The Crosstalk of PCB126 and Hypoxia Sensing Pathways: Effect on Adipocyte Metabolism and Development.

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THESIS ABSTRACT

This thesis evaluated how lipophilic persistent organic pollutants, which accumulate in lipid-rich tissues, and hypoxia, which may develop during adiposity excess, influence adipose tissue functions. A narrative review and two experimental studies were conducted. In the review, we summarize how the hypoxic environment within adipose tissue can modify its functionality, impacting the secretion of adipokines, promoting inflammation, and potentially contributing to metabolic disturbances associated with obesity.

We also examine the interplay between dioxin-like polychlorinated biphenyl-PCB126 and hypoxia, evaluating their combined impact on adipose tissue functions. Both hypoxia and PCBs have demonstrated effects on adipose tissue functions. The signaling pathways induced by PCB126 and hypoxia may crosstalk, as they share a common transcription factor: aryl hydrocarbon receptor nuclear translocator (ARNT). In a first study, using primary human subcutaneous adipocytes (hSA) acutely co-exposed to different levels of PCB126 (48 h) and hypoxia (24 h), a unidirectional crosstalk was confirmed, with hypoxia significantly inhibiting PCB126-induced cytochrome P450 (CYP1A1) transcription. Acriflavine (ACF), an HIF-1α inhibitor, partially restores this induction under hypoxia. Additionally, hypoxia and PCB126 elicit distinct effects on the secretion of key adipokines (leptin, adiponectin, (interleukin)- IL-6 and IL-8), emphasizing a lack of apparent crosstalk between these factors.

Building upon these insights, the second study broadens the scope to examine the combined effects of PCB126 and hypoxia on hSA differentiation, glucose metabolism, and inflammation. Employing pre-differentiation PCB126 exposure and subsequent differentiation under prolonged hypoxia on hSA, results indicate that pre-differentiation PCB126 exposure leads to diminished adenosine triphosphate (ATP) content, reduced glucose uptake, and suppressed leptin expression in mature adipocytes. Under normoxia, PCB126 has limited effects on differentiation, but under hypoxia, preadipocyte differentiation is significantly impaired, marked by decreased lipid accumulation and downregulation of key adipocyte genes including peroxisome proliferator-activated receptor-gamma (PPARγ) and adiponectin. Hypoxia induces increased glucose uptake and glucose transporter 1 (GLUT1) expression, coupled with a loss of adipocytes' insulin response and GLUT4 expression. The combined exposure to PCB126 and hypoxia exhibits additive effects

on adipose tissue inflammation, evidenced by increased expression of pro-inflammatory adipokines IL-6 and IL-8. While PCB126 does not directly impact human preadipocyte differentiation, it does influence subsequent adipocyte populations, as indicated by lower ATP levels and absolute glucose uptake. This research provides valuable insights into the complex interactions of environmental factors with adipose tissue physiology, contributing to our understanding of potential links between persistent organic pollutant (POP) exposure, hypoxia, and the development of chronic metabolic disorders.

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LIST OF ABBREVIATIONS

ACC : Acetyl-CoA carboxylase

ACF : Acriflavine

ADP : Adenosine diphosphate

AhR : Aryl hydrocarbon receptor

AhRR : Aryl hydrocarbon receptor repressor

AMPK : 5′ adenosine monophosphate-activated protein kinase

ARNT : Aryl hydrocarbon receptor nuclear translocator

ASCs : Adipose tissue stem cells

ATBF : Adipose tissue blood flow:

ATP : Adenosine triphosphate

bHLH/PAS : Basic helix-loop-helix/PER-ARNT-SIM

ChREBP : Carbohydrate response element binding protein

$CoCl_2$: Cobalt chloride

COL13A1 : Collagen type XIII alpha

CREB : cAMP response element-binding protein

CVD : Cardiovascular diseases

CYP1A1 : Cytochrome P450 1

DGAT1 and DGAT2 : Diacylglycerol acyl transferase 1 and 2

DLCs : Dioxin-like compounds

DMSO : Dimethyl sulfoxide

DNA : Deoxyribonucleic acid

ECM : Extracellular matrix

ER : Estrogen receptor

ERE : Estrogen response element

FASN : Fatty acid synthase

FIH : Factor-inhibiting HIF

GLUT : Glucose transporter

HIF-1α : Hypoxia-inducible factor-1α

HIFs : Hypoxia-inducible factors

HRE : Hypoxia response element

hSA : Human subcutaneous adipocytes

hsp90 : Heat shock protein 90

IARC : International Agency for Research on Cancer

ICR mice : Institute of Cancer Research mice

IGF-1 : Insulin-like growth factor 1

IL-6 and IL-8 : Interleukin-6 and 8

LDH : Lactate dehydrogenase

LOX : Lysyl oxidase

LPL : Lipoprotein lipase

MCP-1 : Monocyte chemoattractant protein 1

MCT : Monocarboxylate transporter

MeSO2-PCBs : Polychlorinated biphenyl methyl sulfones

MetS : Metabolic syndrome

MMP : Matrix metalloproteinase

MMPs : Metalloproteinases

mRNA : Messenger ribonucleic acid

MTC : Monocarboxylate transporters

NAFLD : Non-alcoholic fatty liver disease

NEFA : Non-esterified fatty acid

NF-κB : Nuclear factor kappa-light-chain-enhancer

NPAD cells : Human adipogenic immortalized cell line

NPADs : Normal Pre-Adipocytes

O_2: molecular oxygen

OH-PCB : Hydroxylated PCB metabolites

PAI-1 : Plasminogen activator inhibitor

PBS : Phosphate-Buffered Saline

PCB : Polychlorinated biphenyl

PCB126 : 3,3',4,4',5-pentachlorobiphenyl PCB

PCDF : Polychlorinated dibenzofurans

PDGF : Platelet-derived growth factor

PHDs : Prolyl hydroxylase domain-containing enzymes

PI3K : Phosphatidylinositol 3-kinase

PIK3R1 : Phosphatidylinositol 3-kinase regulatory subunit 1

PO_2 : Partial pressure of oxygen

POPs : Persistent organic pollutants

PPARγ : Peroxisome proliferator-activated receptor-gamma

sWAT : Subcutaneous white adipose tissue

TCDD : 2,3,7,8-tetrachlorodibenzo-p-dioxin

TEQ : Total toxic equivalence

TG : Triglyceride

TNF-α : Tumor-necrosis factor-alpha

VEGFA : Vascular endothelial growth factor-A

VHL : von Hippel-Lindau

WAT : White adipose tissue

XRE : Xenobiotic response element

CHAPTER 1- BACKGROUND AND SIGNIFICANCE

1.1. INTRODUCTION

Adipose tissue is an essential player involved in whole-body energy and glucose homeostasis through its action on lipid storage/mobilization and its secretion of numerous bioactive factors called adipokines (Harwood, 2012; Luo & Liu, 2016; Vegiopoulos et al., 2017). Dysfunction of adipose tissue can arise in response to prolonged high caloric intake where it is marked by anomalies in adipocyte proliferation and hypertrophy, insufficient vascularization, infiltration of immune cells and increased production of pro-fibrotic extracellular matrix (ECM) proteins (Matafome & Seiça, 2017). Adipose tissue dysfunction as a result of excessive adiposity (i.e., obesity) is often associated with chronic, low-grade inflammation and insulin resistance (de Ferranti & Mozaffarian, 2008). During adipose tissue inflammation several inflammatory pathways are upregulated triggering a cascade of inflammatory adipokine secretions, such as tumor-necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1).

Excessive adiposity is associated with hypertrophied adipocytes where a condition of reduced oxygen tension (hypoxia) at the adipocyte level can build up contributing to its altered function (Trayhurn & Wood, 2004). The response to hypoxia is mediated transcriptionally by hypoxia-inducible factors (HIFs). HIF-1α regulates various target genes involved in cellular responses to adapt to lower oxygen levels, among which is VEGFA (Vascular endothelial growth factor-A), a gene which product is involved in angiogenesis (Herold & Kalucka, 2021), **Figure 1.** Evidence indicates that hypoxia affects adipocyte differentiation and alters their inflammatory and adipokine profile (Lawler et al., 2016; Trayhurn, 2013). Trayhurn and Wood (2004), who pioneered research on adipose tissue hypoxia, were the first to suggest that may be a potential driver of inflammation in adipose tissue during obesity.

Another adipose tissue stressor, besides hypoxia, consists of environmental pollutants. Several studies have indeed linked high adiposity with the accumulation of lipophilic persistent organic pollutants (POPs). POPs are hypothesized to disrupt adipose tissue function and act as endocrine disruptors by modulating the expression of crucial genes involved in adipogenesis, lipid

metabolism, and inflammatory factors associated to obesity-induced inflammation (Heindel et al., 2017; M. J. Kim et al., 2012; Pereira-Fernandes et al., 2014). Polychlorinated biphenyls (PCBs), which are a subgroup of the original twelve POPs covered by the Stockholm convention, are synthetic chlorinated hydrocarbons (Van den Berg et al., 2006).

Dioxin-like PCBs share mechanistic similarities with the potent environmental toxicant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as they can bind to the aryl hydrocarbon receptor (AhR) and activate transcriptional response that is usually associated with the activation of cytochrome P450 1 (CYP1A1, involved in toxins detoxification) **Figure 1** (Cock et al., 2014; M. J. Kim et al., 2012; Regnier & Sargis, 2014), triggering a cascade of metabolic and endocrine

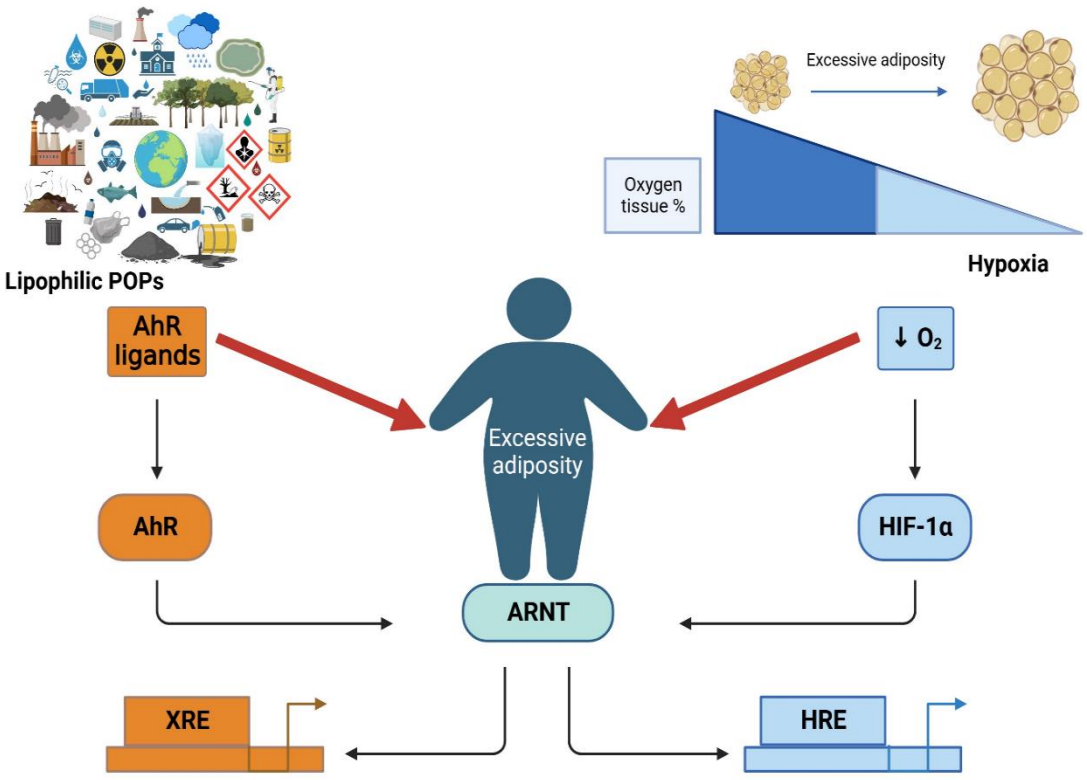

Figure 1. Excessive adiposity is associated with the bioaccumulation of lipophilic pollutants and development of hypoxic patches within adipose tissue. AhR ligands and lower oxygen level are two stressors that activate AhR and HIF induced pathway which both share a common factor, ARNT. Consequently, transcriptional response is activated, and genes are regulated.

Abbreviations: POPs: persistent organic pollutants, AhR: aryl hydrocarbon receptor, HIF-1α: hypoxia inducible factor 1 subunit alpha, ARNT: aryl hydrocarbon receptor nuclear translocator, XRE: xenobiotic response element, and HRE: hypoxia response element.

alterations that may have deleterious effect on adipose tissue development and function (E. L. Dirinck et al., 2016; Heindel et al., 2017; Lubrano et al., 2013). Findings in *in vitro* and *in vivo* studies imply a potential link between pollutants and the development of low-grade adipose tissue inflammation, which often accompanies the onset of obesity-related metabolic disorders (Joffin et al., 2018; M. J. Kim et al., 2012).

Crosstalk between HIF-1α and AhR pathway

Interestingly, both HIF-1α and AhR signaling share a common transduction factor, namely the aryl hydrocarbon nuclear translocator (ARNT) (Myre & Imbeault, 2014). Mammalian bHLH-PAS (basic HLH (helix-loop-helix)-PER-ARNT-SIM) proteins belong to the bHLH superfamily of transcription factors which have a broad spectrum of functions. The transcriptional activation of these proteins involves sensing and triggering cellular response to physiological signals (hypoxia, HIF-1α), or environmental signals (toxins, AhR). HIF-1α and AhR are signal-induced subunits in the bHLH-PAS proteins that both dimerize with ARNT. The two dimers (HIF-1α/ARNT and AhR/ARNT) bind to DNA, and accordingly induce the activation of a cascade of target genes (VEGFA and CYP1A1, respectively), **Figure 2** (Y.-Z. Gu et al., 2000; Kewley et al., 2004).

The regulatory crosstalk between the HIF-1α and AhR signaling pathways through ARNT has been highlighted in animal study of conditional ARNT knockout mice where TCDD is unable to induce various AhR target genes. Also, under mimicked hypoxic conditions (Cobalt chloride, $CoCl_2$), HIF-1α is not able to induce the expression of one of its target gene, GLUT1, highlighting the critical role of ARNT in both AhR and HIF-1α signaling (Tomita et al., 2000). Interestingly, it has been shown that once HIF-1α is activated, it predominates over AhR to recruit ARNT (Gradin et al., 1996). Consequently, CYP1A1 (marker gene of AhR pathway) expression and enzyme activity are inhibited by hypoxia. However, CYP1A1 inhibition by hypoxia is also observed in HIF-1α null hepatocytes, suggesting that HIF-1α might not be the only factor involved in the negative regulation of AhR signaling under hypoxia (Allen et al., 2005). Evidence also supports a hypoxia-dependent ARNT up-regulation depending on cell-line specificity (Mandl & Depping, 2014). Thus, AhR, and HIF-1α subunits competition for ARNT recruitment may depend on cell type, ARNT bioavailability and/or on the respective affinity of AhR and HIF-1α subunits for

ARNT. Further explanation on the induced-signaling pathways of hypoxia and dioxin, and their crosstalk is provided in section 2.4.

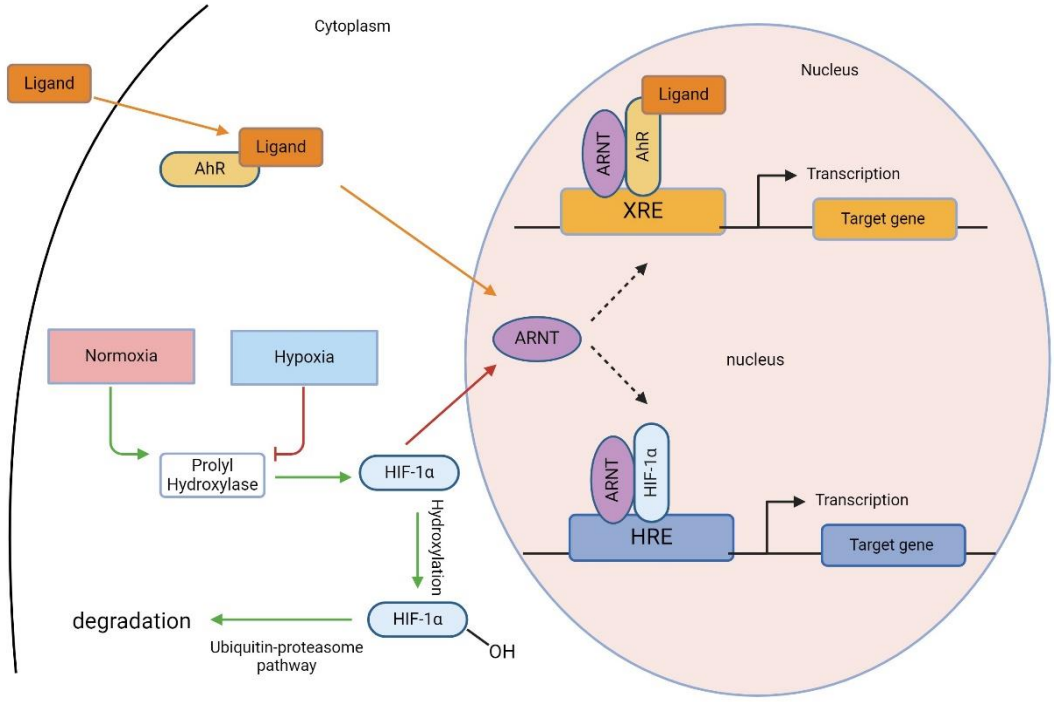

Figure 2. Xenobiotic and hypoxic signaling pathways. (a) AhR/Arnt mechanism: In its inactive state, the AhR is bound to various repressive factors in the cytoplasm. AhR ligands (*i.e.* dioxin-like coplanar PCB126) pass through the cell membrane and bind to AhR. The complex then translocates into the nucleus and dimerizes with ARNT. Binding of the AhR/ARNT complex to xenobiotic-response elements (XRE) induces gene expression.

