Regulating Adipocyte Oxidative Stress: The Effects of Exogenous Ketones on Human Adipocytes in a Hypoxic Environment

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Thesis Abstract

Background: Ketone bodies, particularly β-hydroxybutyrate (βOHB), have been proposed to exert antioxidant and cytoprotective effects by modulating mitochondrial function and antioxidant gene expression. In adipocytes, redox homeostasis is tightly regulated and strongly influenced by oxygen availability. Hypoxia, a condition of low oxygen pressure in the cell environment, has been associated with increased reactive oxygen species (ROS) production and impaired antioxidant defenses. However, findings remain inconsistent, particularly in human adipocyte models. The potential for exogenous ketones to mitigate hypoxia-induced oxidative stress in human adipocytes remains poorly characterized. Accordingly, the overall objective was to determine the impact of exogenous ketones on adipocyte redox homeostasis under varying oxygen conditions, with a focus on ROS production and expression of key antioxidant genes. We hypothesized that hypoxia would increase ROS and suppress antioxidant gene expression in a dose-dependent manner in human differentiated adipocytes, whereas exogenous βOHB would dose-dependently reduce ROS and increase antioxidant gene expression, thereby attenuating hypoxia-induced oxidative stress.

Methods: Human subcutaneous preadipocytes were differentiated into mature adipocytes and exposed for 48 hours to varying oxygen concentrations (21%, 10% and 3%) followed by a 24-hour treatment with increasing concentrations of exogenous βOHB (0 to 10 mM). Intracellular ROS levels were assessed using a DCFDA assay, while lipid accumulation was measured by Oil Red O staining. Gene expression of key antioxidants (catalase, FOXO3, Mt2, SOD1 and SOD2) was quantified by rt-qPCR. Statistical analyses were performed using linear mixed-effects models or ANOVA, where appropriate.

Results: Hypoxic exposure did not significantly increase intracellular ROS levels in differentiated human adipocytes ($p=0.307$), regardless of glucose or βOHB concentration. Despite the absence of elevated ROS, hypoxia induced selective, oxygen-dependent changes in antioxidant expression, with FOXO3 ($p<.001$) and Mt2 ($p<.001$) significantly upregulated under severe hypoxia (3% oxygen). In contrast, catalase ($p<.001$) and SOD1 ($p=0.033$) expression decreased under hypoxic conditions, while SOD2 expression remained unchanged ($p=0.409$). Exogenous βOHB did not significantly modulate ROS levels ($p=0.361$) or antioxidant gene expression (p values between 0.547 and 0.944 for all genes) under normoxic or hypoxic conditions.

Conclusion: These findings indicate that hypoxia elicits adaptive transcriptional responses in human adipocytes without inducing sustained oxidative stress. Contrary to our initial hypothesis, exogenous βOHB did not attenuate hypoxia-associated redox responses under the conditions tested. Together, the results suggest that human adipocytes may possess robust mechanisms to maintain redox homeostasis under reduced oxygen availability, and that ketone-mediated antioxidant effects are highly context-dependent.

Contribution to advancement of knowledge: This thesis provides evidence that challenges the assumption that hypoxia universally induces oxidative stress in adipocytes, and that exogenous ketones act as broad antioxidant modulators. By demonstrating selective redox adaptation in the absence of increased ROS, our work refines current models of adipocyte hypoxia and highlights the importance of temporal dynamics, cellular context, and experimental design when evaluating ketone-mediated redox effects.

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Preface

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List of Abbreviations

Acetyl-CoA	acetyl coenzyme A
Acoc	acetoacetate
AGE	advanced glycation end product
βOHB	beta-hydroxybutyrate
C/EBPα	CCAAT/enhancer binding protein α
CPT1	carnitine palmitoyltransferase 1
DCFDA	2',7'-dichlorodihydrofluorescein diacetate
ERK	extracellular signal-regulated kinase
ETC	electron transport chain
FOXO3	forkhead box O3
HDAC	histone deacetylase
HIF-1α	hypoxia-inducible factor-1 alpha
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2
mtROS	mitochondrial reactive oxygen species
Mt2	metallothionein 2
NAC	n-acetyl cysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOX	NADPH oxidases
PI3K/Akt	phosphatidylinositol 3-kinase / protein kinase B pathway
PPARγ	peroxisome proliferator-activated receptor gamma
Qo	outer quinone binding site
ROS	reactive oxygen species
SCOT	succinyl-CoA:3-ketoacid CoA transferase
SOD1	superoxide dismutase 1
SOD2	superoxide dismutase 2
WAT	white adipose tissue

List of Definitions

(in alphabetical order)

Adipose tissue: A metabolically active tissue involved in energy storage, endocrine signaling, and metabolic regulation, whose function is influenced by redox balance and oxygen availability.

Antioxidants: Enzymatic and non-enzymatic systems that neutralize reactive oxygen species (ROS) to maintain cellular redox homeostasis.

Electron transport chain (ETC): A mitochondrial protein complex system responsible for ATP production that can generate reactive oxygen species (ROS) when electron transfer is disrupted.

Hypoxia: A condition of reduced oxygen availability.

Ketogenesis: The mitochondrial process by which acetyl-CoA derived from fatty acid β-oxidation is converted into ketone bodies, primarily in hepatocytes, and regulated by the rate-limiting enzyme HMGCS2 (Puchalska & Crawford, 2021; Asif et al., 2022).

Ketone bodies: Water-soluble metabolites, mainly beta-hydroxybutyrate (βOHB) and acetoacetate (Acoc), that serve as alternative energy substrates and act as signaling molecules influencing redox homeostasis (Puchalska & Crawford, 2021; Shimazu et al., 2013).

Lipogenesis: The synthesis and storage of fatty acids as triglycerides within adipocytes.

Lipolysis: The breakdown of triglycerides in adipocytes, releasing fatty acids that serve as substrates for hepatic β-oxidation and ketone body production (Rui, 2014).

Oxidative stress: A state in which reactive oxygen species (ROS) production exceeds antioxidant capacity, resulting in mitochondrial dysfunction and metabolic impairment (Castro et al., 2016; Kietzmann et al., 2017).

Reactive oxygen species (ROS): Highly reactive oxygen-derived molecules generated primarily during mitochondrial respiration that act as signaling mediators at low levels and promote cellular damage when excessive (Tormos et al., 2011; Guzy & Schumacker, 2006).

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no-insulin, NI; normoxia with insulin (1μM), HN; hypoxia (3% oxygen) no-insulin, HI; hypoxia with insulin (1μM).

Chapter 1: Introduction

Ketone bodies, primarily β-hydroxybutyrate (βOHB) and acetoacetate (Acoc), are water-soluble metabolites produced mainly by hepatocytes and used as alternative energy substrates during periods of prolonged fasting, pregnancy, high-intensity exercise, and low-carbohydrate intake (Puchalska & Crawford, 2021). Ketogenesis occurs predominantly in hepatic mitochondria, whereas ketone utilization occurs in extrahepatic tissues throughout the body (Dhillon & Gupta, 2023; Edson & Leloir, 1936). Hepatic ketone production is enabled by the presence of 3-hydroxy3-methylglutaryl-CoA synthase 2 (HMGCS2), the rate-limiting enzyme of ketogenesis (Asif et al., 2022). The substrate supply for ketogenesis is driven largely by adipose lipolysis: fatty acids released from white adipose tissue are transported to the liver, imported into mitochondria via carnitine palmitoyltransferase 1 (CPT1), and converted through β-oxidation into acetyl coenzyme A (acetyl-CoA), which is subsequently transformed into ketone bodies through a series of enzymatic reactions (Li et al., 2021; Lopes-Cardozo et al., 1975; Malewiak et al., 1983; Mooli & Ramakrishnan, 2022; Rui, 2014).

Beyond their role as fuels, ketones have been increasingly recognized as signaling molecules capable of influencing cellular metabolism and gene expression (Newman & Verdin, 2014; Puchalska & Crawford, 2017). In adipocyte models, exogenous ketone exposure has been associated with changes in metabolic gene programs and redox-related outcomes, including increased expression of antioxidant genes and reductions in intracellular reactive oxygen species (ROS) (Nishitani et al., 2022). However, interpreting ketone effects in adipose biology requires careful attention to the experimental model, as murine 3T3-L1 adipocytes differ from human adipocytes in mitochondrial abundance, metabolic flexibility, and oxidative characteristics (Cedikova et al., 2016; Sadie-Van Gijsen, 2019). Importantly, species-specific differences in ROS handling and the relative contribution of enzymatic versus mitochondrial ROS (mtROS) sources may influence the extent to which ketone-driven redox effects observed in murine systems generalize to human adipocytes (Jornayvaz et al., 2024; Newton et al., 2011).

A key rationale for investigating ketones in my thesis is their proposed antioxidant and cytoprotective activity. Mechanistically, βOHB has been shown to inhibit class I histone deacetylases, thereby increasing transcription of antioxidant genes implicated in cellular redox defense (e.g., forkhead box O3 (FOXO3), catalase, and superoxide dismutases) (Shimazu et al.,

2013). In parallel, βOHB may modulate mitochondrial physiology in ways that reduce ROS generation, including effects on mitochondrial membrane potential and electron leak (Brookes, 2005; Jones IV et al., 2016). These converging mechanisms suggest that ketones could support redox homeostasis under metabolic stress (Glorieux et al., 2015; Puchalska & Crawford, 2017).

Redox homeostasis is particularly relevant in adipocytes because ROS have a dual, context-dependent role in adipose biology. Controlled ROS production is required for normal adipocyte differentiation and signaling, including mtROS derived from complex III that participate in adipogenic transcriptional programs (Tormos et al., 2011). In contrast, excessive ROS production disrupts mitochondrial function, impairs insulin signaling, disrupts lipid homeostasis, and contributes to adipocyte dysfunction (Castro et al., 2016; Kietzmann et al., 2017). The oxidative state of adipocytes is also influenced by multiple environmental and metabolic factors, such as glucose availability, antioxidant capacity, and oxygen tension, which together determine whether ROS act as signaling mediators or damaging agents (Bhatti et al., 2022; Ighodaro & Akinloye, 2018).

Among these determinants, oxygen availability is particularly important. Hypoxia can occur in expanding adipose tissue as adipocyte hypertrophy outpaces vascularization, and it is frequently linked to elevated oxidative stress (Engin, 2024). At the cellular level, hypoxia may increase ROS through impaired mitochondrial electron transport and enhanced electron leak, particularly at complex III, while also engaging hypoxia-responsive signaling pathways that alter redox regulation (Engin, 2024; Guzy & Schumacker, 2006). Hypoxia has also been associated with reduced antioxidant capacity through suppression of antioxidant gene expression and related defenses, thereby amplifying oxidative stress (Gou et al., 2022; Kim et al., 2014). Despite broad agreement that hypoxia perturbs adipocyte redox balance, the magnitude and sources of hypoxia-induced ROS remain debated, emphasizing the need for controlled studies that evaluate ROS and antioxidant gene responses under graded oxygen conditions (Engin, 2024; Guzy & Schumacker, 2006).

Taken together, current evidence underscores the need to explore whether exogenous ketones modulate hypoxia-induced oxidative stress pathways in human adipocytes. Accordingly, the main research question addressed in my thesis is whether exogenous βOHB modulates ROS levels and antioxidant gene expression in human adipocytes exposed to hypoxia. The overall

objective is to determine the impact of exogenous ketones on adipocyte redox homeostasis under varying oxygen conditions, with a focus on ROS production and expression of key antioxidant genes (FOXO3, catalase, metallothionein 2 (Mt2), superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2)). Based on the mechanisms and gaps identified in the literature, the central hypothesis is that hypoxia will increase ROS and suppress antioxidant gene expression in a dose-dependent manner in human differentiated adipocytes, whereas exogenous βOHB will dose-dependently reduce ROS and increase antioxidant gene expression, thereby attenuating hypoxia-induced oxidative stress in human adipocytes.

Chapter 2: Literature Review

Ketone bodies

Ketone bodies, or ketones, are water-soluble molecules mainly produced by hepatocytes in the liver and serve as an alternative energy source, particularly for organs such as the brain and muscles (Puchalska & Crawford, 2021). Notably, they provide an essential source of energy during periods of prolonged fasting, pregnancy, high-intensity exercise, and low-carbohydrate diets. (Puchalska & Crawford, 2021). The synthesis of ketones, known as ketogenesis, occurs mainly in hepatocyte mitochondria, whereas the degradation of ketone bodies occurs in extrahepatic cells throughout the body (Dhillon & Gupta, 2024; Edson & Leloir, 1936).

Ketone body production

The production of ketone bodies is primarily driven by the breakdown of lipid droplets in white adipose tissue (WAT), leading to the accumulation of fatty acids that enter the bloodstream (Rui, 2014). These fatty acids circulate to hepatocytes, where they enter the mitochondrial matrix using the CPT1 transporter (Li et al., 2021). Within the mitochondrial matrix, fatty acid βoxidation-derived acetyl-CoA is used as the main substrate for ketogenesis (Lopes-Cardozo et al., 1975; Mooli & Ramakrishnan, 2022). Acetyl-CoA is subsequently transformed through a series of enzymatic reactions into two main ketone bodies: βOHB and Acoc (Malewiak et al., 1983). It is the presence of HMGCS2, the rate-limiting enzyme of ketogenesis, that enables ketone body production by hepatocytes (Asif et al., 2022). Once produced, ketones are sent into the bloodstream and are mainly channelled for terminal oxidation through the action of the Succinyl-CoA:3ketoacid CoA transferase (SCOT) enzyme in extrahepatic tissues (Puchalska & Crawford, 2021) (Figure 1).

