Endothelial-derived Erythropoietin: A Novel Player in Regulating Carbohydrate Metabolism

by

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ABSTRACT

ENDOTHELIAL-DERIVED ERYTHROPOIETIN: A NOVEL PLAYER IN REGULATING CARBOHYDRATE METABOLISM

Laura Farquharson
University of Guelph, 2018

Erythropoietin (EPO), known as the master regulator of erythropoiesis, has emerged as a pleiotropic cytokine with non-hematopoietic roles (e.g., metabolic regulation). The kidney is well recognized as the major source of EPO; however, non-renal sources of EPO have been identified. As endothelial cells are pervasive and stabilize metabolic regulators, they are well suited for metabolic regulation. The aim was to determine the physiological significance of endothelial-derived EPO. We hypothesized that the endothelium is a source of EPO that functions in a paracrine fashion to maintain energy homeostasis. Deletion of endothelial-derived EPO resulted in exercise intolerance that was not a result of impairments intrinsic muscle function, and exhibited reduced glycogen content of glycolytic muscles. The modification in local carbohydrate metabolism did not result in changes to whole-body substrate metabolism at rest. This thesis demonstrates that endothelium is a non-renal source of physiologically relevant EPO that is necessary for maintenance of carbohydrate metabolism.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ActB</td>
<td>β Actin</td>
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<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>EPOR</td>
<td>EPO receptor</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia Inducible factor</td>
</tr>
<tr>
<td>JAK2</td>
<td>Janus family protein tyrosine kinase 2</td>
</tr>
<tr>
<td>ND</td>
<td>Non-detectable</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator 1-alpha</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
</tr>
<tr>
<td>PPARα</td>
<td>Peroxisome proliferator-activated receptor alpha</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>rhEPO</td>
<td>Recombinant human EPO</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
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<td>STZ</td>
<td>Streptozotocin</td>
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CHAPTER ONE

REVIEW OF THE LITERATURE
1.1 EPO Regulation by Hypoxia Inducible Factor

While much of the current understanding of the role of erythropoietin (EPO) is related to promoting erythropoiesis, accumulating evidence suggests that EPO has diverse non-erythropoietic functions. In response to decreases in cellular oxygen (O₂), hypoxia inducible factors (HIF) enable transcription of hypoxic-response genes. HIF is a heterodimer consisting of an O₂-regulated α subunit and a constitutively expressed β subunit. The HIF-α subunit is continuously synthesized and degraded under normoxic cellular conditions via the ubiquitin-proteasome pathway. O₂ dependent proteins (e.g., prolyl-4- hydroxylase domain proteins) target the O₂ dependent degradation domain within the α subunit for hydroxylation, which allows for downstream recruitment of ubiquitin-proteasome complex that mediates proteolysis. Under hypoxic conditions, HIF-α subunit escapes ubiquitination, via inactivation of the O₂ dependent proteins, and translocate into the nucleus. Here, HIF-α will form a complex with HIF-β, subsequently binding to the Hypoxic Response Element of target genes, facilitating transcription of hypoxia response genes (e.g., vascular endothelial growth factor, EPO).

Recently, a number of findings indicate that HIF-α stabilization occurs under normoxic conditions. Phosphatidylinositol 3-kinase (PI3K)/Protein Kinase B (PKB)(also known as AKT) pathway induces HIF-α stabilization through phosphorylation. No evidence, thus far, has displayed direct phosphorylation by AKT; instead, most evidence indicates that glycogen synthase kinase-3 (GSK3), a target of the AKT pathway, acts directly on HIF-α stabilization. GSK3 exhibits direct phosphorylation of HIF-α in the O₂ dependent degradation domain under normoxic conditions. Protein kinase A inhibits the proteasomal degradation of HIF-α in an O₂-independent manner through phosphorylation, thus enhancing the transcriptional activity of target genes. These data suggests that HIF-α stabilization through phosphorylation is independent of cellular O₂, indicating potential physiologically relevant roles of HIF targets during normoxia.
Stabilization of HIF results in upregulation of genes to increase O₂ delivery (e.g., erythropoiesis, angiogenesis) and a shift towards anaerobic metabolism. Since the discovery of HIF⁶, two distinct alpha isoforms have been well characterized; HIF-1α and HIF-2α, which are differentially expressed. For example, HIF-1α is expressed ubiquitously in all nucleated cells¹⁴ whereas HIF-2α expression is limited to the endothelium, lung, heart, brain, liver and kidney¹⁵. HIF-1α and HIF-2α share certain gene targets, including EPO, vascular endothelial growth factor and glucose transporter 1¹⁶,¹⁷,¹⁸, but also behave independently. For instance, HIF-1α regulates genes associated with glycolytic metabolism (e.g., phosphofructokinase, phosphoglycerate kinase 1)¹⁷,¹⁹, whereas HIF-2α is involved with genes that promote iron absorption (e.g., divalent metal transporter 1)²⁰. Although both HIF-1α and HIF-2α are proficient in regulating erythropoiesis, HIF-2α is the principal regulator of hypoxia-induced EPO expression²¹,²²,²³.

1.2 Structure of EPO and the EPOR

The gene for EPO is located on chromosome 7 for humans and chromosome 5 for mice. EPO consists of 5 exon-coding regions and 4 introns²⁴. The EPO protein is made up of 165 amino acids²⁴,²⁵ and is heavily glycosylated with four oligosaccharide side chains (Figure 1.1): 3 N-linked (Asp24, Asp 38 and Asp 83)²⁶,²⁷ and one O-linked side chain (Ser126)⁴ that is found in human but not murine; whether this is unique to humans is unknown. It has a molecular weight of ~34kDa²⁴, of which 40% of the total molecular weight is accounted for by glycosylation, which is critical for receptor binding, stability within serum and solubility of the protein²⁷,²⁸. Interestingly, EPO is highly conserved with 80-82% sequence homology in mouse, rat, sheep and pig when compared to the human gene²⁹ suggesting potential for similar hormone action between species.
To assess the non-erythropoietic roles of EPO, studies typically rely on supraphysiological dosages of recombinant human EPO (rhEPO). While endogenous EPO and rhEPO have identical amino acid sequences, isoelectric focusing shows they differ in glycosylation\(^\text{31}\) (Figure 1.2). Isoelectric focusing is an electrophoresis technique that separates proteins based on their isoelectric point (pH at which a protein has no net charge); therefore, this technique is used to assess differences in post-translational modifications (e.g., phosphorylation, glycosylation\(^\text{32}\)). Thus, investigation into the results seen with rhEPO treatment is necessary for the consideration of rhEPO as a pharmaceutical agent.

Considering the importance of the glycosylation for receptor binding, the role of endogenous EPO – at physiological levels – remains to be established and is a goal of this thesis.

During normoxia, multiple glycoforms of EPO exist within human blood, which raises the likelihood of tissue-specific glycoforms\(^\text{27}\). Interestingly, as compared to the kidney, the astrocytes produced a different glycoform of EPO\(^\text{33}\). As glycosylation of EPO is important for receptor binding, tissue-specific isoforms of EPO could have altered physiological relevance by modifying receptor affinity or selectivity.
Figure 1.2 | Differences in glycosylation pattern of endogenous EPO compared to recombinant EPO shown by isoelectric focusing
Isoelectric focusing image of recombinant erythropoietin (rEPO), endogenous urinary erythropoietin (uEPO) and the erythropoiesis-stimulating agent Aranesp (Darbepoietin alpha)\textsuperscript{34}.

The EPO receptor (EPOR) consists of an extracellular ligand binding domain, a single transmembrane domain and an intracellular domain\textsuperscript{35}. EPO exerts its effects by binding to the EPOR that is expressed in several forms (e.g., full length, truncated\textsuperscript{36} and soluble\textsuperscript{37}) as either a homodimer or heterodimer with the \(\beta\)-common receptor. Previous reports observe EPOR physically associating\textsuperscript{38} and interacting\textsuperscript{39} with the \(\beta\)-common receptor subunit. However, mice with the \(\beta\)-common receptor knocked out exhibit normal erythropoiesis. This suggests that the heterodimer complex with the EPOR and \(\beta\)-common receptor could mediate, at least partially, the non-erythropoietic functions\textsuperscript{40}. Alternative mRNA splicing yields a soluble form of the EPOR circulating in human blood\textsuperscript{41,37,42}. The soluble EPOR directly competes for unbound, circulating EPO with membrane-bound EPOR\textsuperscript{43}, thus altering the interaction between EPO and membrane-bound EPOR. While the soluble EPOR is able to bind EPO, the physiological role remains largely unknown.
Binding of EPO to the extracellular domain of the EPOR causes a conformational change of the intracellular domain, which results in activation of Janus family protein tyrosine kinase 2 (JAK2) (Figure 1.3). JAK2 is constitutively associated with the intracellular domains of the EPOR. Once JAK2 is phosphorylated, tyrosine residues on EPOR are phosphorylated initiating downstream signaling cascades. The phosphorylated tyrosine residues act as docking sites for the Signal transducers and activators of transcription (STAT) family of proteins, PI3K/ AKT, and Mitogen-activated protein kinase (MAPK), depending on the cell type.

![Figure 1.3 | EPOR Signaling](image)

Binding of EPO causes a conformational change of the EPOR dimers resulting in transphosphorylation (P) of JAK2 this allows for phosphorylation of the EPOR and subsequent activation of downstream signaling proteins.

### 1.3 Extra Renal Sources of EPO and Non-Erythroid Expression of the EPOR

The kidney is well recognized as the major source of EPO; however, non-renal sources of EPO mRNA have been identified, although the physiological relevance has yet to be elucidated. During development, the major site of fetal EPO production is the liver while after birth, the kidney becomes the principal EPO producing organ. EPO mRNA is detectable in cardiomyocytes and astrocytes of the brain and spinal cord. In females, there is evidence of both hypoxia and estrogen-
dependent synthesis of EPO in reproductive organs (e.g., uterus\textsuperscript{63,64}, oviducts placenta\textsuperscript{65}). In addition, EPO expression is observed in the spleen, skeletal myoblasts and skeletal muscle tissue\textsuperscript{66–68,69}. The expression of EPOR in non-erythroid cells provides support for EPO as a pleiotropic cytokine. Unfortunately, the functions of these extra renal sources of EPO have yet to be investigated. EPOR expression is pervasive with mRNA identified in endothelial cells\textsuperscript{70–73}, brain\textsuperscript{33,60,61,74,75}, adipose tissue\textsuperscript{76,77}, proopiomelanocortin (POMC) neurons\textsuperscript{76}, pancreases\textsuperscript{78}, heart\textsuperscript{58,79,80} and skeletal muscle\textsuperscript{66,68,81,82}. The expression of the EPOR beyond erythroid cells in other tissues further provides evidence for the possibility of multiple physiologically relevant roles of EPO.

1.4 Non-Erythropoietic Roles of EPO

1.4.1 EPO and Cardiac Function

In anemic patients treated with rhEPO, observations of improved cardiac output and cardiac exercise performance\textsuperscript{83} suggest the possible role of EPO in modulating cardiac function. Interestingly, rhEPO exhibits positive inotropic and lusitropic activity. Treatment of isolated atria with rhEPO from normoxic mice demonstrated increases in contractility, while treatment with anti-rhEPO antibody reduced contractility\textsuperscript{84}. Similarly, Kaygisiz et al. (2006) observed that rhEPO increased cardiac contractility, in a dose-dependent manner, in isolated rat hearts\textsuperscript{85}. These findings were confirmed using intact muscle strips from both murine and human left ventricle, where rhEPO treatment increases twitch tension and peak sarcomere shortening\textsuperscript{86}. rhEPO treatment of the intact muscle strips also exhibits increases in maximum relengthening velocity (index of relaxation), therefore, adding lusitropic agent to the list of rhEPO effects on cardiomyocyte and ventricular muscle preparations\textsuperscript{86}. Thus, at supraphysiological levels, rhEPO is both an inotropic and lusitropic agent \textit{in vitro}. However, whether endogenous EPO is an inotropic agent \textit{in vivo}, in response to physiological levels, remains to be investigated.
1.4.2 EPO and Skeletal Muscle

1.4.2.1 EPO and Skeletal Muscle Function

Patients suffering from severe renal failure exhibit reductions in circulating EPO levels, which leads to chronic anemia. Severe renal failure patients are exercise intolerant that cannot be fully accounted for by reductions in oxygen carrying capacity. Indeed, there are structural and functional abnormalities of their skeletal muscle, which partially contributes to impaired exercise capacity and increased fatigability. Interestingly, anemic patients treated with rhEPO experience improvements in quadriceps contraction, once hemoglobin levels are normalized (average 13.5wks of rhEPO therapy to reach 11g/dL hemoglobin). The duration and amplitude of contraction are increased suggesting that some of the improvements observed in exercise tolerance, following correction of anemia, is due to enhancements in skeletal muscle function. However, skeletal muscle EPO-overexpressing mice, with increased hematocrit, exhibit no changes in muscle force production or fatigue resistance. Similarly, no effect on resistance to fatigue and maximal twitch tension is observed in EPO deficient mice (EPO-Tg mice), despite hematocrit levels similar to anemic patients. Therefore, this suggests EPO signaling within skeletal muscle is not necessary for baseline function but could play a role in improving function indirectly by increasing oxygen delivery to impaired muscle.