(b) HIF-1α/Arnt mechanism: Under normal oxygen conditions (21% oxygen), HIF-1α proteins are proline hydroxylated by prolyl hydroxylase, ubiquitinated by the ubiquitin-proteasomal pathway and eventually leading to HIF-1α degradation. Under hypoxic conditions (< 6% oxygen), prolyl hydroxylase is diminished and non-hydroxylated HIF-1α translocates to the nucleus and dimerizes with ARNT. HIF-1α/ARNT complex then activates gene transcription by binding to hypoxia- response elements (HRE). Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; HIF-1α, hypoxia-inducible factor

In sum, it seems that a complex regulatory relationship governs the crosstalk between AhR and HIF-1α proteins (D. Lee, 2012). The role of ARNT in both AhR and HIF-1α signaling is well established, however, many of the observed findings seem to be dependent on the cell type, the exposure agent, and the duration of exposure.

To our knowledge, the interaction between the AhR and HIF pathways has not been explored in adipose tissue, despite it being the primary storage site for lipophilic pollutants. Such crosstalk in adipose tissue could potentially, in the context of hypertrophied adipocytes, shift cells

to a metabolically inactive phenotype with more pro-inflammatory status. Additionally, the response of adipocytes to simultaneous exposure to dioxin-like PCBs and hypoxia remains uninvestigated. These knowledge gaps underscore the necessity of enhancing our understanding of the impact of bioaccumulated lipophilic PCB pollutants on adipocyte metabolism within the context of cellular hypoxia.

1.2. RATIONALE AND SIGNIFICANCE

The effect of HIF and AhR pathways and their crosstalk through their common subunit ARNT have been studied extensively. Most of these studies were carried out on hepatocytes (the main site of pollutants detoxification) or cancer cells (since pollutants are shown to be carcinogenic) that usually reside in a hypoxic environment. Lipophilic persistent organic pollutants accumulate in lipid-rich tissues such as human adipose tissue. An excessive accumulation of adipose tissue is also characterized by local hypoxia. The impact of dioxin-like PCBs and hypoxia on adipocyte metabolism, differentiation and inflammatory profile is well documented, nevertheless, the crosstalk between these sensing pathways and ultimately the effects of this crosstalk on adipocytes have never been investigated. This knowledge would further our understanding of the biological outcomes and physiological significance of the regulatory crosstalk between hypoxia and certain environmental pollutants on adipose tissue metabolism and ultimately on human health.

Objectives

Thesis objectives were established as follows:

1. **Assess the crosstalk of hypoxia and AhR pathway through ARNT in human subcutaneous differentiated adipocytes (hSA) under co-exposure of hypoxia and PCB126 and its associated inflammatory response.**

   The first objective (Article 1) was accomplished by co-exposing fully differentiated hSA to 2 different severities of hypoxia (10% O_2 and 3% O_2) and 3 different PCB126 concentrations (0.1, 1 and 10µM). Then, we used ARNT dimerization inhibitor of the pathway that oppositely supressed the other pathway transcriptional response to assess the ARNT role as a common factor. We assessed the transcriptional response of hypoxia and AhR induced pathway by measuring the gene expression of key gene markers as well as some inflammatory adipokines.

2. **Assess the effect of PCB126 exposure on hSA ability to differentiate, glucose uptake and gene expression of some adipokines in the context of hypoxia.**

   The second objective (Article 2) was accomplished by exposing preadipocytes to PCB126 (10 μM) during proliferation and subsequently exposing them to hypoxia (3% O_2) throughout differentiation (14 days). This study design permitted to assess the differentiation ability of hSA by measuring the gene expression of PPARγ, triglyceride content, glucose uptake, and other inflammatory adipokines.

1.3. HYPOTHESIS

General hypothesis of the thesis:

Using primary culture of hSA simultaneously exposed to PCB126 and hypoxia, the general hypothesis of the thesis was that HIF-1α and AhR induced pathways would crosstalk in adipocytes, and consequently exert pro-inflammatory status and metabolic alterations. More specifically, this would translate into exacerbated inflammation, reduced ability to differentiate, altered glucose uptake and gene expression of key factors in adipocytes metabolism. The mechanism of this crosstalk through ARNT would translate when one of the pathways prevents the transcriptional response of the other. Since HIF-1α in most cell lines showed a higher affinity to ARNT, it is hypothesized that hypoxia would suppress the transcriptional response of the AhR induced pathway. However, the inflammatory response triggered by PCB126 on adipocytes co-exposed to hypoxia would still be observed since previous studies showed AhR-independent PCB-induced inflammatory response. Finally, we expected that hypoxia would have a greater impact on adipocytes' ability to differentiate than PCB126.

Hypothesis 1

The HIF and AhR pathways would crosstalk through ARNT in fully differentiated hSA exposed simultaneously to PCB126 and hypoxia. The hypoxia-induced pathway would therefore suppress the PCB126 induced pathway, consequently suppressing its transcriptional response by decreasing CYP1A1 expression. The use of HIF-inhibitor would reverse this

inhibitory effect. However, the inflammatory response of HIF and AhR induced pathway would be additive with altered adipokine profile.

Hypothesis 2

Human subcutaneous preadipocytes exposed to PCB126 prior to differentiation would have a decreased ability to differentiate and would display an inflammatory profile. Prolonged exposure to hypoxia for the 14 days of differentiation would decrease differentiation of adipocytes, favor an inflammatory status and increase glucose uptake. Also, the key adipokine, leptin and adiponectin, would be altered with hypoxia, leptin production being increased, and adiponectin decreased.

1.4. RELEVANCE OF THE THESIS

Although previous research has investigated the crosstalk between dioxin and hypoxia induced pathways, such interactions have not been explored in adipocytes. Examining the interplay between AhR and HIF-1α in response to PCB126/hypoxia exposure in adipocytes is expected to provide mechanistic insights into PCB126 toxicity and its potential implications for human toxicology, obesity, and related disorders. Given the bioaccumulation and extended half-lives of PCBs in adipose tissue, assessing their adverse effects on physiological processes will help us better understand their effects on public health, especially on chronic diseases such as type 2 diabetes and cardiovascular disease (CVD). The long-term objective of the research described in this thesis is to enhance our comprehension of how environmental pollutants, such as dioxin-like PCBs, function as metabolic disruptors within adipocytes in the context of cellular hypoxia. By investigating their activation and regulation of cellular responses in the context of varying oxygen environments, this thesis aims to further our knowledge of the involvement of environmental pollutants and cellular hypoxia on human health.

CHAPTER 2 - REVIEW OF THE LITERATURE

2.1 OBESITY AND ADIPOSE TISSUE, THE ENERGY RESERVOIR

Obesity is a public health epidemic affecting a substantial portion of the global population as 38% of individuals are currently living with either overweight or obesity (Koliaki et al., 2023). Obesity causes severe yet preventable comorbidities, notably cardiovascular diseases (CVD), diabetes, and cancer, a condition known as metabolic syndrome (MetS) (Goossens & Blaak, 2015). It is currently well acknowledged that adipose depots function as energy reservoirs as well as dynamic organs that secrete a multitude of bioactive molecules, hormones, and inflammatory cytokines and that obesity comorbidities are likely due to dysfunctional adipose tissue (Blüher, 2009). Adipose tissue comprises progenitor preadipocytes, differentiated adipocytes, and multipotent mesenchymal stem cells, known as adipose tissue stem cells (ASCs) (Rutkowski et al., 2015). While both ASCs and preadipocytes can differentiate into white adipocytes, preadipocytes are more committed to the adipose lineage (Tang & Lane, 2012). Preadipocytes constitute a substantial portion (15-50%) of fat tissue (Tchkonia et al., 2010). Adipose tissue development occurs from gestation to adolescence, driven by increased preadipocyte proliferation and differentiation. Post-adolescence, changes in fat mass primarily involve lipid accumulation, with minimal alterations in cell numbers (Spalding et al., 2008). During adulthood, adipocyte turnover involves a balance between adipocytes death and the proliferation/differentiation of preadipocytes (Tchkonia et al., 2010). Adipose tissue mass is dependent on both the average adipocyte volume and the number of adipocytes in the adipose tissue. When there is an energy surplus, neutral triglycerides (TGs) are stored in adipose tissue through the lipogenic pathway (adipogenesis). When energy surplus is repeated or prolonged, then neutral TGs deposition in adipocytes increases the lipid droplet size (*i.e.,* hypertrophied adipocytes), leading to obesity in the long term (Tan & Vidal-Puig, 2008). On the other hand, when energy demand exceeds energy intake, TGs reserved in adipocytes are broken down into glycerol and fatty acids through the lipolytic pathway (lipolysis) (Lafontan & Langin, 2009). The released glycerol and fatty acids from adipose tissue enter the blood stream and are subsequently delivered into muscle, liver, kidney and other organs to satisfy the energy need (Coelho et al., 2013; Frayn, 2002). Hence, the ability of preadipocytes to differentiate into mature adipocytes (adipogenesis) is crucial for adipose tissue to store lipids and orchestrate energy homeostasis. Failure to store lipids in adipose tissue

promotes ectopic lipid storage in non-adipose tissues such as the liver, pancreas, heart, and skeletal muscles, which adversely affect the metabolism of these tissues and organs, a process termed lipotoxicity (Borén et al., 2013; Morelli et al., 2013).

Adipogenesis is triggered by hormones and growth factors, with PPARγ serving as the master regulator (Gregoire et al., 1998; Rosen & Spiegelman, 2014). PPARγ activation drives the transcription of genes crucial for mature adipocyte development. Altered adipogenesis may lead to adipocyte dysfunction, increasing the risk of insulin resistance and type 2 diabetes (Acosta et al., 2016; Smith & Kahn, 2016). As stated above, PPARγ is expressed predominantly in adipose tissue where it plays a key role in adipocyte differentiation, enhancing insulin sensitivity, as well as promoting FA uptake by adipocytes; the net effect of these processes is an increase of TGs storage in adipocytes. Also, adipose tissue is a major endocrine organ secreting several key hormones, outstandingly leptin and adiponectin among several other adipokines, many of which are linked to the inflammatory response (Blüher & Mantzoros, 2015; Luo & Liu, 2016; Unamuno et al., 2018).

Adipose tissue dysfunction during obesity is still the subject of active research. The following sections will explore the possible contribution of hypoxia development as adiposity becomes excessive and lipophilic pollutants bioaccumulation in adipose tissue to altered metabolism and inflammation.

2.2 HYPOXIA

2.2.1 Hypoxia Hypothesis and Obesity

Obesity frequently gives rise to a state of hypoxia, particularly within the overly expanding adipose tissue, a phenomenon substantiated by clinical and preclinical inquiries (Trayhurn, 2013). In the early 2000s, the hypoxia hypothesis was postulated suggesting that the excessive accumulation of lipids in adipose tissue, characteristic of obesity, could induce hypoxia within adipose depots.

Research conducted in rodent models has indicated that the expansion of adipose tissue mass lacking sufficient neovascularization can induce adipose tissue hypoxia, resulting in

inflammation, fibrosis, and systemic insulin resistance (Crewe et al., 2017). However, the impact of excess adiposity on adipose tissue oxygenation in humans remains uncertain, given conflicting findings across various studies. Some studies have reported lower adipose tissue partial pressure of oxygen (PO_2) in individuals with obesity compared to lean individuals (Cifarelli et al., 2020; Kabon et al., 2004; Lawler et al., 2016; Pasarica et al., 2009, 2010), while others have found no significant differences (Goossens et al., 2011, 2018). Additionally, inconsistent results exist regarding adipose tissue PO_2 in individuals with metabolically healthy obesity compared to those with metabolically unhealthy obesity, with some studies linking obesity to higher adipose tissue PO_2 (Vink et al., 2017), lower adipose tissue PO_2 (Goossens et al., 2018), or not significantly different adipose tissue PO_2 (Lawler et al., 2016; Pasarica et al., 2010). Recent evidence associated hypoxia in adipose tissue of individuals with obesity to insulin insensitivity independently of adiposity (Lempesis et al., 2020). Discrepancies among studies may stem from variations in methods used to assess adipose tissue oxygen content, inadequate participant numbers for detecting statistically significant effects, variations in study populations such as the onset and physical history of obesity (e.g., weight cycling), and other individual characteristics (e.g., age, sex, ethnicity, presence of type 2 diabetes), as well as the specific subcutaneous white adipose tissue (sWAT) depot studied.

Several mechanistic inquiries have proposed that adipose tissue hypoxia may serve as the underlying mechanism responsible for the various molecular inflammatory processes typically observed in the adipose tissue of metabolically compromised individuals with obesity, such as inflammatory cellular infiltration, adipocyte apoptosis, endoplasmic reticulum stress, and perturbations in mitochondrial function (Ye, 2009).

2.2.2 Hypoxia and Adipocytes Metabolism

Note: The following section is succinct to avoid any redundancy with Chapter 3.1 entitled ‘White Adipose Tissue Metabolic Responses to Hypoxia’ which is a narrative review we published as a book chapter. Chapter 3.1 will provide additional information for the reader regarding hypoxia and its impact on the metabolism of adipocytes.

As mentioned previously, the response to hypoxia depends mainly on HIF-1α, a transcription factor that controls the expression of genes regulated by hypoxia-response elements.

HIF-1α targets genes that are involved in glucose metabolism, lipid metabolism and cell survival. Amongst these adaptive responses is the HIF-1α dependent reprogramming of glucose metabolism to reduce reliance on oxidative energy production (Papandreou et al., 2006). Basal glucose uptake into cells is governed by glucose transporters, GLUT1 and GLUT3, both of which are subject to HIF-1α regulation (Kido et al., 2020; Mamun et al., 2020). The mRNA expression levels of glucose transporters GLUT1 and GLUT3 are upregulated in adipocytes under hypoxia, however, only the protein level of GLUT1 was shown to be increased accordingly (Mamun et al., 2020; I. S. Wood et al., 2007). Pharmacological inhibition of HIF-1α significantly attenuated hypoxia-induced GLUT1 and GLUT3 expression. It has also been shown that hypoxia upregulates genes encoding glycolytic enzymes such as pyruvate dehydrogenase kinase 1 and lactate dehydrogenase A (G. L. Semenza, 2011). Combined, these observations indicate that hypoxia increases glucose uptake and induces anaerobic metabolism in adipocytes (H. S. Park et al., 2016; I. S. Wood et al., 2007) . The increased glucose uptake and its subsequent metabolism under conditions of hypoxia help maintain cellular ATP production concomitant with reduced oxidative metabolism.

Recent evidence indicates that hypoxia can alter lipid metabolism mainly in a HIF-dependent manner, contributing significantly to metabolic disorders and lipotoxicity (Mylonis et al., 2019). More specifically, hypoxia can contribute to elevate circulating free fatty acids levels through the induction of insulin resistance and inflammatory response, leading to the activation of lipolysis in adipocytes (Netzer et al., 2015; Jun Yin et al., 2009). In obesity, hypoxia and free fatty acids activates the nuclear factor kappa-light-chain-enhancer NF-κB pathway, which induces expression of inflammatory cytokines such as TNF-α and IL-6 in adipose tissue. NF-κB inhibits PPARγ function through nuclear corepressor, resulting in the suppression of the transcription of genes which products are involved in the insulin-induced GLUT4 translocation (*i.e.* insulin sensitivity) (Ye, 2009). In brief, the inhibition of adipogenesis and triglyceride synthesis by hypoxia leads to elevated levels of circulating free fatty acids, inflammation, and insulin resistance.

2.3 POLYCHLORINATED BIPHENYLS

2.3.1 Origins and Chemistry

Polychlorinated biphenyls (PCBs) are synthetic chlorinated hydrocarbons that drawn significant attention in the field of toxicology because of their environmental persistence, human exposure

routes, and diverse toxicological effects (Cock et al., 2014; E. Dirinck et al., 2011; M. J. Kim et al., 2012; Vorkamp, 2016; Walford et al., 1999).

Structurally, PCBs are compounds consisting of biphenyl rings with the molecular formula, C12H10-nCln, where "n" represents the number of chlorine atoms attached to the rings (Figure 3). PCBs contain 209 congeners in total. PCBs congeners differ according to the degree of chlorination and chemical distribution, ranging from monochlorinated to fully chlorinated forms. These structural variations give rise to diverse congeners and metabolites, each exhibiting unique interaction with biological targets, leading to distinct toxicological profiles (Safe, 1993). PCBs are broadly classified into "dioxin-like" and "non-dioxin-like" compounds based on their level and chlorination position (non or mono-ortho/ ortho congeners) and conformational structure (coplanar and non-coplanar congeners). Most toxicity studies related to PCBs at molecular and cellular levels focus on coplanar dioxin homologs due to their high toxicity (Dutta et al., 2012). Dioxin-like compounds (DLCs) exert their toxic effects primarily by their ability to bind and activate AhR, a protein that regulates the expression of certain genes involved in critical biological processes. Persistent activation of the AhR by these compounds can lead to disruption of AhR endogenous functions. As a result, a wide range of toxic outcomes are exerted, including cancer, reproductive and developmental problems, immune system disruption, and endocrine effects (White & Birnbaum, 2009).

In our studies, we used PCB126, known as 3,3',4,4',5-pentachlorobiphenyl, which is a coplanar non-ortho-substituted PCB congener. PCB126 lacks chlorine substitutions in the ortho positions of its phenyl rings, resulting in a planar structure that enhances its affinity for the AhR, making it the most potent dioxin-like PCB congener in the environment. Also, PCB126 is characterized by its highly chlorinated structure depicted in **Figure 3** (Walker et al., 2006). Owing to its high chlorination, PCB126 exhibits remarkable stability, accumulating persistently in the environment and food chain (Xing et al., 2011), contributing to 40% to 90% of the total toxic potency of PCBs exhibiting "dioxin-like" activity. This compound also demonstrates bioaccumulation in human and animal tissues, eliciting biological responses similar to those induced by TCDD (Černá et al., 2007; Walker et al., 2006).