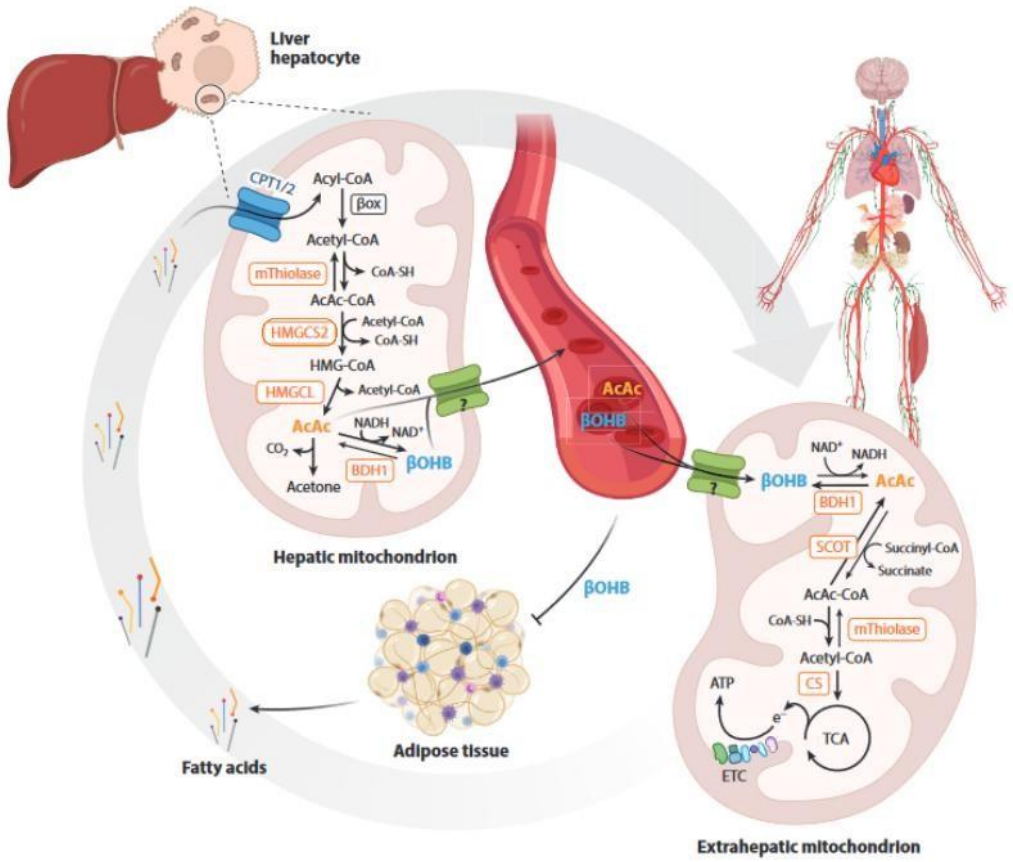

Figure 1. Overview of ketone metabolism in the body. Ketone body production begins with lipolysis in white adipose tissue, releasing fatty acids that are transported to the liver and imported into hepatocyte mitochondria via CPT1. Mitochondrial β-oxidation generates acetyl-CoA, which is converted into the ketone bodies βOHB and acetoacetate (AcAc) through HMGCS2-dependent ketogenesis. Once synthesized, ketones are released into circulation and utilized by extrahepatic tissues through SCOT-mediated oxidation. AcAc, acetoacetate; ATP, adenosine triphosphate; BDH1, D-β-hydroxybutyrate dehydrogenase 1; βOHB, β-hydroxybutyrate; βox, β-oxidation; CoA, coenzyme A; CoA-SH, CoA sodium salt hydrate; CPT, carnitine palmitoyltransferase; e−, electron; CS, citrate synthase; ETC, electron transport chain; HMGCL, 3-hydroxymethylglutaryl-CoA lyase; HMGCS2, 3hydroxymethylglutaryl-CoA synthase 2; mThiolase, mitochondrial thiolase; SCOT, succinyl-CoA:3-oxoacid-CoA transferase; TCA, tricarboxylic acid. Adapted from Puchalska & Crawford, 2021.

Historically, hepatocytes were believed to be the exclusive site of physiologically relevant ketone production (Williamson & Whitelaw, 1978). However, emerging evidence challenges this assumption. Nishitani et al. (2022) reported that differentiated 3T3-L1 adipocytes express HMGCS2 and secrete βOHB, suggesting that adipocytes may possess a limited ketogenic capacity. In their study, HMGCS2 expression increased markedly following adipocyte differentiation, accompanied by elevated βOHB concentrations in both medium and lysates. These findings introduced the possibility that adipocytes contribute modestly to local ketone signalling and redox regulation, even if their contribution to systemic ketogenesis remains negligible.

Mechanistic considerations support the idea that adipocyte ketogenesis, if present, is likely modest. Adipocytes contain relatively few mitochondria compared to hepatocytes, limiting their β-oxidation capacity and thus their ability to generate the acetyl-CoA required for ketogenesis (Cedikova et al., 2016). Furthermore, ketogenesis in hepatocytes is tightly regulated by hormonal cues such as insulin, which suppresses fatty acid flux and ketone production (Alberti et al., 1978). Whether the same regulatory mechanisms operate in adipocytes remains unclear, and exploratory work from our laboratory suggests that insulin does not significantly alter βOHB accumulation in human adipocyte cultures (see Appendix I – Exploratory Findings).

Although the physiological significance of adipocyte-derived ketones is not well established, their potential signalling role is noteworthy. Extrahepatic ketogenesis has been documented in other specialized tissues, for example, retinal pigment epithelial cells produce βOHB to support local mitochondrial function (Adijanto et al., 2014). If adipocytes similarly generate small quantities of βOHB, these ketones may act in an autocrine or paracrine fashion to influence redox homeostasis, mitochondrial metabolism, or epigenetic regulation of gene expression.

Based on the work of Nishitani et al. (2022) on 3T3-L1 cells, we conducted an exploratory experiment (see Appendix I – Exploratory Findings) to assess and validate their claim that adipocytes are capable of ketogenesis using human differentiated preadipocytes. We also aimed to determine whether this process is influenced by oxygen availability, given that hepatocyte ketogenesis is modulated by hypoxia (Behari et al., 2014). In brief, our findings show that human adipocytes did undergo ketogenesis, but modulation of oxygen partial pressure or insulin concentration did not impact the production of ketone bodies by human adipocytes. Furthermore, HMGCS2, the key rate-limiting enzyme of ketogenesis, was not highly expressed when compared to other related genes such as SCOT, as opposed to what is observed by Nishitani et al., 2022. The differences observed between our findings and that of Nishitani and colleagues (2022) might be explained by the cellular model used (3T3-L1 vs. human adipocytes).

Physiological and Metabolic Differences Between 3T3-L1 and Human Adipocytes

Much of the literature examining adipocyte ketogenesis, oxidative stress, and metabolic signalling derives from studies using 3T3-L1 murine adipocytes. These cells are convenient, highly

proliferative, and differentiate efficiently; however, important physiological differences limit their generalizability to human adipocytes (Sadie-Van Gijsen, 2019). A nuanced understanding of these differences is essential when interpreting findings related to ketone biology, ROS generation, and hypoxia.

One of the most significant distinctions lies in mitochondrial abundance and metabolic flexibility. Murine 3T3-L1 adipocytes are more metabolically active and exhibit greater reliance on oxidative metabolism compared to human adipocytes, which tend to adopt a more glycolytic phenotype (Cedikova et al., 2016; Sadie-Van Gijsen, 2019). This disparity implies that pathways requiring robust mitochondrial function, such as β-oxidation, ketogenesis, and ROS buffering, may be more active or detectable in 3T3-L1 cells than in their human counterparts (Sadie-Van Gijsen, 2019).

Another difference concerns ketolytic capacity. Murine adipocytes dynamically modulate expression of the ketolytic enzyme SCOT, which prevents futile cycling of ketones and facilitates ketone oxidation (Mei et al., 2021; Puchalska & Crawford, 2021). In contrast, SCOT expression in human adipocytes appears less flexible, suggesting that βOHB may function more as a signalling molecule than an oxidative substrate in human cells (Clemons et al., 2024, Jones IV et al., 2016).

Species-specific differences in ROS metabolism also complicate comparisons. 3T3-L1 cells generate substantial mtROS during differentiation and metabolic stress, whereas human adipocytes rely more heavily on nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) enzymes for ROS production and exhibit lower mtROS overall (Newton et al., 2011; Jornayvaz et al., 2024). These differences may partially explain why βOHB robustly modulates ROS in murine cells (Nishitani et al., 2022), whereas the effects in human adipocytes remain poorly defined.

For these reasons, I chose to investigate ketone effects specifically in primary human adipocytes, allowing for more physiologically relevant conclusions. Understanding how exogenous βOHB modulates hypoxia-induced ROS production in human adipocytes requires moving beyond murine models, which may overestimate or misrepresent the antioxidant potential of ketones due to their more oxidative metabolic profile.

Ketone Antioxidant Roles

While ketone bodies have long been recognized as alternative energy substrates, a substantial body of evidence now demonstrates that βOHB also exerts direct antioxidant and cytoprotective effects (Newman & Verdin, 2014; Puchalska & Crawford, 2017). This literature provides the mechanistic foundation for my hypothesis that exogenous ketones can attenuate hypoxia-induced ROS in human adipocytes.

One of the most influential discoveries was made by Shimazu et al. (2013), who identified βOHB as an endogenous class I histone deacetylase (HDAC) inhibitor. HDAC inhibition leads to chromatin relaxation and increased transcription of antioxidant genes, including FOXO3, SOD2, catalase, and Mt2 (Shimazu et al., 2013). This mechanism operates at physiologically relevant βOHB concentrations (1-3 mM), meaning that modest elevations in ketone levels, such as those induced by fasting or exogenous supplementation, may meaningfully enhance antioxidant gene expression (Shimazu et al., 2013).

Ketone bodies also regulate oxidative stress through mitochondrial mechanisms. Jones IV et al. (2016) demonstrated that βOHB increases mitochondrial oxygen consumption and proton leak, both of which reduce mitochondrial membrane potential. Lower membrane potential diminishes electron backflow at complexes I and III, thereby reducing mtROS generation (Brookes, 2005). These findings support a model in which βOHB buffers oxidative stress by improving mitochondrial efficiency and reducing electron leakage.

Additional evidence comes from studies showing that βOHB improves mitochondrial membrane potential stability and reduces oxidative injury in various cell types, including adipose-derived stem cells (Gan et al., 2026). Likewise, Nishitani et al. (2022) found that βOHB supplementation upregulated multiple antioxidant genes and decreased intracellular ROS in adipocytes, although their interpretation was confounded by simultaneous using very low glucose concentrations (2.5mM) in their culture media.

Together, these findings suggest that βOHB exerts antioxidant effects through multiple complementary pathways, including epigenetic regulation, mitochondrial modulation, and enhancement of intrinsic antioxidant defenses (Glorieux et al., 2015). These mechanistic insights

strongly support the central objective of my thesis: to determine whether exogenous ketones can counteract the oxidative stress imposed by hypoxia in human adipocytes.

Normal ROS Levels and Adipocyte Function

ROS play a dual and context-dependent role in adipocyte biology. During adipogenesis, controlled ROS production functions as an essential signalling mechanism (Jones IV et al., 2016). Tormos et al. (2011) demonstrated that complex III–derived mtROS facilitate adipocyte differentiation by activating transcriptional pathways required for lipid accumulation and mitochondrial biogenesis. These findings emphasize that physiological levels of ROS are necessary for normal cellular development.

However, in mature adipocytes, ROS must be tightly regulated to maintain metabolic homeostasis (Castro et al., 2016). Excess ROS disrupts insulin receptor signalling, impairs βoxidation, damages mitochondrial DNA, and promotes lipid peroxidation (Castro et al., 2016; Kietzmann et al., 2017). Antioxidant enzymes such as SOD1, SOD2, catalase, and glutathione peroxidase play crucial roles in buffering these radicals and maintaining redox equilibrium (Jones IV et al., 2016). Dysregulation of this balance contributes to adipocyte hypertrophy, chronic inflammation, and metabolic dysfunction (Castro et al., 2016).

Given that hypoxia increases ROS levels (Kim et al., 2014), restoring balance through enhanced antioxidant gene expression is essential for protecting adipocyte function. This understanding provides a conceptual link between oxidative biology and ketone metabolism. If βOHB can increase transcription of antioxidant genes, as demonstrated in several models (Shimazu et al., 2013; Nishitani et al., 2022), then exogenous ketones may serve as a metabolic tool to restore redox balance in hypoxic adipocytes.