1.4.2.2 EPO response with Exercise

Dependent on the duration and severity of exercise, skeletal muscle can become transiently hypoxic. In exercising skeletal muscle, acute exercise results in elevated HIF-1α protein levels in human vastus lateralis muscle. The HIF-1α mRNA expression is unchanged, which suggests post-translational mechanisms account for the up-regulation in this protein. In response to exercise-induced hypoxia, stabilization of HIF-1α results in a subsequent improvement in metabolic capacity and oxygen supply in skeletal muscle. HIF-1α-targeted genes increase oxygen delivery through EPO-mediated erythropoiesis and vascular endothelial growth factor-induced angiogenesis.
muscle function is improved through increases in glucose transporters and expression of glycolytic enzymes\textsuperscript{94}. The mRNA of the downstream target of HIF-1\(\alpha\), \textit{EPO}, is significantly increased six hours post exercise\textsuperscript{93}. Despite this findings, a number of studies conclude that circulating EPO concentrations are not increased with exercise, and this is not affected by duration, intensity or type of training in humans\textsuperscript{96,97}. Rundqvist and colleagues observe that during 60 minutes of cycling, the exercising leg releases EPO into circulation, while during recovery EPO is absorbed into the exercising leg (arterial vs venous EPO concentration)\textsuperscript{68}. No changes in \textit{EPO} expression or protein content within the skeletal muscle are detectable two hours after exercise\textsuperscript{68}. The post exercise time point chosen for evaluation of \textit{EPO} expression could be why no change was detected. Rundqvist and colleagues did detect EPOR activation in the skeletal muscle with acute exercise suggesting signaling through EPOR is involved in exercise-induced skeletal muscle adaptation, thus extending the biological role of EPO into the skeletal muscle\textsuperscript{68}. In contrast to what is observed in humans, mice gastrocnemius muscle exhibits increases in the expression of \textit{EPO} immediately post-exercise\textsuperscript{98}. In mice, endurance training increases muscle \textit{EPO} expression but, surprisingly, increases in EPO protein is not detected\textsuperscript{99}. As stated, investigation into tissue specific EPO production is hindered by poor efficiency of anti-EPO antibodies. Commercially available anti-EPO antibodies have poor specificity for endogenous EPO due to its glycosylation, which interferes with epitope recognition. This could obstruct the identification of physiological significant tissue-specific isoforms of EPO, for example, in skeletal muscle in response to exercise.

\subsection*{1.4.2.3 EPO and Angiogenesis}

During exercise, stabilization of HIF-1\(\alpha\) in response to severe oxygen stress occurs in skeletal muscle. The stabilization of HIF-1\(\alpha\) results in subsequent increases in angiogenesis by upregulating angiogenic factors (e.g., vascular endothelial growth factor)\textsuperscript{94,95}. The angiogenic factors increase capillarization to provide additional oxygenated blood to the muscle\textsuperscript{100}. Similar to vascular endothelial
growth factor, EPO has been described as exerting pronounced angiogenic effects\(^{101-104}\). One of the suggested mechanisms by which EPO promotes angiogenesis is by increasing the level of vascular endothelial growth factor within the tissue\(^{105,106}\). In both marrow stromal cells and brain, treatment with rhEPO enhances the release of vascular endothelial growth factor\(^{101,102,103,107}\). As vascular endothelial growth factor is vital for skeletal muscle capillary growth\(^{100}\), it is possible that EPO can mediate angiogenic affects by promoting vascular endothelial growth factor within muscle. In healthy male participants, rhEPO treatment did not affect capillarization (capillaries per fiber)\(^{82,108}\). In agreement, 3 weeks of rhEPO treatment in rats did not affect angiogenesis in the rat’s soleus muscle\(^{109}\). As well, no increase in vascular endothelial growth factor protein expression is observed in both the human and rat muscle\(^{108,109}\). Previous studies have used models where the tissues are undergoing growth or repair (e.g., traumatized muscle, myocardial infarction, cerebral infarction)\(^{101,102-104}\) in which expression of other angiogenic compounds would be enhanced relative to maintenance. These data suggests that rhEPO treatment alone cannot induce angiogenesis when the tissue is healthy.

### 1.4.2.4 EPO as a proliferative agent

Evidence of an additional role of EPO in skeletal muscle is in muscle fiber growth. EPO binding to the EPOR stimulates STAT5, where STAT5 activation is known to control cell proliferation\(^{110}\). STAT5 activates the PI3K-AKT signaling pathway\(^{80,111}\) that is vital in regulation of skeletal muscle hypertrophy\(^{112-114}\). Thus, activation of the EPOR may contribute in regulating skeletal muscle fiber growth through activation of the downstream PI3K-AKT signaling pathway. Ogilvie and colleagues first observed the expression of the \textit{EPOR} mRNA and protein in mouse C2C12 myoblasts and primary satellite cells\(^{66}\). Several groups have more recently demonstrated \textit{EPOR} mRNA and protein expression in isolated human primary myoblasts and satellite cells\(^{68,81}\) as well as in human muscle tissue\(^{68,82,115}\). However, is it unknown if activation of skeletal muscle EPOR stimulates the same signaling cascade as in erythroid progenitor cells. Treatment of mouse C2C12 myoblasts with
rhEPO demonstrates increases in JAK2, STAT5 and AKT phosphorylation. However, similar effects are not seen in human skeletal muscle in vivo. In accordance with its role in erythroid progenitor cells, EPO promotes proliferation in mouse myoblasts. These findings are not confirmed in human and rat myoblasts. The lack of consistency across tissues and species emphasizes the need for further investigations.

Modifying endogenous EPO signaling results in changes to muscle fiber growth. EPO-overexpression in mouse hind limb induces an increase in muscle mass under baseline conditions. No difference in total protein concentration (per gram of muscle) indicates the increased muscle mass is due to muscle fiber hypertrophy or hyperplasia and not the formation of edema. Conversely, myoblasts isolated from mouse muscle with the EPOR restricted to erythroid cells (TgEPOR) did not proliferate in culture. Interestingly, TgEPOR mice have normal skeletal muscle development when compared to Wt. However, with muscle damage/ injury, the loss of EPO signaling within skeletal muscle becomes apparent when reductions in satellite cells and impaired myoblast proliferation that inhibits muscle repair in TgEPOR mice. These data suggest that in muscle, EPO might contribute more importantly to the stress response/muscle repair rather than muscle development during embryogenesis.

1.4.3 EPO and Metabolism in Health and Disease

While EPO is well known for its erythropoietic effects, it is emerging as a cytokine with roles in metabolic homeostasis. Over 20 years ago, indicators of improved metabolism (e.g., increased insulin sensitivity, normalized serum insulin levels, improved fasting glucose) were observed following rhEPO therapy in hemodialysis patients and in diabetic patients with repeated phlebotomies, which causes anemia and a resultant increase in endogenous EPO. These initial results led to the investigation of EPO’s role in regulating metabolic homeostasis in health and disease.
More than one fifth of Canadians are obese, costing an estimated $5 billion annually. Furthermore, rates of obesity are projected to increase to $8.8 billion by 2021. Obesity, defined as excess adipose tissue, is linked with an increased risk of developing diabetes mellitus; specifically type 2 diabetes mellitus. Diabetes is a multi etiological disease (e.g., genetics, diet) with the unifying characteristic of chronic hyperglycemia (i.e., impaired glucose tolerance). In healthy subjects, postprandial blood glucose level increase, which stimulates the pancreas to secrete insulin. This, in turn, promotes insulin-induced glucose uptake within the primary storage sites, skeletal muscle and the liver, thereby lowering blood glucose levels back to baseline. This process is dysregulated in obese patients, who experience insulin resistance in part due high levels of circulating fatty acids, which leads to development to type 2 diabetes mellitus. Therefore, more insulin must be secreted postprandial to ensure adequate glucose uptake within the cells of insulin resistant individuals. Over time, β-cells’ (insulin secreting cells in the pancreas) insulin production becomes blunted due to the constant strain. Subsequently, β-cells are unable to meet the insulin demand, leading to chronic hyperglycemia. Therefore, therapeutic agents (e.g., exogenous insulin) are needed to help maintain blood glucose levels. Patients unable to control their blood sugar risk severe complications of this disease, as chronic hyperglycemia damage blood vessels and can result in cardiovascular disease, neuropathy, nephropathy and retinopathy. While insulin is well regarded as a key regulator of glucose homeostasis, all cytokines involved in glucose metabolism remain to be identified. Further research into other cytokines could provide insight into potential therapeutic targets for patients who are insulin resistant.

1.4.3.1 Phenotype of Mice with EPOR Restricted to Erythroid Tissue

EPO/EPOR signaling is vital for the maintenance of metabolic homeostasis. EPOR knockout mice are embryonically lethal, although, expression of the EPOR specifically in erythroid cells (Tg mice) can rescue the pathology, restoring normal erythropoiesis and preventing mortality. Tg mice
exhibit no gross abnormal morphology, although a disproportionate increase in body weight occurs from the first week postnatal. Obesity is evident by 4 months in female and 6 months in male Tg mice, which is attributed to the increase of fat mass with no change in lean mass. The accelerated obese phenotype, evident in female Tg mice compared to male Tg mice, suggests estrogen-dependent EPO signaling. The development of obesity in the Tg mice suggests EPO is involved in the regulation of fat mass accumulation. Frequently, insulin resistance and glucose intolerance are associated with accumulation of white adipose tissue. Interestingly, on a normal chow diet, the Tg mice are already obese with insulin resistance and glucose intolerance, which suggests loss of EPO signaling is sufficient to induce a pre-diabetic state regardless of food intake. The obese phenotype observed in the Tg mice precedes the onset of insulin resistance, which suggests a type 2 diabetes-like phenotype. Examination of the white adipose tissue revealed increased expression of pro-inflammatory adipokines (e.g., tumour-necrosis factor-α) and decreased expression of interleukin-10 (anti-inflammatory cytokine). These data suggest that loss of EPOR in non-erythroid tissues contributes to elevated inflammation, leading to the development of insulin resistance.

Expression of the EPOR in erythroid cell precursors is vital for the erythropoietic role of EPO. To evaluate if non-erythroid cell EPOR expression is associated with the fat mass accumulation, Wt and Tg mice are treated with rhEPO for 3 weeks. Both Wt and Tg mice exhibit the expected increase in hematocrit, while Tg mice do not display the reductions in fat mass observed in Wt mice. This suggests that the effect of EPO on fat mass accumulation is dependent on EPOR signaling in non-erythroid tissues and is not associated with changes in hematocrit. When investigating the role of EPO in regulating energy homeostasis, Tg mice exhibit reductions in total oxygen consumption and respiratory quotient. This suggests a shift towards increased fat metabolism. When normalized to food intake, Tg mice gain more body weight suggesting decreased energy expenditure compared to Wt mice. These data suggest EPO contributes to the central regulation of energy homeostasis.
The murine hypothalamus expresses genes involved in regulating activity, food intake and energy homeostasis\textsuperscript{135}. In mice, the EPOR localizes in the proopiomelanocortin (POMC) neurons of the hypothalamus\textsuperscript{76}. The POMC neurons sense change in peripheral regulatory hormones, leptin and insulin, which, in turn, secrete hormones (e.g., \(\alpha\)-melanocyte stimulating hormone) that decrease food intake and increase energy expenditure\textsuperscript{136,137}. In rhEPO-treated Wt mice, hypothalamus expression of POMC increases, whereas \(Tg\) mice exhibit reductions in POMC expression\textsuperscript{76}. In both rhEPO-treated Wt mice and \(Tg\) mice, expression of other hypothalamic neuropeptides are not affected\textsuperscript{76}. In agreement, Wt neuronal cultures treated with rhEPO show increases in POMC expression that is in response to JAK/STAT\textsubscript{3} activation\textsuperscript{138}. Dey et al. observes that the \(Tg\) mice have blunted leptin-induced STAT\textsubscript{3} activation, which is thought to be due to the 4-fold decrease in POMC expression\textsuperscript{138}. These findings suggest EPO may have a central regulatory role in energy metabolism by targeting the hypothalamus.

\subsection*{1.4.3.2 Fat specific deletion of EPOR}

Adipose tissue is recognized as a highly active metabolic and endocrine organ\textsuperscript{139}. Adipocyte function is disrupted in obesity, suggesting adipose tissue plays a role in both development and progression of insulin resistance\textsuperscript{140}. The EPOR, which is expressed in multiple non-erythroid tissues, is highly expressed in white adipose tissue\textsuperscript{76}. The expression of the EPOR in adipose tissue suggests endogenous EPO signaling might be involved in maintaining metabolic homeostasis, which could protect against obesity. When comparing to Wt, adipose specific deletion of the EPOR (\(\Delta\)EPOR\textsuperscript{Adipose}) leads to subsequent increases in body weight and adipose accumulation\textsuperscript{141}. The increase in body weight gain in \(\Delta\)EPOR\textsuperscript{Adipose} mice are comparable to that observed in the \(Tg\) mice\textsuperscript{76}. This suggests that the loss of EPOR signaling in adipose tissue is a large contributor to the increase in fat mass in the \(Tg\) mice. \(\Delta\)EPOR\textsuperscript{Adipose} mice were less active and exhibited reductions in oxygen consumption and respiratory quotient\textsuperscript{141}. When challenged by a high fat diet, the \(\Delta\)EPOR\textsuperscript{Adipose} mice become glucose
intolerant and insulin resistant, an expected result with an obese phenotype\textsuperscript{141}. Although body weight was further accentuated on the high fat diet, food intake was comparable with high fat diet controls\textsuperscript{141}, suggesting reduced energy expenditure. In line with the results from \textit{Tg} mice (EPOR resisted to erythroid tissue)\textsuperscript{76}, these data demonstrate the ΔEPOR\textsuperscript{Adipose} mouse have reductions in energy expenditure\textsuperscript{141}. Insulin contributes to glucose and energy homeostasis through insulin-stimulated protein kinase B (also known as AKT) activation (phosphorylation)\textsuperscript{142}. AKT phosphorylation is reduced in ΔEPOR\textsuperscript{Adipose} mice, while rhEPO treatment in control mice increases AKT activity\textsuperscript{141}. This suggests AKT activation is modified by EPOR signaling, which may have an effect on insulin signaling. These data suggests energy homeostasis is, at least partially, regulated by EPOR signaling within white adipose tissue.