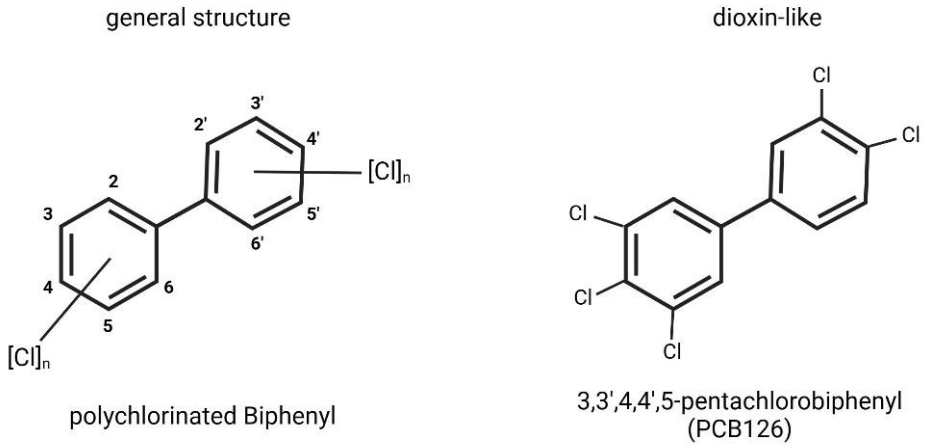

Figure 3. Structure of PCBs. PCBs have biphenyl core (left) with chlorines attached to it. PCBs without chlorines at ortho positions (2, 2', 6, 6') are called "dioxin-like" PCBs (right).

PCBs were originally manufactured on a large scale from 1929 to the early 1980s for various industrial applications due to their chemical stability, insulating properties, and non-flammability (Breivik et al., 2007; Safe, 1993). Commercial mixtures of PCBs were manufactured and sold as mixtures under different trade names (Arochlor, Fenchlor, Clophen, Kanechlor) and were widely used (Safe, 1993). PCBs were later banned worldwide for their toxicity. However, despite restrictions on PCBs manufacturing and use, these compounds continue to be released into the environment through sources such as hazardous waste sites, leakages, and spills, as well as the improper disposal of PCB-containing materials (Safe, 1993). As the climate warms, there is also evidence that POPs, including PCBs, deposited in sinks such as water and ice are expected to revolatilize into the atmosphere. More specifically, it is estimated that an increase in climate change by 1 °C would increase the volatility of POPs such as PCBs by 10 to 15% and thus increase their mobility in the ecosystem (Hung et al., 2022; Ma et al., 2011). Global warming could therefore undermine global efforts to reduce environmental and human exposure to these toxic chemicals. PCBs have a global spread that is found in food items like fish, sediments, and the air. They bioaccumulate in every living organism owing to their lipophilicity and resistance to biodegradation (Jackson et al., 2017), where PCB126 has an estimated half-life of 4–5 years in humans (Milbrath et al., 2009).

Despite regulatory guidelines to limit human exposure in various settings, including drinking water and occupational environments, PCBs, with their complex chemistry and wide-

ranging environmental and health impacts, remain a persistent environmental hazard, necessitating ongoing efforts to monitor, regulate, and mitigate their presence and effects (Hung et al., 2022; Vorkamp, 2016); and despite these intensive efforts, it remains clear that the population will continue to be exposed to these persistent environmental toxicants.

2.3.2 PCB Toxicity

The toxicity of PCBs congeners varies with their structural variation and their metabolites, leading to differential interactions with various biological targets, such as the nervous system, the endocrine systems (thyroid, thymus, pancreas and gonads), the reproductive system, the cardiovascular system and the immune system (Hestermann et al., 2000; Montano et al., 2022; Vorkamp, 2016).

The toxicity of dioxin-like PCBs is measured in toxic equivalency factor (TEF) relative to TCDD based on various factors such as the degree of structural similarity with dioxin, the binding affinity to AhR, dioxin-specific biochemical/toxicological response and, persistence and bioaccumulation in the environment (Hestermann et al., 2000). Total toxic equivalence (TEQ) is a method used to quantify the toxicity of a mixture of chemicals that exhibit similar toxicological effects. It is commonly used in the assessment of dioxins, furans, and PCBs.

As mentioned before, PCB126 influences transcription through the AhR pathway (Fukuzawa et al., 2003; Hassoun et al., 2001) and stands out as the most potent AhR agonist within the PCB family, denoted by a toxic equivalency factor (TEF) of 0.1 (Davis & Safe, 1990). This implies that its toxicity is one tenth that to the most toxic form of dioxin, TCDD (TEF=1). Hence, dioxin-like PCBs, sharing mechanistic similarities with TCDD, can activate the AhR pathway and upregulate CYP1A1 gene expression, which leads to their hydroxylation and subsequent excretion (Cock et al., 2014; M. J. Kim et al., 2012; Regnier & Sargis, 2014). However, exceptional cases exist, such as TCDD and PCB126, where the positioning of chlorine atoms confers resistance to hydroxylation by CYP1A1. This unique trait results in slow metabolism, leading to their accumulation in tissues. As these compounds persist in organs and tissues, prolonged AhR activation ensues, intensifying toxicities. Consequently, TCDD and PCB126 associated toxicities develop gradually over days to weeks (Walker et al., 2006).

2.3.3 Routes of Exposure for PCBs in Humans

Human exposure to PCBs occurs through multiple routes, including ingestion, inhalation, and dermal contact (Breivik et al., 2007; Lyche et al., 2010; Myrmel et al., 2016; Roos et al., 2012). The primary route of human exposure to PCBs, including the highly toxic PCB126, is dietary intake. PCB126 is present in various food sources, with concentrations ranging from 0.05 to 0.83 pg/g accounting for more than half of total consumption of DLCs/day/person with food intake of 13 pg TEQ/day (Walker et al., 2006). Studies on human intake of PCBs through food primarily focus on fish, with salmon containing the highest levels of PCBs, followed by canned tuna, beef steak, butter, and fried chicken. Despite this, PCB concentrations in food have declined over the past 20 years, indicating reduced dietary exposure. However, exposure susceptibility to PCBs can vary among different human populations due to differences in lifestyle, environment, and dietary habits (Montano et al., 2022). Due to their lipophilicity, ingested PCBs mainly bioaccumulate in adipose tissue. Their accumulation is proportional to the amount of triglycerides stored in adipocyte cells (Bourez et al., 2013). The average level of TCDD and PCB126 in human adipose tissue are 5.5 pg TEQ/g and 22.4 pg TEQ/g, respectively. TCDD, PCB126 and other dioxins combined (32.9 pg TEQ/g lipid) represent 48% of the total level of DLCs present in human adipose tissue (Walker et al., 2006). Levels and congeners distribution of DLCs and PCBs in buffaloes adipose tissue sampled *in vivo* and milk showed that PCB126 is the highest dioxin congener contributing to this contamination due to its high resistance to metabolic degradation (Chirollo et al., 2018).

The concentration range of dioxin-like PCBs was found to be between 0.034–5.676 pg-TEQ/g wet weight which exceeds the maximum permissible levels for "food for infants and young children" set by Food and Agriculture Organization (Commission Regulation (EU), 2011). The detection of PCBs in human milk, known to potentially impede child development, indicates that exposure to these compounds persists beyond the prenatal period (via placental transfer) and extends into breastfeeding, during which infants are exposed to the highest concentration of xenobiotics (dioxin-like PCBs among others), particularly in the first two months of life (Witczak et al., 2022). In a recent longitudinal study, the concentrations of dioxins and dioxin-like PCBs found in samples of human milk collected between 2013 and 2015 from 81 donor residents in the five provinces of Lazio region in Italy showed a concentration average of 13.3 pg/g of fat for

PCB126. The levels of dioxin-like PCBs detected in human milk samples from Lazio appeared to be lower than those previously reported in national and European averages. This confirms a decrease in these contaminants in human milk over the past decades (80% reduction). However, these levels still exceed the maximum limits permitted by European regulations for baby food. (Busico, 2019). Among the 1,017 human milk samples collected in the Maternal – Infant Research on Environmental Chemicals (MIREC) study, 298 were analyzed for PCBs. WHO toxic equivalency concentrations (WHO TEQ2005) for dioxin-like PCBs ranged from 2.2 to 27 pg TEQ_{2005} g^{-1} lipid. Significantly higher PCB concentrations were found in milk from women born in Europe compared to those born in Canada, with elevated levels also observed in older women (>30 years) and primiparous women. PCB concentrations in Canadian human milk have continued to decline since previous studies (Rawn et al., 2017).

These dioxins tend to bioaccumulate in the adipose tissue of every living organism and with notably higher body burden in the expanded adipose tissue of individuals with obesity. During periods of weight loss, these pollutants are continuously released into the bloodstream (internal exposure route). This underscores the potential health risks associated with the storage and release of dioxins in and from adipose tissue (Jackson et al., 2017; Roos et al., 2012).

2.3.4 PCB Metabolism

Genes with xenobiotic response elements (XREs) in their promoters are regulated by the AhR pathway. These genes include phase I detoxifying enzymes (CYP1 family members) and phase II detoxifying enzymes (aldehyde dehydrogenase 3, UDP-glucuronosyltransferase 1A1, and glutathione S-transferase) (Beischlag et al., 2008; Larigot et al., 2018). CYP enzymes play a crucial role in drug metabolism and detoxification. The CYP1 family, which includes CYP1A1, CYP1A2, and CYP1B1, is regulated by AhR and is responsible for metabolizing various compounds (Zanger & Schwab, 2013). As mentioned previously, the metabolism of PCBs varies according to their chlorination levels, with decreased metabolism observed as chlorination increases. These compounds have the ability to induce hepatic enzymes, particularly xenobiotic-metabolizing enzymes, and can undergo biotransformation to yield hydroxylated or other metabolites, detectable in human blood and in other tissues (Grimm et al., 2015; Vondráček et al., 2005). In more detail, PCBs can be metabolized by consecutive phases of detoxification process via hydroxylation by

cytochrome P450 (CYP) enzymes (phase I), and subsequent conjugation with glucuronic acid or sulfates by various enzymes (phase II), which ultimately results in excretion in bile, urine or feces (Nebert & Dalton, 2006; Registry, 2000).

Dioxin and dioxin-like compounds are mainly metabolized in the liver. Consistently, CYP1A1, a major member of the CYP1 family, is primarily expressed in the liver, but can also be found in other organs, such as in adipose tissue (Ellero et al., 2010; Yoshinari et al., 2004). Interestingly, the highly prevalent dioxin-like PCB126 exhibits distinctive characteristics compared to other coplanar PCBs. As mentioned earlier, it does not undergo hepatic metabolism but instead accumulates in the liver, partly due to its lipophilic properties and its ability to block the active site of the cytochrome P450 enzyme, CYP1A2. Unmetabolized PCB126 is subsequently transported through the bloodstream and accumulates in adipose tissue during both acute and chronic exposures (Denison et al., 2011). However, during weight loss, adipose tissue may release these unmetabolized dioxin-like congener into the bloodstream, causing induction of the AhR pathway. Excessive induction of xenobiotic metabolic enzymes in the liver can result in oxidative stress and the production of toxic metabolites. Dioxin-like PCBs metabolism is generally considered a detoxification process because a significant portion of hydroxylated PCB metabolites (OH-PCBs) are excreted from the body either directly or after conjugation. However, it is noted that PCB metabolism can also produce metabolites that present increased toxicity compared to the parent compound. Electrophilic metabolic intermediates, such as arene oxides, may cause harm by reacting with proteins, DNA, or lipids. Furthermore, if OH-PCBs are further oxidized to (semi)quinones, these highly reactive species may form covalent adducts with proteins, DNA, and other endogenous compounds. Additionally, certain metabolites like hydroxylated PCBs, PCB sulfates, and PCB methyl sulfones can persist in the body similar to the original PCB congener exerting their own further toxic effects (Grimm et al., 2015; Guarnieri et al., 2020; Safe, 1993; Vondráček et al., 2005).

2.3.5 Effects of PCB Exposure on Health

PCBs exposure is associated with a spectrum of adverse health effects, including carcinogenesis, developmental abnormalities, endocrine disruption, and metabolic perturbations (Ghosh et al., 2011; Grimm et al., 2015; Registry, 2000; Vitalone et al., 2010). In 2013, the

International Agency for Research on Cancer (IARC) reclassified PCBs as Group 1 carcinogens, up scoring their carcinogenic potential in humans (Registry, 2000).

Accidental or occupational human exposure to DLCs has been associated with a range of health effects, encompassing chloracne, reproductive deficits, developmental abnormalities, and various cancers, such as rectal, stomach, skin, lung, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, myeloid leukemia, and liver cancer (White & Birnbaum, 2009). PCBs have emerged as significant environmental contaminants associated with diverse health effects, notably metabolic disorders such as diabetes, obesity, dyslipidemia, hypertension, and non-alcoholic fatty liver disease (NAFLD) (Ghosh et al., 2014; Nadal et al., 2017; Shan et al., 2020). The observation that these chemicals disrupt hormonal regulation of energy homeostasis led to what is referred to as the “endocrine disruptor” theory. PCBs, in particular, have been categorized as "metabolic disruptors" due to their involvement in various metabolic disorders, extending beyond obesity (Burgio et al., 2015; Heindel et al., 2017; Petrakis et al., 2017). Studies have revealed positive associations between PCB levels in adipose tissue and the risk of obesity, with this risk being influenced by factors including sex, age and diet. Accumulating evidence also shows that low-level exposures may influence not only the development of diabetes in adults (strongest positive correlations occurred for PCBs, TCDD and TCDD-like chemicals), but also the health of their offspring (Jackson et al., 2017; Muscogiuri et al., 2017). A systematic review and meta-analysis study including 23 studies explored the association between PCBs measured in blood or adipose tissue samples and the risk of MetS and diabetes. It showed that dioxin-like PCBs were positively associated with MetS and diabetes, while non-dioxin-like PCBs were negatively associated with diabetes. In a subgroup analysis of PCBs homologs, dioxin-like PCB126 and PCB118 were risk factors for MetS and diabetes, respectively (Gao et al., 2023).

In sum, PCB congeners are implicated in a complex array of health effects, prominently featuring metabolic disorders. Recent research has unveiled their role as metabolic disruptors, connecting these chemicals to a spectrum of metabolic diseases. This evolving understanding underscores the necessity for further investigations into the mechanisms through which PCB126 may induce metabolic disruption in adipose tissue, particularly concerning adipocytes ability to differentiate, secrete adipokine and/or regulate metabolic homeostasis.

2.3.6 Effects of Dioxin and Dioxin-Like Compounds Exposure on Adipose Tissue

DLCs are known to decrease adipocytes ability to differentiate and alter adipogenesis through AhR (Alexander et al., 1998; Phillips et al., 1995). Interestingly, the dose of PCBs appears to dictate this effect. As seen in a study by Arsenescu *et al.*, low concentrations of PCB77 or TCDD increased adipocyte differentiation, glycerol-3-phosphate dehydrogenase activity, and expression of PPARγ, whereas higher concentrations inhibited adipocyte differentiation in 3T3-L1 cells. These effects were shown to depend on AhR signaling given that they were abolished by the AhR antagonist α-naphthoflavone (Arsenescu et al., 2008). Also, in both *in vitro* and *in vivo* experimental studies, it has been demonstrated that AhR ligands like TCDD, PCB126 and PCB77, are able to promote the expression and secretion of a variety of pro-inflammatory adipokines such as leptin, TNF-α and IL-6 and decreases the expression of adiponectin, an anti-inflammatory adipokine (Arsenescu et al., 2008). Notably, these studies used much higher doses than the average human exposure level (Alexander et al., 1998; Arsenescu et al., 2008; Baker, Karounos, et al., 2013; M. J. Kim et al., 2012; Matsumura & Vogel, 2006; Valentino et al., 2013).

Interestingly, Caron *et al*, (2020) showed that only insulin resistant 3T3-L1 adipocytes treated with PCB126 had significantly higher adiponectin, IL-6, monocyte chemoattractant protein 1 [MCP-1] and TNF-α secretion and lower mitochondrial function, glucose uptake, and glycolysis with no significant effect on insulin sensitive cells. These results suggest that adipocytes with altered glucose metabolism may be more sensitive to the deleterious effect of DLCs on their adipokine profile.

Subcutaneous adipocytes are the main source of the key adipokines, leptin and adiponectin (Wronska & Kmiec, 2012). The inability to produce leptin or insensitivity to its effects can result in increased fat mass, while decreased levels of adiponectin can lead to insulin resistance and elevated circulation of free fatty acids. It has been shown that 3T3-L1 cells exposed to PCB77 reduced adiponectin gene expression and its basal release (Arsenescu et al., 2008). In agreement, *in vitro* experiment using human mesenchymal stem cells exposed to a mixture of dioxin-like PCBs led to a significant reduction in the transcript levels of both PPARγ and adiponectin (Behan-Bush et al., 2023). In a recent Japanese study, serum levels of leptin were significantly lower in

the victims of the Yusho incident that were highly exposed to dioxin-like PCBs (Uchi et al., 2013). These findings suggest an endocrine disrupting role of dioxin-like PCBs in adipocytes.

Moreover, dioxin-like PCB77 was reported to cause AhR-dependent inflammation in adipose tissue and impair glucose homeostasis as reflected by increased blood glucose levels (Baker, Karounos, et al., 2013). Interestingly, genetically modified mice with deficiency of AhR in adipocytes showed augmented development of obesity, indicating that endogenous ligands for AhR may be involved in adipose homeostasis (Baker, Karounos, et al., 2013). PCB77 exposure has been shown to inhibit insulin-stimulated glucose uptake in adipocytes (Baker, English, et al., 2013). In accordance, multiple former work by Matsumura labs, using *in vivo* and *in vitro* experiments documented TCDD-mediated inhibition of glucose uptake and established the sensitivity of GLUT4 to TCDD in 3T3-L1 preadipocyte. Experimental evidence highlighted AhR dependent TCDD glucose uptake inhibition by down regulation of GLUT4 and, to a lesser extent, GLUT1 (P. C. C. Liu & Matsumura, 2006; Olsen et al., 1994).