Factors Affecting the Oxidative State of Adipocytes

Multiple metabolic and environmental inputs influence ROS production and antioxidant capacity in adipocytes. Understanding these regulatory factors is essential for contextualizing hypoxia-induced oxidative stress and predicting how ketone supplementation may counteract it.

Insulin

Insulin influences oxidative balance through its effects on glucose uptake, lipogenesis, and mitochondrial activity (Alberti et al., 1978). Chronic hyperinsulinemia increases lipogenic flux and mitochondrial substrate load, thereby elevating ROS production in adipose tissue (Engin, 2024). Conversely, insulin resistance in adipose tissue is associated with impaired mitochondrial function and increased oxidative stress through inflammatory signalling pathways (Engin, 2024). The lack of an insulin effect on endogenous ketone production in my exploratory results (see Appendix I) suggests that human adipocytes may regulate ketogenesis differently from hepatocytes, supporting the need to study exogenous rather than endogenous ketones in this context.

Glucose

Glucose availability strongly modulates ROS generation. High glucose elevates mitochondrial electron flux and increases electron leakage, while hyperglycemia contributes to oxidative stress through advanced glycation end product (AGE) formation in multiple cell types, including adipocytes (Bhatti et al., 2022). Conversely, low glucose conditions may reduce ROS formation, complicating interpretation of ketone-supplementation studies in which glucose levels differ between experiments, such as in Nishitani et al. (2022). However, other studies have stipulated an alternative hypothesis, by showing that the production of ROS was greater in a low glucose medium, although the exact mechanisms for this have yet to be elucidated (Gan et al., 2026).

Antioxidants

Endogenous antioxidants, including glutathione, SOD, and catalase, form the core defense system that protects adipocytes from oxidative injury (Ighodaro & Akinloye, 2018). Exogenous antioxidants such as n-acetyl cysteine (NAC) can dramatically reduce ROS levels; indeed, Kim et al. (2014) found that NAC completely abolished hypoxia-induced ROS accumulation in human adipocytes. These findings highlight the sensitivity of adipocyte redox state to variations in antioxidant availability.

Oxygen Availability

Oxygen tension profoundly influences ROS production. Under normoxia, oxygen serves as the final electron acceptor in the mitochondrial electron transport chain (ETC) (Zhao et al., 2019). Hypoxia disrupts this balance by reducing oxygen availability, leading to increased electron leak and ROS formation (Brookes, 2005). This mechanism explains why hypoxia is consistently associated with increased ROS and serves as a primary driver of oxidative stress in adipose tissue.

Impact of Hypoxia on Adipocyte Differentiation

Oxygen availability plays a critical role in adipocyte biology, particularly during the differentiation of preadipocytes into mature adipocytes (El Amine et al., 2023; Famulla et al., 2012; Mahat et al., 2021). Differentiation is an energetically demanding process that requires robust mitochondrial biogenesis, increased oxidative phosphorylation, lipid droplet formation, and transcriptional activation of adipogenic pathways such as peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα) (Famulla et al., 2012). These processes depend on adequate oxygen to sustain mitochondrial activity and minimize cellular stress (Famulla et al., 2012). Thus, hypoxia during differentiation disrupts normal adipose tissue development.

Our group has demonstrated that exposing human preadipocytes to 3% oxygen over the entire 14-day differentiation period nearly abolished both adipocyte proliferation and lipid accumulation. Hypoxia markedly suppressed the expression of adipogenic transcription factors and prevented the acquisition of mature adipocyte morphology (El Amine et al., 2023; Mahat et al., 2021). These findings underscore that oxygen is a necessary component for normal adipogenesis, without which the metabolic and structural maturation of adipocytes is severely impaired.

Mechanistically, hypoxia exerts its inhibitory effects through multiple pathways. A primary mediator is the stabilization of hypoxia-inducible factor-1 alpha (HIF-1α), which redirects cellular metabolism toward glycolysis and represses adipogenic gene networks (Engin, 2024). Hypoxia also induces ROS accumulation through mitochondrial dysfunction, creating oxidative stress that interferes with adipogenic signalling (Kim et al., 2014). ROS can impair PPARγ activation, disrupt mitochondrial DNA integrity, and interfere with cytoskeletal remodeling required for lipid droplet formation (Castro et al., 2016).

Because hypoxia disrupts adipocyte differentiation so profoundly, it is essential that hypoxic exposure in my study is applied only after adipocyte maturation. This avoids confounding my results with impaired differentiation and ensures that any observed changes in ROS levels or antioxidant gene expression reflect effects on mature adipocytes rather than developmental abnormalities. This methodological distinction is central to interpreting how hypoxia and exogenous ketones interact to shape the oxidative state of human adipocytes.

Hypoxia and Oxidation in Adipocytes: Controversies

Although hypoxia is widely recognized as a driver of oxidative stress in adipocytes, the specific mechanisms and magnitude of ROS elevation remain subjects of debate. Conflicting models in the literature reflect the complexity of redox regulation and the variability in experimental systems.

One model posits that hypoxia increases mtROS production by limiting oxygen's availability as the final electron acceptor in the ETC (Zhao et al., 2019). Reduced oxygen tension slows electron transfer at complexes III and IV, increasing the probability of electron leakage and superoxide formation, particularly at the outer quinone binding site (Qo) of complex III (Guzy & Schumacker, 2006). This mechanism explains the dose-dependent increases in ROS observed when oxygen tension is reduced from 21% to physiological (10%) or hypoxic (3–5%) levels, as seen in Kim et al. (2014).

A competing model suggests that hypoxia-induced ROS are primarily the result of HIF1α–controlled signalling cascades rather than changes in mitochondrial oxygen availability (Engin, 2024). HIF-1α can activate phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) and extracellular signal-regulated kinase (ERK) pathways, which in turn stimulate NADPH oxidases (NOX enzymes) that generate cytosolic superoxide (Engin, 2024). This mechanism implies that ROS elevation under hypoxia may be driven by regulated signalling events rather than passive mitochondrial dysfunction.

Another controversy concerns the magnitude and consistency of ROS elevations under hypoxia. Some studies report dramatic increases, while others observe modest effects or transient responses (Alva et al., 2024; Arias et al., 2024). Differences in cell type, differentiation state, culture density, glucose concentration, or ROS detection methods (e.g.,

2',7'dichlorodihydrofluorescein diacetate's (DCFDA) preferential detection of hydrogen peroxide derivatives) contribute to inconsistencies across studies (Dikalov & Harrison, 2014).

These controversies emphasize the need to carefully control oxygen levels, exposure duration, and metabolic conditions when studying hypoxia-induced ROS. In the context of my thesis, they reinforce the value of examining whether exogenous ketones can attenuate ROS production across a range of oxygen tensions (21%, 10%, and 3%), thereby reducing the variability typically associated with hypoxia-driven oxidative stress.

Impact of Dysregulated Oxidant/Antioxidant Balance on Adipocytes

An imbalance between ROS production and antioxidant defenses, known as oxidative stress, has profound implications for adipocyte function and systemic metabolic health (Ighodaro & Akinloye, 2018). When ROS production exceeds the capacity of antioxidant systems such as SOD1, SOD2, catalase, Mt2, and glutathione peroxidase, damage to cellular structures and metabolic pathways ensues (Ighodaro & Akinloye, 2018).

In adipocytes, excess ROS impair mitochondrial function by damaging mitochondrial DNA, oxidizing lipids, and disrupting respiratory chain complexes, leading to further ROS generation in a self-reinforcing cycle (Kietzmann et al., 2017). Oxidized lipids and proteins interfere with insulin signalling pathways, thereby contributing to adipocyte insulin resistance, a hallmark of metabolic dysfunction in obesity (Castro et al., 2016). Oxidative stress also promotes inflammation by activating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and other pro-inflammatory transcription factors, which in turn amplify ROS through cytokine-dependent signalling loops (Ran et al., 2025).

At the tissue level, chronic oxidative stress contributes to adipocyte hypertrophy, extracellular matrix remodeling, and fibrosis, reducing adipose tissue expandability and exacerbating systemic metabolic dysfunction (Engin, 2024). Hypoxia itself, which often arises in hypertrophic adipose tissue, further amplifies ROS production and diminishes antioxidant capacity (Kim et al., 2014; Gou et al., 2022), creating a worsening cycle of oxidative injury.

Understanding the destructive consequences of dysregulated oxidant/antioxidant balance highlights the importance of identifying strategies that restore redox homeostasis. This provides

the rationale for investigating whether ketone bodies, through their ability to enhance antioxidant gene transcription and improve mitochondrial function, can counteract the oxidative insults caused by hypoxia in human adipocytes.

ROS as a Modulator of the Hypoxic Response

ROS serve not only as damaging oxidants but also as critical signalling molecules in the cellular response to hypoxia. Under reduced oxygen conditions, cytosolic and mtROS act as modulators of hypoxia-responsive pathways, particularly those governed by HIF-1α (Engin, 2024).

Two major mechanistic hypotheses have been proposed. The first posits that mtROS production increases under hypoxia, generating localized redox changes that influence the stability and transcriptional activity of HIF-1α (Chandel et al., 1998). These ROS promote the inhibition of prolyl hydroxylases that normally target HIF-1α for degradation, thereby stabilizing HIF-1α and enabling it to activate downstream genes involved in angiogenesis, glycolysis, and survival (Chandel et al., 1998).

The second hypothesis centers on HIF-1α–dependent activation of signalling cascades that subsequently stimulate ROS production. Engin (2024) describes how hypoxia-induced activation of PI3K/Akt and ERK signalling can increase ROS generation through NADPH oxidase activity. In this model, ROS are generated downstream of hypoxia signalling, amplifying or fine-tuning transcriptional responses rather than initiating them.

Intriguingly, applying exogenous ROS such as hydrogen peroxide under normoxic conditions does not mimic hypoxia-induced HIF-1α stabilization, suggesting that ROS alone are insufficient and that mitochondrial respiratory inhibition under hypoxia produces unique redox signatures (Guzy & Schumacker, 2006). Furthermore, antioxidant treatments often only partially reduce hypoxia-induced gene expression, indicating that ROS are necessary but not singular drivers of the hypoxic response (Engin, 2024).

For my thesis, this dual role of ROS is particularly important. If hypoxia increases ROS in a manner that reinforces metabolic stress and reduces antioxidant gene expression, then interventions capable of decreasing ROS, such as exogenous ketone supplementation, may modulate hypoxia signalling and restore redox homeostasis.

Ketones as a Mitigator of Hypoxia-Induced ROS in Adipocytes

The central objective of my thesis is to investigate whether exogenous ketones can mitigate the oxidative stress induced by hypoxia in human adipocytes. Based on the cumulative evidence, βOHB possesses several properties that make it a promising candidate for reducing hypoxia-induced ROS and enhancing antioxidant defenses.

First, βOHB enhances transcription of antioxidant genes (FOXO3, catalase, SOD1, SOD2, Mt2) through epigenetic mechanisms involving HDAC inhibition (Shimazu et al., 2013). Because hypoxia reduces antioxidant capacity, partly by suppressing glutathione synthesis and downregulating antioxidant genes (Kim et al., 2014; Gou et al., 2022), βOHB may counteract these effects by reinforcing endogenous antioxidant pathways.

Second, βOHB modulates mitochondrial function in ways that reduce ROS production. By increasing proton leak and lowering mitochondrial membrane potential, βOHB reduces electron leakage and superoxide formation (Jones IV et al., 2016). This is particularly relevant in hypoxia, where electron transport is already compromised and prone to ROS overproduction (Engin, 2024).

Third, βOHB has been shown to improve mitochondrial membrane potential stability and reduce oxidative damage in adipose-derived cells (Gan et al., 2026). Improved mitochondrial function under hypoxic stress may attenuate ROS formation and enhance cell viability (Gan et al., 2026).

Rationale and Statement of the Problem

Adipose tissue is a metabolically active organ whose function is tightly regulated by redox balance and oxygen availability (Castro et al., 2016; Kietzmann et al., 2017). Under physiological conditions, ROS serve essential signalling roles in adipocyte differentiation and metabolic regulation, particularly during adipogenesis where controlled mtROS production is required for proper cellular maturation (Tormos et al., 2011). When ROS production exceeds antioxidant capacity, however, oxidative stress develops, leading to mitochondrial dysfunction, impaired insulin signalling, inflammation, and adipose tissue dysfunction (Castro et al., 2016; Kietzmann et al., 2017).

Hypoxia frequently arises in expanding adipose tissue as adipocyte hypertrophy outpaces vascularization, resulting in reduced oxygen availability within the tissue microenvironment (Engin, 2024; Trayhurn, 2013). Reduced oxygen tension disrupts mitochondrial electron transport, increasing electron leak and promoting ROS generation in adipocytes (Carrière et al., 2004; Guzy & Schumacker, 2006; Kim et al., 2014). Concurrently, hypoxia suppresses antioxidant defences by downregulating antioxidant gene expression and depleting intracellular antioxidant capacity, thereby exacerbating oxidative stress (Kim et al., 2014; Gou et al., 2022). Together, increased ROS production and diminished antioxidant defences contribute to adipocyte dysfunction and are implicated in the progression of metabolic disease (Castro et al., 2016; Kietzmann et al., 2017).