Obesity and type 2 diabetes are linked to mitochondrial dysfunction in adipose tissue\textsuperscript{143,144,145}. An indicator of mitochondrial content is citrate synthase activity, the first component of the citric acid cycle. rhEPO treatment in obese mice increase citrate synthase activity in white adipose tissues, as well citrate synthase activity in ΔEPOR\textsuperscript{Adipose} mice is decreased compared with Wt\textsuperscript{141}. This suggests that the decrease in energy expenditure observed in ΔEPOR\textsuperscript{Adipose} mice is linked to reductions in mitochondrial function in the adipose. Mitochondrial oxidative metabolism can be determined through the measurement of oxygen consumption rate, a measure of mitochondrial respiration. In line with whole body metabolic results, isolated ΔEPOR\textsuperscript{Adipose} mice adipocytes exhibit reductions in the rate of oxygen consumption\textsuperscript{141}. In comparison, isolated adipocytes from rhEPO-treated obese mice exhibit increases in the rate of mitochondrial oxygen consumption. When challenged with palmitate, a substrate for fatty acid oxidation, rhEPO-treated adipocytes increase the oxygen consumption rate\textsuperscript{141}. These data demonstrate that loss of EPO in adipose tissue decreases energy expenditure and could impair fatty acid metabolism. Together this may promote the storage of excess lipids thus accelerating the development of obesity.
To rule out the possibility of other tissues involvement in EPO/EPOR signaling in the maintenance of energy homeostasis, Wang et al. treated Wt and ΔEPOR\textsuperscript{Adipose} mice with rhEPO\textsuperscript{141}. Wt mice treated with rhEPO (on a chow diet) exhibit the expected increase in hematocrit and a significant decrease in body weight\textsuperscript{141}. The ΔEPOR\textsuperscript{Adipose} mice, similar to the Tg mice, demonstrate the expected increase in hematocrit without the reductions in body weight\textsuperscript{141}. These findings suggest that adipose specific deletion of EPOR is sufficient to develop metabolic dysfunction (e.g., obesity, glucose intolerance, insulin resistance). This supports the idea that EPO signaling in adipose contributes to energy homeostasis; however, indirect effects of EPO on whole body metabolism cannot be entirely excluded.

Differences in the background strain of the mice provide conflicting results\textsuperscript{146,147,148}. Luk et al. observed contradicting results to Wang et al. where ΔEPOR\textsuperscript{Adipose} mice are metabolically similar to the controls\textsuperscript{149}. Luk et al. use mice on a mixed background (mixed 129J-C57BL6L/6-FVB/N) while the strain commonly used as a model of obesity is the C57BL/6 mouse\textsuperscript{150}. ΔEPOR\textsuperscript{Adipose} mice, on the mixed background, exhibit no differences body weight gain and adipose tissue mass when compared to control mice\textsuperscript{149}. The ΔEPOR\textsuperscript{Adipose} mice, on the mixed background, demonstrate no changes in total oxygen consumption, suggesting no difference in energy expenditure and respiratory quotient compared to controls\textsuperscript{149}. Unlike the results from Wang et al.\textsuperscript{141}, ΔEPOR\textsuperscript{Adipose} mice, on the mixed background, do not become glucose intolerant and insulin resistant when fed a high fat diet\textsuperscript{149}. This suggests that adipocyte EPOR signaling is not required for maintenance of glucose homeostasis. The difference between strains emphasizes the complexity of EPO signaling. Interestingly, EPOR expression in diabetic patients did not differ compared to healthy human, suggesting the receptor is not induced with chronic metabolic stress\textsuperscript{149}. The authors indicate that the observed results are consistent with the proposed mechanism of Teng et al.\textsuperscript{76} and that rather than through direct EPO signaling in adipose, EPO regulates energy metabolism by inducing hypothalamic POMC expression\textsuperscript{149}. 

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Table 1.1 | Various transgenic EPO receptor mice

<table>
<thead>
<tr>
<th>Reference</th>
<th>Model</th>
<th>Receptor Knockout</th>
<th>Circulating EPO</th>
<th>Hematocrit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wu et al. (1995)</td>
<td>EPOR\textsuperscript{\text{-/-}}</td>
<td>Homozygous for EPOR</td>
<td>Embryonically lethal</td>
<td></td>
</tr>
<tr>
<td>Suzuki et al. (2002)</td>
<td>Tg Mice</td>
<td>(EPOR restricted to erythroid cells)</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Wang et al. (2013)</td>
<td>\text{\Delta}EPOR\textsuperscript{Adipose Mice}</td>
<td>Adipose specific EPOR deletion</td>
<td>No change</td>
<td>No change</td>
</tr>
</tbody>
</table>

1.4.3.3 Chronic elevation of EPO

Evidence demonstrating improved metabolic homeostasis (e.g., increased insulin sensitivity, normalized serum insulin levels) in patients treated with rhEPO\textsuperscript{119}, led to the investigation of EPO’s role in regulating metabolism. The results from treatment with rhEPO are valuable, however, considering the importance of the glycosylation for receptor binding, the role of endogenous EPO remains to be elucidated. A useful model of chronic EPO administration is the EPO-\textit{tg6} mouse. The EPO-\textit{tg6} mouse expresses a 12-fold increase in circulating EPO compared to Wt. Although, the higher circulating levels correspond to human, not murine EPO\textsuperscript{151}. On a normal chow diet, EPO-\textit{tg6} mice exhibit reductions in body weight and fat mass as compared to Wt\textsuperscript{152}. This would suggest increased energy expenditure as the EPO-\textit{tg6} mice consume increased amounts of food per week in comparison to the Wt. The EPO-\textit{tg6} mice exhibit lower fasting blood glucose and serum insulin levels\textsuperscript{152}. In accordance, glucose tolerance and insulin sensitivity are improved suggesting enhanced glucose metabolism with elevated serum EPO levels\textsuperscript{152}. EPO-Tg skeletal muscle (skeletal muscle EPO-overexpressing) mice have 100-fold increase in serum EPO, unlike Katz et al.\textsuperscript{152}, the high circulating levels are murine EPO\textsuperscript{91}. However, unlike the EPO-\textit{tg6} mice\textsuperscript{152}, the skeletal muscle EPO-overexpressing mice exhibit no change in body weight compared to controls when fed a normal chow diet\textsuperscript{91}. Contradicting results suggest a difference in the binding affinities to the EPOR within a mouse
model between murine and human EPO. When challenged with a high fat diet, the skeletal muscle EPO overexpressing mice have reductions in adipose mass accumulation compared to the Wt. The decrease in adipose mass accumulation is accompanied by normalization of insulin sensitivity and glucose tolerance in EPO skeletal muscle overexpressing mice on high fat diet. At 1 week of EPO overexpression, while on a high fat diet, increases in the expression of genes involved in lipid metabolism are exhibited, while there was a decrease in expression genes involved in glucose transport and insulin signaling. This suggests the normalization observed in glucose tolerance is most likely an indication of reduced adipose tissue mass rather than a direct influence on glucose clearance rate (i.e., insulin-induced glucose uptake). These data propose clinical implications of supraphysiological levels of EPO in maintaining metabolic homeostasis during diet-induced obesity.

Table 1.2 | Summary of transgenic EPO mice

<table>
<thead>
<tr>
<th>Reference</th>
<th>Model</th>
<th>Form of EPO</th>
<th>Circulating EPO</th>
<th>Cerebral EPO</th>
<th>Hematocrit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vogel et al. (2003)</td>
<td>EPO-Tg6 [Systemic constitutive transgenic EPO overexpression]</td>
<td>Human</td>
<td>↑</td>
<td>Not Determined</td>
<td>90%</td>
</tr>
<tr>
<td>Schuler et al. (2012)</td>
<td>EPO-Tg21 [CNS constitutive transgenic EPO overexpression]</td>
<td>Human</td>
<td>No Change</td>
<td>↑</td>
<td>44%</td>
</tr>
<tr>
<td>Hojman et al. (2009)</td>
<td>Skeletal muscle transient conditional transgenic EPO overexpression</td>
<td>Mouse</td>
<td>↑</td>
<td>Not Determined</td>
<td>65%*</td>
</tr>
</tbody>
</table>

* Hematocrit approximately calculated from hemoglobin
Table 1.3 | Summary of metabolic responses in transgenic EPO/EPOR mice

<table>
<thead>
<tr>
<th>Reference</th>
<th>Model</th>
<th>Energetic Stress</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Teng et al. (2011)</strong>&lt;sup&gt;116&lt;/sup&gt;</td>
<td>Tg Mouse (EPOR restricted to erythroid cells)</td>
<td>Normal Chow Diet</td>
<td>↑ Body weight,↑ Accumulation of adipose,↓ Insulin sensitivity,↓ Glucose tolerance,↑ Inflammatory cytokines</td>
</tr>
<tr>
<td><strong>Wang et al. (2013)</strong>&lt;sup&gt;141&lt;/sup&gt;</td>
<td>ΔEPOR&lt;sub&gt;Adipose&lt;/sub&gt; Mice</td>
<td>Normal Chow Diet</td>
<td>↑ Body weight,↑ Accumulation of adipose,↓ Respiratory quotient</td>
</tr>
<tr>
<td><strong>Wang et al. (2013)</strong>&lt;sup&gt;141&lt;/sup&gt;</td>
<td>ΔEPOR&lt;sub&gt;Adipose&lt;/sub&gt; Mice</td>
<td>High Fat Diet</td>
<td>↑ Body weight,↓ Insulin sensitivity,↓ Glucose tolerance,↔ Food Intake,↓ Energy Expenditure</td>
</tr>
<tr>
<td><strong>Katz et al. (2010)</strong>&lt;sup&gt;152&lt;/sup&gt;</td>
<td>EPO-tg6 Mice</td>
<td>Normal Chow Diet</td>
<td>↓ Body weight,↓ Accumulation of adipose,↑ Energy Expenditure,↑ Insulin sensitivity,↑ Glucose tolerance</td>
</tr>
<tr>
<td><strong>Hojman et al. (2009)</strong>&lt;sup&gt;91&lt;/sup&gt;</td>
<td>Skeletal Muscle Erythropoietin Overexpressing Mice</td>
<td>Normal Chow Diet</td>
<td>↔ Body Weight</td>
</tr>
<tr>
<td><strong>Hojman et al. (2009)</strong>&lt;sup&gt;91&lt;/sup&gt;</td>
<td>Skeletal Muscle Erythropoietin Overexpressing Mice</td>
<td>High Fat Diet</td>
<td>↓ Accumulation of adipose,↑ Insulin sensitivity,↑ Glucose tolerance</td>
</tr>
</tbody>
</table>

1.4.3.4 EPO treatment in models of diet induced obesity

Obesity is a complex, chronic disease often involving decades of pathophysiological changes and adaptations. Several surrogate models are available that explore its etiology, including genetic mutation (e.g., leptin knockout) and environmental exposure models (e.g., diet-induced obesity)<sup>155</sup>. The mouse
model of diet-induced obesity is consistent with identified dietary factors (high fat) distinct in human obesity trends. Accordingly, mice are fed diets consisting of high fat (~60% total calories from fat) to induce this state. Obesity is associated with an increased risk of developing hyperglycemia and insulin resistance, specifically type 2 diabetes mellitus. Mice with chronic elevations of circulating EPO exhibit reductions in adipose tissue mass accumulation paired with normalization of fasting glucose and insulin sensitivity when fed a high fat diet. This data propose that supraphysiological levels of rhEPO could improve metabolic status (e.g., fasting glucose, insulin sensitivity) of obese mice. When treated with rhEPO for 2 weeks Wt mice, fed a high fat diet, demonstrate attenuations in body weight gain compared to saline treated high fat diet controls. Typically obese mice have elevated fasting blood glucose and serum insulin levels, associated with the increased adipose tissue accumulation. However, rhEPO-treated high fat diet fed Wt mice exhibit improvements in fasting blood glucose and serum insulin levels. This suggests rhEPO mitigates the weight gain and glucose intolerance classically linked with high fat diet. In contrast, Alnaeeli et al., observes that only prolonged rhEPO administration (>3 weeks) reduces body weight gain in Wt mice fed a high fat diet. A 2-week treatment regimen at the same dosage as Meng et al. found this does not affect body weight or fat mass. Although, Alnaeeli et al. observe that 2 weeks of rhEPO treatment is able to improve blood glucose levels and glucose tolerance. This is mediated by increases in glucose uptake in skeletal muscle and higher glycogen synthesis rates. Alnaeeli et al. are unable to detect the EPOR in the skeletal muscle, therefore suggesting that rhEPO indirectly stimulates glucose uptake. In agreement with Alnaeeli et al., rats fed high fat diet and treated with rhEPO for 2 weeks ameliorates glucose metabolism (i.e., fasting blood glucose and glucose tolerance) independently of improvements in body weight and fat mass.

AKT phosphorylation leads to glucose transportation, glycogen synthesis and inhibition of gluconeogenesis. Impairments in AKT signaling are connected with glucose intolerance and
insulin resistance, as well as increased expression of hepatic glucose production enzymes\textsuperscript{159,160,161}. rhEPO treatment partially rescues the insulin-induced AKT phosphorylation in the soleus muscle of rats on a high fat diet\textsuperscript{158}. However, in agreement with Alnaeeli et al., expression of the EPOR is not detectable in soleus\textsuperscript{158}, thus suggesting rhEPO indirectly enhances AKT phosphorylation in muscle. Typically, elevated fasting blood glucose in diabetic patients is correlated with accelerated liver glucose production\textsuperscript{162}. The treatment with rhEPO enhances AKT phosphorylation while inhibiting glycogen-6-phosphorylase and phosphoenolpyruvate carboxykinase (PEPCK) expression (i.e., glucose production enzymes) in the liver of Wt mice fed high fat diets\textsuperscript{157}. Therefore, activation of AKT with rhEPO treatment could inhibit gluconeogenesis, which would help to regulate glucose homeostasis. Peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)) is a transcription factor that is expressed in adipose tissue, liver and muscle\textsuperscript{163–165}. It is vital for controlling the expression of genes involved in glucose homeostasis, including PEPCK and PI3K\textsuperscript{166}. In line with Meng et al.\textsuperscript{157}, rhEPO treatment promoted AKT activation in the liver of high fat diet fed mice\textsuperscript{167}. Inhibition of PPAR\(\gamma\) signaling, via selective PPAR\(\gamma\) antagonist (GW9662)\textsuperscript{168}, suggests it is required for rhEPO AKT activation\textsuperscript{167}. These data suggests there are potential therapeutic implications for rhEPO in alleviating liver insulin resistance through AKT activation\textsuperscript{167}.