Interestingly, in animal study using ICR mice, PCB126 exposure via nursing during early postnatal life showed significant impaired glucose tolerance at 3 and 9 weeks of age in both male and female offspring. However, at 12 weeks of age, no effect on body composition nor glucose tolerance were observed in the offspring ($p > 0.05$) demonstrating that exposure to PCB126 through the mother's milk had impaired glucose tolerance only in the short-term (Rice et al., 2023). Previous work of the same group showed that PCB126 exposure prior to and during gestation (not during nursing) significantly impairs glucose disposal after a glucose challenge in animals (Rice et al., 2023).

The effects of DLCs on adipose tissue will depend on factors such as the type and concentration of the compounds, the window and duration of exposure, and individual variability. The mechanisms through which these compounds exert their effects on adipose tissue metabolism are complex and may involve interactions with multiple signaling pathways. Research in this area is ongoing, and understanding the molecular mechanisms underlying the effects of DLCs on adipose tissue metabolism is an active area of investigation in the field of environmental health and metabolic disorders.

2.4 DIOXIN AND HYPOXIA INDUCED PATHWAY: CROSSTALK THROUGH ARNT

2.4.1 AhR Pathway

TCDD and dioxin-like PCBs share mechanistic similarities as they can bind to the aryl hydrocarbon receptor (AhR) (M. J. Kim et al., 2012). The AhR is a latent signal-regulated transcription factor that belongs to the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) protein family. This family of proteins includes important regulators of gene expression. AhR must form heterodimers with other bHLH proteins to create functional DNA binding complexes (Kewley et al., 2004; Larigot et al., 2018). AhR contains various domains with specific functions. The bHLH domain is involved in dimerization and DNA binding, while the PAS A and PAS B domains function as secondary dimerization domains. The PAS domains are highly conserved through evolution. The transactivation region plays a role in interactions with other transcription coactivators and affects transcriptional transactivation (Hankinson, 1995).

The AhR is normally bound to repressive factors like heat shock protein 90 (hsp90) in the cytoplasm, keeping it in an inactive state. Ligand binding occurs in the PAS B region, leading to a conformational change and exposing the nuclear localization signal. This exposure allows the AhR to translocate to the nucleus, where it forms a heterodimer with ARNT. The AhR:ARNT heterodimer serves as an active transcription factor complex. This complex binds to xenobiotic response elements (XREs) in the promoters of specific genes, inducing their transcription (Beischlag et al., 2008; Hankinson, 1995). Various endogenous and exogenous compounds can bind to and activate the AhR. Endogenous activators include bilirubin and tryptophan metabolites, while synthetic compounds such as TCDD, benzo(a)pyrene, and PCBs are known to activate the AhR (Larigot et al., 2018). Genes with xenobiotic response elements (XREs) in their promoters are regulated by the AhR pathway.

The transcriptional targets of the AhR can vary across species and cell types due to epigenetic factors, promoter accessibility, and ligand affinity (Hestermann et al., 2000; Pohjanvirta, 2011; Simon et al., 2015). The AhR pathway is a critical molecular pathway involved in the regulation of genes related to detoxification and xenobiotic metabolism when activated by various exogenous ligands, leading to adverse cellular and metabolic effects.

2.4.2 HIF Pathway

The hypoxia pathway stands as a pivotal mechanism for sensing alterations in oxygen levels that are encountered across various physiological and pathological contexts. Hypoxia transcriptional response is triggered by the actions of hypoxia-inducible factors (HIFs), a family of proteins that orchestrate hypoxia-induced gene expression. Three distinct hypoxic proteins isoforms are recognized, collectively referred to as HIF-α, all belonging to the class I bHLH/PAS protein family: HIF-1α, HIF-2α, and HIF-3α (Kewley et al., 2004). While HIF-1α and HIF-2α are transcription factors, our understanding of HIF-3α remains somewhat limited, with indications that it may function as a negative regulator of HIF-1α and HIF-2α (Yang et al., 2015). Each isoform exhibits distinct patterns of expression within human tissues. HIF-1α is ubiquitously expressed across all cell types, whereas HIF-2α and HIF-3α selectively manifest in specific cell types, including endothelial cells (Majmundar et al., 2010).

The regulation of HIF-1α predominantly occurs at the post-translational level. At 21% O_2 in cell culture, HIF-1α proteins undergo targeted degradation via the actions of prolyl hydroxylase domain-containing enzymes (PHDs). These enzymes carry out oxygen-dependent hydroxylation at two proline residues, a modification that allows for subsequent ubiquitination by von Hippel-Lindau (VHL) proteins and eventually its proteasomal degradation (Déry et al., 2005; Kewley et al., 2004; Ohh et al., 2000). Additional hydroxylation, mediated by oxygen-sensitive enzyme, the asparaginyl hydroxylase FIH (factor-inhibiting HIF), modifies HIF-α subunits at the C-terminal transactivation domain, thereby blocking the recruitment of co-factors essential for transactivation and impairing residual HIF transcriptional activity (Pugh & Ratcliffe, 2003). However, when oxygen level drops below 6%, PHD activity diminishes, inhibiting HIF-1α hydroxylation causing HIF-1α accumulation, and then non-hydroxylated HIF-1α proceeds to translocate to the nucleus. Within the nucleus, it forms heterodimers with ARNT (Kewley et al., 2004).

Similar to AhR:ARNT heterodimers, HIF-1α:ARNT complex is involved in gene transcription by recognizing specific similar sequences known as hypoxia response elements (HREs). Transcriptional response is activated after the binding of HIF-1α:ARNT heterodimer to HREs that comprise the sequence 5'–G/ACGTG–3' (Jaakkola et al., 2001; Majmundar et al., 2010).

2.4.3 Interplay Between AhR and HIF-1α: Crosstalk in Environmental Sensing

AhR and HIF-1α proteins are pivotal sensors of environmental changes, governing distinct responses to external stimuli. AhR primarily orchestrates responses to environmental chemicals, while HIF-1α regulates reactions to low oxygen conditions. To facilitate their respective functions, both proteins must form heterodimers with their common binding partner, ARNT (Kewley et al., 2004). ARNT binds to the nucleotide sequence 5'–GTG–3', found in both the XRE (Xenobiotic Response Element) and HRE (Hypoxia Response Element) core sequences (Chan et al., 1999).

The critical role of ARNT is highlighted in both AhR and HIF-1α signaling by the findings of studies that used knockout ARNT models. S. M. Wood *et al*. (1996) demonstrated that ARNT-deficient mouse hepatoma cells failed to induce HIF-1α transcriptional activity under hypoxia conditions. Gassmann *et al.* (1997) corroborated these findings by showing that ARNT-deficient mouse hepatoma cells were unable to respond to hypoxia, highlighting the necessity of ARNT as key co-factor for HIF-1α function. In conditional ARNT knockout mice, TCDD, a classical AhR ligand, could not induce various AhR target genes. Additionally in this study, cobalt chloride ($CoCl_2$), a chemical inducer of HIF-1α, failed to induce the HIF-1α target gene GLUT1 in these mice (Tomita et al., 2000).

This interplay between AhR and HIF-1α signaling via ARNT can result in alterations in their respective responses (Nie et al., 2001). For instance, hypoxic conditions may lead HIF-1α to sequester ARNT, thus inhibiting the robust AhR transcriptional response in the presence of co-planar dioxin-like PCBs. Conversely, exposure to PCBs may attenuate HIF-1α-mediated responses in hypoxic environments. Extensive research demonstrated that HIF-1α- and AhR-activated gene expression is mutually or partly inhibited under simultaneous exposure to xenobiotic and hypoxia stress (Vorrink & Domann, 2014). However, the debate regarding the antagonistic competition for ARNT recruitment between AhR and HIF-1α remains unresolved. Some studies have suggested mutual inhibition between the HIF-1 and AhR pathways (Chan et al., 1999; K. Y. Lee et al., 2011; Nie et al., 2001; Schults et al., 2010), while others has indicated that activation of the HIF-1 pathway inhibits the AhR pathway where activated AhR does not inhibit HIF-1 (Gassmann et al., 1997; Gradin et al., 1996; J. E. Kim & Sheen, 2000; Zhang et al., 2022). In concordant, hypoxic responses, induced by exposing rats to 0.1% carbon monoxide (CO), negatively impacted TCDD-

induced CYP1A1 mRNA expression levels while sustaining VEGFA mRNA expression, a marker gene of hypoxic pathway (Hofer et al., 2004). Similarly, hypoxia reduced TCDD-induced CYP1A1 mRNA expression in fish cells, while TCDD did not affect hypoxia-induced heme oxygenase expression (Prasch et al., 2004). Together, these findings suggest that hypoxia pathway has higher affinity to ARNT than AhR, and consequently has inhibitory effect over AhR-induced transcriptional response. On the other hand, Kraemer and Schulte found that the dioxin-like PCB77 decreased hypoxia-induced glycolytic enzyme activity in fish, further emphasizing crosstalk between AhR and hypoxia pathways (Kraemer & Schulte, 2004). Also, Terzuoli *et al.* (2010) reported inhibition of HIF-1α target gene expression in human cells treated with the AhR ligand aminoflavone under physiologic hypoxia.

While numerous studies have explored the crosstalk between AhR and hypoxia pathways using AhR ligands such as TCDD and others, few have investigated the effect of polychlorinated biphenyls (PCBs) on this interplay. Fleming *et al.* (2009) employed fish cells to evaluate ARNT role in AhR signaling induced by various AhR ligands, including PCB126, in the presence and absence of hypoxia. They discovered that PCB-induced reporter gene activity was diminished in hypoxia, an effect abolished by ARNT overexpression, suggesting that AhR and HIF-1α crosstalk is dependent on ARNT. Furthermore, PCB126 and benzo[k]fluoranthene failed to inhibit HIF-1α reporter activity, affirming the dominance of HIF-1α over AhR in ARNT competition (Fleming et al., 2009). The physiological, pharmacological, or toxicological implications of these interactions have not been thoroughly evaluated. Furthermore, most studies have focused on liver cancer cells (e.g., Hepa-1 cells, B-1 cells), cervical carcinoma cells (e.g., HeLa cells), zebrafish liver cells (e.g., PLHC-1 cells), etc., with limited investigation in adipocyte cells.

These diverse findings emphasize the complexity of the crosstalk between AhR and hypoxia pathways which is subject to cell type, exposure agents, and duration of exposure. The complex interplay between these two pivotal signaling pathways warrants further investigation, especially considering their significance in various cellular functions. More specifically, future research is required to shed light on the antagonistic relationship between AhR and HIF-1α signaling via ARNT in human adipocytes cells under conditions more closely mirroring physiological hypoxia and employing the dioxin-like PCB126, a relatively understudied AhR ligand.

CHAPTER 3 - THESIS ARTICLES

3.1 THESIS ARTICLE 1

White Adipose Tissue Metabolic Responses to Hypoxia

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ABSTRACT

White adipose tissue (WAT) is a key organ where energy in excess of needs is stored, mainly in the form of triglycerides (TGs). When needed, adipose tissue TGs can be mobilized as fatty acids that are released into circulation to sustain systemic energy requirements. A fine balance must be exerted between WAT lipid storage and mobilization functions to avoid an excessive fatty acid flux to non-adipose tissue, which could result in metabolic abnormalities. Besides its role in energy metabolism, WAT also secretes diverse proteins involved in several biological functions, conferring to this organ an endocrine status. Both the energy storage and endocrine functions of WAT are affected when exposed to low levels of oxygen (or hypoxia), a phenomenon that occurs consequently to excessive WAT hypertrophy in rodents and potentially in humans. This chapter focuses on the responses of WAT and its constituent, adipocytes, to hypoxia and how these hypoxia-induced responses may contribute to the development of obesity-associated disorders.

Keywords: Adipogenesis, Adipokines, Adipose tissue, Fibrogenesis, Hypoxia, Lipogenesis, Lipolysis.

Number of tables: 4; Number of figures: 2

INTRODUCTION

Over the last few decades, the rise in the prevalence of obesity and its implications for public health in developed and developing countries has fueled a growing interest in white adipose tissue (WAT) physiology. In humans, WAT is one of the largest and most plastic organs. The size of WAT can indeed range from a few kilograms in severely malnourished individuals to contributing to more than half of the weight of a person suffering from severe obesity. For several years, WAT has been perceived as a passive fat storage organ in which energy surplus is stored as triglycerides (TGs), a process termed lipogenesis. Conversely, upon increased energy need as during fasting or exercising, adipose tissue TGs can be mobilized into fatty acids and glycerol, a process called lipolysis. It was long believed that the metabolic role of the adipose tissue was limited to lipolysis and lipogenesis, a belief that may find its origin in the rather simple morphology of adipose tissue and its major constitutive cells, the mature adipocytes (Trayhurn, 2013). However, over the last 25 years, WAT has been demonstrated to fulfill more complex biological functions. The recognition that WAT is a full-fledged endocrine organ secreting hormones and peptides involved in a wide range of metabolic processes has drastically changed our perception of the biological function of this organ (Frühbeck et al., 2001).

Recently, it has been recognized that hypoxic patches may develop in the WAT of rodents with obesity, a result of impaired oxygen diffusion caused by adipocyte hypertrophy. Adipose tissue hypoxia has been shown to induce changes in adipocyte function that may underlie some features associated with obesity-related disorders such as insulin resistance, type 2 diabetes, and/or cardiovascular diseases. Whether hypoxia and its physiological effects occur in the context of human obesity is still, however, a matter of debate. In this book chapter, we discuss the basis of adipose tissue hypoxia and its multiple effects on adipose tissue functions that may contribute to, or even be the root cause of, the development of metabolic disorders associated with excessive fat mass.

1. Definitions of Words and Terms?

Adipogenesis: The regulated cellular differentiation process by which preadipocytes are differentiated into mature adipocytes for fat storage.

Adipokines: Signaling proteins that are secreted by adipocytes, the main cellular constituent of adipose tissue.

Angiogenesis: Formation of new blood vessels from preexisting vascular network.

Deoxyribonucleic acid (DNA): The double-stranded, helix-shaped molecule that carries the genetic information of all living cells.

Fibrosis: Condition that refers to an excessive accumulation of fibrillar components due to an imbalance between the synthesis and the degradation of the extracellular matrix.

Hypoxia: Condition in which the body or region of the body is deprived of oxygen. Hypoxia can be the result of environmental causes, such as high altitude, or pathological conditions such as chronic obstructive pulmonary disease, obstructive sleep apnea, or severe anemia.

Inflammation: A protective cellular reaction in response to injuries or harmful stimuli. It involves or affects immune cells, blood vessels, and molecular mediators to overcome the pathogen or injury and initiate the affected tissue repair process.

Insulin resistance: Condition where cells from insulin-sensitive tissues such as skeletal muscles, liver, and/or adipose tissues fail to fully respond to insulin, thus leading to, among other abnormalities, a diminished capacity to utilize glucose from the bloodstream.

Messenger ribonucleic acid (mRNA): Molecules issued of gene expression and DNA transcription that transport genetic information to the ribosome, where it serves as a template for protein synthesis.

Primary cell culture: Laboratory technique where cells are obtained from intact animal tissue and grown in vitro. By opposition to continuous cell lines, cells used in primary cell culture have a finite number of replication cycles, but are generally recognized to better reflect the physiology of cells in vivo.

Transcription factor: Protein that binds to a specific region of the DNA sequence and controls the rate of transcription of genes from DNA to mRNA.

2. Hypoxia and Adipose Tissue: The Hypoxia Hypothesis

As compared to other organs such as the liver, the heart, or the brain, WAT is poorly vascularized (Fleischmann et al., 2005). Angiogenesis is nonetheless essential for adipose tissue expansion and development (Rupnick et al., 2002). A classical study conducted on mouse cell lines proposed that hypoxic environment stimulates the development of new blood vessels during adipose tissue expansion via an increase in the expression of angiogenic genes such as the vascular endothelial growth factor-A (VEGFA) (Lolmède et al., 2003). These findings led Trayhurn & Wood to elaborate the "hypoxia hypothesis" (Trayhurn & Wood, 2004), which proposes that areas of relative hypoxia occur in regions of the WAT as the tissue expands during the development of obesity. It was also proposed that hypoxia could trigger an inflammatory response in the tissue, which could lead to the development of the metabolic disorders associated with obesity. This inflammatory response would ultimately favor an increase in blood flow and stimulate angiogenesis.

The hypoxia hypothesis in obesity is further supported by the following arguments: the proportion of the cardiac output and the amount of the blood flow to the adipose tissue are not augmented in individuals with obesity in spite of the considerable enlargement of adipose tissue mass (Blaak et al., 1995; Kabon et al., 2004; Virtanen et al., 2002); individuals with obesity do not exhibit a postprandial rise in adipose tissue blood flow (ATBF) as observed in individuals with normal weight (Goossens et al., 2011; Karpe et al., 2002); hypertrophied adipocytes can reach diameters up to 150–200 μm, while the accepted diffusion range of O_2 within tissues is between 100 and 200 μm (Brahimi-Horn & Pouysségur, 2007). In support of this last argument, partial pressure of oxygen (PO_2) close to zero has been experimentally observed at only 100 μm from the vasculature in some tissues under various circumstances (Brahimi-Horn & Pouysségur, 2007; Folkman et al., 2000; Gatenby & Gillies, 2004)..

2.1 Blood Circulation and Oxygenation of Adipose Tissue

In addition to being important for WAT development, vasculature and oxygen supply are critical requirements for the proper functioning of WAT (Frayn & Karpe, 2014; Heinonen et al., 2016). The adipose tissue PO_2 reflects the balance between the oxygen delivered to the adipose tissue from the blood and the oxygen consumed by the adipose tissue. From an anatomical

standpoint, oxygen-rich blood is supplied to the tissues through the arteries (arterial blood) and the oxygen-depleted blood leaves the tissue through the venous system (venous blood). Adipocytes are surrounded by an extensive network of capillaries. The density of this capillary network varies between fat depot and appears to depend on the mass as well as the localization of the depot (Wertheimer & Shapiro, 1948).