Ketone bodies, particularly βOHB, have been shown to exert antioxidant and cytoprotective effects that extend beyond their role as alternative metabolic substrates (Puchalska & Crawford, 2021). βOHB inhibits class I HDACs, resulting in increased transcription of antioxidant genes such as FOXO3, catalase, and superoxide dismutase (Shimazu et al., 2013). In addition, βOHB modulates mitochondrial function by reducing mitochondrial membrane potential and electron leak, thereby lowering ROS production (Brookes, 2005; Jones IV et al., 2016). These complementary mechanisms position ketones as potential regulators of redox homeostasis under conditions of metabolic and oxidative stress (Puchalska & Crawford, 2021).

Despite this evidence, the role of ketones in regulating oxidative stress in human adipocytes exposed to hypoxia remains poorly characterized (Nishitani et al., 2022). Much of the existing literature relies on murine 3T3-L1 adipocyte models, which differ substantially from human adipocytes in mitochondrial abundance, metabolic flexibility, and ROS handling (Cedikova et al., 2016; Nishitani et al., 2022). These species-specific differences limit the direct translation of ketone-mediated antioxidant mechanisms observed in murine adipocytes to human adipose tissue physiology (Cedikova et al., 2016).

Furthermore, studies examining hypoxia-induced oxidative stress in adipocytes report variable findings regarding both the magnitude and sources of ROS production, reflecting ongoing mechanistic controversies in the field (Guzy & Schumacker, 2006; Engin, 2024). Differences in oxygen tension, experimental duration, and methodological approaches to ROS detection contribute to inconsistent interpretations of hypoxia-driven oxidative responses (Engin, 2024). This variability underscores the need for carefully controlled investigations examining oxidative

stress responses across graded hypoxic conditions in human adipocytes (Kim et al., 2014; Gou et al., 2022).

Exploratory findings from our laboratory (see Appendix I – Exploratory Findings) suggest that endogenous ketone production in human adipocytes is minimal and not significantly modulated by oxygen availability or insulin, consistent with the low mitochondrial content of these cells (Cedikova et al., 2016). This supports the interpretation that any observed effects of βOHB supplementation in human adipocytes are likely attributable to exogenous ketone action, rather than endogenous ketogenesis (Nishitani et al., 2022).

Therefore, the central problem addressed in this thesis is the exploration of how exogenous ketones modulate oxidative stress responses in human adipocytes exposed to hypoxia. Addressing this gap is essential for clarifying the potential role of ketones as metabolic signals capable of mitigating hypoxia-induced adipocyte dysfunction.

Research Questions, Objectives and Hypotheses

Research Questions

Based on the gaps identified in the literature, this thesis addresses the following research questions:

1. Does hypoxia increase ROS levels and alter antioxidant gene expression in human adipocytes in a dose-dependent manner?
2. Do exogenous ketones reduce ROS production in human adipocytes exposed to hypoxic conditions?
3. Do exogenous ketones modulate the expression of key antioxidant genes in human adipocytes under normoxic and hypoxic conditions?
4. Are the effects of exogenous ketones on ROS levels and antioxidant gene expression dose-dependent?

Objectives

The overall objective of this thesis is to investigate the impact of exogenous ketones on oxidative stress in human adipocytes exposed to hypoxia.

The specific objectives are to:

1. Quantify intracellular ROS levels in human adipocytes exposed to varying oxygen concentrations.
2. Determine the effect of exogenous βOHB on ROS production under normoxic and hypoxic conditions.
3. Assess the expression of antioxidant and redox-regulatory genes (FOXO3, catalase, Mt2, SOD1, and SOD2) in response to hypoxia.
4. Evaluate whether exogenous βOHB alters antioxidant gene expression in a dose-dependent manner.
5. Examine the interaction between oxygen availability and ketone supplementation in regulating adipocyte redox homeostasis.

Hypotheses

Based on the existing literature and the rationale outlined above, the following hypotheses are proposed:

1. Hypoxia will increase ROS production in human adipocytes in a dose-dependent manner.
2. Hypoxia will reduce the expression of antioxidant genes, including FOXO3, catalase, Mt2, SOD1, and SOD2, in a dose-dependent manner.
3. Exogenous βOHB will reduce ROS levels in human adipocytes under both normoxic and hypoxic conditions.
4. Exogenous βOHB will increase the expression of antioxidant genes in a dose-dependent manner.
5. βOHB will partially or fully attenuate hypoxia-induced oxidative stress in human adipocytes by restoring redox balance.

Chapter 3: Methodology

Cell culture

Human preadipocytes were obtained from ZenBio (Cryopreserved subcutaneous preadipocytes, ZenBio Inc., Research Triangle Park, NC, USA). We obtained human subcutaneous preadipocytes from a single 46-year-old female donor with a body mass index of 29.9 kg/m^2 (Lot# LM070622B). Cells were seeded in ZenBio Preadipocyte Proliferation Media (PM-1) according to the manufacturer's instructions in 96-well plates at a density of at least 40,000 $cells/cm^2$. The plates were then placed in a HeraCell 150iO2 incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 100% humidity and 5% CO2. Once confluence was visually confirmed using a Zeiss Axiovert 40 C microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), usually 3 days after the start of incubation, culture medium was replaced with ZenBio Preadipocyte Differentiation Medium (DM-2) to induce differentiation. After 7 days of differentiation, the medium was partially replaced with adipocyte maintenance medium, either at a low glucose concentration (5 mM) or high glucose concentration (17.5mM), where 5mM acted both as a control and a physiological approximate, while 17.5mM is a common cell culture medium concentration and based on optimisation research done by the manufacturer (ZenBio Inc., Research Triangle Park, NC, USA). After discussion with the manufacturer, we decided to create the maintenance medium in lab using an expired patent (#US6153432A) due to our specifications on the glucose concentration. Based on the patent recommendations, our maintenance medium was formulated using the following concentrations: regular and glucose-free DMEM/F12 with HEPES were used to achieve 17.5mM and 5mM of glucose for each condition (Innovative Research, Inc., Novi, MI, USA, SKU: IDMEMF120310500 and SKU: IDMEMF120323500), 10nM of insulin (Sigma-Aldrich, Inc., St. Louis, MO, USA, Product No. I9278-5ML), 10nM of dexamethasone (Sigma-Aldrich, Inc., St. Louis, MO, USA, Product No. D4902-25MG), 17uM of D-Pantothenic acid hemicalcium salt (Sigma-Aldrich, Inc., St. Louis, MO, USA, Product No. P5155-100G), 33uM of biotin (Sigma-Aldrich, Inc., St. Louis, MO, USA, Product No. B4639-100MG), and a 1:1000 dilution of Penicillin-Streptomycin-Amphotericin B Solution (ATCC, Product No. PCS-999-002).

After 7 days of incubation in maintenance medium, the cells were considered mature adipocytes, displaying several lipid droplets. The cells were loaded with specific ketone body concentration for 24 hours: 0mM as a control, 0.25mM (higher physiological range), 3mM

(prolonged fasting), and 10mM (diabetic ketoacidosis) (Laffell, 1999). To do this, βOHB (Sigma-Aldrich, Inc., St. Louis, MO, USA, Product No. 298360-1G) was added to the previously described maintenance medium.

Following this 24-hour incubation, the plates were separated and exposed to one of three oxygen concentrations for an additional 24 hours: 21%, 10% and 3%. We chose these concentrations as 21% not only represents ambient air, but is also commonly used in cell culturing, therefore acting as a control condition. The 3 and 10% oxygen concentrations represent the range of physiological oxygen tension found in the adipose tissue of humans with varying adiposity levels (Cifarelli et al., 2020; Fleischmann et al., 2005; Kabon et al., 2004; Lawler et al., 2016; Pasarica et al., 2009). Furthermore, 3% is a standard hypoxia level in cell culture experiments (Mahat et al., 2021).

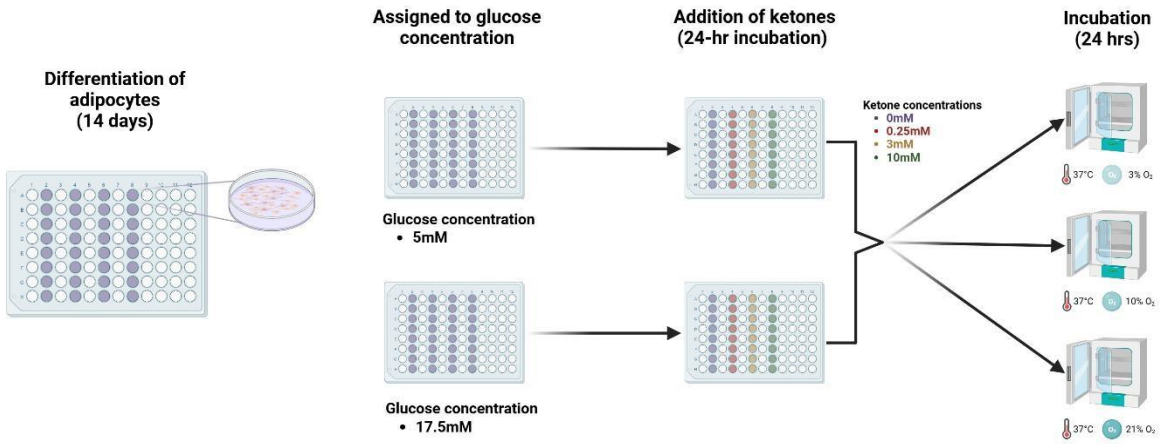

Figure 2. Schematic representation of the study methodology. Created with BioRender.com.

rt-qPCR

After incubation, half the cells were rapidly washed 3 times with ice-cold PBS and lysed using Qiagen RLT buffer with 1% beta-mercaptoethanol, then promptly put in -80 °C until further processing. Upon thawing, lysates were spun on Qiagen QiaShredder columns to fully break down cell membranes and increase RNA yield. Total RNA was extracted using Qiagen RNeasy Mini Kit (Cat. No. 74104) and concentrated using Qiagen MinElute clean up kit when necessary (Cat. No. 74204). Complementary DNA (cDNA) was obtained using the Qiagen Quantitect Reverse

transcription kit and the maximum volume of RNA template allowed by the kit (12 µL) on a Tpersonal Combi thermocycler (Biometra, Gottingen, Germany) and real-time quantitative polymerase chain reaction (RT-qPCR) was conducted using Quantitect primers (Qiagen, Germantown, MD, USA) and MBI EVOlution EvaGreen rtPCR mix (Montreal Biotech Inc., Dorval, QC, Canada) on a RG-3000 Rotor-Gene (Corbett Research Ltd., Mortlake, Australia). The Quantitect primers used had the following Qiagen catalog numbers: Beta-Actin QT00095431; Catalase QT00079674; Metallothienin-2 QT02317819; FOXO3 QT00031941; SOD1 QT01671551; SOD2 QT01008693. Melting curve analyses were performed to ensure that amplification yielded single products. Amplification curves were analyzed using the Rotor-Gene Analysis software 6.1 version 1.93. Amplification efficiency was determined for each individual amplification reaction using the comparative quantitation feature of the Q software, which is based on the fluorescence increase over 4 cycles following "take-off" of the fluorescence signal. The "take-off" point corresponds to the point at which the second derivative of the amplification curve reaches 20% of its maximum (determined automatically by the Rotor-Gene Analysis software). Average amplification factors were very stable within and across genes (averaging 1.65 to 1.8). Fluorescence thresholds for CT determination were determined for each gene in the first experiments and arbitrarily assigned the value corresponding to half the average fluorescence at take-off cycle. Fold-changes were calculated using the formula (Pfaffl, 2001):

$$Fold\ \ change = \frac{E_{tar}^{(CT_{tar\ ctl} - CT_{tar\ treat})}}{E_{ref}^{(CT_{ref\ ctl} - CT_{ref\ treat})}}$$

where E corresponds to the average amplification efficiency of each gene within the same experiment (i.e., E was averaged from individual reactions for each gene for each experiment).

DCFDA and Oil Red O Stain

The other half of the cells were assayed for ROS levels by DCFDA fluorescence emission, as well as lipid content by oil red O stain. Given the many limitations with DCFDA assays, we conducted a preliminary positive control experiment with hydrogen peroxide (H2O2) to simulate

high ROS levels, which showed a high fluorescence from DCFDA readings, confirming the ability of this assay to react to ROS levels in our cell cultures (data not shown).