Inflammation, a hallmark of obesity, contributes to the development of insulin resistance\textsuperscript{169,170}. A systemic and local increase of the inflammatory cytokine tumor necrosis factor-\(\alpha\) is found in experimental models of obesity, where neutralization improves insulin sensitivity in adipose tissue and muscle\textsuperscript{171–173}. As well, interleukin-6 has emerged as a potential mediator linking obesity-derived chronic inflammation with insulin resistance\textsuperscript{174}. rhEPO treatment in high fat diet fed Wt mice reduces circulating tumor necrosis factor-\(\alpha\) and interleukin-6 levels\textsuperscript{157}. This suggests mitigation of high fat diet induced inflammation with rhEPO treatment that could lead to improvements in insulin sensitivity.
1.4.3.5 EPO treatment in genetic models of obesity

A well-studied genetic model of obesity is the leptin deficient (ob/ob) mouse that becomes grossly overweight, hyperphagic and develops severe insulin resistance\textsuperscript{175}. Leptin is a hormonal product of the obesity gene and primarily secreted by adipocytes. Leptin is known to regulate food intake and energy homeostasis through signaling in the hypothalamus\textsuperscript{175}. Treatment of ob/ob mice with leptin is able to reduce body weight, fat mass, food intake, and improve blood glucose and insulin\textsuperscript{176–178}. EPO and leptin are both members of the class-I cytokine superfamily, signaling through JAK/STAT pathways\textsuperscript{179–181}. Interestingly, ob/ob mice treated with rhEPO show attenuation of weight gain, while not affecting food intake\textsuperscript{152}. The rhEPO-treated mice also exhibit reductions in fasting blood glucose with improvements in glucose tolerance\textsuperscript{152}. A more recent study shows that the metabolic status improvement (i.e., reduction in weight gain, specifically fat mass) is not a result of an increase in hematocrit seen when ob/ob mice are treated with rhEPO\textsuperscript{76}. Teng et al., maintained normal hematocrit levels using repeated phlebotomies in ob/ob mice treated with rhEPO and saw the same reductions in weight gain\textsuperscript{76}. This suggests that the increase in oxygen carrying capacity was not responsible for the reduction in fat mass. These findings propose the effect of rhEPO, on a genetic model of obesity, is capable of improving metabolic homeostasis.

1.4.3.6 EPO treatment in experimental models of obesity

Streptozotocin (STZ) is an antibiotic that induces pancreatic β-cell destruction. It is commonly used to generate experimental models of type 1 diabetes mellitus that display impairments in insulin sensitivity and hyperglycemia\textsuperscript{182}. Chronic rhEPO treatment (4wks) in high fat diet fed STZ rats demonstrates reductions in fasting blood glucose with improvements in glucose tolerance (decrease in AUC for oral glucose tolerance test). Furthermore, reactive oxygen species play an important role in the development of diabetes\textsuperscript{144,183,184}. In diabetes, the activity of antioxidant enzyme glutathione peroxidase is lower than in controls\textsuperscript{183}. The increased activity of antioxidant enzymes, superoxide
dismutase and glutathione peroxidase, seen in the STZ rats treated with rhEPO suggests increased antioxidation leads to the improved glucose homeostasis. In accordance with the results from Chen et al., STZ rats treated with varying dosages of rhEPO demonstrate attenuations in hyperglycemia in a dose dependent manner. In line with the study by Teng et al., the reduction in hyperglycemia is seen without altering erythropoietic parameters. When blocking EPO/EPOR signaling in STZ rats with EPOR specific antibodies, the plasma glucose of rhEPO-treated STZ rats remains at control levels, suggesting EPO/EPOR signaling is required for the attenuation. The rhEPO-treated STZ rats have increases in glucose transporter isoform 4 in the skeletal muscle paired with reductions in PEPCK in the liver. This is suggestive of enhanced glucose uptake with concurrent decreases in gluconeogenesis leading to improved metabolic status of the STZ rats.

1.4.3.7 Metabolic response to EPO treatment in patients

In rodent studies, rhEPO treatment in obese models repeatedly elicits reductions in body weight while improving glucose tolerance and insulin sensitivity. While genetic models of EPO overexpression or EPOR knockout provide insight into endogenous EPO’s contribution to metabolic homeostasis, small clinical studies suggest that rhEPO administration influence metabolism.

rhEPO was first introduced as an FDA approved agent in 1989 to treat anemia associated with chronic renal failure. In recent years, studies have assessed the benefits of rhEPO on metabolic homeostasis.

All cause mortality rates in hemodialysis patients are increased when patients exhibit higher cholesterol levels. This increased rate of mortality is correlated to cholesterol only in the absence of inflammation and malnutrition. A study of hemodialysis patients’ report that rhEPO treatment, for two years, results in no changes to the lipid profile (e.g., total cholesterol, low-density and high-density lipoproteins and triglycerides) of the patients. In contrast, a more recent study of hemodialysis patients reports that 7 months of rhEPO treatment positively affects the lipid profile. The patients who positively respond to the rhEPO treatment (~80%) show increases in high-density lipoprotein and
decreases in triglyceride levels during the duration of the therapy. This suggests rhEPO therapy reduces mortality by improving the lipid profile in patients who respond to treatment. Another benefit observed with chronic rhEPO treatment in hemodialysis patients is increases in muscle glycogen content paired with lower fat content. These patients also exhibited increase in muscle strength, both greater force production and longer duration of contraction. This would suggest that some of the physical benefits seen with rhEPO treatment are a result of improvements in skeletal muscle function.

Moderate to severe renal disease patients are often insulin resistant. Improvements in insulin sensitivity correlates with duration of rhEPO treatment in hemodialysis patients and are proposed to occur by altering glucose homeostasis. This suggests the increase in insulin sensitivity with rhEPO treatment might reduce the risk of associated cardiovascular mortality in patients with end-stage renal disease.

Only a handful of studies have investigated the effects of rhEPO treatment on substrate metabolism in health. This is imperative in order to understand the mechanisms for the changes observed in patients. Promotion of fat loss and fat oxidation is a significant observation from EPO overexpression mice models. Indirect calorimetry analysis of healthy human subjects, given a single dose of rhEPO, report increases in resting energy expenditure along with increases in fat oxidation and decreases in respiratory quotient. A more recent study observes that 4 weeks of rhEPO treatment in young males increases fat oxidation in skeletal muscle during exercise and reduces respiratory quotient post exercise. In partial accordance, 8 weeks of rhEPO treatment in healthy males demonstrate increases in mitochondrial oxidation without changes in whole body maximal fat oxidation. This suggests that in humans, similar to results found in rodent models, rhEPO treatment can enhance fat oxidation. In conclusion, the confounding metabolic effects of rhEPO treatment in individuals may differ based on duration and health status of the patient.
The discovery of non-renal sources of EPO, non-erythropoietic functions and alternative receptors triggers the demand for further investigation into erythropoietin biology and EPO’s physiological relevance.

1.5 Glucose Metabolism

Blood glucose concentration is a balance between glucose disposal in peripheral tissues (i.e., liver, skeletal muscle and adipose) and liver glucose production (the sum of glycogenolysis and gluconeogenesis). Blood glucose is transported into the liver through the glucose transporter isoform 2 (GLUT2). GLUT2 is a low affinity, bi-directional glucose transporter with a fundamental role in maintaining glucose homeostasis. Insulin is released from pancreatic β cells in response to an increase in blood glucose. Insulin does not directly control the activity of the GLUT2 transporter; instead increased circulating insulin modulates the post-uptake fate of glucose. Within the liver, insulin inhibits glycogen phosphorylase that prevents the unnecessary release of glucose from glycogen stores, activates glucokinase to promote the transition of glucose to glucose-6-phosphate and stimulates glycogen synthase to promote the storage of glucose as glycogen. Glucose that bypasses the liver is delivered to the brain and to the periphery to be taken up by tissues such as skeletal muscle.

Two isoforms of the facilitative glucose transporter family, glucose transporter isoform 4 (GLUT4) and glucose transporter isoform 1 (GLUT1) are expressed in skeletal muscle. The less abundant GLUT1 isoform resides primarily in the plasma membrane and is not activated by insulin, suggesting it may be responsible for basal glucose uptake. Circulating insulin stimulates glucose uptake into skeletal muscle via insulin-induced translocation of GLUT4 to the plasma membrane. Upon binding of insulin to its receptor, insulin receptor autophosphorylates and begins a phosphorylation cascade from insulin receptor substrate proteins to protein kinase B (also known as AKT). Finally, AKT substrate 160 kDa (AS160) is phosphorylated, removing
inhibition thus allowing GLUT4-containing vesicles in the cytosol to translocate to the plasma membrane\textsuperscript{208,205}.

In skeletal muscle, glucose transport is also induced by contractile activity, independent of insulin action\textsuperscript{209}. Muscle contraction stimulates translocation of GLUT4 to the plasma membrane\textsuperscript{203,210}. Evidence that contraction-mediated glucose transport is normal in insulin resistant muscle suggests that insulin and contraction facilitated glucose transport are distinct pathways\textsuperscript{211}. Exercise and isolated muscle contractions can induce GLUT4 translocation in the complete absence of insulin via pathways that bypass insulin signaling proteins, such as AMP activated protein kinase (AMPK), and Ca\textsuperscript{2+}/calmodulin dependent kinase (CaMK)\textsuperscript{212,213,214}.

Muscle glucose transport occurs by facilitated diffusion, and is directly linked to the number of glucose transporters in the plasma membrane\textsuperscript{215,216}. Translocation of GLUT4 to the plasma membrane stimulates the uptake of circulating glucose down its concentration gradient into the cytosol\textsuperscript{217}, ultimately leading to reductions in blood glucose levels.

Upon entering the cell, glucose is promptly converted to glucose-6-phosphate by the enzyme hexokinase\textsuperscript{218}. Hexokinase is inhibited by glucose-6-phosphate (end product inhibition)\textsuperscript{219}. Glucose-6-phosphate is then converted to glucose-1-phosphate by phosphoglucomutase. Subsequently, uridine triphosphate and glucose-1-phosphate form uridine diphosphate-glucose. Glycogen synthase catalyzes the reaction that transfers the glucose attached to the uridine diphosphate-glucose to the terminal glucose residue at the nonreducing end of an amylose chain to form a \(\alpha (1\rightarrow 4)\) glycosidic linkage\textsuperscript{218}. Glycogen synthase activity can be inhibited by phosphorylation via GSK3 or stimulated by insulin-induced AKT phosphorylation. AKT phosphorylation will inactivate GSK3, thus increasing glycogen synthesis\textsuperscript{218}. The end product formed is glycogen, a readily mobilized multibranched polysaccharide of glucose residues. When the amount of glucose exceeds the glycogen storage capacity (i.e., glycogen
stores are saturated), excess glucose is channeled to the *de novo* lipogenic pathway to generate triacylglycerides\(^{220}\).

In response to increases in energy requirements (e.g., exercise) glycogen is broken down into glucose to produce ATP. Glycogen can be mobilized and converted back to glucose-1-phosphate by glycogen phosphorylase. Phosphoglucomutase catalyzes the reaction that converts glucose-1-phosphate to glucose-6-phosphate. Available glucose-6-phosphate will enter glycolysis, where the primary outcomes are the net production of ATP, nicotinamide adenine dinucleotide and formation of the end product pyruvate. The resulting ATP can be immediately used as an energy source, while nicotinamide adenine dinucleotide can carry electrons to the electron transport chain where they will be used to produce more ATP.

As exercise duration and intensity increase, or during periods of prolonged fasting, glycogen stores within skeletal muscle diminish, thus requiring alternative glucose production pathways to maintain energy requirements. The liver is able to produce glucose that can be transported to peripheral tissues in times of energy stress. Glucagon, released from the pancreas during conditions of low blood glucose, promotes liver glucose output thus maintaining circulating glucose levels\(^{221}\). Liver glucose production stems from glycogen breakdown and from *de novo* synthesis of glucose (i.e., gluconeogenesis). In order to be transported out of the liver, glucose-6-phosphate that was derived from either gluconeogenesis or glycogenolysis is dephosphorylated to free glucose in the endoplasmic reticulum\(^{222}\). Gluconeogenesis substrates can also be delivered to the liver through circulation from other tissues such as the skeletal muscle and adipose\(^{223}\). This function of the liver to make glucose available for other tissues is vital during periods of exercise or fasting.
Figure 1.4 | Overview of glucose uptake, storage and utilization in skeletal muscle
CHAPTER TWO

AIMS OF THESIS
EPO is emerging as a pleiotropic cytokine with both erythropoietic and non-erythropoietic roles, including regulation of metabolism\textsuperscript{76,141}. EPOR expression solely in erythroid cells (Tg mice) results in a disproportionate increase in body weight starting from birth\textsuperscript{76}. Tg mice exhibit an obese phenotype that is associated with the development of glucose intolerance and a shift toward increased fat metabolism\textsuperscript{76}. This suggests a critical role for EPO signaling in maintenance of energy homeostasis. While deletion of the EPOR in non-erythroid cells results in the outlined phenotype, whether a non-renal source of EPO is responsible for the metabolic effects remains to be elucidated.

Endothelial cells are pervasive in all tissues throughout the body. The endothelium, in response to hypoxia stabilizes HIF\textsuperscript{14,15,224}, the upstream gene regulator of EPO\textsuperscript{16}. As recently reported, HIF is not solely regulated by hypoxia dependent, but also hypoxia independent mechanisms\textsuperscript{12,13}. This suggests that the endothelium has the potential to express EPO in response to a hypoxic or metabolic stress (e.g., exercise, fasting, obesity). As the endothelial cell behaves as both a receptor, sensing physical or chemical stimuli that occur inside the vessel of a tissue, and an effector, expressing response genes, it can communicate with the tissue to maintain homeostasis. Therefore, the endothelium is well suited for metabolic regulation.

**Aim:** What is the physiological significance of endothelial derived EPO and is it required for maintenance of energy homeostasis?