In humans, ATBF is regulated by the sympathetic nervous system and changes markedly upon diverse physiological states. More precisely, following an overnight fast, ATBF is close to 6 mL/100 g/min in individuals with normal adiposity and increases by about twofold after a meal (Frayn & Karpe, 2014). In individuals with obesity, fasting ATBF is half that observed in individuals with normal adiposity and is unresponsive to calorie intake (Frayn & Karpe, 2014).

There is convincing evidence that adipose tissue hypoxia occurs in animal models of obesity. Using pimonidazole, a hypoxic marker that can be detected by immunostaining or western blotting, it has been shown that mouse models of genetic and dietary-induced obesity have more hypoxic areas in adipose tissue than their lean counterparts (Hosogai et al., 2007; Rausch et al., 2008; Ye et al., 2007). Similar observations were also reported in mouse models of both genetic and dietary-induced obesity using direct measurements of adipose tissue PO_2 with needle-type optical-fiber oxygen sensors (Rausch et al., 2008; Ye et al., 2007; Jun Yin et al., 2009). Evidence for such reduction in adipose tissue PO_2 in humans with obesity is not as convincing. Significantly lower oxygen levels were observed in individuals with obesity as compared to individuals with normal weight through the use of a commercially available electrode inserted in subcutaneous abdominal adipose tissue (Kabon et al., 2004; Pasarica et al., 2009). Conversely, no difference in adipose tissue PO_2 was observed between individuals with normal weight versus obesity using a microdialysis catheter inserted into the subcutaneous abdominal region (Goossens et al., 2011). Of note, this last study even reported that individuals with obesity display adipose tissue hyperoxia despite lower ATBF, a fact likely explained by lower adipose tissue oxygen consumption. Concordant with these findings, Hodson *et al.* reported that both adipose tissue oxygen delivery and consumption are reduced in individuals with obesity (Hodson et al., 2013). Goossens *et al.* have suggested that the discrepancies between animal and human studies may be due to the fact that animal models of obesity experience relatively greater weight gain at a faster rate compared to humans. Moreover, few human studies had groups matched for factors that may affect tissue

oxygenation such as age, gender, ethnicity, and the presence of type 2 diabetes, which is likely to introduce noise and compromise the power of these studies (Goossens & Blaak, 2015) (**Table 1**).

Table 1. Key Facts of Hypoxia and Adipose Tissue Metabolism

• At sea level, the partial pressure of oxygen (PO_2) in inspired air is 160 mmHg, roughly ~21% of 760 mmHg, the normal atmospheric pressure at this altitude. Tissue oxygenation represents the oxygen available for use in a given tissue and reflects the balance between the oxygen supplied to and the oxygen consumed by the tissue. The PO_2 in WAT of lean rodents is ~47 mmHg while it is ~15 mmHg in rodents with obesity. This demonstrates that, in rodents, excessive adipose tissue or obesity is clearly associated with lower oxygenation or hypoxia in WAT. Whether hypoxia occurs in adipose tissue in humans living with obesity is not as clear.
• Cellular response to hypoxia is signaled by hypoxia-inducible factors (HIFs) a family of many isoforms amongst which HIF-1 is the most studied. Under normoxia, HIF-1 is not stable, readily oxidized by prolyl hydroxylase and labeled for degradation. Under hypoxia, prolyl hydroxylase is inactivated. Hence, HIF-1 is stabilized and induces the transcription of specific genes.

2.2 Oxygen Sensing System: Hypoxia-Inducible Transcription Factor-1

Key transcription factors signaling the cellular response to hypoxia are members of the hypoxia-inducible factors (HIFs) family (Gregg L. Semenza, 2003). There are three major HIF isoforms: HIF-1, HIF-2, and HIF-3, HIF-1 being the most studied. HIF-1 is a heterodimeric transcription complex composed of an O_2-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit. As shown in Figure 1, in condition of normoxia a family of prolyl hydroxylases (PHDs) is activated and hydroxylate (addition of a hydroxyl group, –OH) HIF-1α. Hydroxylation of HIF-1α generates sites for the binding of the von Hippel–Lindau protein (Gregg L. Semenza, 2003), which in turn favors the ubiquitination of HIF-1α and its subsequent proteosomal degradation. PHDs are inactivated under hypoxia. HIF-1α accumulates and migrates to the nucleus where it associates with HIF-1β and the cofactors p300/CBP. This heterocomplex then binds to specific promoter regions called hypoxia-response elements favoring the activation of specific genes. Overall, HIF-1α targets genes that encode secreted factors and cell surface receptors that promote O_2 delivery, on the one hand, and reduces O_2 utilization, on the other. It is important to note that HIFs are not the only transcription factors directly implicated in the hypoxic response. NF-κB, CREB (cAMP response element-binding protein that binds to specific DNA

sequences to regulate certain genes in the cell) and other factors are involved in the cellular responses to hypoxia (Cummins & Taylor, 2005; van Uden et al., 2008).

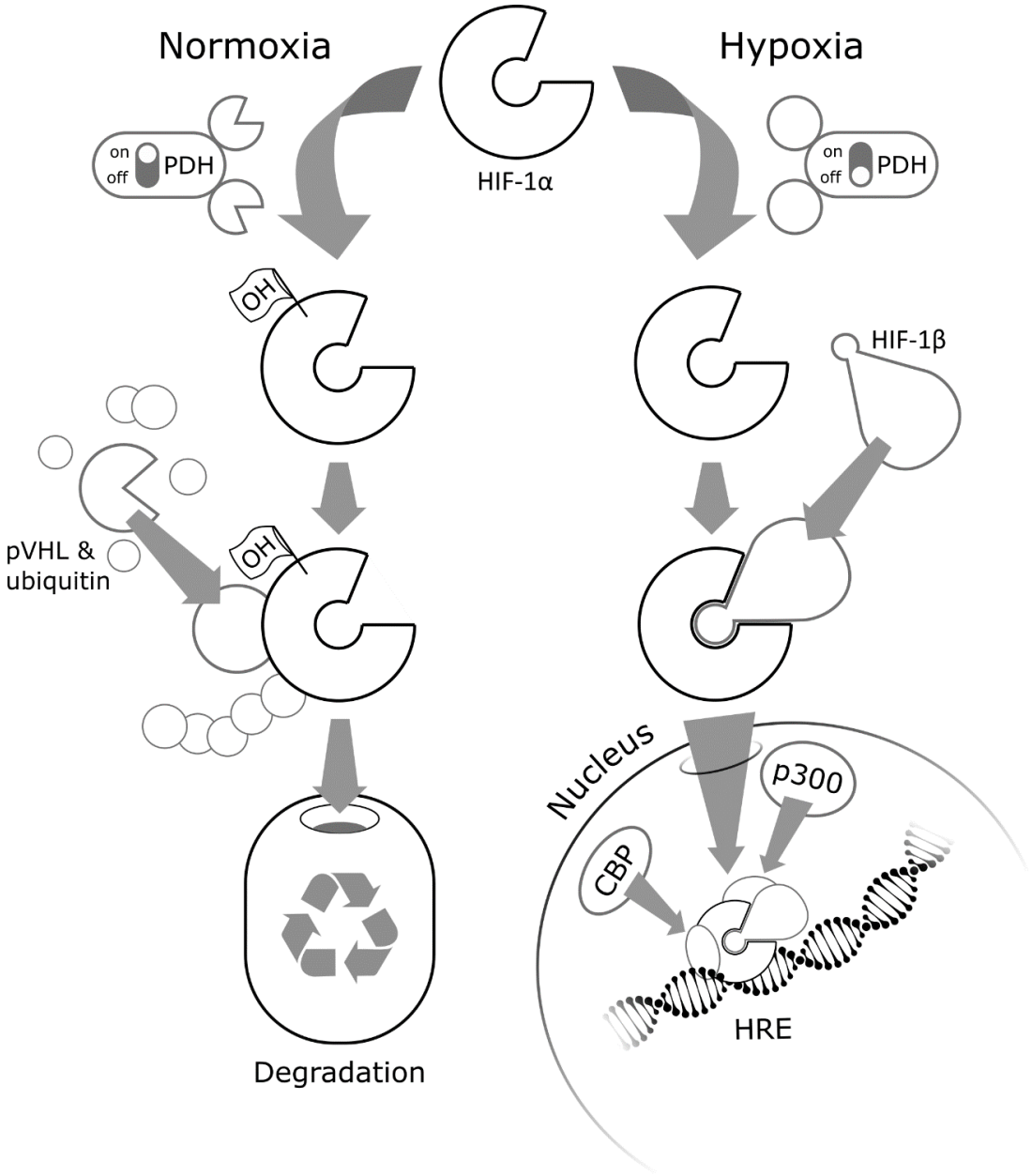

Figure 1. Hypoxia-inducible factor 1 alpha (HIF-1α) metabolism. Under normoxia, HIF-1α is rapidly hydroxylated by prolyl hydroxylase-domain (PHD) enzymes. This allows HIF-1α recognition and ubiquitination by the von Hippel–Lindau tumor suppressor (pVHL), leading to its proteasomal degradation. Under hypoxia, PHDs are inhibited and HIF-1α can migrate to the nucleus where it interacts with its cofactor HIF-1β. The complex further interacts with the transcription coactivators CBP and p300, which induces the transcription of genes containing hypoxia-response elements (HRE) in their promoter.

3. Hypoxia and Adipocyte Metabolic Functions

The adipocytes are the main cells within adipose tissue where the excess of energy substrates is stored as TGs, a process named lipogenesis, but commonly referred to simply as fat storage. In periods of energy surplus such as after a meal, adipocytes store fatty acids derived from the hydrolysis of circulating lipoprotein-bound TGs synthesized by the small intestine through the action of the lipoprotein lipase (LPL) (Olivecrona, 2016; Young & Zechner, 2013). The activity of this enzyme is stimulated by insulin and favors the entry of fatty acids within adipocytes through fatty acid transporters. Insulin also favors glucose uptake through the translocation of a specific glucose transporter (GLUT4) to the plasma membrane. Fatty acids can then be re-esterified using glycerol 3-phosphate derived from glucose as a backbone to form TG within adipocytes.

In time of increased metabolic need, such as during fasting or physical activity, stored lipids can be mobilized by converting adipocytes TG into fatty acids using a process called lipolysis. The lipolysis depends mainly on the activation of two specific enzymes, the adipose triglyceride lipase and the hormone-sensitive lipase (Lafontan & Langin, 2009; Lass et al., 2011). Fatty acids derived from intracellular lipolysis are released into circulation and delivered to peripheral tissues to sustain energy demand. Catecholamines, natriuretic peptides, and insulin are the major regulators of lipolysis.

In addition to storing or mobilizing substrate, adipocytes produce numerous bioactive proteins, termed adipokines, which communicate with other organs and modulate a wide range of biological functions (**Table 2**). The following subsections briefly cover how hypoxia impacts the storage, mobilization, and secretory functions of adipocytes.

Table 2. Key Facts of Adipose Tissue Metabolism

• WAT mass depends on the number and the size of its constituent adipocytes. Key functions of adipocytes are to store/mobilize lipids, depending on the energy needs of the body and to produce key proteins, termed adipokines, that convey signals to peripheral organs and modulate a wide range of biological functions.
• WAT is poorly vascularized in comparison to other organs, and its blood flow is regulated by the sympathetic nervous system.

3.1 Hypoxia and Glucose Utilization/Lactate Production

Glucose transporters (GLUTs), which belong to the family of facilitated glucose transporters, are required for the transport of glucose within adipocytes. The adipocytes express a number of the facilitative (energy-independent) GLUTs such as GLUT1, GLUT3, GLUT4, GLUT5, and GLUT12 (I. S. Wood et al., 2003). Despite the complexity of the process of glucose delivery into cells, GLUT1 is recognized to be the main transporter responsible for basal glucose import. Compared to the other GLUTs found in adipocytes, GLUT4 operates as an insulin-sensitive GLUT, e.g., under low-insulin conditions, GLUT4 resides primarily in intracellular membrane compartments. When insulin levels rise, intracellular GLUT4 redistributes to plasma membrane, thus favoring glucose uptake.

Under hypoxic conditions, the production of the primary cellular energy "currency," adenosine triphosphate (ATP), by oxidative phosphorylation, is altered. Exposure to hypoxia leads to a change in energy production strategy: the breakdown of glucose via non-oxygen-dependent pathways is increased to optimize ATP production per mole of oxygen consumed. In this line, reduced oxygen tension markedly increases basal glucose uptake in differentiated preadipocytes (Regazzetti et al., 2009; I. S. Wood et al., 2007; Jun Yin et al., 2009). This increase in basal glucose uptake upon low O_2 tension is likely attributable to the marked upregulation of GLUT1 gene/protein expression, as reported from experiments using murine and human adipocytes (Geiger et al., 2011; Hosogai et al., 2007; Lolmède et al., 2003; Mazzatti et al., 2012; B. Wang et al., 2007; I. S. Wood et al., 2007; Ye et al., 2007; Jun Yin et al., 2009). Conversely, hypoxia has no effect on the insulin-sensitive GLUT4 gene/protein expression (I. S. Wood et al., 2007). It is, however, important to note that following 24 h of exposure to hypoxia, insulin-stimulated glucose uptake is significantly inhibited in white adipocytes (Regazzetti et al., 2009; Jun Yin et al., 2009). Overall, these results indicate that hypoxia increases basal glucose uptake but creates a state of insulin resistance in adipocytes.

As a consequence of both the increased basal glucose flux within the adipocyte and the increased glycolytic flux under hypoxia, an increase in lactate production has been reported in studies using murine or human adipocytes exposed to reduced O_2 tension (Lolmède et al., 2003; I. S. Wood et al., 2009). Monocarboxylate transporters comprise 14 members, four of which (MCT1-

MCT4) are responsible for transporting lactate across the plasma membrane (Meredith & Christian, 2008). Perez de Heredia *et al.* previously documented that MCT1, MCT2, and MCT4 genes are expressed in human adipocytes (De Heredia et al., 2010). These authors also reported that under low PO_2, the gene expression level of MCT1 and MCT4 was increased while the one of MCT2 was decreased. Upon low O_2 tension, there is an increase in the expression of specific lactate transporters, MCT1 and MCT4, which favors the release of lactate adipocytes. Once released, lactate is involved in a variety of biological processes (for review, see (Brooks, 2009; S. Sun et al., 2017). At the adipocyte level, lactate has been shown to mediate, in an autocrine fashion, the insulin-dependent inhibition of lipolysis through G-protein-coupled receptor 81 (Ahmed et al., 2010).

3.2 Hypoxia and Triglyceride Mobilization and Storage (Lipolysis/Lipogenesis)

A tight control between TG hydrolysis and NEFA esterification for the maintenance of appropriate circulatory NEFA concentration must be exerted. This is evidenced by the observation of excessive lipid deposition in non-adipose tissues when fat cells are unable to effectively uptake and store plasma NEFA or display an increased TG mobilization capacity, a phenomenon that has been linked to lipotoxicity and a greater prevalence of metabolic disorders (Defronzo, 2004; Lelliott & Vidal-Puig, 2004). Hypoxia at the adipose tissue level may act as a potent perturbator of this fine balance between fat mobilization and storage. O'Rourke *et al.* previously reported an increased basal lipolysis in isolated human visceral and subcutaneous adipocytes following a 24-h exposure to 1% O_2 (O'Rourke et al., 2013). These results are consistent with our recent results showing a dose-dependent stimulating effect of hypoxia on basal lipolysis in differentiated subcutaneous human preadipocytes (Mahat et al., 2017). Acute hypoxia does not, however, affect the isoproterenol-stimulated lipolytic response of isolated human adipocytes (O'Rourke et al., 2013) or differentiated human preadipocytes (Mahat et al., 2017). Of note, increased basal lipolysis but normal isoproterenol-stimulated lipolysis are also observed in isolated adipocytes from individuals with obesity (Mauriege et al., 1991; O'Rourke et al., 2013). It was proposed that this pattern of lipolytic response to acute hypoxia could be due to the inhibition of hexosamine biosynthesis (O'Rourke et al., 2013), the increase production of interleukin-6 (IL-6), and/or the potentiation of proinflammatory cytokines such as tumor necrosis factor alpha (Bhattacharya et al., 2015), which are further detailed in Section 1.3.3.

We have previously reported that acute hypoxia had a sixfold inhibiting effect on LPL activity of differentiated human preadipocytes, a key enzyme involved in the breakdown of dietary TG (Mahat et al., 2016). The mechanism underlying this observation is still unknown but could be related to the significant increase in the expression of angiopoietin-like proteins-4, a major posttranslational regulator of LPL activity that inactivates LPL at the plasma membrane of adipocytes, in response to hypoxia (Makoveichuk et al., 2013; I. S. Wood et al., 2011). The inhibiting effect of LPL activity upon hypoxia complements evidence showing that hypoxia impedes the expression of lipogenic genes in subcutaneous and visceral human adipocytes (García-Fuentes et al., 2015; O'Rourke et al., 2013).

Overall, these results indicate that hypoxia diverts adipocyte lipid metabolism toward an increase in basal lipid mobilization capacity and a reduction in storage function. Together, these effects may expose organs such as the heart, liver, and skeletal muscles to an excess of lipids, and favor ectopic fat storage, a condition recognized to lead to the development of metabolic disorders (Defronzo, 2004; Lelliott & Vidal-Puig, 2004).