The cells were washed with warmed PBS twice and a new culture media was added, made from a mixture of DMEM at concentrations of 5mM or 17.5mM of glucose (Innovative Research, Inc., Novi, MI, USA, SKU: IDMEMF120310500 and SKU: IDMEMF120323500), βOHB at concentrations of 0mM, 0.25mM, 3mM and 10 mM (Sigma-Aldrich, Inc., St. Louis, MO, USA, Product No. 298360-1G), and DCFDA at a concentration of 5uM dissolved in 100% EtOH (Thermo Fisher Scientific, Waltham, MA, USA, Cat. No. C400). The plates were incubated in their respective oxygen concentrations for 30 minutes and promptly washed twice with warmed PBS. A mixture of Hank's Balanced Salt Solution (HBSS) 1X (ZenBio Inc., Research Triangle Park, NC, USA, Cat. No. HBSS-500), βOHB at concentrations of 0mM, 0.25mM, 3mM and 10mM (Sigma-Aldrich, Inc., St. Louis, MO, USA, Product No. 298360-1G), and dextrose at concentrations of 5mM and 17.5mM were added to the cells. A first reading was immediately taken at 485nm using Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, Vermont, USA). The cells were then incubated at their respective oxygen concentrations (3%, 10%, or 21%) for 1.5 hours, with readings taking place every 15 minutes.

The 0.5% oil red O stain stock solution (Sigma-Aldrich, Inc., St. Louis, MO, USA, Product No. 01391-250ML) was diluted in 100% isopropanol. For 1 volume of this solution, 1.5 volume of distilled water was added to give a 0.2% oil red O solution in 40% isopropanol. The solution was then briefly filtered through a 0.2um Basix syringe filter (Lot #2411132201) and kept at room temperature for a maximum of one week before use. The culture medium was removed from the wells by gentle aspiration, and cells were fixed with 100 ul of 10% buffered formalin (Thermo Fisher Scientific, Waltham, MA, USA, Product No. 245-684) for 15 minutes at room temperature. The formalin was then removed from the wells, and 50ul of the oil red O solution was added to each well. Following a 30-minute incubation at room temperature, the solution was removed and the cells were washed 4-5 times with distilled water. Special care was taken to ensure that any dye aggregates that may have formed during the incubation period were properly aspirated without touching the cells directly. Next, 100ul of 100% isopropanol was added to the wells, and the plates were then set on a 4625 Titer Plate Shaker (Lab-Line Instruments, Melrose Park, IL, USA) at a slow shaking speed (speed setting 2 out of 10) for 10 minutes at room temperature. After

incubation, 80ul of each well was transferred to a new clear bottom 96-well plate, with 100% isopropanol added as a control. Absorbance was measured at 510nm using Synergy HT MultiMode Microplate Reader (BioTek Instruments, Winooski, Vermont, USA).

Statistical Analyses

Lipid accumulation measured by Oil Red O stain was analyzed using an ANOVA, given that only one set of measurements was used. All other variables were analysed using a linear mixed-effects model to evaluate the effect of glucose concentration (5mM × 17.5mM) and ketone concentration (0mM x 0.25mM x 3mM x 10mM) within each set of oxygen conditions (21% x 10% x 3%), while replicates were modelled as a random effect. Reported P-values were adjusted for multiplicity using the Tukey's test correction. Given that no interactions were found between the variables in the model, we opted to present our data with the main effects only. All statistical analyses were conducted using jamovi (v.2.2.5, with the gamlj module, v.2.6.6) and α set at 0.050 to establish statistical significance. Figures were created using GraphPad Prism (v.9.51; GraphPad Software, USA).

Chapter 4: Results

Cell Viability

Visual examination of the cells showed no obvious anomaly such as reduced cell proliferation or cell detachment from culture ware in response to treatments throughout the experiment duration (17 days total), regardless of oxygen pressure (Figure 3).

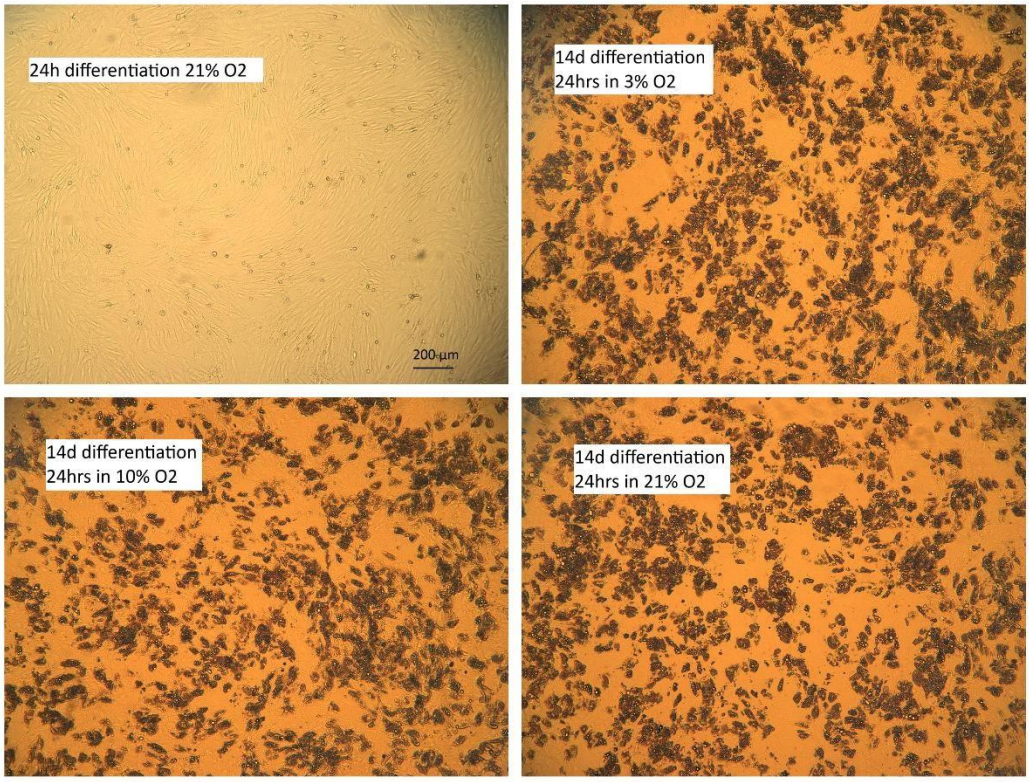

Figure 3. Differentiating preadipocytes exposed to varying oxygen pressures, during proliferation (24hours) and after maturation (14days). The top left picture was taken after the first 24 h of differentiation under 21% O2. The other 3 pictures were taken after 14 days of differentiation at 21% O2, followed by a 24 h exposure to either 3%, 10% or 21% O2. All pictures were taken using a light microscope at 5× magnification.

Oil Red O stain was conducted after 48 hours of exposure to varying βOHB concentrations and 24 hours of exposure to either normoxia or hypoxia to quantify lipid accumulation in adipocytes, and to further confirm cell viability. Exposure to 3% and 10% oxygen concentrations significantly reduced lipid accumulation in comparison to 21% oxygen ($p<.001$), with no differences between 3% and 10% oxygen ($p= 0.140$) (Figure 4). Glucose concentration in the media had no impact on lipid accumulation ($p= 0.598$). The addition of ketones did not significantly affect lipid accumulation ($p= 0.294$).

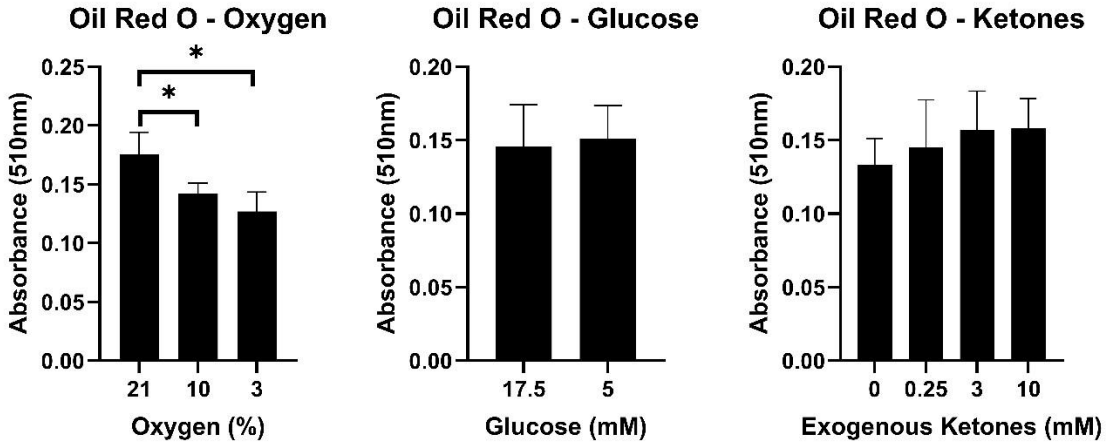

Figure 4. Cellular lipid accumulation measured by Oil Red O Stain (OD 510nm) in differentiated human adipocytes under varying glucose concentrations after exposure to different oxygen (24h) and exogenous ketone (48h) concentrations (n=1). Data are presented as the mean (SD). Data were compared using an ANOVA with α set at 0.050.

Effect of hypoxia, glucose and exogenous ketone on ROS levels expression

DCFDA assays were conducted to evaluate ROS levels in adipocytes after 48 hours of exposure to varying βOHB concentrations and 24 hours of exposure to either normoxia or hypoxia (n=3). The levels did not change significantly across the 3 oxygen concentrations (p= 0.307), the 2 glucose concentrations (p= 0.192), nor the 4 ketone concentrations (p= 0.361) (Figure 5). No interactions were identified across the three variables, even when controlling for each one separately (oxygen x glucose p= 0.836; oxygen x ketone p= 0.120; glucose x ketone p= 0.290; oxygen x glucose x ketone p= 0.429).

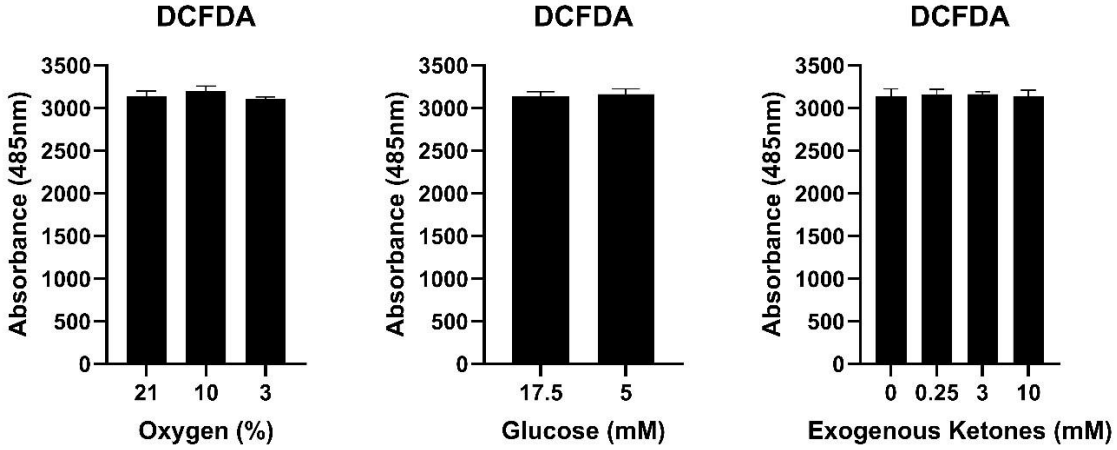

Figure 5. Cellular ROS detected by 2',7'-dichlorofluorescein diacetate (DCFDA) assay (OD 485nm) in differentiated human adipocytes under varying glucose concentrations after exposure to different oxygen (24h) and exogenous ketone (48h) concentrations (n=3). Data are presented as the mean (SD). Data were compared using a linear mixed-effects model with α set at 0.050.

Effect of hypoxia, glucose and exogenous ketone on antioxidant gene expression

Catalase

Catalase gene expression (n=4), after 48 hours of exposure to varying βOHB concentrations and 24 hours of exposure to either normoxia or hypoxia, significantly decreased upon exposure to a hypoxic environment (3% and 10% oxygen) when compared to normoxia of 21% (p<.001) (Figure 6). There were no differences between the 3% and 10% hypoxic conditions (p=0.879).

Exogenous ketones (p=0.825) and glucose concentration (p=0.935) had no impact on catalase expression (Figure 6), even when controlling for oxygen concentration (data not shown). There were no interactions between ketones, glucose, and oxygen (p=0.477) across the 4 experiments.

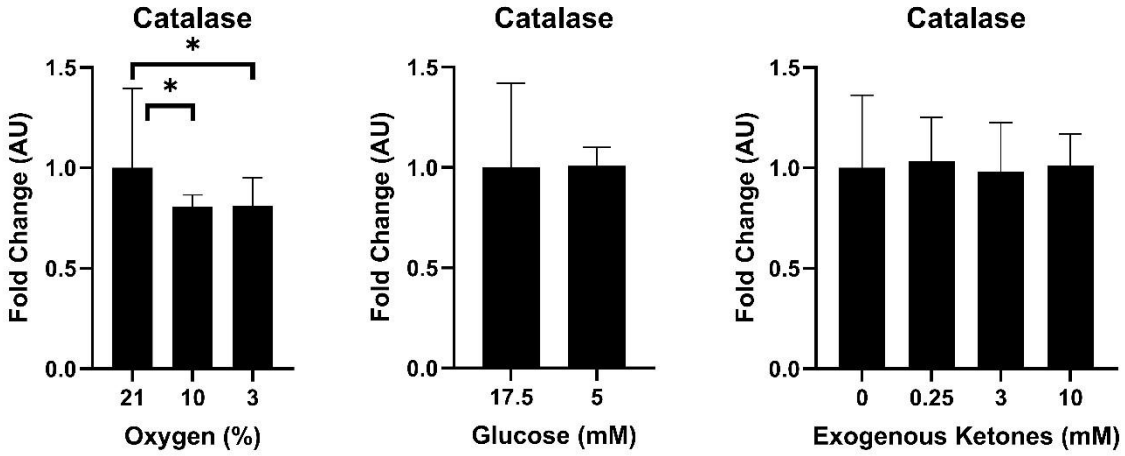

Figure 6. qRT–PCR of catalase in differentiated human adipocytes under varying glucose concentrations after exposure to different oxygen (24h) and exogenous ketone (48h) concentrations (n=4). Data are presented as the mean (SD). Data were compared using a linear mixed-effects model with α set at 0.050. AU; arbitrary units.