We hypothesize that the endothelium is a source of EPO that acts in a paracrine fashion to increase carbohydrate metabolism. Further, loss of endothelium EPO will result in a decrease in carbohydrate metabolism.
CHAPTER THREE

MATERIALS AND METHODS
3.3.1 Animals

Generation of endothelial-specific EPO\(^{fl/fl}\):Tek-cre\(^{+/-}\) transgenic mice was completed by crossing EPO loxP mice (as previously described\(^{225}\)) with mice expressing Cre recombinase under the control of the mouse endothelial-specific promoter tyrosine kinase (Tek) (Jackson Laboratories B6.Cg-Tg(Tek-cre)1Ywa/J). For the purpose of this study, animals positive for endothelial-specific EPO deletion (EPO\(^{fl/fl}\):Tek-cre\(^{+/-}\)) are denoted EPO\(^{fl/fl}\)-ENDO. Control animals included the following genotypes: EPO\(^{fl/fl}\):Tek-cre\(^{-/-}\); EPO\(^{+/+}\):Tek-cre\(^{+/-}\); and EPO\(^{+/+}\):Tek-cre\(^{-/-}\). No differences were observed in these groups and thus were combined and subsequently referred to as EPO\(^{WT}\). Genotyping was completed via tail clips by PCR (REDExtract-N-Amp Tissue PCR Kit; Sigma-Aldrich, Oakville, ON, Canada) using the following primer sets: EPO-KO-F, 5’AGTGAAGTTTGCCGAGAAG-3’; EPO-KO-R, 5’-AGATCGAACTTGGCTCCTCA-3’; EPO-TA-R, 5’-GTGGGACGTTCTGGGAAGAAA-3’; Cre-F, 5’-ATCCGAAAAAGAAAAACGTTGA-3’; Cre-R, 5’-ATCCAGGTTACGGATATAGT-3’. The following protocol was used with the EPO primer set: 1 cycle at 95C for 2 min, 39 cycles at 95C for 45 sec, 1 min at 59C (primer set specific annealing temperature), and 72C for 5 min. The following protocol was used with the Cre primer set: 1 cycle at 95C for 2 min, 39 cycles at 95C for 30 sec, 30 sec at 54C (primer set specific annealing temperature), and 72C for 1 min.

Housing was maintained at 24C, 45% humidity and kept to a 12h light-dark cycle (Lights on 8:00am – 8:00pm). Animals were housed in groups (2-4) with food and water provided ad libitum. Housing and experimental procedures were approved by the Animal Care Committee at the University of Guelph in conformity with the guidelines of the Canadian Council on Animal Care.

3.3.2 Hemodynamic and echocardiographic analyses

For both echocardiogram and cardiac catheterization, 12-week adult mice were anesthetized using isoflurane (2:98% isoflurane:oxygen) and maintained at 37C using a heating pad. Transthoracic 2D
and motion-mode echocardiography was obtained using the VisualSonics Vevo 770 imaging system (VisualSonics, Toronto, ON, Canada) equipped with a RMV707B transducer (30MHz). Imaging was performed from the long axis view of the heart at the level of the papillary muscle. Heart rate and left ventricle dimensions were measured and standard cardiac parameters were calculated. Measurements were averaged from 3 separate cardiac cycles.

The right carotid artery was isolated and a 1.2F solid-state pressure catheter (Transonic Scisense Inc., London, ON, Canada) was inserted and advanced into the left ventricle. Hemodynamic signals were recorded following a 15 min acclimatization period and cardiac function analyzed using iWorx analytical software (LabScribe2; iWorx Systems Inc., Dover, NH, USA). Subsequently, blood was collected via cardiac puncture and centrifuged in 1.5mL eppendorf tubes (4000 rpm for 20mins at 4C), the supernatant was removed and stored at -80C for further analysis. A portion of blood collected was centrifuged in heparinized microcapillary tubes (10 min at 5000 x g) to determine hematocrit. Animals were sacrificed via cardiac excision and tissues were harvested for either histological analysis (described in 3.3.3) or snap-frozen and stored at -80C for subsequent qPCR analysis (described in 3.3.11).

3.3.3 Histological analysis

Tissue was coated in optimal cutting temperature compound (TissueTek; VWR International, Mississauga, ON, Canada) and rapidly frozen in -80C isopentane. Sections 7 µm were prepared and stained with wheat germ (Alexa488 wheat germ agglutinin, 10µg/ml; Invitrogen, Burlington, ON, Canada) or isolectin (Alexa568 GS-IB4 conjugate, 10µg/ml; Invitrogen, Burlington, ON, Canada). Fluorescent sections were imaged using an Olympus FSX 100 light microscope (Olympus Canada Inc., Toronto, ON, Canada). Cross sectional area (CSA) and capillary density were analyzed using ImageJ (National Institutes of Health, Bethesda, Maryland, USA).
### 3.3.4 Exercise Protocol

EPO$^{WT}$ and EPO$^{fl/fl}$-ENDO mice (12-16 week adult mice) were acclimatized to the treadmill (Model Exer-3/6R; Columbus Instruments, Columbus, OH, USA) for 2 sessions; each session consisted of 10 min of running at 15m/min at a 5° incline. Mice rested for 48 hours following the acclimation sessions before undergoing an endurance capacity test. The maximal running capacity test involved mice running at 12 m/min with a 20° incline; where speed was subsequently increased by 1 m/min at 2, 5, 10, and every 10 min thereafter until mice remained at the back of the treadmill and are unresponsive to encouragement via prodding 3 consecutive times. Mice were also evaluated using a sprint-interval protocol (previously described in Imamura et al. 2014, The Biochemical Journal$^{226}$) were mice were warmed up for 3 minutes at 6 m/min at 0° incline and then 2 more minutes at 15° incline. Speed was first increased to 14 m/min for 30 s, followed by a short 1.5 min of low speed running at 6 m/min. Speed was then increased to 18 m/min for 30 s, then to 20 m/min for 15 s, then 22 m/min for 15 s then 24 m/min for 15 s (total 15 x 4= 60 s). After the speed was reduced to 6 m/min for 3 min, followed by an increase in the speed from 20 m/min to 32 m/min with an increment of 4 m/min at 15 s intervals. After the speed was reduced to 6 m/min for 3 min. This pattern of exercise continues with a subsequent increase of 2 m/min in the starting sprint speed (next interval starts at 22 m/min and increasing to 34 m/min) until mice remained at the back of the treadmill and were unresponsive to encouragement via prodding 3 consecutive times.

### 3.3.5 ELISA

EPO protein concentration was quantified using a quantikine mouse EPO ELISA (MEP00B; R&D Systems, Minneapolis, Minnesota, USA), as per manufacturer’s instructions; expect samples were not diluted. ELISA plate was read on a BioTek Synergy Mx microplate reader (Biotek, Winooski, Vermont, USA) at 540nm and 450nm. Readings at 540nm were subtracted from the readings at 450nm (correction for optical imperfections in the plate).
3.3.6 Metabolic Analysis

EPO\textsuperscript{WT} and EPO\textsuperscript{fl/fl-ENDO} mice (12-week adult mice) were individually housed in Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA) units for a 48 hour period. The light:dark cycle was maintained as described above and water and food were provided \textit{ad libitum} while in the CLAMS. Mice were monitored every 15 min for metabolic (VO\textsubscript{2}, VCO\textsubscript{2}, feed) and locomotor (physical activity) measurements. The CLAMS calculates RER, as such: RER= VCO\textsubscript{2}/ VO\textsubscript{2}. Activity was estimated by summing the number of beam brakes (counts) in the X and Z planes. Z beam brakes measure rearing activity, while X-ambulatory measures mouse ambulation in the caging. X-total is calculated as the total of all the beam brakes in the X-plane and X-ambulatory measures.

3.3.7 Fasted Blood Glucose

Adult EPO\textsuperscript{WT} and EPO\textsuperscript{fl/fl-ENDO} mice were fasted for 12 hours (overnight). Blood glucose was assessed using a glucometer (Countour next EZ; Bayer AG, Leverkusen, Germany) through tail vein sampling. Fasted blood glucose was also evaluated after a 6 hour fast (8:00am- 2:00pm).

3.3.8 Glucose Tolerance Test

Both an intraperitoneal (IP) glucose tolerance test (IPGTT) and oral glucose tolerance test (OGTT) were performed following a 12-hour (overnight) fast. Blood glucose was assessed using a glucometer (Countour next EZ; Bayer AG, Leverkusen, Germany) through tail vein sampling at 0, 15, 30, 60, 90 and 120 min after either the IP injection or oral dosage of glucose (2 g/kg body weight). Area under the curve was calculated using Prism6 (GraphPad, La Jolla, CA, USA).

3.3.9 Skeletal Muscle Dissociation

Tibialis anterior (TA) was harvested from EPO\textsuperscript{WT} and EPO\textsuperscript{fl/fl-ENDO} mice (n=1 is combined muscles from six mice). Enzyme mix was prepared following kit instructions (skeletal muscle
dissociation kit for mouse and rat 130-098-305; Miltenyi Biotec, Auburn, CA, USA). Tissue was transferred into gentleMACs tubes containing enzyme mix and incubated for 30 mins at 37°C under continuous rotation (MACsmix tube rotator; Miltenyi Biotec, Auburn, CA, USA). Tissue was dissociated using gentleMACs dissociator (program m_muscle_01) and then incubation was repeated. Dissociation step was repeated before sample was applied to MACs smartstrainer. Strainer was washed with 8mL of DMEM and the sample was then centrifuged at 300 x g for 20 min at 6°C. Supernatant was completely aspirated and sample was resuspended with 90μL of PEB buffer (Solution containing phosphate buffered saline pH7.2, 0.5% bovine serum albumin and 2mM EDTA).

3.3.10 Skeletal Myocyte and Endothelial Isolation

Depletion of CD45+ Cells.

Resuspend the sample from the skeletal muscle dissociation (collected from section 3.3.9) and add 10μL of CD45 microbeads (130-052-301; Miltenyi Biotec, Auburn, CA, USA). Sample was then incubated for 15 min at 4°C. Cells were then washed with PEB buffer (1mL per 10^7 cells; approximately 10^8 cells per gram of tissue) and centrifuged at 300 x g for 5 min. Supernatant was aspirated from sample. In 500μL of PEB buffer, resuspend cells then apply to the LS column (130-042-401; Miltenyi Biotec, Auburn, CA, USA). Unlabeled cells (CD45- fraction) will pass through the LS column and were collected for later isolation.

Enrichment of CD31+ Cells.

The CD45- fraction was centrifuged at 300 x g for 10 min, then supernatant was aspirated. To the resuspended sample, add 10μL of CD31 microbeads (130-097-418; Miltenyi Biotec, Auburn, CA, USA). The sample was then incubated for 15 min at 4°C. Cells were washed with 1mL PEB buffer and centrifuged at 300 x g for 5 min. Supernatant was aspirated then sample was resuspend in 500μL of PEB buffer. Resuspended cells were applied to the MS column (130-042-201; Miltenyi Biotec,
Auburn, CA, USA). The unlabeled cells (CD45-/CD31-; skeletal myocyte fraction) that eluted from column were snap frozen for later qPCR analysis. The MS column was removed from the MACs separator and flushed (CD45-/CD31+; Endothelial cell fraction) then snap frozen for later qPCR analysis.

3.3.11 qPCR

Changes in mRNA expression of EPO were determined using real time quantitative PCR (qPCR). RNA was isolated using TRIzol (Invitrogen, Burlington, ON, Canada) with the Qiagen RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA concentrations were quantified (NanoDrop, ND1000; Thermo Fisher Scientific, Waltham, Massachusetts, USA) prior to cDNA synthesis. Protein contamination was assessed by measuring absorbance at 280nm. Generation of cDNA was completed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer’s instructions, using 1000 ng of RNA per sample. qPCR was performed for detection of EPO using Platinum SYBR Green qPCR supermix with ROX (Invitrogen, Burlington, ON, Canada) and the following primers:

EPO-F, 5’-CATCTGCGACAGTGCAGTCTG-3’;
EPO-R, 5’-CACACCCATCGACATTTTC-3’;
ActB-F, 5’-TGTGATGGTGAGGATGGTCAGAA-3’;
ActB-R, 5’-TGTGCTGCCAGATCTTCTCCATGT-3’.

The qPCR was carried out using a 7500 Real Time PCR detection system (Applied Biosystems, Foster City, CA, USA). The following protocol was used: 1 cycle at 50C for 2 min, 1 cycle at 95C for 5 min, then 40 cycles at 95C for 15 sec, 1 min at 58C (EPO specific primer annealing temperature), followed by a dissociation curve to assess specificity of the reaction. Samples were run in duplicate 25 µL reactions. The primers for EPO were designed to span an exon-exon junction as to eliminate the possibility of contamination with genomic DNA. There
was no difference in ActB (β Actin) expression between EPO\textsuperscript{WT} and EPO\textsuperscript{fl/fl-ENDO} mice. Cycle thresholds (CT) greater than 35 were regarded as non-detectable. Results were analyzed according to the ΔCT method using ActB as a reference gene.

3.3.12 In Vitro Muscle Function

Intact EDL and soleus were excised from the animal and placed in Krebs-Henseleit solution ((mM/L): 118 NaCl, 4.7 KCl, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 1.2 MgSO\textsubscript{4}· 7 H\textsubscript{2}O, 27.26 NaHCO\textsubscript{3}, 11.1 glucose, 2.3 CaCl\textsubscript{2}·2H\textsubscript{2}O, 10 units/L insulin and 3.0x10\textsuperscript{-4} g/L curare) at room temperature and pH 7.4. Silk ties were secured to tendons on both ends of the muscle and attached to a force transducer (Grass instruments, Warwick, RI, USA) and stationary hook, respectively. Stainless steel stimulating electrodes connected to a Grass electrical stimulator were placed on either side of the muscle section and used to elicit contractions via field stimulation. Tissue baths were placed such that each muscle was immersed in Krebs-Henseleit bicarbonate buffer continuously warmed to 27C and aerated with 95% O\textsubscript{2}:5%CO\textsubscript{2}. Following setting optimal muscle length and a 45-minute thermoequilibration period, peak force and rate of force production (+dT/dt max) were determined during a force-frequency test (1 contraction every 30 s with 250 ms train duration). Additionally, skeletal muscle fatigability and passive force production were determined during repeated submaximal stimulations (EDL 20Hz, 60 contractions per minute with 100 ms train duration; Soleus 40Hz, 60 contractions per minute with 100 ms train duration). Isometric contractility data was collected using AcqKnowledge software (BIOPAC systems, Goleta, CA, USA) and analyzed using Spike2 software (Cambridge Electronic Design Limited, Milton, Cambridge, England). Force production and contractility were normalized to muscle weight (mg) to account for differences in tissue size between mice.

