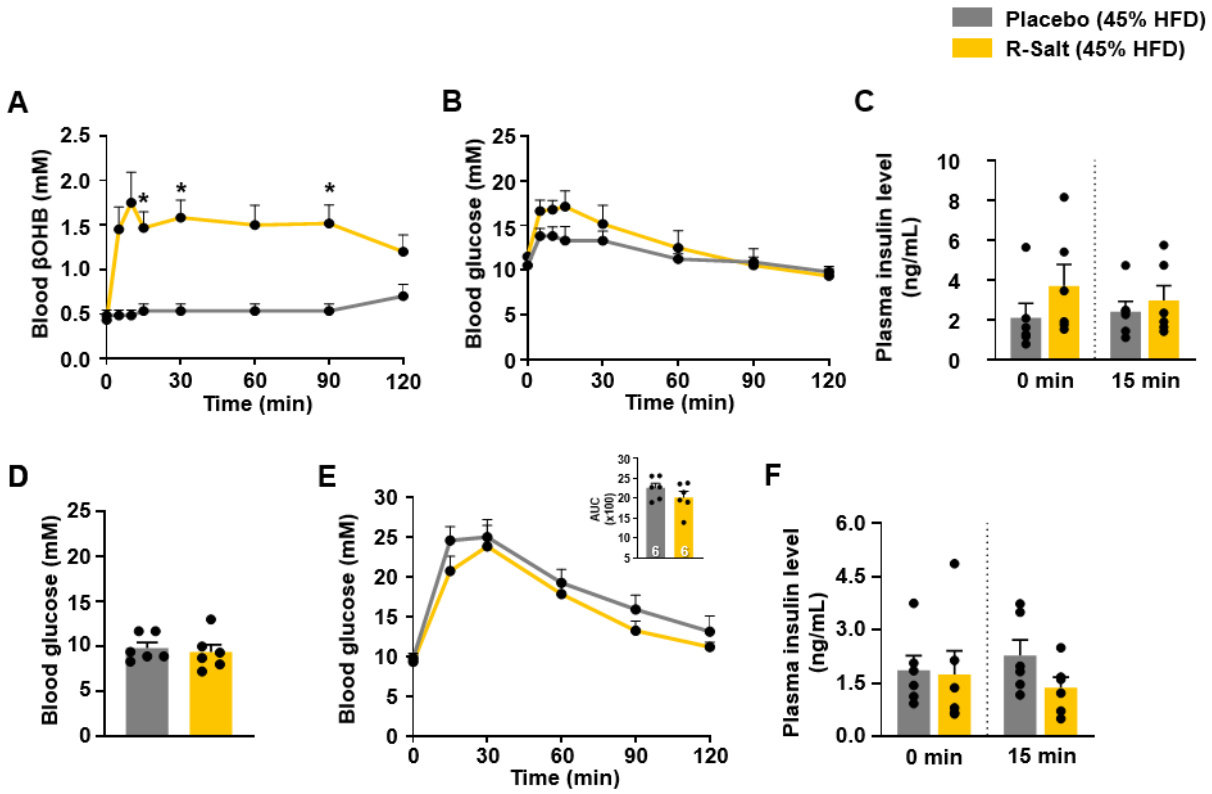
Appendices



Appendix 1 *Streptozotocin (STZ) injected C57BL6/J Mice on 60% High-fat Diet Treated with Ketone Ester*.

(A-C) Circulating βOHB levels (A), circulating glucose levels (B) (n=8) and corresponding plasma insulin levels (C) (n=7-8) in Streptozotocin (STZ) injected C57BL/6J mice on 60% high-fat diet that are treated with either placebo or ketone ester through oral gavage. (D-F) Starting glucose levels prior to glucose tolerance (D), glucose tolerance (performed 2hr following the gavage) with the calculated AUC (E) (n=8), and corresponding plasma insulin levels (F) (n=7-8). Values represent means ± SEM.



Appendix 2 *C57BL6/J Mice on 45% High-fat Diet Treated with (R)-3-Hydroxybutanoic Acid (R-Salt)*.

(A-C) Circulating βOHB levels (A), circulating glucose levels (B) and corresponding plasma insulin levels (C) in C57BL/6J mice on 45% high-fat diet that are treated with either placebo or (R)-3-Hydroxybutanoic acid through oral gavage (n=6). (D-F) Starting glucose levels prior to glucose tolerance (D), glucose tolerance (performed 2hr following the gavage) with the calculated AUC (E) and corresponding plasma insulin levels (F) (n=6). Values represent means ± SEM.