FOXO3

Exposure to 3% oxygen for 24 hours significantly increased the gene expression of FOXO3 (n=4) when compared to 10% and 21% (p<.001) (Figure 7). There were no differences between 10% and 21% oxygen (p=0.617).

A 48-hour exposure to exogenous ketones (p=0.664) had no significant impact on FOXO3 expression, as well as glucose concentration (p=0.097) (Figure 7). No interactions were found between ketones, glucose and oxygen across the 4 replicates (p=0.521).

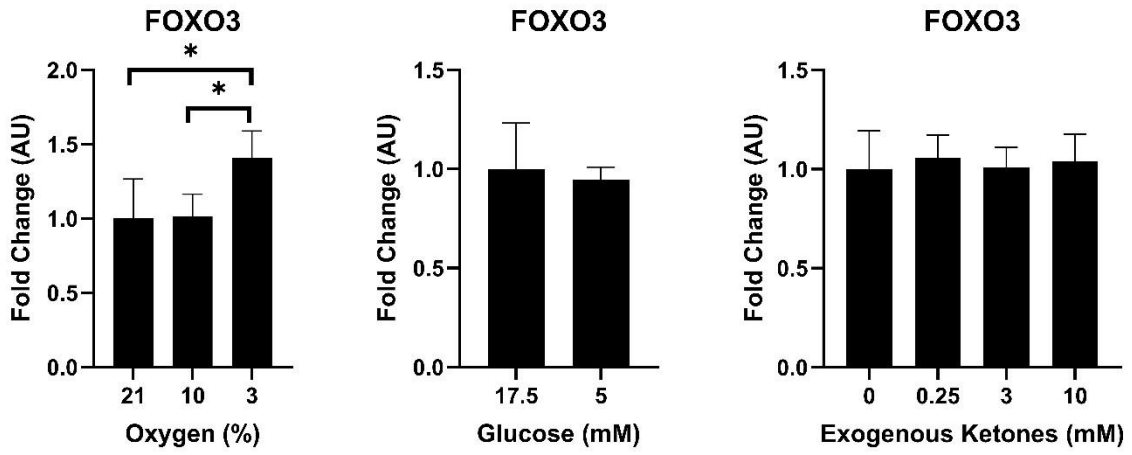

Figure 7. qRT–PCR of FOXO3 in differentiated human adipocytes under varying glucose concentrations after exposure to different oxygen (24h) and exogenous ketone (48h) concentrations (n=4). Data are presented as the mean (SD). Data were compared using a linear mixed-effects model with α set at 0.050. AU; arbitrary units.

Mt2

As with FOXO3, Mt2 gene expression (n=4) after 48 hours of exposure to varying βOHB concentrations and 24 hours of exposure to either normoxia or hypoxia was significantly increased in the 3% oxygen condition when compared to 10% and 21% (p<.001) (Figure 8), with no differences between 10% and 21% oxygen (p=0.712).

There were no significant changes on Mt2 expression with the addition of ketones (p=0.944), or with differing glucose concentrations (p=0.454) (Figure 8). Across all 4 experiments, no interactions were found between ketones, glucose and oxygen conditions for Mt2 expression (p=0.783).

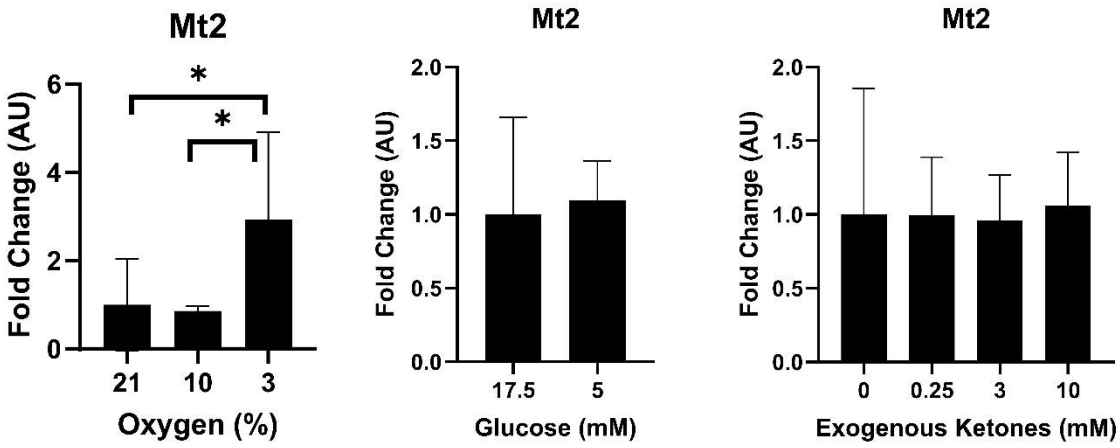

Figure 8. qRT–PCR of Mt2 in differentiated human adipocytes under varying glucose concentrations after exposure to different oxygen (24h) and exogenous ketone (48h) concentrations (n=4). Data are presented as the mean (SD). Data were compared using a linear mixed-effects model with α set at 0.050. AU; arbitrary units.

SOD1

SOD1 gene expression (n=4), after 48 hours of exposure to varying βOHB concentrations and 24 hours of exposure to either normoxia or hypoxia, significantly decreased when exposed to 10% hypoxic condition (p=0.033), in comparison to a normoxic environment (21%) (Figure 9). There was also a reduction when exposed to 3% oxygen, although not statistically significant (p= 0.097).

The addition of ketones did not impact SOD1 expression (p= 0.876), nor did the variation in glucose concentrations (p= 0.897) (Figure 9). No interactions were found between ketones, glucose and oxygen conditions for the 4 experiments (p= 0.362).

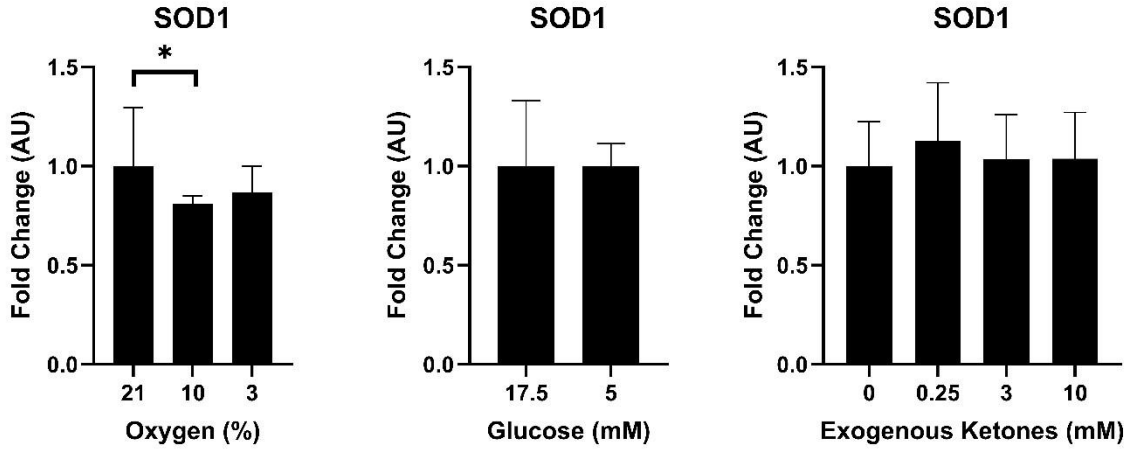

Figure 9. qRT–PCR of SOD1 in differentiated human adipocytes under varying glucose concentrations after exposure to different oxygen (24h) and exogenous ketone (48h) concentrations (n=4). Data are presented as the mean (SD). Data were compared using a linear mixed-effects model with α set at 0.050. AU; arbitrary units.

SOD2

SOD2 gene expression levels (n=4), after 48 hours of exposure to varying βOHB concentrations and 24 hours of exposure to either normoxia or hypoxia, did not change in the two hypoxic environments when compared to normoxia (p= 0.409) (Figure 10). The glucose (p= 0.350) and ketone conditions (p= 0.547) did not impact SOD2 expression (Figure 10). As with all the other genes measured, there were no interactions between ketones, glucose and oxygen conditions for SOD2 expression across all experiments (p= 0.492).

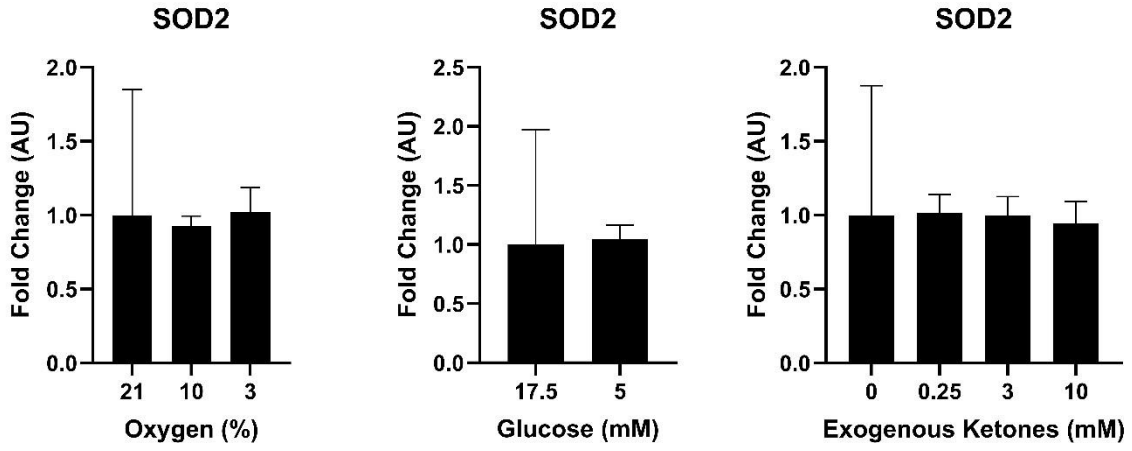

Figure 10. qRT–PCR of SOD2 in differentiated human adipocytes under varying glucose concentrations after exposure to different oxygen (24h) and exogenous ketone (48h) concentrations (n=4). Data are presented as the mean (SD). Data were compared using a linear mixed-effects model with α set at 0.050. AU; arbitrary units.

Chapter 5: Discussion

Hypoxia and ROS production

Hypoxia is widely recognized as a key regulator of adipocyte metabolism and redox balance, largely through its effects on mitochondrial function and oxidative stress pathways (Castro et al., 2016; Kietzmann et al., 2017). Reduced oxygen availability limits ETC efficiency, increasing the likelihood of electron leak and ROS generation, while also activating hypoxia-responsive signaling pathways that reshape cellular metabolism (Guzy & Schumacker, 2006; Engin, 2024). Although controversial, previous studies have shown that hypoxia might inhibit mitochondrial electron transport, which could provoke redox changes in the electron carriers that leads to an increase in ROS production (Carrière et al., 2004). Based on this literature, the first hypothesis of this thesis proposed that reducing oxygen availability would induce a dose-dependent increase in ROS production in human adipocytes.

In the present study, hypoxic exposure of differentiated human adipocytes did not lead to a significant increase in intracellular ROS levels (Figure 5), suggesting that mature adipocytes may exhibit a distinct oxidative response compared to other cell types under low oxygen conditions. This finding contrasts with previous reports in which differentiated 3T3-F442A preadipocytes exposed to 1% hypoxia for one hour displayed a marked elevation in ROS generation (Carrière et al., 2004). Similarly, Kim et al. (2014) demonstrated that hypoxia significantly enhanced mtROS, as indicated by increased mito-SOX fluorescence, in differentiated human adipose-derived stem cells. These discrepancies may reflect differences in cellular phenotype, metabolic activity, and mitochondrial dynamics between cells. Furthermore, variations in experimental conditions, including oxygen tension, exposure duration, and ROS detection methods, could contribute to divergent outcomes. Collectively, these observations underscore the complexity of hypoxia-induced oxidative stress in adipose tissue and highlight the need for further studies to delineate the mechanisms governing ROS regulation across different stages of adipocyte differentiation.