3.3 Hypoxia and Adipokines

The effect of hypoxia on the expression and secretion of adipokines has been well investigated. Several studies using primary cell culture reported the induction of the immunoreactive HIF-1α by hypoxia in differentiated human preadipocytes or clonal cell adipocyte such as 3T3-F442A or 3T3-L1. Lolmède *et al.* were the first to examine the effects of low PO_2 on adipokine production (Lolmède et al., 2003). Results indicated that under hypoxic conditions (5% O_2) for 24 h, 3T3-F442A adipocytes showed an increase in HIF-1α protein levels as well as increased protein levels of leptin, VEGFA, and gelatinase matrix metalloproteinases (MMPs), which are key molecules stimulating angiogenesis. Another key study performed in 3T3-L1 cells revealed that hypoxia (1% O_2) induced a modulation in inflammation-related proteins by increasing plasminogen activator inhibitor (PAI-1) expression and by reducing adiponectin gene expression and protein secretion (B. Chen et al., 2006). Further studies have confirmed the upregulation of PAI-1 and the downregulation of adiponectin gene expression by low PO_2 in differentiated human preadipocytes (Hosogai et al., 2007; B. Wang et al., 2007). The inhibition of adiponectin expression and release by low PO_2 is of importance since as mentioned earlier

adiponectin has important anti-inflammatory and insulin-sensitizing functions (Ouchi et al., 1999; Yamauchi et al., 2001). Other studies have also reported an effect of hypoxia on the gene expression and secretion of several proinflammatory adipokines such as IL-6, macrophage migration inhibitory factor, and serum amyloid A (Mendes De Oliveira et al., 2013; B. Wang et al., 2007; I. S. Wood et al., 2011). Of note is the fact that the invalidation of a key nuclear receptor involved in the development of adipose tissue, peroxisome proliferator-activated receptor gamma (PPARγ), was shown to attenuate the usual robust induction of inflammation-related HIF-1α targets in white adipocytes exposed to low O_2 tension, suggesting that PPARγ is required to facilitate the expression of hypoxia-related genes (Pino et al., 2012), **Figure 2**.

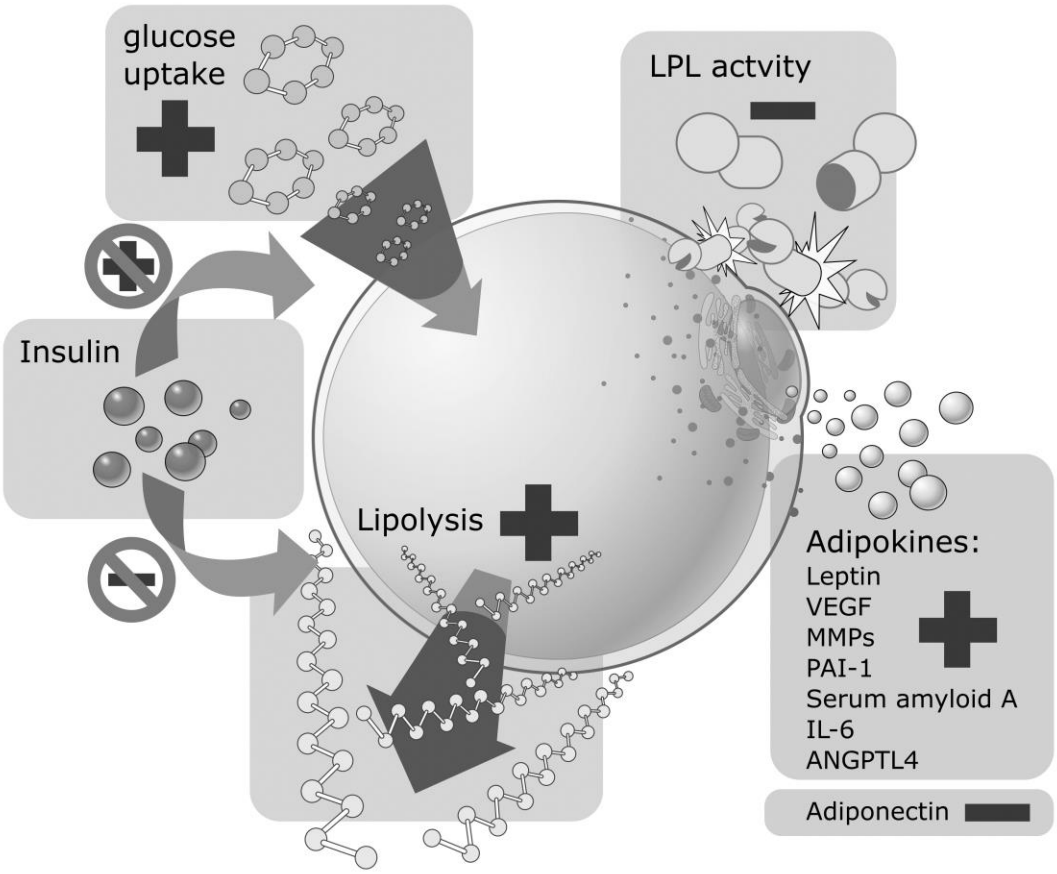

Figure 2. Features of the adipocyte response to hypoxia. Hypoxia stimulates insulin-independent glucose uptake and constitutive lipolysis. Lipoprotein lipase (LPL) activity is reduced, purportedly by conversion of the active LPL dimers to inactive monomers. The adipocyte's capacity to further increase its glucose uptake or inhibit its lipolytic activity in response to insulin is impaired. Finally, the adipokines profile is adversely altered. (Abbreviated adipokines: VEGFA, vascular endothelial growth factor-A; MMPs, matrix metalloproteinases; PAI-1, plasminogen activator inhibitor-1; IL-6, interleukin-6; ANGPTL4, angiopoietin-like 4).

4. Adipose Tissue Hypoxia and Adipogenesis

Adipose tissue mass depends on the average volume and the number of adipocytes. The number of adipocytes is dependent on adipogenesis, a process that entails two steps: (1) the generation of progenitor adipocytes or preadipocytes from stem cells, and (2) the differentiation of preadipocytes into mature adipocytes (Gregoire et al., 1998). In comparison to the processes involved in differentiation, the mechanisms underlying the generation of preadipocytes remain largely unknown. For this reason, this section will focus mainly on the potential impact of hypoxia on adipocyte differentiation. One of the key transcription factors involved in adipogenesis is PPARγ (Spiegelman et al., 1997). PPARγ is essential for the transcriptional activity of PIK3R1 (regulatory subunit 1 of phosphatidylinositol 3-kinase (PI3K), which is essential for adipogenesis (Y. J. Kim et al., 2014). It has been demonstrated that hypoxia, through the induction of a member of HIF-1 target genes, namely Stra13, decreases PPARγ expression and inhibits adipogenesis (Yun et al., 2002). Cell culture conditions mimicking hypoxia have also been shown to reduce adipocyte differentiation by inhibiting PPARγ and by stopping clonal expansion of preadipocytes through the activation of AMPK (5′ adenosine monophosphate-activated protein kinase), an enzyme that plays a key role in cellular energy homeostasis (Ban et al., 2014). Together, these results indicate that hypoxia inhibits the differentiation of preadipocytes into adipocytes mainly by inhibiting the expression of PPARγ through HIF-1 signaling and/or the activation AMPK (Table 3).

Table 3. Key Facts of Adipogenesis

• During adipogenesis, progenitor adipocytes or preadipocytes are generated from stem cells and preadipocytes differentiate into mature adipocytes.
• In vitro systems are used to study adipocyte differentiation. This favors the dissection of the molecular and cellular events occurring during the transition from undifferentiated preadipocytes into mature round fat cells or adipocytes.
• PPARγ is a nuclear factor that plays a crucial role in adipogenesis. Hypoxia inhibits PPARγ and therefore adipogenesis.

5. Hypoxia and Adipose Tissue Fibrosis

Adipocytes are surrounded by a network of proteins forming the extracellular matrix (ECM) that provides mechanical support and responds to different signaling cues (K. Sun et al., 2013) (**Table 4**). Adipose tissue mass development involves the remodeling of the ECM, which requires the degradation of the existing ECM and the production of new ECM components. The imbalance between the synthesis and the degradation of ECM can lead to an excessive accumulation of fibrillar components and the development of fibrosis (Halberg et al., 2009; Henegar et al., 2008). It is now well recognized that many ECM components are altered in adipose tissue of individuals with obesity compared to individuals with normal weight (Henegar et al., 2008).

Table 4. Key Facts of Fibrosis

• Adipose tissue mass development involves the remodeling of the extracellular matrix. An excessive accumulation of the extracellular matrix in the adipose tissue leads to fibrosis.
• Hypoxia upregulates the expression of adipocyte genes encoding proteins of the extracellular matrix.
• Macrophages play a role in remodeling the extracellular matrix, contributing thus to fibrosis of adipose tissue.

As previously mentioned, ECM proteins respond to different cues, and several lines of evidence suggest that hypoxia is a key initiator of ECM induction. Low PO_2 affects the expression of genes coding for proteins that compose or affect the ECM. More precisely, upon exposure to reduced O_2 tension, human adipocyte expression of COL13A1, a gene encoding collagen type XIII alpha, and LOX, which encodes lysyl oxidase responsible for initiating the cross-linking of collagen and elastin, are upregulated dependently on HIF-1 (Mazzatti et al., 2012). In line with these observations, adipose tissues collected from individuals with obesity show increased collagen V levels while elastin levels are reduced (Spencer et al., 2011). This study also reported a reduction in elastin expression and an increase in collagen production in adipocytes cultured with M2 macrophages (Spencer et al., 2011), indicating that immune cells like macrophages play a role in the development of adipose tissue fibrosis by remodeling the extracellular matrix. Concordant

with this, an upregulation of several MMP genes (MMP1, MMP3, MMP9, MMP10, and MMP12) was observed in human adipocytes exposed to macrophage-conditioned medium (O'Hara et al., 2009). Together, these observations lend credit to two potential models underlying the development of WAT fibrosis, as suggested by K. Sun *et al.* (2013). The hypoxic state noted during excessing WAT expansion could induce a profibrotic transcription program resulting in an abnormal development of ECM and, in turn, local fibrosis. Local fibrosis would trigger adipocyte cell death, which in turn, would attract macrophages secreting proinflammatory markers. Alternatively, hypoxia could directly induce proinflammatory factors that would favor the infiltration of proinflammatory macrophages that could interact with adipocytes and generate fibrotic components.

CONCLUSION

In order to accommodate its expansion and functions, WAT relies on the recruitment of preadipocytes that are differentiated into adipocytes as well as on the remodeling of the vasculature and the extracellular matrix (K. Sun et al., 2011). Studies on animal models (Rausch et al., 2008; Ye et al., 2007; Jun Yin et al., 2009) as well as some human studies (Kabon et al., 2004; Pasarica et al., 2009) provide convincing evidence that WAT hypoxia may occur with excessive WAT expansion. Under hypoxic conditions, the cellular mechanisms ensuring WAT expansion and functions are compromised. Specifically, hypoxia (1) impairs lipid storage, (2) modulates the secretion of adipokines associated with inflammation, (3) impairs adipogenesis, and (4) favors the development of WAT fibrosis. These features are well recognized to be involved in the development of obesity-related disorders such as insulin resistance and cardiovascular diseases (K. Sun et al., 2011). However, it is still unclear whether WAT hypoxia does occur in individuals living with obesity. Such a question warrants further studies … so let's hold our breath!

Summary Points

1. White adipose tissue (WAT) is a key organ in which energy surplus is stored as TGs. TGs are stored within adipocytes, the main cell constituent of WAT. WAT is also recognized as an endocrine organ, e.g., it secretes hormones, termed adipokines, which are involved in a wide range of metabolic processes. It has been hypothesized that WAT presents areas of relatively low oxygen levels or hypoxia as the tissue excessively expands. Based on animal models with

obesity, such a hypoxic status alters the storage and secretory functions of WAT and triggers an inflammatory response in the tissue. The alteration in WAT metabolism in response to obesity-induced hypoxia is thought to explain, at least partly, some of the metabolic abnormalities associated with obesity.

2. The partial pressure of oxygen (PO_2) in WAT is critical for this tissue to exert its physiological functions. WAT PO_2 depends on blood O_2 delivery to adipose tissue and adipose tissue O_2 consumption. In individuals living with obesity, fasting adipose tissue blood flow (ATBF) is significantly lower than in individuals with normal adiposity. In rodents, excessive adipose tissue or obesity is clearly associated with WAT hypoxia using different PO_2 assessing methods. Some studies in humans have confirmed a reduction in adipose tissue PO_2 in individuals with obesity while others did not. Whether hypoxia occurs in adipose tissue of humans living with obesity therefore needs further investigation.

3. HIF-1 is the key transcription factor signaling the cellular response to hypoxia. Under hypoxia, HIF-1α accumulates and migrates to the nucleus and associates with HIF-1β and the cofactor p300/CBP to form a heterocomplex that binds to specific gene promoter regions called hypoxia-response elements (HRE). This allows the transcription and expression of specific genes encoding secreted factors and cell surface receptors that promote O_2 delivery and reduce O_2 utilization.

4. Exposure to hypoxia leads to a shift in metabolic strategy aiming at optimizing energy production per mole of O_2 consumed. Accordingly, reduced oxygen tension markedly increases basal glucose uptake in adipocytes by upregulating GLUT1 gene expression and presence at the plasma membrane. On the other hand, insulin-stimulated glucose uptake is significantly inhibited in white adipocytes exposed to hypoxia, creating a hypoxia-induced state of insulin resistance in WAT.

5. A tight control between the mobilization and storage of lipids must be exerted to maintain appropriate circulatory non-esterified fatty acids (NEFAs). Hypoxia at the adipose tissue level increases the mobilization capacity of adipocytes, which release more NEFAs. Hypoxia also impedes the storage capacity of lipids. Together, these effects may expose organs such as the

heart, liver, and skeletal muscles to an excess of lipids, a condition known to favor the development of metabolic disorders.

6. Under hypoxia, adipocytes adapt their secretory function and produce adipokines such as leptin, VEGFA, gelatinase matrix metalloproteinases (MMPs), and plasminogen activator inhibitor-1 (PAI-1) that are involved in angiogenesis and inflammation.

7. WAT tissue mass depends on the average volume and the number of adipocytes. The number of adipocytes is dependent on adipogenesis, a process that implies the differentiation of preadipocytes into mature adipocytes. Hypoxic conditions inhibit the differentiation of preadipocytes into adipocytes mainly by inhibiting the expression of peroxisome proliferator-activated receptor gamma (PPARγ).

8. Adipocytes are surrounded by a network of extracellular matrix (ECM) proteins that provide mechanical support and respond to different signaling cues. Hypoxia increases human adipocytes' expression of ECM by inducing the expression of certain genes encoding proteins of the ECM such as collagen and lysyl oxidase (LOX). Immune cells like macrophages also play a role in the development of adipose tissue fibrosis by remodeling the extracellular matrix and upregulating several MMPs genes, increasing collagen production and reducing elastin expression.

Author Contributions

N.Y. and Z.E.A. contributed equally to the writing of the book chapter. J.-F.M. conceived the figures. All authors have reviewed the chapter and have read and agreed to the published version of this letter.

3.2 THESIS ARTICLE 2

CYP1A1, VEGFA and Adipokine Responses of Human Adipocytes Co-exposed to PCB126 and Hypoxia

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ABSTRACT

It is increasingly recognized that hypoxia may develop in adipose tissue as its mass expands. Adipose tissue is also the main reservoir of lipophilic pollutants, including polychlorinated biphenyls (PCBs). Both hypoxia and PCBs have been shown to alter adipose tissue functions. The signaling pathways induced by hypoxia and pollutants may crosstalk, as they share a common transcription factor: aryl hydrocarbon receptor nuclear translocator (ARNT). Whether hypoxia and PCBs crosstalk and affect adipokine secretion in human adipocytes remains to be explored. Using primary human adipocytes acutely co-exposed to different levels of hypoxia (24 h) and PCB126 (48 h), we observed that hypoxia significantly inhibits the PCB126 induction of cytochrome P450 (CYP1A1) transcription in a dose-response manner, and that Acriflavine (ACF)—an HIF-1α inhibitor—partially restores the PCB126 induction of CYP1A1 under hypoxia. On the other hand, exposure to PCB126 did not affect the transcription of the vascular endothelial growth factor-A (VEGFA) under hypoxia. Exposure to hypoxia increased leptin and interleukin-6 (IL-6), and decreased adiponectin levels dose-dependently, while PCB126 increased IL-6 and IL-8 secretion in a dose-dependent manner. Co-exposure to PCB126 and hypoxia did not alter the adipokine secretion pattern observed under hypoxia and PCB126 exposure alone. In conclusion, our results indicate that (1) hypoxia inhibits PCB126-induced CYP1A1 expression at least partly through ARNT-dependent means, suggesting that hypoxia could affect PCB metabolism and

toxicity in adipose tissue, and (2) hypoxia and PCB126 affect leptin, adiponectin, IL-6 and IL-8 secretion differently, with no apparent crosstalk between the two factors.

Keywords: hypoxia; polychlorinated biphenyl; inflammatory adipokines; human adipocytes; AhR; ARNT.

Number of figures: 8

INTRODUCTION

There is accumulating evidence that as adipose tissue mass expands, insufficient angiogenesis and cellular hypertrophy may limit oxygen availability to adipocytes, a concept that has been called 'adipose tissue hypoxia' (Andrei et al., 2017; Trayhurn & Wood, 2004). Studies conducted in rodent models have shown that adipose tissue oxygenation is lower in rodent models with obesity compared to their lean counterparts (Rausch et al., 2008; Ye et al., 2007; Jun Yin et al., 2009). Lower adipose tissue oxygenation in the context of excess adiposity has also been confirmed in several human studies (Cifarelli et al., 2020; Fleischmann et al., 2005; Kabon et al., 2004; Lawler et al., 2016; Pasarica et al., 2009; Seo et al., 2019) but not all of them (Goossens et al., 2018). On the other hand, many in vitro studies have shown that hypoxia negatively alters the lipid metabolism in adipocytes, mainly by decreasing their lipogenic capacity and increasing their basal lipolytic rate (García-Fuentes et al., 2015; Mahat et al., 2016; Netzer et al., 2015).