3.3.13 Glycogen Content Analysis

For glycogen analysis, muscles were snap frozen, dried, powdered, and glucose monophosphates were degraded using sodium hydroxide (0.1M) at 80C.
for 10 min. Samples were neutralized in a buffer containing HCL (0.1M), citric acid (0.2M), and Na2HPO4.7H2O (0.2M). Total glycogen was analyzed using spectrophotometric methods (previously described in Harris et al. 1974, in the Scandinavian Journal of Clinical and Laboratory Investigation\textsuperscript{227}).

### 3.3.14 Statistical Analyses

Graphical and statistical analyses were completed using Prism6 (GraphPad, La Jolla, CA, USA). Data presented as mean ± standard deviation, unless otherwise stated. Gaussian distribution was tested using D’Agostino-Pearson test for normality. The skeletal muscle histology data of EPO\textsuperscript{fl/fl}-ENDO mice was analyzed using an unpaired, two-tailed Mann-Whitney (nonparametric) t-test. The survival curve of the sprint exercise test was analyzed using Mantel Cox test. Comparison of glycogen content and glucose tolerance between EPO\textsuperscript{WT} and EPO\textsuperscript{fl/fl}-ENDO mice was determined using a two-way ANOVA for main effects, multiple comparisons were determined using a Tukey's multiple comparisons test. For all other statistical analyses, an unpaired, two-tailed parametric t-test was used. Differences were considered significant at p<0.05. Outliers were determined by a Grubbs outlier test (\(\alpha = 0.05\)).
CHAPTER FOUR

RESULTS
4.1 Characterization of the EPO\textsuperscript{fl/fl-ENDO} mice

The ability of endothelial cells to sense hypoxia and metabolic stressors, as well as their pervasive nature, makes them well suited to regulate metabolic homeostasis; therefore, we generated an endothelial-specific deletion of EPO using Cre recombinase expression driven by the Tek2 promoter\textsuperscript{228}. EPO\textsuperscript{fl/fl-ENDO} mice were viable and fertile (yielding expected Mendelian inheritance), with no obvious differences in appearance and no changes in body (Figure 1A), liver, kidney, heart, adipose (subcutaneous, epididymal and retroperitoneal) and skeletal muscle weight between EPO\textsuperscript{WT} and EPO\textsuperscript{fl/fl-ENDO} mice (Table 1). The kidney is known as the primary EPO producing organ, however, the cell type responsible remains to be fully understood. Unexpectedly, EPO\textsuperscript{fl/fl-ENDO} mice had reductions in renal EPO expression (Figure 1B). This reduction in kidney EPO expression was not accompanied by changes in circulating EPO levels (Figure 1C) or hematocrit (Figure 1D). This suggests that under baseline conditions, endothelial-derived EPO is not required to maintain normal erythropoiesis. The liver EPO expression, responsible for EPO production during embryogenesis, was non-detectable in both the adult EPO\textsuperscript{fl/fl-ENDO} and EPO\textsuperscript{WT} mice (Figure 1E). As skeletal muscle tissue exhibits EPO expression\textsuperscript{66,68}, and is comprised of multiple cell types (e.g. skeletal myocytes, endothelial cells, satellite cells), we investigated if differences were detectable in the EPO\textsuperscript{fl/fl-ENDO} compared to EPO\textsuperscript{WT} mice. EDL EPO expression (Figure 1F) was reduced in the EPO\textsuperscript{fl/fl-ENDO} mice, however, no differences were observed in the soleus muscle (Figure 1G). This suggests that endothelial-derived EPO accounts for a portion of total EPO expression in glycolytic, but not oxidative, skeletal muscle. For proof of knockout, EPO expression was evaluated in isolated endothelial cells from tibialis anterior demonstrating detectable levels in EPO\textsuperscript{WT} mice, however, was non-detectable (ND) in the EPO\textsuperscript{fl/fl-ENDO} mice (Figure 1H). We were intrigued to determine if the skeletal myocytes, the most abundant cell type within muscle, would compensate for the loss of endothelial-derived EPO expression within a glycolytic muscle. We investigated the EPO expression in the isolated skeletal myocytes from tibialis anterior demonstrating no difference between EPO\textsuperscript{WT} and EPO\textsuperscript{fl/fl-ENDO} mice (Figure 1H).
## Table 4.1 | Comparison of tissue weights between EPO\textsuperscript{WT} and EPO\textsuperscript{fl/fl-ENO} mice

<table>
<thead>
<tr>
<th>n</th>
<th>EPO\textsuperscript{WT}</th>
<th>EPO\textsuperscript{fl/fl-ENO}</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDL (mg)</td>
<td>9 ± 2</td>
<td>9 ± 1</td>
<td>0.743</td>
</tr>
<tr>
<td>TA (mg)</td>
<td>46 ± 2</td>
<td>45 ± 5</td>
<td>0.629</td>
</tr>
<tr>
<td>SOL (mg)</td>
<td>11 ± 5</td>
<td>8 ± 2</td>
<td>0.213</td>
</tr>
<tr>
<td>iWAT (mg)</td>
<td>391 ± 233</td>
<td>401 ± 120</td>
<td>0.913</td>
</tr>
<tr>
<td>eWAT (mg)</td>
<td>674 ± 311</td>
<td>729 ± 248</td>
<td>0.717</td>
</tr>
<tr>
<td>rpWAT (mg)</td>
<td>193 ± 94</td>
<td>198 ± 58</td>
<td>0.901</td>
</tr>
<tr>
<td>Liver (mg)</td>
<td>1093 ± 204</td>
<td>1145 ± 157</td>
<td>0.644</td>
</tr>
<tr>
<td>Kidney (mg)</td>
<td>161 ± 15</td>
<td>164 ± 18</td>
<td>0.765</td>
</tr>
<tr>
<td>Heart (mg)</td>
<td>126 ± 18</td>
<td>138 ± 16</td>
<td>0.173</td>
</tr>
</tbody>
</table>

Data is presented as mean ± SD. n, number of animals; EDL, extensor digitorum longus; TA, tibialis anterior; iWAT, inguinal white adipose tissue; eWAT, epididymal white adipose tissue; rpWAT, retroperitoneal white adipose tissue. *p<0.05 compared to EPO\textsuperscript{WT}, as determined by an unpaired, two-tailed parametric t-test.
Figure 4.1 | Characterization of EPO[β/β-ENDO] mice

(B) Kidney EPO expression in adult EPO[WT] and EPO[β/β-ENDO] mice (n=5, EPO[β/β-ENDO] n=6).
(C) Serum EPO levels (EPO[WT] n=8, EPO[β/β-ENDO] n=9) and (D) hematocrit (EPO[WT] n=10, EPO[β/β-ENDO] n=11) in adult EPO[β/β-ENDO] compared to EPO[WT] mice. (E) Liver (n=5, EPO[β/β-ENDO] n=6), (F) EDL, (G) soleus (EPO[WT] n=3, EPO[β/β-ENDO] n=4), (H) endothelial cells and (I) skeletal myocyte EPO (n=2) expression in adult EPO[WT] and EPO[β/β-ENDO] mice. Data is presented as mean ± SD. *p<0.05 compared to EPO[WT], as determined by an unpaired, two-tailed parametric t-test.
4.2 No change in the cardiovascular parameters of the EPO^{fl/fl-ENDO} mice

Treatment of isolated atria and ventricular muscle preparations with supraphysiological levels of rhEPO exhibits both inotropic and lusitropic affects\(^{84,86}\). However, whether endogenous endothelial EPO equates to significant \textit{in vivo} contribution to cardiac function is unknown. To evaluate cardiac function, echocardiographic and invasive hemodynamic analyses were performed. There were no differences in chamber dimension, diastolic and systolic function of EPO^{fl/fl-ENDO} as compared to EPO\(^{WT}\) mice (Table 2). Loss of endothelial EPO did not affect cardiac structure or function as assessed by echocardiography and invasive hemodynamics. While knockout of endothelial EPO led to reductions in EPO expression in limb muscles there was no change in EPO expression (Data not shown) in the heart of the EPO^{fl/fl-ENDO} as compared to EPO\(^{WT}\) mice. The lack of changes in cardiac phenotype is consistent with the molecular data and indicates endothelium within the heart does not produce EPO. Therefore, endothelium EPO expression is not ubiquitous in all muscle.

Table 4.2 | Comparison of cardiovascular parameters between EPO\(^{WT}\) and EPO^{fl/fl-ENDO} mice

<table>
<thead>
<tr>
<th>Echocardiographic parameters</th>
<th>EPO(^{WT})</th>
<th>EPO^{fl/fl-ENDO}</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textbf{n}</td>
<td>10</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>\textbf{End systolic dimensions (mm)}</td>
<td>2.6 ± 0.4</td>
<td>2.6 ± 0.3</td>
<td>0.853</td>
</tr>
<tr>
<td>\textbf{End diastolic dimensions (mm)}</td>
<td>4.0 ± 0.3</td>
<td>4.0 ± 0.2</td>
<td>0.622</td>
</tr>
<tr>
<td>\textbf{Posterior wall thickness (mm)}</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>0.344</td>
</tr>
<tr>
<td>\textbf{Stroke volume (µL/beat)}</td>
<td>44.7 ± 4.4</td>
<td>43.5 ± 3.8</td>
<td>0.511</td>
</tr>
<tr>
<td>\textbf{Ejection fraction (%)}</td>
<td>64.5 ± 7.4</td>
<td>64.3 ± 6.5</td>
<td>0.951</td>
</tr>
<tr>
<td>\textbf{Fractional shortening (%)}</td>
<td>35.0 ± 5.3</td>
<td>35.0 ± 4.7</td>
<td>0.916</td>
</tr>
<tr>
<td>\textbf{Cardiac output (mL/min)}</td>
<td>22.6 ± 2.8</td>
<td>21.9 ± 2.9</td>
<td>0.553</td>
</tr>
<tr>
<td>\textbf{HR (bpm)}</td>
<td>506 ± 45</td>
<td>502 ± 33</td>
<td>0.784</td>
</tr>
</tbody>
</table>
Hemodynamic parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EPO\textsuperscript{WT}</th>
<th>EPO\textsuperscript{fl/fl-ENDO}</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>110 ± 13</td>
<td>110 ± 5</td>
<td>0.954</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>74 ± 11</td>
<td>72 ± 5</td>
<td>0.661</td>
</tr>
<tr>
<td>LV pressure (mmHg)</td>
<td>115 ± 10</td>
<td>115 ± 4</td>
<td>0.858</td>
</tr>
<tr>
<td>dp/dt\textsubscript{max} (mmHg/s)</td>
<td>8506 ± 1575</td>
<td>9151 ± 1425</td>
<td>0.405</td>
</tr>
<tr>
<td>dp/dt @ LVP40 (mmHg/s)</td>
<td>7701 ± 1395</td>
<td>8203 ± 1150</td>
<td>0.442</td>
</tr>
<tr>
<td>dp/dt\textsubscript{min} (mmHg/s)</td>
<td>-9114 ± 1406</td>
<td>-9765 ± 888</td>
<td>0.276</td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>8.9 ± 2.6</td>
<td>9.1 ± 2.4</td>
<td>0.887</td>
</tr>
<tr>
<td>Tau (Glantz) (s)</td>
<td>9.7 ± 1.9</td>
<td>9.1 ± 1.1</td>
<td>0.343</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>478 ± 59</td>
<td>454 ± 30</td>
<td>0.300</td>
</tr>
</tbody>
</table>

Values are means ± SD. \( n \), number of animals; HR, heart rate; BP, blood pressure; \( \text{dp/dt}_{\text{max}} \), maximal rate of change of systolic pressure during systole; \( \text{dp/dt}_{\text{min}} \), maximal rate of change of diastolic pressure during diastole; LV, left ventricle; EDP, end diastolic pressure. \(* p < 0.05\) compared to EPO\textsuperscript{WT}, as determined by an unpaired, two-tailed parametric t-test.

4.3 Reduced exercise tolerance in EPO\textsuperscript{fl/fl-ENDO} mice not due to alterations in intrinsic muscle function

EPO has been identified as a cytokine that can regulate energy homeostasis\textsuperscript{76,77,91,152}. Using treadmill exercise as an energetic stressor, we evaluated the exercise tolerance of the EPO\textsuperscript{fl/fl-ENDO} mice in comparison to EPO\textsuperscript{WT}. EPO\textsuperscript{fl/fl-ENDO} mice exhibited reductions in both the endurance and sprint exercise protocols (Figure 2A & 2B), demonstrating exercise intolerance. To determine if the exercise intolerance was due to intrinsic muscle dysfunction, \textit{in vitro} muscle preparation was performed on a glycolytic (EDL) and oxidative (soleus) muscle of the EPO\textsuperscript{fl/fl-ENDO} and EPO\textsuperscript{WT} mice. There was no difference in the maximal force produced at a given frequency (Figure 2C & 2D) or the fatigability (Figure 2E & 2F) in both the glycolytic (EDL) and oxidative (soleus) hind limb muscle of the EPO\textsuperscript{fl/fl-ENDO} and EPO\textsuperscript{WT} mice. Changes in the ability of the sarcoplasmic reticulum to uptake Ca\textsuperscript{2+} can result in impaired relaxation and therefore diminished muscle function. There was no difference in the peak minimum force (Figure 2G & 2H), which would indicate an issue with Ca\textsuperscript{2+} uptake and/or muscle
relaxation (e.g., myofilament dissociation impairment), in the EPO^{fl/fl-ENDO} mice as compared to EPO^{WT} for both the glycolytic (EDL) and oxidative (soleus) hind limb muscles.
**Figure 4.2 |** EPO^β/β-ENDO^ mice exhibit altered exercise tolerance

(A) Comparison of single bout exhaustive treadmill running distances in adult EPO^WT^ and EPO^β/β-ENDO^ mice (EPO^WT^ n=12, EPO^β/β-ENDO^ n=15). (B) Comparison of single bout sprint treadmill running interval exhaustion in adult EPO^WT^ and EPO^β/β-ENDO^ mice (n=16). (C) EDL (EPO^WT^ n=5, EPO^β/β-ENDO^ n=4) and (D) soleus (n=5) force-frequency curves in adult EPO^WT^ and EPO^β/β-ENDO^ mice. (E) EDL (EPO^WT^ n=5, EPO^β/β-ENDO^ n=4) and (F) soleus (n=5) fatigue curves in adult EPO^WT^ and EPO^β/β-ENDO^ mice. (G) EDL (EPO^WT^ n=5, EPO^β/β-ENDO^ n=4) and (H) soleus (n=5) peak minimum force calculated from fatigue curves in adult EPO^WT^ and EPO^β/β-ENDO^ mice. Data is presented as mean ± SD. *p<0.05 compared to EPO^WT^, as determined by an unpaired, two-tailed parametric t-test. #p<0.05 compared to EPO^WT^, as determined by Mantel Cox test. Outliers (O) were determined by a Grubbs outlier test (α = 0.05).