Hypoxia and antioxidant gene expression

Although hypoxia did not induce an increase in ROS levels, it produced clear changes in antioxidant gene expression. Hypoxia has been reported to modify antioxidant defenses through transcriptional mechanisms linked to hypoxia-inducible signaling and metabolic adaptations (Gou

et al., 2022; Kim et al., 2014). Consistent with this literature, hypoxic exposure in the present study resulted in oxygen-dependent, gene-specific alterations in antioxidant gene expression (Figure 610). Notably, FOXO3 and Mt2 gene expression increased significantly under pronounced hypoxia (3% oxygen) (Figures 7-8). FOXO3 modulates gene transcription associated with oxidative stress resistance and cellular adaptations, while Mt2 is a redox-sensitive stress-response gene involved in intracellular redox buffering (Gou et al., 2022; Shimazu et al., 2013). Their induction indicates activation of adaptive antioxidant pathways, even in the absence of significant ROS accumulation.

On top of their role in antioxidant defenses, FOXO3 and Mt2 both play important roles in cellular function. FOXO3 is involved in many processes such as cell growth, DNA repair, and cell death (Hagenbuchner & Ausserlechner, 2013). Under hypoxia, FOXO3 has been shown to reduce oxygen consumption as an important cellular adaptation to lower oxygen availability (Hagenbuchner & Ausserlechner, 2013). As such, its expression can be increased under hypoxia even in the absence of high ROS levels, since its function goes beyond ROS-scavenging.

As for Mt2, its overexpression has been shown to decrease oxygen consumption, downregulate cellular ATP levels, and reduce oxidative phosphorylation capacity, possibly via indirect interactions with mitochondrial complexes and metal binding–mediated inhibition of respiratory enzymes (Ling et al., 2016). This in turn might explain its upregulation under hypoxia, even in the absence of increased ROS levels.

In contrast, catalase and SOD1 showed a decrease under 10% and 3% hypoxia, although SOD1 reduction under 3% hypoxia was not statistically significant (Figures 6, 9). The main roles of both catalase and SOD1 are that of antioxidant defenses (Glorieux et al., 2015; Wang et al., 2018). Given the lack of increase in oxidative stress in our adipocytes under hypoxia (Figure 5), catalase and SOD1 are not being solicited, which could explain our results. Further to this, El Amine et al. (2023) have shown that adipocytes exposed to hypoxia exhibit a significant reduction in ATP levels (approximately 20%, $p = 0.003$) along with a modest increase in the ADP/ATP ratio. This metabolic shift may indicate enhanced proton leak across the mitochondrial inner membrane, a process known to attenuate ROS production by reducing electron pressure within the respiratory chain (Boveris & Chance, 1973; Herrero & Barja, 1997). Consequently, such a mechanism could contribute to lower ROS levels under hypoxic conditions, thereby diminishing the reliance on

classical antioxidant defenses such as catalase and SOD1. Additionally, other studies have suggested that catalase, in the form of catalase-peroxidase, might require oxygen to fulfill its oxidase activity, given that it is heme-dependent (Glorieux et al., 2015). This could in part explain why catalase expression was reduced under low oxygen availability.

Furthermore, hypoxia did not modulate the expression of SOD2 (Figure 10), which codes for superoxide dismutase 2, an enzyme that localizes in the mitochondria (Karnati et al., 2013). Like catalase and SOD1, SOD2 serves as a potent antioxidant in the presence of oxidative stress (Wang et al., 2018), which was not evident in our cellular model as shown by the results from the DCFDA assays (Figure 5). However, instead of being reduced by hypoxia like catalase and SOD1, SOD2 showed no significant changes under varying oxygen pressures (Figure 10). This might indicate that the observed hypoxia-dependent downregulation of catalase and SOD1 are linked to cytosolic-specific pathways that do not implicate SOD2. The precise spatial regulation of ROS homeostasis and signaling achieved by compartmentalization of distinct SOD isoforms might be related to the difference in expression between SOD1 and SOD2 (Wang et al., 2018), although our methodology did not allow us to differentiate the localization of ROS within the cell compartments. Overall, these results suggest that hypoxia induced a selective translational response rather than global modulation of antioxidant defenses.

Exogenous βOHB and ROS levels

Building on the hypoxia-only findings, the next objective was to determine whether exogenous βOHB could modulate ROS production in human adipocytes. Ketone bodies have been proposed to exert antioxidant effects by reducing mitochondrial membrane potential, limiting ETC electron leak, and stabilizing redox balance (Brookes, 2005; Jones IV et al., 2016). Nishitani et al. (2022) have also demonstrated the possibility for βOHB to reduce ROS levels, even in a normoxic environment without a distinct induction in ROS production. Accordingly, it was hypothesized that βOHB supplementation would reduce ROS levels, especially under hypoxic conditions.

Contrary to this hypothesis, 48 hours of βOHB treatment did not significantly alter ROS levels after 24 hours of either normoxic or hypoxic conditions (Figure 5). ROS measurements remained stable across βOHB concentrations and oxygen levels, with no evidence of a dosedependent reduction. This finding is consistent with the modest ROS burden observed under

hypoxia alone (Figure 5) and suggests that human adipocytes tightly regulate ROS levels, limiting the detectability of additional antioxidant effects.

Exogenous βOHB and antioxidant gene expression

Beyond its proposed effects on ROS production, βOHB has been shown to influence redox balance through transcriptional regulation of antioxidant genes via inhibition of class I histone deacetylases (Shimazu et al., 2013). Based on this mechanism, the final hypothesis proposed that BOHB would increase antioxidant gene expression in a dose-dependent manner, particularly under hypoxic conditions.

In contrast to this expectation, 48 hours of βOHB treatment did not induce consistent or statistically significant changes in catalase, FOXO3, Mt2, SOD1, or SOD2 expression after 24 hours of either normoxic or hypoxic conditions (Figures 6-10). This is contrary to what Nishitani et al. (2022) found in 3T3-L1 cells, where the same antioxidant genes measured (catalase, FOXO3, Mt2, SOD1 and SOD2) were increased following a 24h exposure to 10mM BOHB under normoxia. This might be due to the differences between 3T3-L1 (mechanistic, metabolic, etc) that could allow 3T3-L1 to react quicker to βOHB supplementation. A longer exposure to βOHB might be necessary to observe an increase in catalase and SOD1 expression in human adipocytes. These findings indicate that βOHB does not independently activate antioxidant transcription in mature human adipocytes, under the experimental conditions we tested.

Exogenous BOHB and adipocyte redox balance

Taken together, these findings show that hypoxia induces adaptive redox responses in human adipocytes, by selective activation of stress-responsive antioxidant genes without significant ROS accumulation or loss of cell viability. In contrast, exogenous βOHB did not significantly alter ROS levels or antioxidant gene expression under either normoxic or hypoxic conditions.

Limitations

Some limitations should be considered when interpreting our findings. First, we used the DCFDA assay to measure ROS. While this method is widely used, it primarily detects hydrogen peroxide-related species and does not provide information on the specific source or subcellular

localization of ROS (Dikalov & Harrison, 2014). As such, it is possible that more subtle or compartment-specific changes in ROS were not captured in this model. Second, the duration of exposure to both hypoxia and βOHB may have been relatively short. Some of the antioxidant effects described in the literature, particularly those involving transcriptional regulation, may require longer exposure times to become detectable (Kim et al., 2014). Third, this study was conducted in an in vitro model using human adipocytes derived from a single donor. While this improves experimental control and internal validity, it does limit generalizability, especially given known variability in adipose tissue across individuals. Finally, while we controlled key variables such as oxygen and glucose, the simplified cell culture environment does not fully replicate the complexity of in vivo adipose tissue, where factors like inflammation, vascularization, and systemic metabolism also influence redox balance. Taken together, these limitations suggest that while our findings are robust within this model, they should be interpreted within the specific experimental context.

Conclusion

Our results indicate that hypoxia does not increase ROS levels in human adipocytes, regardless of the dose. However, exogenous ketones did increase expression of antioxidant genes such as FOXO3 and Mt2, while conversely reducing levels of other antioxidant genes such as catalase and SOD1. These differences might be related to their respective roles in cellular function adaptation under hypoxia, instead of a response to an increase in oxidative stress. To our knowledge, this constitutes the first experiment to look at the effects of exogenous βOHB on ROS levels and antioxidant expression in human adipocytes exposed to a hypoxic environment.

Chapter 6: Perspective

The findings of this thesis contribute to a better understanding of redox regulation in human adipocytes under hypoxic conditions and refine current assumptions regarding the antioxidant role of ketone bodies. While βOHB has been proposed to have antioxidant effects in various experimental models (Newman & Verdin, 2014; Puchalska & Crawford, 2017; Shimazu et al., 2013), our results indicates that its redox capacities in human adipocytes are highly context dependent. Under the experimental conditions of this thesis, exogenous βOHB did not significantly alter ROS levels or antioxidant gene expression, suggesting that ketone signalling does not universally translate into redox changes in human adipocytes. Rather than contradicting existing literature, our findings emphasize the importance of cellular context, metabolic state, and experimental design in shaping ketone-mediated effects.

A key implication of this thesis is that hypoxia should not necessarily be regarded as a direct driver of overt oxidative stress in human adipocytes. In the present model, 24 hours of hypoxic exposure elicited selective and adaptive transcriptional responses, such as with FOXO3 and Mt2, without a significant accumulation of ROS or a reduction in cell viability. The existing literature remains divided on the relationship between hypoxia and ROS production in adipocytes, with some studies reporting marked ROS increases under hypoxic conditions (Carrière et al., 2004; Kim et al., 2014), while others observe minimal or no changes (Alva et al., 2024; Arias et al., 2024). These discrepancies likely reflect differences in experimental design, including the limitations of DCFDA-based ROS measurements (as discussed by Dikalov & Harrison, 2014), the timing of ROS assessment following hypoxic exposure, the subcellular compartment examined (cytosolic versus mitochondrial), and the cellular model used (murine vs human adipocytes, preadipocytes vs fully differentiated adipocytes), among other factors.

Taken together, the findings of this thesis support the interpretation that human adipocytes may not exhibit sustained increases in ROS following a 24-hour hypoxic exposure, at least within the constraints of our experimental model. To further investigate this question, future studies could examine ROS levels following more acute hypoxic exposure (1-2 hours) to determine if human adipocytes have a more transient oxidative response that is quickly mitigated through adaptive redox regulation. Additionally, exploring the potential role of SOD1 and SOD2 in compartmentalizing ROS production, especially between the cytosol and mitochondria, could

provide more insight into the redox control mechanisms in human adipocytes, as suggested by previous research (Wang et al., 2018). Finally, integrating measurements of ATP levels as well as oxygen consumption could help clarify whether the hypoxia-induced expression of FOXO3 and Mt2 we observed reflects broader metabolic adaptations to reduced oxygen availability, as we proposed in this thesis.

Overall, this thesis highlights the capacity of human adipocytes to maintain redox balance under hypoxic stress and suggests that ketone-mediated antioxidant effects are not uniformly expressed across tissues or conditions. By putting an emphasis on context-dependent regulation, the effect of time, and the importance of human-relevant models, this work contributes to a more refined framework for interpreting adipocyte redox responses and informs future investigations into the metabolic and therapeutic implications of ketone signalling.

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Appendix I – Exploratory Findings

Exploratory experiment and findings

Methods

Human preadipocytes were obtained from ZenBio (Cryopreserved subcutaneous preadipocytes, ZenBio Inc., Research Triangle Park, NC, USA). We obtained human subcutaneous preadipocytes from a single 46-year-old female donor with a body mass index of 29.9 kg/m^2 (Lot# LM070622B). Cells were seeded in ZenBio Preadipocyte Proliferation Media (PM-1) according to the manufacturer's instructions in 96-well plates at a density of at least 40,000 cells/cm^2. The plates were then placed in a HeraCell 150iO2 incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 100% humidity and 5% CO2. Once confluence was visually confirmed using a Zeiss Axiovert 40 C microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), usually 3 days after the start of incubation, culture medium was replaced with ZenBio Preadipocyte Differentiation Medium (DM-2) to induce differentiation. After 7 days of differentiation, the medium was partially replaced with ZenBio Adipocyte Maintenance Medium (AM-1) for another 7 days, after which cells were considered mature adipocyte, displaying several lipid droplets.

The mature adipocytes were separated into two groups for each plate: one group received basal medium (BM-1, ZenBio Inc.), and the other received basal medium supplemented with 1µM of insulin (Nishitani et al., 2022). One plate was kept in a normoxic environment at 21% oxygen, and the other was kept in a hypoxic environment at 3% oxygen, using the HeraCell 150iO2 incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 100% humidity and 5% CO2. The samples of cell culture medium and cell lysates were collected at 4 different time points: 12h, 24h, 48h and 72h after starting the initiation of experimental treatments. Culture medium was collected by gentle aspiration and immediately stored at -80°C until future analyses.