The cellular response to hypoxia is mainly induced through the hypoxia-dependent stabilization of the transcriptional factor hypoxia-inducible factor-1α subunit (HIF-1α). Stabilized HIF-1α dimerizes with its cofactor, aryl hydrocarbon receptor translocator (ARNT), and the complex induces the transcription of several genes containing hypoxia response elements (HRE) (Taabazuing et al., 2014; Zimna & Kurpisz, 2015). Many genes expressed in adipose tissue have been reported to contain HRE, including leptin (Ambrosini et al., 2002), VEGFA (Zimna & Kurpisz, 2015), glucose transporter 1, GLUT1 (M. Hayashi et al., 2004), and interleukin-6 (IL-6) (Famulla et al., 2012; I. S. Wood et al., 2011; Ye et al., 2007), implying that hypoxia affects critical adipose tissue features such as inflammation, insulin resistance, glucose intolerance, and angiogenesis (Lempesis et al., 2020; Netzer et al., 2015; Trayhurn & Alomar, 2015; B. Wang et al., 2007; Ye et al., 2007). Moreover, the expression and release of adipocytes' signature hormones, leptin and adiponectin, are remarkably altered during obesity, an effect that has been

shown to be HIF-1α-dependent (O'Rourke et al., 2011; Trayhurn et al., 2008; Unamuno et al., 2018; B. Wang et al., 2007; Ye et al., 2007). The fact that the alterations induced by hypoxia in the adipose tissue mirror the adipose tissue alterations observed in the context of obesity strongly suggests that adipose tissue hypoxia could play an important role in the development of the health complications associated with obesity (K. Sun et al., 2011; Trayhurn et al., 2008).

Adiposity levels have also been strongly correlated with the accumulation of lipophilic persistent organic pollutants (POPs) (Cock et al., 2014; E. Dirinck et al., 2011; Jackson et al., 2017; Regnier & Sargis, 2014), such as polychlorinated biphenyls (PCBs), which in turn have been correlated with many of the complications linked to obesity (Fierens et al., 2003; Hong et al., 2012; Valvi et al., 2012; Van den Berg et al., 2006). Among PCBs, coplanar dioxin-like congeners such as PCB77 and PCB126 are among the most toxic and widespread in the environment (Heindel et al., 2017). While the classical cellular response to dioxin-like compound exposure consists of an increased expression of detoxifying cytochrome enzymes, namely cytochrome P450 (an enzyme regulated by the CYP1A1 gene) (Larigot et al., 2018), these pollutants have also been reported to alter adipose tissue metabolism and endocrine function. As such, PCB126 exposure has been linked to the increased secretion of leptin and pro-inflammatory cytokines such as interleukin, IL-6 and IL-8, and the decreased secretion of adiponectin (Baker et al., 2015; Caron et al., 2020; Gadupudi et al., 2015; Hankinson, 1995; M. J. Kim et al., 2012; Klingelhutz et al., 2018; Lubrano et al., 2013; Sato et al., 2008). Most of these effects are thought to arise from the binding of PCBs to the aryl hydrocarbon receptor (AhR), a transcription factor that modulates the expression of genes bearing dioxin- and xenobiotic-response elements (DRE/XRE). Interestingly, the genomic action of AhR requires its dimerization with ARNT, the same co-factor involved in the hypoxia signaling pathway. This dual role of ARNT is best illustrated by the fact that conditional ARNT knockout mice are not able to induce AhR target genes in response to TCDD exposure, nor HIF-1α target genes under hypoxic-like conditions (Tomita et al., 2000).

Hence, ARNT is required as a common co-factor for these two signaling pathways, which can lead to crosstalk and interference (Nie et al., 2001). The crosstalk between the hypoxia-induced and the xenobiotic-induced pathways has been reviewed in detail by Vorrinck and Domann (Vorrink & Domann, 2014); it appears that, in most cases, co-exposure to dioxin-like POPs and hypoxia results in the induction of the hypoxia response and a repression of the xenobiotic response

(Vorrink & Domann, 2014). However, this crosstalk has been almost exclusively studied in liver cell lines. This is likely to be because the liver is a highly metabolically active organ in which hypoxia may develop, and it is also the main organ responsible for xenobiotic detoxification and excretion. However, because excessive adiposity can give rise to adipose tissue hypoxia and lipophilic xenobiotics accumulation, this crosstalk is also most likely to occur in adipocytes, especially in individuals with obesity.

The aim of the present study was to characterize the interaction between hypoxia and dioxin-like PCB126 exposure on the expression of genes induced by hypoxia (VEGFA) and dioxin (CYP1A1), as well as on the secretion of IL-6, IL-8, leptin and adiponectin in human adipocytes.

METHODS AND MATERIALS

1. Human Subcutaneous Preadipocyte Culture

Human subcutaneous preadipocytes and complete media were obtained commercially (ZenBio Inc., Research Triangle Park, NC, USA), and were used according to the manufacturer's instructions. Preadipocytes were plated in Preadipocyte Medium (PM-1) at a density of 40,000 cells/cm^2 in 24-well plates (Corning Life Science, Corning, NY, USA); these were incubated in a sterile, humidified incubator at 37 °C with 5% CO_2 until the cells reached full confluence (24–48 h). PM-1 was then replaced with Differentiation Medium (DM-2) to induce differentiation. Seven days post-induction, the cells were fed using Adipocyte Maintenance Medium (AM-1). Then, 14 days post-induction, the cells displayed multiple lipid droplets; these were considered mature adipocytes, and treatments were initiated.

2. Human Adipocytes' Exposure to PCB126 and Hypoxia

A 10 mM stock solution of 3,3',4,4',5-pentachlorobiphenyl (PCB126, Ultra Scientific, North Kingstown, RI, USA) was prepared with sterile-filtered dimethyl sulfoxide (DMSO). Mature adipocytes were exposed to either DMSO (control), 1 µM PCB126 or 10 µM PCB126 in AM-1 medium for 24 h. The control and PCB126 treatments were prolonged for another 24 h under either 21%, 10% or 3% O_2. Cell culturing has traditionally been conducted under 21% O_2; as such, this O_2 concentration was chosen as a control condition. We used 3 and 10% O_2 because these cover the physiological O_2 tension range observed in the human adipose tissue of individuals with

different adiposity levels (Cifarelli et al., 2020; Fleischmann et al., 2005; Goossens et al., 2018; Kabon et al., 2004; Lawler et al., 2016; Pasarica et al., 2009). Hypoxia treatments were conducted using a HERA cell 150iO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA) and medical nitrogen. The cells were therefore exposed to PCB126 for a total of 48 h, and to hypoxia for 24 h. Acriflavine (ACF, Millipore Sigma, Oakville, ON, Canada), an HIF-1α-inhibitor that binds directly to HIF-1α and HIF-2α and inhibits HIF-1α dimerization without affecting cell proliferation or survival (K. Lee et al., 2009), was prepared in sterile Phosphate-Buffered Saline (PBS) and added (at a final concentration of 5 µM) 4 h prior to the 24-h hypoxia/normoxia exposure. After the 48-h treatment phase the media were collected, and the cells were rapidly washed with sterile ice-cold PBS and kept on ice until further processing. Each experiment was repeated 3 times (n = 3), with all treatments being run in triplicate each time.

3. Adipokine Quantification

The media were stored at −80 °C until they were analyzed. The adipokine concentrations were determined by commercially available colorimetric ELISAs (R&D Systems, Minneapolis, MN, USA). The assays were performed following the manufacturer's protocols. According to the manufacturer, the assays' sensitivities were: leptin, 7.8 pg/mL; IL-6, 0.09 pg/mL; and adiponectin, 0.9 ng/mL, IL-8 7.5 pg/mL. In order to avoid freeze–thaw cycles, all of the adipokines were measured on the same day for samples from the same experiment. The measurements were carried out in singlets.

4. RNA Extraction and Quantification by qPCR

Immediately after washing, the cells were lysed in RLT buffer (Qiagen, Germantown, MD, USA) supplemented with B-mercaptoethanol and shredded using Qiagen QiaShredder columns. The lysates were then kept at −80 °C until they were processed further. Total RNA extraction was performed using Qiagen's RNeasy kit, following the manufacturer's protocol. The extracted RNA was reverse transcribed using Qiagen's QuantiTect Reverse Transcription kit, and real-time polymerase chain reactions (rtPCR) were performed for VEGFA, CYP1A1 and B-actin using 20 ng cDNA, Qiagen's QuantiTect Primers and Montreal Biotech's (Dorval, QC, Canada) MBI EVOlution EvaGreen qPCR master mix on a RotorGene (Corbett Research, Australia) thermocycler. The Ct values for VEGFA and CYP1A1 were normalized to β-actin, and the ΔΔCt

method was used to calculate fold changes in gene expression for each experimental condition using the DMSO-21% O_2 exposure as the reference treatment.

5. Statistical Analysis

All data are expressed as a mean ± standard error. Three-way ANOVAs were conducted with the PCB126 concentration, oxygen concentration, and ACF as fixed factors for all the variables. For gene expression, statistical analyses were performed on the ΔCt values. Post hoc pairwise comparisons between levels of significant main factors or interaction terms were conducted using the Tukey HSD test. Significance was set at $p < 0.05$. All of the statistical analyses were performed using JAMOVI statistical software version 2.2.5 (Jamovi project, Sydney, Australia).

RESULTS

1. Effects of Hypoxia and PCB126 on CYP1A1 and VEGFA Expression

PCB126 exposure induced CYP1A1 expression in a comparable and dose-dependent fashion under normoxia and 10% O_2 (**Figure 1**). This effect was still present but greatly attenuated under 3% O_2 (the main effect of O_2 $p < 0.001$). ACF increased the expression of CYP1A1, especially when combined with 10 µM PCB126 (ACF × PCB126 interaction $p = 0.012$) and restored the CYP1A1 response to PCB126 at 3% O_2 to levels comparable to that observed under normoxia and 10% O_2 without ACF.

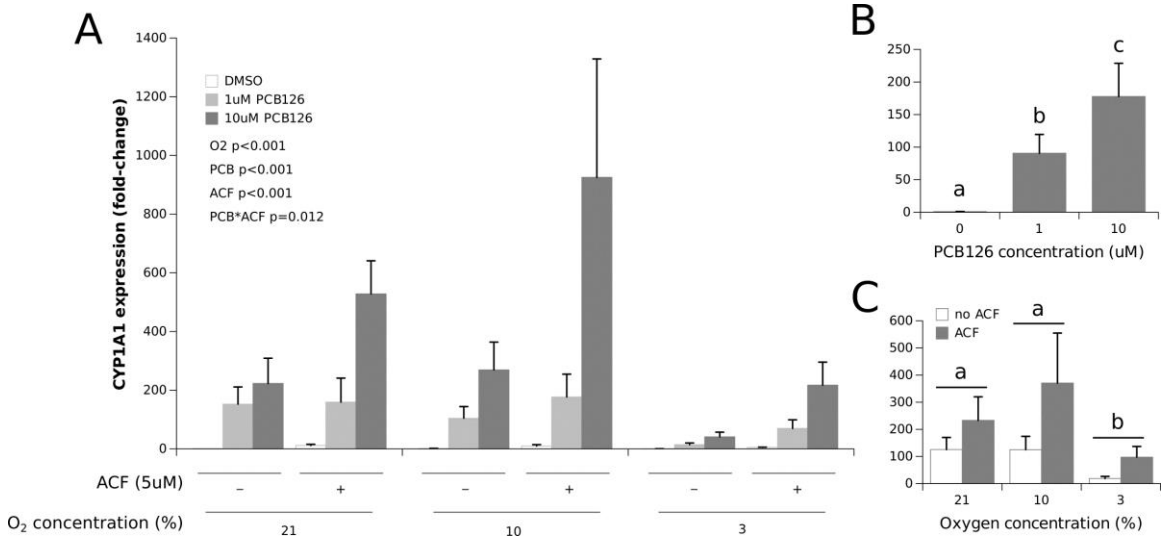

Figure 1. Effect of PCB126 and hypoxia on CYP1A1 expression in human adipocytes. Adipocytes were treated with DMSO (vector) or 1 and 10 µM PCB126 for 24 h and then subjected to 21, 10 or 3% O_2 for another 24 h. Acriflavine (ACF) 5 µM was used as an HIF-1α-inhibitor. Panel (**A**) illustrates the whole model. Markers of statistical difference

for individual bars were omitted due to the complexity of the model. Panel (**B**) summarizes the main effect of PCB126 on CYP1A1 expression. Panel (**C**) summarizes the effects of hypoxia and ACF treatment on CYP1A1 expression. In panels B and C, bars or groups of bars not sharing a common letter are statistically different at $p < 0.05$. The letters in panel C refer to the effect of hypoxia alone, although the effect of ACF is also significant. Each bar represents the mean ± SEM of three separate experiments for each treatment group.

As a reference point for O_2 tension exposure, VEGFA gene expression was measured. VEGFA expression was significantly increased in a dose-dependent manner by hypoxia (the main effect of O_2 $p < 0.001$) (**Figure 2**). PCB126 exposure did not affect this response (the main effect of PCB126 $p = 0.476$). The increase in VEGFA expression under hypoxia was completely abolished by HIF-1α inhibition with ACF (O_2 × ACF interaction $p < 0.001$).

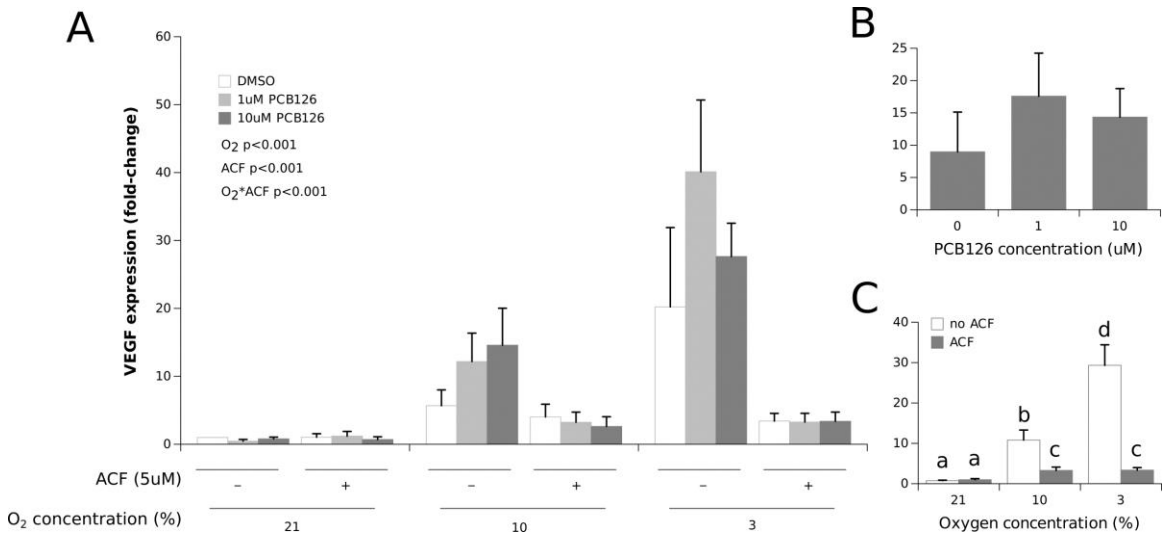

Figure 2. Effect of PCB126 and hypoxia on VEGFA expression in human adipocytes. Adipocytes were treated with DMSO (vector) or 1 and 10 µM PCB126 for 24 h, and then subjected to 21, 10 or 3% O_2 for another 24 h. Acriflavine (ACF) 5 µM was used as the HIF-1α-inhibitor. Panel (**A**) illustrates the whole model. Markers of statistical difference for individual bars were omitted due to the complexity of the model. Panel (**B**) summarizes the main effect of PCB126 on VEGFA expression. Panel (**C**) summarizes the effects of hypoxia and ACF treatment on VEGFA expression. In panel C, bars not sharing a common letter are statistically different at $p < 0.05$. Each bar represents the mean ± SEM of three separate experiments for each treatment group.

2. Effects of Hypoxia and PCB126 on Adipokine Secretion

Leptin secretion was significantly increased under hypoxia in a dose-dependent fashion (roughly 300% and 500% at 10% and 3% O_2, respectively, with a main effect of O_2 $p < 0.0001$) (**Figure 3**). This hypoxia-induced effect on leptin levels was not affected by PCB126 exposure. HIF-1α inhibition with ACF completely abolished the stimulating effect of hypoxia on leptin secretion (O_2 × ACF interaction $p < 0.001$).