**4.4 Reduced glycogen storage in glycolytic muscle of EPO^β/β-ENDO^ mice**

In order to further gain insight into the cause of the exercise intolerance, carbohydrate substrate availability was determined. The glycogen content of the muscle in the EPO^β/β-ENDO^ mice at baseline in comparison to the EPO^WT^ was evaluated to determine if substrate availability could be a factor in the increased fatigability of the exercising mice. In comparison to EPO^WT^, EPO^β/β-ENDO^ mice had reduced muscle glycogen in EDL at baseline (Figure 3A). Although no glycogen content difference was observed in tibialis anterior (Figure 3B) or soleus (Figure 3C), the liver of the EPO^β/β-ENDO^ had increased glycogen content at baseline (Figure 3D). To investigate if reduced substrate availability in EPO^β/β-ENDO^ mice is a contributing factor to the exercise intolerance, we evaluated the post exhaustion muscle glycogen content. In comparison to EPO^WT^, EPO^β/β-ENDO^ mice exhibit no change in the glycogen content of EDL (Figure 3A), tibialis anterior (Figure 3B), soleus (Figure 3C) and liver (Figure 3D) post-exhaustive exercise. Two-way ANOVA confirms exercise reduces oxidative muscle and liver glycogen, regardless of genotype; as well, liver glycogen of EPO^β/β-ENDO^ mice is higher than EPO^WT^ before exercise. No change in pre- (baseline) and post-exhaustive blood glucose (Figure 3I) was observed in EPO^β/β-ENDO^ mice compared to EPO^WT^. 

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**Figure 4.3** | EPO^{fl/fl-ENDO} mice display impaired glycogen storage in glycolytic skeletal muscle

(A) EDL (EPO^{WT} n=6-9, EPO^{fl/fl-ENDO} n=10) glycogen content at baseline and post exhaustive exercise in EPO^{WT} and EPO^{fl/fl-ENDO} mice. (B) Tibialis Anterior (EPO^{WT} n=6-7, EPO^{fl/fl-ENDO} n=7-11) glycogen content at baseline and post exhaustive exercise in EPO^{WT} and EPO^{fl/fl-ENDO} mice. (C) Soleus (EPO^{WT} n=7, EPO^{fl/fl-ENDO} n=7-9) glycogen content at baseline and post exhaustive exercise in EPO^{WT} and EPO^{fl/fl-ENDO} mice. (D) Liver (EPO^{WT} n=6-7, EPO^{fl/fl-ENDO} n=7-10) glycogen content at baseline and post exhaustive exercise in EPO^{WT} and EPO^{fl/fl-ENDO} mice. (E) Blood glucose pre- and post-exhaustive exercise in adult EPO^{WT} and EPO^{fl/fl-ENDO} mice (n=7-10). Data is presented as mean ± SD. Two-way ANOVA main effects are shown on the top of the figures (main effect of group † p<0.05)(main effect of treatment ‡ p<0.05)(interaction δ p<0.05). #p<0.05 compared to baseline for each group; *p<0.05 compared to EPO^{WT}, as determined using a Tukey's multiple comparisons test.
4.5 Skeletal myocyte hypertrophy in glycolytic muscle of EPO^fl/fl-ENO^ mice

Mice with the EPOR restricted to erythroid cells (Tg mice) display reduced skeletal muscle proliferation post-injury resulting in impaired recovery^{116}. However, under baseline conditions, Tg mice experience normal skeletal muscle development^{116}. Interestingly, skeletal muscle EPO overexpression mice exhibit increases in angiogenesis under baseline conditions^{91}. To evaluate if oxygen delivery to the skeletal myocytes is a contributing factor to the exercise intolerance, skeletal muscle morphology was investigated. Gross skeletal morphology was not different between EPO^{WT} and EPO^{fl/fl-ENO} mice and there were no differences in skeletal muscle weights (EDL and soleus)(Figure 4A & 4B). In contrast, the glycolytic muscle (EDL) of the EPO^{fl/fl-ENO} mice exhibit greater CSA, compared to EPO^{WT} (Figure 4D). While there was no difference in overall capillary density (data not shown) between EPO^{WT} and EPO^{fl/fl-ENO} mice, the ratio of capillaries per myocyte, when normalized to CSA (Figure 4F), was reduced. This indicates that angiogenesis was affected in the developing glycolytic muscle and suggests that the cellular hypertrophy of the EPO^{fl/fl-ENO} mice, which could impair oxygen delivery during exercise. No differences in the CSA (Figure 4E) and normalized capillary density (Figure 3G) were observed in the soleus muscle.
Figure 4.4 | EPO^{fl/fl-ENDO} mice show glycolytic skeletal myocyte hypertrophy
(A) EDL and (B) soleus muscle weights in adult EPO^{WT} and EPO^{fl/fl-ENDO} mice (EPO^{WT} n=12, EPO^{fl/fl-ENDO} n=15). (C) Representative images of EDL and soleus from adult EPO^{WT} and EPO^{fl/fl-ENDO} mice stained with wheat germ agglutinin (WGA) and isolectin. (D) EDL (EPO^{WT} n=5, EPO^{fl/fl-ENDO} n=7) and (E) soleus (EPO^{WT} n=4, EPO^{fl/fl-ENDO} n=5) cross sectional area (CSA) in adult EPO^{WT} and EPO^{fl/fl-ENDO} mice. (F) EDL and (G) soleus capillary density per cell normalized to CSA in adult EPO^{WT} and EPO^{fl/fl-ENDO} mice (n=5). Magnification: 20X; bar represents 200µm. Data is presented as mean ± SD. *p<0.05 compared to EPO^{WT}, as determined by an unpaired, one-tailed nonparametric t-test.
4.6 No change in whole body substrate selection in EPO^{fl/fl-ENDO} mice

To determine whether the observed glycogen storage impairments resulted in changes to energy homeostasis, we investigated whether EPO^{fl/fl-ENDO} mice had altered metabolic parameters (e.g., volume of O2 consumed) and activity, as compared to EPO^{WT}. To assess metabolism, EPO^{WT} and EPO^{fl/fl-ENDO} mice were housed in CLAMS metabolic cages for 48 hours. No difference in total oxygen consumption (Figure 5A & 5B), total carbon dioxide production (Figure 5C & 5D) and respiratory exchange ratio (Figure 5E & 5F) were observed, indicating that the EPO^{fl/fl-ENDO} mice when compared to EPO^{WT} do not have altered substrate selection. EPO^{fl/fl-ENDO} mice did not display changes in food intake (Figure 5G) or total activity levels (Figure 5H) when compared to EPO^{WT}.
4.7 Increased fasting blood glucose with no change in glucose tolerance in EPO<sup>fl/fl-ENDO</sup> mice

Deletion of EPORs in non-erythroid cell impairs glucose homeostasis in both TgEPOR<sup>76</sup> and ΔEPOR<sup>adipose</sup> <sup>141</sup> mice. Whether deletion of endothelium EPO results in a similar effect, we evaluated circulating blood glucose levels. No difference in the fed and 6 hour fasted blood glucose levels between groups. In contrast, blood glucose levels after 12 hours of fasting (overnight) were increased in the EPO<sup>fl/fl-ENDO</sup> mice compared to the EPO<sup>WT</sup> (Figure 6A). Glucose tolerance was investigated
using both an intraperitoneal (IP) and oral (O) glucose tolerance test (GTT). Mice baseline glucose levels were tested prior to injection/oral gavage with a glucose dosage of 2g/kg body weight. No difference in glucose tolerance in response to the IPGTT (Figure 6B & 6C) and the OGTT (Figure 6D & 6E) in the EPO^{fl/fl-ENDO} mice in comparison to EPO^{WT}.

**Figure 4.6 | Comparison of glucose tolerance between EPO^{WT} and EPO^{fl/fl-ENDO} mice**

(A) Fed (n=5), 6hr fasted (n=7) and 12hr fasted (EPO^{WT} n=15, EPO^{fl/fl-ENDO} n=24) blood glucose levels in adult EPO^{WT} and EPO^{fl/fl-ENDO} mice. (B) Intraperitoneal glucose tolerance test (IPGTT) in adult EPO^{WT} and EPO^{fl/fl-ENDO} mice with relative changes in blood glucose over time plotted, and (C) AUC was calculated (n=9). (D) Oral glucose tolerance test (OGTT) in adult EPO^{WT} and EPO^{fl/fl-ENDO} mice with relative changes in blood glucose over time plotted, and (E) AUC was calculated (n=7). Data is presented as mean ± SD. Two-way ANOVA main effects are shown on the top of the figure (main effect of time ‡ p<0.05). #p<0.05 compared to fed state for each group; *p<0.05 compared to EPO^{WT}, as determined using a Tukey's multiple comparisons test.
CHAPTER FIVE

DISCUSSION
Here we show the first non-renal endogenous source of EPO that exhibits a physiological relevant role in carbohydrate metabolism. EPO expression is not ubiquitous amongst striated muscle, but rather specific to glycolytic muscle. The salient findings demonstrate that deletion of endothelial EPO results in exercise intolerance that is not caused by impairments to intrinsic muscle function but to a combination of alterations in substrate storage and utilization. This change in local substrate metabolism does not result in differences to whole body substrate selection under baseline conditions. We show that the endothelium is a non-renal source of physiologically relevant EPO expression that regulates carbohydrate metabolism in murine. These data suggest a new role of the endothelium in the regulation of muscle metabolism in a hypoxia independent environment that has implications for chronic metabolic diseases, such as obesity, and aging, which are associated with endothelial dysfunction.

While the kidney is a significant source of EPO, the specific cell type that produces EPO remains elusive. EPO production within the kidney is localized to the corticomedullary region\textsuperscript{229}. These peritubular cells are distinctive as they exhibit both fibroblast- and neuronal-like morphology\textsuperscript{230}. Despite extensive investigation, researchers have yet to conclusively identify a particular cell type responsible for renal EPO production. The possibility of a summation of a number of EPO producing cells within the kidney has not been explored. Interestingly, the EPO\textsuperscript{flo/flo-ENDO} mice have a reduction (~30\%) in renal EPO expression in comparison to EPO\textsuperscript{WT} controls (Figure 1C). This suggests that the endothelium within the kidney accounts for a physiological significant portion of EPO expression. Despite this reduction, EPO\textsuperscript{flo/flo-ENDO} mice do not experience changes in circulating EPO (Figure 1F) and/or hematocrit (Figure 1E). This indicates that endothelial-derived EPO is not required to maintain hematocrit under baseline conditions. Investigation into the hypoxic response of endothelial EPO could be accomplished through a hypoxic chamber of adult mice or cobalt chloride treatment of isolated endothelial cells, both of which are known to promote an increase in EPO expression\textsuperscript{231}. As
the change in EPO expression within the kidney does not result in changes to circulating EPO levels, we suggest that endothelial-derived EPO remains local within the expressing tissue and elicits paracrine effects.

Investigation into the effects of EPO on cardiac function suggests EPO is an inotropic agent \textit{in vitro}^{84-86}. Two critical limitations of using rhEPO to investigate the non-erythropoietic roles of EPO is: 1) the concentrations used are at a supraphysiological level and 2) the artificial glycosylation of the rhEPO. It is unknown whether a supraphysiological dosage of rhEPO results in non-specific receptor binding (i.e., non-EPOR), which could exhibit non-endogenous EPO outcomes. Particularly, supraphysiological dosages of rhEPO have been used exclusively to evaluate the role of EPO on cardiac function\textsuperscript{84-86}. Using echocardiography and invasive hemodynamics the cardiac function of EPO$^{fl/fl}$-ENDO mice exhibited no change when compared to EPO$^{WT}$ (Table 4.2). As there is no change in cardiac morphology (data not shown) and serum EPO levels (Figure 4.1C) the preservation of cardiac function at baseline of the EPO$^{fl/fl}$-ENDO mice was not unexpected. This suggests that endothelial-derived EPO in the heart is not required to maintain normal cardiac function at baseline. Investigation into the role of endothelial EPO in response to cardiac stress could be evaluated using a Dobutamine stress test, which would demonstrate if endothelial-derived EPO has a critical role in contractile reserve. As the heart becomes hypoxic with strenuous exercise, and expression of EPO increases in response to hypoxia, differences in the cardiac function of the EPO$^{fl/fl}$-ENDO mice could occur when stress to the system is applied (e.g., exercise). This data would allow for exclusion of cardiac impairments contributing to the exercise intolerance observed in the EPO$^{fl/fl}$-ENDO mice.

Due to the extensive glycosylation of EPO, which interferes with epitope recognition, commercially available antibodies have poor specificity for endogenous EPO. Unfortunately, the poor effectiveness of anti-EPO antibodies hinders the investigation of tissue-specific EPO production. Limited knowledge on the antibodies in the ELISA, which are designed to bind rhEPO, may impair our
ability to detect changes in tissue-specific EPO. However, it remains unknown if these ELISAs have the same affinity for endogenous EPO as rhEPO, therefore, we need to better understand what the ELISA is detecting. In doing so, we need anti-bodies that can specifically bind different glycoforms of EPO, which will allow us to track disease and tissue specific production of EPO. It is possible that we are not able to detect changes in circulating endothelial EPO, and that our observation of no change in serum EPO, should be interpreted with causation as it a result of limited binding of EPO glycoforms to the ELISA.