To obtain the cell lysates, cells were rapidly washed 3 times with ice-cold PBS and lysed using Qiagen RLT buffer with 1% beta-mercaptoethanol, then promptly put at -80 °C until further processing. Upon thawing, lysates were spun on Qiagen QiaShredder columns to fully break down cell membranes and increase RNA yield. Total RNA was extracted using Qiagen RNeasy Mini Kit (Cat. No. 74104). Complementary DNA (cDNA) was obtained using the Qiagen Quantitect Reverse transcription kit and the maximum volume of RNA template

allowed by the kit (12 µL) on a T-personal Combi thermocycler (Biometra, Gottingen, Germany) and realtime quantitative polymerase chain reaction (RT-qPCR) was conducted using Quantitect primers (Qiagen, Germantown, MD, USA) and MBI EVOlution EvaGreen rtPCR mix (Montreal Biotech Inc., Dorval, QC, Canada) on a RG-3000 Rotor-Gene (Corbett Research Ltd., Mortlake, Australia). The Quantitect primers used had the following Qiagen catalog numbers: Beta-Actin QT00095431; HMGCS2 QT02400440; SCOT QT00046221. Melting curve analyses were performed to ensure that amplification yielded single products. Amplification curves were analyzed using the Rotor-Gene Analysis software 6.1 version 1.93. Amplification efficiency was determined for each individual amplification reaction using the comparative quantitation feature of the Q software, which is based on the fluorescence increase over 4 cycles following "take-off" of the fluorescence signal. The "take-off" point corresponds to the point at which the second derivative of the amplification curve reaches 20% of its maximum (determined automatically by the Rotor-Gene Analysis software). Average amplification factors were very stable within and across genes (averaging 1.7 to 1.8). Fluorescence thresholds for CT determination were determined for each gene and arbitrarily assigned the value corresponding to half the average fluorescence at take-off cycle. Fold-changes were calculated using the formula (Pfaffl, 2001):

$$Fold\ \ change = \frac{E_{tar}^{(CT_{tar\ ctl} - CT_{tar\ treat})}}{E_{ref}^{(CT_{ref\ ctl} - CT_{ref\ treat})}}$$

where E corresponds to the average amplification efficiency of each gene within the same experiment (i.e., E was averaged from individual reactions for each gene for each experiment).

The cell culture medium samples were analyzed using a colorimetric assay to quantify the concentration of the ketone body βOHB (β-Hydroxybutyrate Colorimetric Assay Kit, Cayman Chemicals, Item No. 700190). Absorbance readings were taken at 445nm using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, Vermont, USA).

Statistical Analyses

The concentrations of βOHB in the cell medium were analyzed using a linear mixed effects model to evaluate the effects of time (12h, 24h, 48h, 72h) and insulin (insulin and no insulin) within each condition (normoxia and hypoxia). A Tukey's post-hoc test was conducted for significant results. All statistical analyses were conducted using R Statistical Software (v4.3.2; R Core Team 2023). Significant p-values were analyzed, and effect sizes were determined using the emmeans R package (v1.10.0; Russell V. Lenth et al., 2023). All graphs were generated using the flexplot R package (v0.20.3; Fife D, 2024). Tables were generated using jamovi (v2.4.11). ***Results*** βOHB concentration in the culture media increased significantly over 72 hours ($p<.001$) (Figure S1). All other individual and combination of factors were not shown to be significant (data not shown). We ran a Tukey's post hoc analysis to investigate the pairwise comparison of the different time points. We found that all time intervals were significant, save for the interval between 24 and 48 hours. When looking at the raw data, we found that in the hypoxic condition, there was a slight decrease in βOHB concentration between 24 hours and 48 hours, as shown in Figure S1. Although this was not the case for the normoxic condition, this might explain why ketone bodies concentration were not different between these 2 time points.

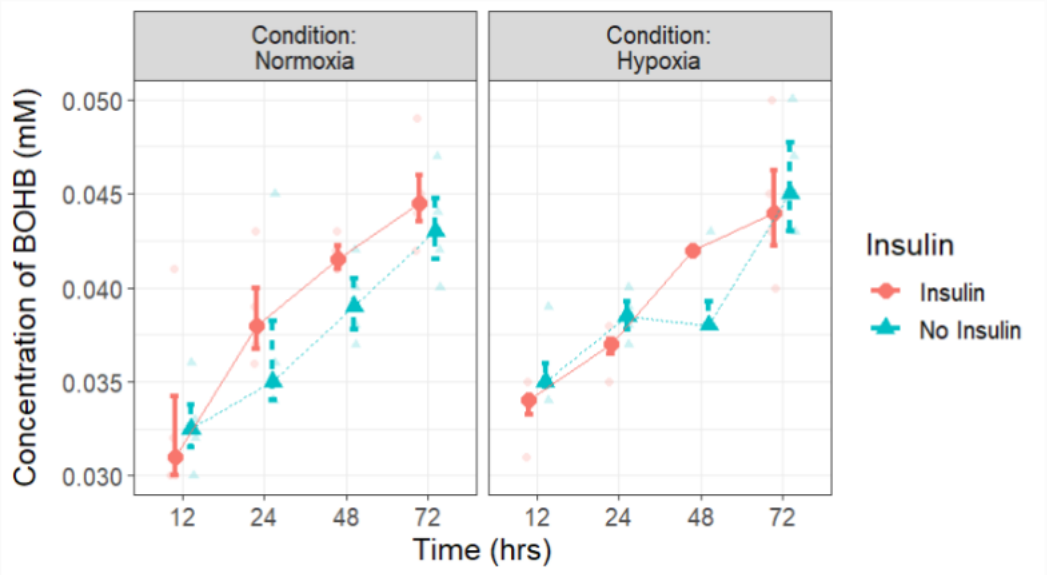

Figure S1. Cell culture medium levels of βOHB over 72 hours following incubation with a basal medium (BM1) with and without insulin in normoxic (21% O2) and hypoxic (3% O2) conditions. Data are presented as the mean (SD) and individual points. Data were compared using a linear model with α set at 0.050.

Overall, the results of the linear model show an increase in βOHB concentration in the cell culture medium over time which is not influenced by the oxygen concentration (hypoxic vs normoxic, p=0.428) nor the presence of 1nM insulin (p=0.531) thus indicating that differentiated human white adipocytes do undergo ketogenesis, albeit without clear modulation by oxygen concentration and insulin. The highest concentration of βOHB measured was approximately 0.045mM after 72 hours of incubation in BM-1 (Figure S1). In comparison, this would fall on the lower end of the physiological range for blood ketone concentration in a healthy adult not following a low carbohydrate diet or a prolonged fast (range 0 to 0.25mM) (Laffell, 1999). In our cell culture model, it is unclear whether ketogenesis increases over time or if another mechanism inhibits the degradation of ketones in the medium, leading to an increase in βOHB concentration over time.

To further quantify the capability of human adipocytes to undergo ketogenesis, we measured the expression of the key rate-limiting enzyme HMGCS2. We observed a low amplification and late take-off (average CT = 32.01) for HMGCS2 with our RT-qPCR analyses, indicating low levels of HMGCS2 RNA in the cell lysates. Furthermore, we were unable to detect HMGCS2 RNA presence in some of our samples, although our control gene (B-actin) did not show any anomalies, indicating no issues with our protocol or cell culture. As for SCOT, we observed an increase in gene expression under normoxia with insulin, and a decrease in hypoxia, with and without insulin (Figure S2).

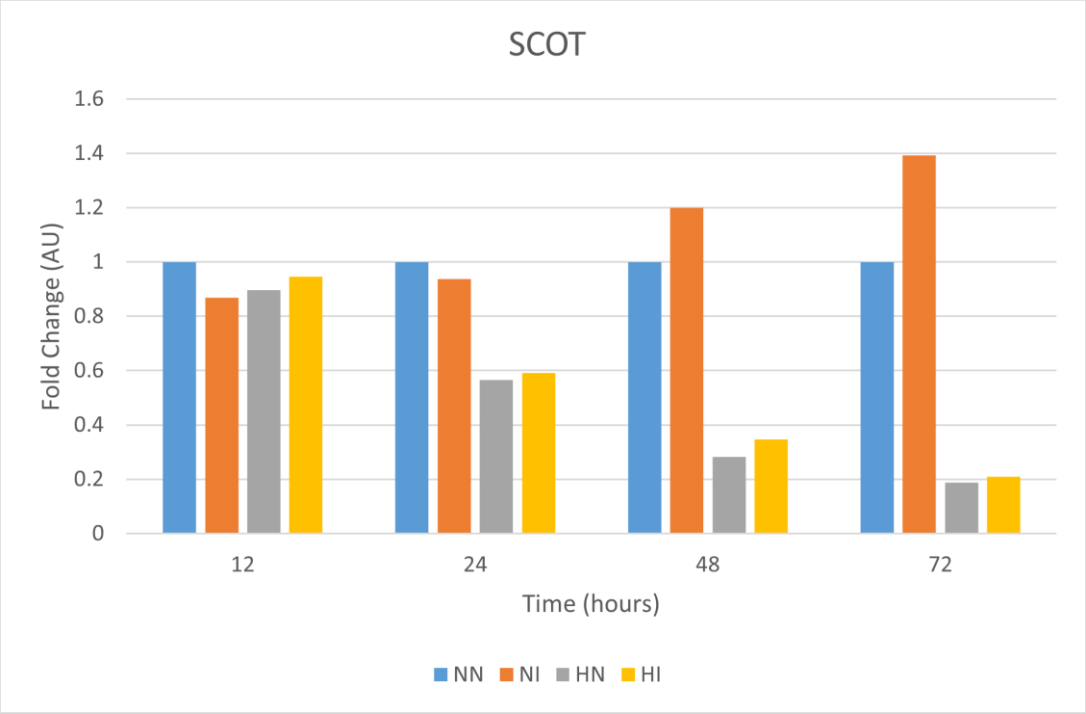

Figure S2. qRT–PCR of SCOT in differentiated human adipocytes under different oxygen and insulin concentrations (n=1). Data are presented as the mean (SD). Data were compared using a linear mixed-effects model with α set at 0.050. AU; arbitrary units, NN; normoxia (21% oxygen) no-insulin, NI; normoxia with insulin (1µM), HN; hypoxia (3% oxygen) no-insulin, HI; hypoxia with insulin (1µM).

These preliminary observations suggest that adipocyte ketogenesis may be modulated by mechanisms that differ from that of hepatocytes. In liver cells, insulin acts as an inhibitor and prevents the production of ketone bodies (Alberti et al., 1978). This is due to the nature of ketogenesis, which is to supply the brain and other vital organs with an alternate fuel source when carbohydrate and glucose stores are low. After consumption of a meal high in carbohydrates or glucose, the pancreas secretes insulin to stimulate glucose uptake and normalize the glycemia. A higher concentration of insulin signals to the liver that there is enough glucose available for the body, and as such ketogenesis is inhibited. Insulin acts by increasing fatty acid synthesis in hepatocytes and increasing production of malonyl-COA, which in turn inhibits the acylcarnitine transferase system that supplies fatty acid to the mitochondria (Alberti et al., 1978). The reduction in fatty acid uptake diminishes the capacity for beta oxidation that creates the substrates needed for hepatocyte ketogenesis.

Since insulin did not significantly lower the concentration of βOHB in the culture medium of our cells, we believe that it has no effect on the rate of ketogenesis in human white

adipocytes. Because insulin is known to modulate many steps in the cascade of ketogenesis reactions in hepatocytes (Alberti et al., 1978), at least one of these steps must be different in adipocyte ketogenesis.

Our results also showed that adipocyte ketogenesis is not modulated by hypoxia, as opposed to hepatocyte ketogenesis. The increase in βOHB concentration over time, even after controlling for insulin (condition x insulin = 0.147), showed no statistical difference between normoxia and hypoxia. Studies in humans have shown that blood ketone levels increase in hypoxic states as opposed to normoxia, although the reasons for this have yet to be elucidated (Marcoux et al., 2022). We believe that the nature of our *in vitro* experiment might explain the difference in our results, as adipocytes were not only exposed to a much stronger hypoxic environment (3% as opposed to 12% in humans), but also since the rate of ketogenesis in adipocytes might be much lower than the capacity of hepatocytes, and thus be less impacted by changes in ketogenesis capacity. Hepatocytes can contain more than 2000 mitochondria per cell (Parente et al., 2023), whilst white adipocytes contain very few mitochondria (Cedikova et al., 2016). Since ketone bodies are mitochondria-produced molecules, it stands to reason that adipocytes produce a low amount in comparison to hepatocytes. As such, small changes in hepatocyte ketogenesis are more likely to be observed *in vivo*, while they may go unnoticed in the low concentration of ketones produced by adipocytes. On top of this, our βOHB concentrations were on the lower end of the range of detection for the colorimetric assay, which might lead to a lower sensitivity in βOHB changes.

Significance

Given that our findings did show the production of βOHB in human adipocytes, but a low expression of the key rate-limiting enzyme HMGCS2 as well as a lack of modulation by oxygen and insulin conditions, we pivoted our research focus to further understanding the role of exogenous ketones on human adipocytes. From the perspective of my thesis, the debate surrounding adipocyte ketogenesis has practical implications. Since ketogenesis in human adipocytes is not influenced by oxygen variations, then exposure to different oxygen partial pressures would not modify βOHB levels in cell culture, thus eliminating this as a potential confounder, ensuring that any changes observed in our experiments can reasonably be attributed to exogenous ketone action. This distinction strengthens the interpretability of my experimental

design, which aims to isolate the direct impact of exogenous ketones in hypoxia-exposed human adipocytes.