Chronic anemic patients are exercise intolerant and display increased fatigability\textsuperscript{89} that is partially attributed to structural and functional abnormalities in the skeletal muscle\textsuperscript{88}. Treatment with rhEPO results in improved quadriceps contraction\textsuperscript{90}. Hemoglobin levels are normalized, thus the enhancement in oxygen carrying capacity, compared to improved intrinsic muscle function, could be the determining factor for the increase in exercise tolerance. Modifying endogenous EPO signaling does not directly change intrinsic muscle function. EPO overexpression\textsuperscript{91} and EPO deficient\textsuperscript{92} mice exhibit no changes in force production or resistance to fatigue. This is in line with the results observed in the EPO\textsuperscript{fl/fl-ENDO} mice in which there was no change in force production (Figure 2C & 2D) or fatigability (Figure 2E & 2F) of both the glycolytic (EDL) and oxidative (Soleus) muscle. This further insinuates that direct EPO signaling within skeletal muscle is not necessary for intrinsic muscle function.

Inconsistent results plead for further investigation into the role of EPO in skeletal muscle development. The developing mouse hind limb originate as a bump on the flank of the embryo, which during a 5-day period from E9.5 to E14.5 grows to form a miniature model of an adult hind limb\textsuperscript{232}. This process of limb development is a balance between cell proliferation and cell death\textsuperscript{232}. The adult EPO\textsuperscript{fl/fl-ENDO} mice have cellular hypertrophy but no changes in muscle mass (Figure 4). The glycolytic EDL muscle of the EPO\textsuperscript{fl/fl-ENDO} displays increased CSA compared to EPO\textsuperscript{WT} (Figure 4D), however
this does not result in changes to total muscle weight (Figure 4A). Taken together, cellular hypertrophy in the absence of changes in muscle weight suggests a reduction in the overall number of myocytes in the EDL of adult EPO^{fl/fl-ENDO} mice. The Tek promoter is expressed ubiquitously in endothelial cells by E9.5^{228}. As the Tek promoter is expressed from the beginning of limb development, the deletion of endothelial EPO has resulted in altered skeletal muscle morphology of the glycolytic muscle fiber type. Our data would suggest that endothelial-derived EPO is required for cell proliferation during limb development of glycolytic but not oxidative muscle fiber type. Further investigation into EPO’s role in the development of skeletal muscle is required as Tg mice (EPOR restricted to erythroid cells) exhibit normal skeletal muscle development under baseline conditions^{67}. This contradicting data, paired with the lack of consistency on expression of the EPOR in skeletal muscle^{66,68,77,81,82} implies EPO could potentially have an indirect role in skeletal muscle development.

There are 3 energy resources utilized in the body: carbohydrates, proteins and lipids. The energy source utilized can be determined by indirect calorimetry, in which oxygen consumed and carbon dioxide produced are measured. The respiratory exchange ratio, calculated by the ratio carbon dioxide produced/ oxygen consumed, is a surrogate of whole body substrate utilization^{233}. With disease, (e.g., obesity) selectivity between energy substrates shifts^{234,235}. As EPO is emerging as a cytokine with roles in metabolic homeostasis, we evaluated the whole body metabolism of the EPO^{fl/fl-ENDO} mice using a comprehensive laboratory animal monitoring system. No differences in metabolic parameters (e.g., oxygen consumed, respiratory exchange ratio) were observed in the EPO^{fl/fl-ENDO} mice (Figure 5). We expected to see a shift towards lipid metabolism as the storage of glycogen within glycolytic skeletal muscle (EDL) was reduced (Figure 4A). This suggests that the impaired storage of carbohydrates within the muscle did not result in changes to whole body substrate selection. Although Tg (EPOR restricted to erythroid cells) and adipose EPOR knockout mice exhibit reductions in respiratory exchange ratio^{76}, contradicting data in healthy humans demonstrates that a single dosage of
rhEPO also results in a decrease in respiratory exchange ratio\textsuperscript{194}. Differences in the metabolic response to EPO could, in part, be due to changes in health (e.g., baseline vs disease), therefore, further investigation into the EPO’s role in energy homeostasis is required. Using the EPO\textsuperscript{fl/fl-ENDO} mice, we could explore the role of endothelial specific EPO in maintaining energy homeostasis in a variety of models that experience metabolic dysregulation such as aging, obesity, and cardiovascular disease.

In rodent studies, rhEPO treatment repeatedly corrects the typical obesity-induced metabolic dysregulation (e.g., adipose tissue accumulation, hyperglycemia, glucose intolerance, insulin sensitivity)\textsuperscript{76,141,152,157,158}. To determine if endothelial-derived EPO plays a role in the maintenance of glucose homeostasis, we used both an oral and intraperitoneal glucose tolerance test. EPO\textsuperscript{fl/fl-ENDO} mice display no differences in glucose tolerance compared to the EPO\textsuperscript{WT} under baseline conditions (Figure 6B & 6D). This suggests that either endothelial EPO is not required for maintenance of glucose homeostasis or the health status (e.g., high fat diet-induced obesity) and/or the supraphysiological dosages of rhEPO used are vital for the changes in glucose tolerance seen in rhEPO-treated mice. To investigate if endothelial-derived EPO plays a role in glucose homeostasis in disease state, high fat diet-induced obesity could be used as a metabolic stressor. High fat diet-induced obesity is one surrogate model available to explore the etiology of obesity as a multifaceted chronic disease involving pathophysiological changes and adoptions.

While our understanding of the role of EPO in metabolism in females is limited, there is clearly a sex-specific difference of EPO in metabolism. A study by Zhang et al. (2017) demonstrates that sex differences in mouse metabolic response to rhEPO are present during health and disease. With either a regular chow or high fat diet, male mice experience reductions in body weight and fat mass accumulation with rhEPO therapy, while female mice do not. However, in both chow and high fat diet fed groups’ male and female mice exhibit decreased fasting blood glucose levels and improved glucose tolerance (Table 5.1). This suggests the improvements in glucose homeostasis are independent of
reductions in body weight and fat mass. Zhang et al. further investigated the role of estrogen in EPO signaling by treating ovariectomized females fed a high fat diet with rhEPO, observing similar reduction in weight gain and fat mass accumulation as in males. As the effect is abrogated with estradiol supplementation, these findings indicate estrogen is associated with the metabolic effects of EPO signaling\textsuperscript{236}. As these results were explored using rhEPO, endogenous EPO signaling in females has yet to be fully elicited. Teng et al. (2011) briefly state that the female Tg mice (EPOR restricted to erythroid cells) exhibit an accelerated obese phenotype compared to litter matched males and attribute the difference to estrogen-dependent EPO signaling (Table 5.1). This gap in the knowledge of endogenous EPO signaling in females proposes future experiments to investigate sex-specific differences in energy homeostasis. Thus, using the EPO$^{fl/fl}$-ENDO mouse model, we can evaluate the effects of endothelial EPO on metabolism regulation in female mice. In relation to published data on endogenous EPO’s role in metabolism, I would predict that female EPO$^{fl/fl}$-ENDO mice, when stressed with a high fat diet, would experience greater metabolic dysfunction (e.g., increase body weight, glucose intolerance) than male EPO$^{fl/fl}$-ENDO mice.

Table 5.1 | Summary of metabolic responses in transgenic EPOR female mice and female mice treated with rhEPO  
<table>
<thead>
<tr>
<th>Reference</th>
<th>Model</th>
<th>Energetic Stress</th>
<th>Results</th>
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<tbody>
<tr>
<td>Teng et al. (2011)\textsuperscript{76}</td>
<td>Tg Mice (EPOR restricted to erythroid cells)</td>
<td>Normal Chow Diet</td>
<td>↑ Body weight, ↑ Accumulation of adipose, ↓ Insulin sensitivity, ↓ Glucose tolerance</td>
</tr>
<tr>
<td>Zhang et al. (2017)\textsuperscript{236}</td>
<td>C57BL/6 treated with rhEPO for 3 weeks</td>
<td>High Fat Diet</td>
<td>↔ Body weight, ↔ Accumulation of adipose, ↓ Fasting blood glucose, ↑ Glucose tolerance</td>
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Repeated activation of muscles results in a progressive decline in performance that largely recovers after a period of rest. This reversible phenomenon is muscle fatigue. Many muscle properties change during fatigue (e.g., intracellular metabolites) and a range of mechanisms have been identified that contribute to the decline in function\textsuperscript{237}. The original explanation, that accumulation of intracellular lactate and hydrogen ions causes impaired function of contractile proteins, which is largely thought to not play a primary role in fatigue\textsuperscript{237}. Instead, alternative explanations (e.g., substrate availability, sarcoplasmic reticulum Ca\textsuperscript{2+} handling, ionic changes on the action potential) are considered the main causes of muscle fatigue\textsuperscript{237}. One accepted root cause of fatigue is reductions in oxygen supply to the working muscle during prolonged aerobic exercise. The value of pO\textsubscript{2} of the working muscles does not change from mild to severe exercise, this indicates that diffusion is maximal and that the only increase in O\textsubscript{2} must come from increasing blood flow\textsuperscript{238}. The EPO\textsuperscript{fl/fl-ENDO} mice have reduced capillary to CSA ratio therefore the glycolytic skeletal muscle could be experiencing hypoxia during exercise due to potential reductions in blood flow.

In skeletal muscle, glucose is stored as glycogen. This substrate store is a major source of energy during muscle activity. Several studies confirm that glycogen depletion is associated with fatigue\textsuperscript{239–241}. The current understanding is that prolonged exhaustive exercise results in depletion of glycogen that contributes to fatigue by decreasing sarcoplasmic reticulum Ca\textsuperscript{2+} release\textsuperscript{237}, yet the mechanism is not fully understood\textsuperscript{242}. EPO\textsuperscript{fl/fl-ENDO} mice exhibit reduced glycogen content in glycolytic muscle (EDL)(Figure 3A) at baseline. No difference in post-exhaustive exercise glycogen content was observed in the skeletal muscle and liver of the EPO\textsuperscript{fl/fl-ENDO} mice compared to EPO\textsuperscript{WT}. The EPO\textsuperscript{fl/fl-ENDO} mice, while starting with less muscle glycogen, deplete glycogen stores to the same level as EPO\textsuperscript{WT}, which would limit energy production within the exercising muscle and could result in the increased fatigability. Further investigation into the association between impaired substrate availability
in the skeletal muscle of the endothelial-derived EPO knockout and the exercise intolerance is necessary to determine EPO’s role in substrate metabolism during energetic stress.

In contrast, recent work by Williams et al. (2013) suggests that hypoglycemia, not depletion of muscle glycogen, results in fatigue\textsuperscript{243}. Animals fed low glycogen diets exhibited reduced voluntary treadmill exercise, as compared to either control (i.e., normal chow diet) or high glycogen diets. However, using \textit{in situ} stimulation, they were unable to detect differences in the rate of fatigue between groups\textsuperscript{243}. This indicates that reduced muscle glycogen does not affect isolated muscle function but markedly impairs voluntary exercise. Williams and colleagues suggest that hypoglycemia, rather than peripheral muscle fatigue, leads to exhaustion. In the EPO\textsuperscript{fl/fl-ENDO} mice no change in blood glucose is seen at exhaustion compared to EPO\textsuperscript{WT} (Figure 3E). This implies that in the case of exercise intolerance in the EPO\textsuperscript{fl/fl-ENDO} mice, hypoglycemia is not the cause of increased fatigability. The cause of fatigue in EPO\textsuperscript{fl/fl-ENDO} mice is likely multiply factorial, but resides, at least in part, with impairments in perfusion, and carbohydrate availability.

The potential for cell specific EPO production and the lack of anti-EPO antibody specificity results in the need for transgenic mouse models. Multiple glycoforms of EPO exist within human blood during normoxia\textsuperscript{27}. This raises the likelihood of tissue-specific glycoforms. As glycosylation is imperative for appropriate receptor binding, tissue-specific glycoforms may have altered physiological relevance owing to modified binding affinities. As anti-EPO antibodies are not known to be specific for the potential tissue-specific glycoforms we utilized the Cre-lox system for tissue-specific investigation of EPO expression to bypass this obstacle. We used a constitutively active promoter that inopportunely comes with both strengths and limitations. As the constitutive deletion of EPO in the endothelium occurs before development of limb muscles, it is difficult to ascribe any changes in adult muscle function to EPO produced from adult endothelium versus EPO produced during embryogenesis resulting in developmental adaptations. For example, the EPO\textsuperscript{fl/fl-ENDO} mice exhibit modifications to
the glycolytic muscle morphology, which in adulthood is most likely contributing to the exercise intolerance. The adult EPO^{fl/fl-ENDO} EDL muscle displays increased CSA without paired increases in the number of capillaries thus resulting in the potential for a hypoxic core of an exercising muscle due to impaired O2 delivery. Using the *in vitro* muscle preparation, no change in force production and fatigability were observed in the EPO^{fl/fl-ENDO} mice compared to EPO^{WT}. The *in vitro* muscle preparation negates blood flow issues as delivery of substrates to the tissue is solely based on diffusion. Taken together, these findings suggest that the reduced capillary to CSA ratio contributes to the exercise intolerance in the EPO^{fl/fl-ENDO} mice. To detangle the developmental adaptations and physiological effects of endothelial EPO deletion, an inducible Tek2 Cre promoter should be used. Inducible deletion would be ideal for exploring the physiological effects of EPO on energy homeostasis in health and disease in an adult murine.

In conclusion, the results presented provide contributions to the field of EPO physiology, specificity focusing on EPO’s non-erythropoietic roles. Although decades of research have fixated on the kidney as the primary source of EPO, findings demonstrated here encourage the shift in our view that there is physiological relevant expression from multiple sources that can exhibit paracrine effects. Investigation into the non-renal sources of EPO would expand our understanding of EPO biology, providing important insights into metabolism – in health and disease – and could lead to new therapeutic avenues to treat muscle dysregulation.
REFERENCES