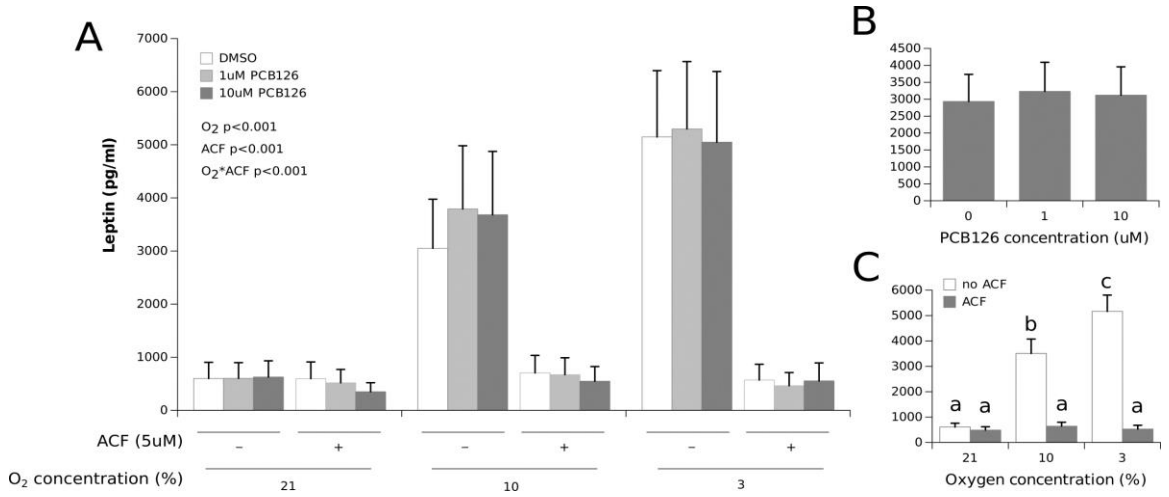

Figure 3. Effect of PCB126 and hypoxia on the media leptin concentration in human adipocytes. Adipocytes were treated with DMSO (vector) or 1 and 10 µM PCB126 for 24 h, and then subjected to 21, 10 or 3% O_2 for another 24 h. Acriflavine (ACF) 5 µM was used as an HIF-1α-inhibitor, then subjected to 21, 10 or 3% O_2 for another 24 h. Acriflavine (ACF) 5 µM was used as an HIF-1α-inhibitor. Panel (**A**) illustrates the whole model. Markers of statistical difference for individual bars were omitted due to the complexity of the model. Panel (**B**) summarizes the main effect of PCB126 on the media leptin concentrations. Panel (**C**) summarizes the effects of hypoxia and ACF treatment on the media leptin concentrations. Bars not sharing a common letter are statistically different at $p < 0.05$. Each bar represents the mean ± SEM of three separate experiments for each treatment group.

The adiponectin concentrations were significantly decreased under hypoxia, but only in the most severe hypoxia condition (−25% at 3% O_2, the main effect of hypoxia $p < 0.0001$). HIF-1α inhibition with ACF partially but not completely restored adiponectin secretion under 3% O_2 (the main effect ACF $p = 0.074$) (**Figure 4**). Adiponectin secretion was not affected by PCB126 exposure.

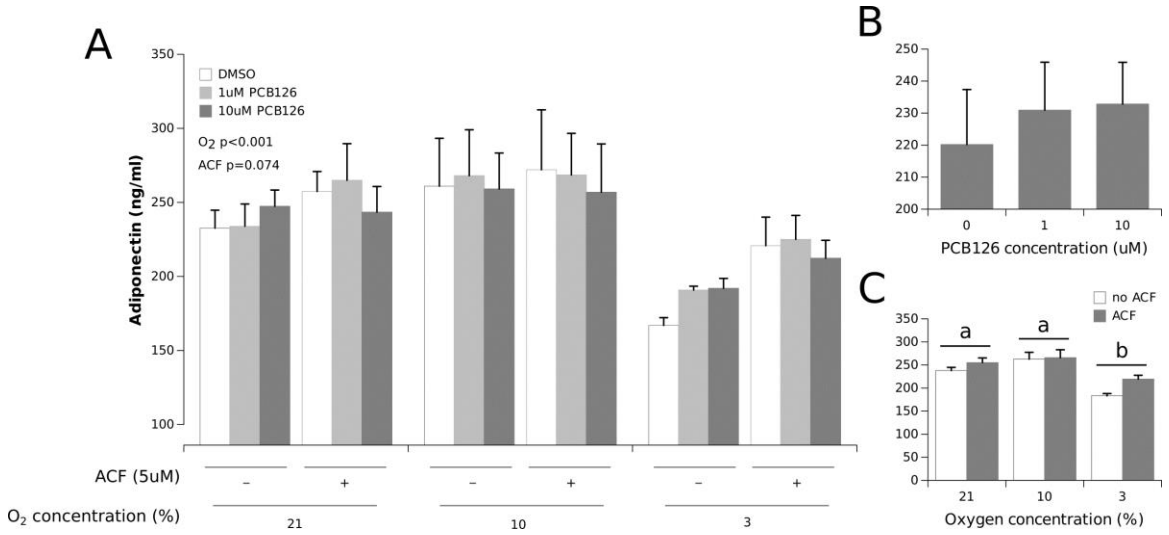

Figure 4. Effect of PCB126 and hypoxia on the media adiponectin concentration in human adipocytes. Adipocytes were treated with DMSO (vector) or 1 and 10 µM PCB126 for 24 h, then subjected to 21, 10 or 3% O_2 for another 24 h. Acriflavine (ACF) 5 µM was used as an HIF-1α-inhibitor. Panel (**A**) illustrates the whole model. Markers of

statistical difference for individual bars were omitted due to the complexity of the model. Panel (**B**) summarizes the main effect of PCB126 on the media adiponectin concentrations. Panel (**C**) summarizes the effects of hypoxia and ACF treatment on the media adiponectin concentrations. Pairs of bars (representing the main effect of oxygen) not sharing a common letter are statistically different at $p < 0.05$. Each bar represents the mean ± SEM of three separate experiments for each treatment group.

IL-6 secretion was increased by hypoxia in a dose-dependent fashion (**Figure 5**) (the main effect of O_2 $p = 0.002$). This effect was not repressed by ACF; rather, it was increased by 35% in all conditions (the main effect of ACF $p = 0.001$). A dose-dependent trend was observed between PCB126 and IL-6 secretion (the main effect of PCB126 $p = 0.068$).

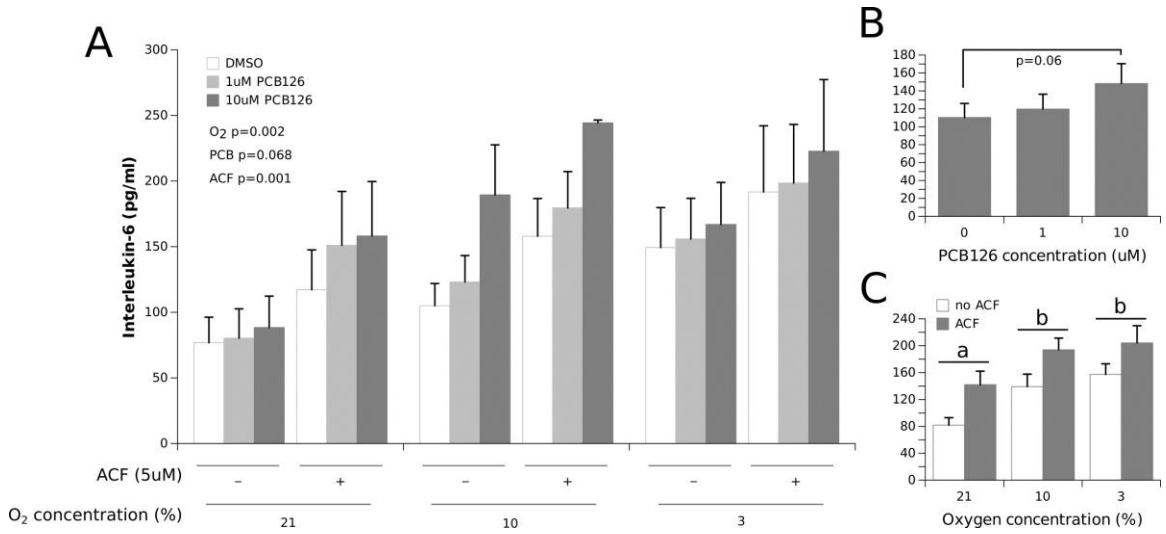

Figure 5. Effect of PCB126 and hypoxia on the media IL-6 concentration in human adipocytes. Adipocytes were treated with DMSO (vector) or 1 and 10 µM PCB126 for 24 h, then subjected to 21, 10 or 3% O_2 for another 24 h. Acriflavine (ACF) 5 µM was used as an HIF-1α-inhibitor. Panel (**A**) illustrates the whole model. Markers of statistical difference for individual bars were omitted due to the complexity of the model. Panel (**B**) summarizes the trend between 0 and 10 µM PCB126 on media IL-6 concentrations. Panel (**C**) summarizes the effects of hypoxia and ACF treatment on media IL-6 concentrations. In panel C, pairs of bars (representing the main effect of oxygen) not sharing a common letter are statistically different at $p < 0.05$. Each bar represents the mean ± SEM of three separate experiments for each treatment group.

IL-8 secretion was not significantly affected by hypoxia but was increased overall by 50% and 60% at 1 µM and 10 µM PCB126, respectively (the main effect PCB126 $p = 0.003$) (**Figure 6**). IL-8 concentrations were also significantly increased by 25% with ACF treatment (the main effect ACF $p = 0.031$).

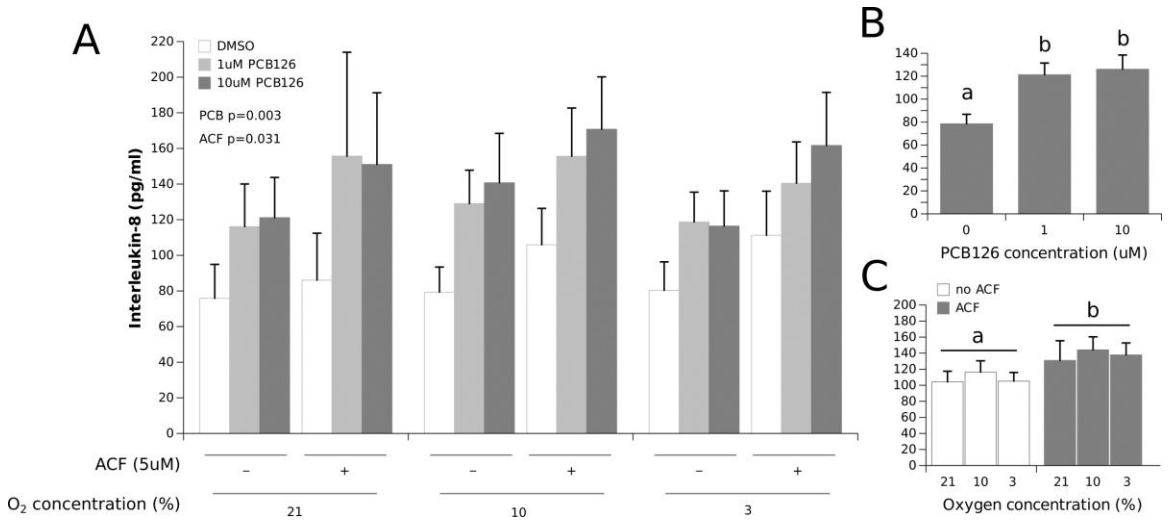

Figure 6. Effect of PCB126 and hypoxia on the media IL-8 concentration in human adipocytes. Adipocytes were treated with DMSO (vector) or 1 and 10 µM PCB126 for 24 h, then subjected to 21, 10 or 3% O_2 for another 24 h. Acriflavine (ACF) 5 µM was used as an HIF-1α-inhibitor. Panel (**A**) illustrates the whole model. Markers of statistical difference for individual bars were omitted due to the complexity of the model. Panel (**B**) summarizes the main effect of PCB126 on media IL-8 concentrations. Panel (**C**) summarizes the effects of hypoxia and ACF treatment on media IL-8 concentrations. In panels B and C, bars or groups of bars not sharing a common letter are statistically different at $p < 0.05$. Each bar represents the mean ± SEM of three separate experiments for each treatment group.

DISCUSSION

The main objective of the present study was to characterize the interaction between hypoxia and dioxin-like PCB126 exposure, two stressors to which the responses involve the heterodimerization partner ARNT, in primary human adipocytes. We found that exposure to PCB126 significantly increased CYP1A1 expression, a classical element of the dioxin response, in a dose-response manner, and this response was significantly repressed after 24 h of exposure to 3% O_2. In contrast, exposure to hypoxia induced VEGFA expression, a classical element of the hypoxia response, but this response was not altered by PCB126 exposure. Together, these observations suggest that hypoxia interferes with the xenobiotic sensing pathway, and that highly potent dioxin-like compounds such as PCB126 do not interfere with the hypoxia response.

To our knowledge, this is the first study showing that CYP1A1 induction by PCB126 is significantly inhibited in human adipocytes exposed to hypoxia (**Figure 1**). This finding is in line with several other studies using different cell models co-exposed to dioxin/dioxin-like POP and hypoxia (Fleming et al., 2009; Gassmann et al., 1997; Khan et al., 2007; J. E. Kim & Sheen, 2000; Matson et al., 2008; Prasch et al., 2004). Still, some studies reported a downregulation of the hypoxia signaling pathway by POPs (Kraemer & Schulte, 2004; Seifert et al., 2008; Takacova et

al., 2009; Terzuoli et al., 2010); and in one instance, using aminoflavone, the inhibition was shown to be independent of AhR activation (Terzuoli et al., 2010). Furthermore, some studies even reported dioxin-like compounds and hypoxia to mutually inhibits each other pathways (Nie et al., 2001; Schults et al., 2010; Vorrink et al., 2014). Most of these previous studies were almost exclusively conducted in human and rodent hepatic cell lines using TCDD or benzo(a)pyrene as an AhR ligand, and with different hypoxic modalities of various intensities and durations (Vorrink & Domann, 2014). These experimental disparities in the body of work on POPs and hypoxia co-exposure make fine comparisons between studies almost impossible, but they emphasize the robustness with which hypoxia appears to interfere with the xenobiotic response. The present study extends our understanding of the xenobiotics–hypoxia interaction to human adipose tissue by showing that, as far as the traditional gene markers of hypoxia (VEGFA) and xenobiotic (CYP1A1) responses are concerned, acute co-exposure to hypoxia and PCB126 results in the full activation of the HIF-1α signaling pathway and the downregulation of the AhR signaling pathway in human adipocytes.

Whether hypoxia interferes with the xenobiotic response solely because HIF-1α stabilization prevents AhR dimerization with ARNT is, however, still unclear. The fact that ACF restored CYP1A1 induction by 10µM PCB126 under 3% O_2 to levels comparable to normoxia and 10% O_2 without ACF suggests so. However, because ACF significantly increased CYP1A1 expression in the presence of 10 µM PCB126 at both 21% and 10% O_2, CYP1A1 expression with ACF treatment still appeared restrained at 10 µM PCB126 and 3% O_2 (compared to normoxia and 10% O_2 with ACF) (**Figure 1**). The incomplete inhibition of HIF-1α by ACF appears unlikely because ACF treatment completely abolished VEGFA and leptin induction under hypoxia (**Figure 2** and **Figure 3**). Therefore, our observations suggest that factors other than HIF-1α stabilization may interfere with CYP1A1 induction by xenobiotics under severe hypoxia in human adipocytes. As such, it is noteworthy that ARNT has additional partners that may be activated under stress conditions and affect the AhR induced transcriptional response (Simon et al., 2015). Further studies are warranted to confirm the presence and identity of such factors that may adversely affect adipocyte xenobiotic metabolism under hypoxia independently of HIF-1α signaling.

1. Secretion under Co-Exposure to Hypoxia and PCB126

Because hypoxia and dioxin-like xenobiotics have been reported to affect the secretion of many adipokines, we sought to determine how co-exposure to hypoxia and PCB126 would affect leptin, IL-6, IL-8 and adiponectin secretion compared to exposure to hypoxia and PCB126 alone. We observed that leptin secretion was increased dose-dependently by hypoxia, an effect that was mitigated by ACF (**Figure 3**). These results are in line with previous evidence demonstrating that the transcriptional activity of the leptin gene is upregulated by the HIF-1α-ARNT complex under hypoxia (Ambrosini et al., 2002; Alexandra Grosfeld et al., 2002). PCB126 exposure had no effect on leptin secretion per se and did not affect the leptin response to hypoxia. Contrary to the well-described effects of hypoxia on leptin secretion, whether PCB126 exposure or POP exposure in general affect adipose tissue leptin secretion is still obscure. In humans, one study reported a correlation between the plasma concentrations of some POPs and leptinemia, especially in women, but this relationship was not observed with any PCB congeners (Pereira-Fernandes et al., 2014). In rodents, exposure to PCB126 at doses from 0.1 to 10 µg/kg for 15 d was reported to have no effect on leptinemia (Loiola et al., 2016); while in vitro, leptin secretion was not altered in 3T3-L1 cells exposed for 24 h to low doses of PCB126 (between 1 and 100 nM) (Caron et al., 2020). Our observations, combined with previous reports, support the notions that acute PCB126 exposure does not seem to impact leptin secretion and that PCB126 does not affect the increase in leptin secretion under hypoxia, at least under the experimental conditions we tested.

IL-6 secretion increased dose-dependently in response to hypoxia (**Figure 5**), which is consistent with previous studies conducted in vitro on cultured human adipocytes (Famulla et al., 2012; I. S. Wood et al., 2011; Ye et al., 2007). IL-6 secretion was also increased dose-dependently by PCB126, with increases of 11% at 1 µM PCB126 and 34% at 10 µM PCB126 compared to DMSO, but this did not reach statistical significance ($p = 0.068$). Previous reports on the effect of PCB126 exposure on IL-6 secretion are conflicting. Gourronc *et al.* (2018) observed a twofold increase in IL-6 secretion in cultured adipocytes following a 48-h exposure to 10 µM PCB126. On the other hand, Loiola *et al.* (2016) showed that a 15-day exposure to PCB126 up to 10 µg/kg decreased retroperitoneal adipose tissue IL-6 mRNA levels in rats, while Caron *et al.* (2020) showed no effect of a 24-h exposure to low PCB126 doses up to 100 nM on IL-6 secretion in 3T3-L1. Interestingly, TCDD, the most potent dioxin, has been demonstrated to repress LPS-induced

IL-6 production in bone marrow stromal cells, suggesting that the alleged binding of activated AhR to the IL-6 XRE may, in some cases, downregulate IL-6 expression (Jensen et al., 2003). As for our observations, they suggest that acute exposure to PCB126 and hypoxia may have additive effects on IL-6 secretion in human adipocytes, such that the combination of both PCB126 accumulation and hypertrophy-induced hypoxia may lead to greater inflammatory responses and metabolic disturbances in human adipose tissue than either stressor alone.

IL-8, another pro-inflammatory adipokine, behaved differently from leptin and IL-6 in that its secretion was induced by PCB126 exposure but was unaffected by hypoxia (**Figure 6**). The increase in IL-8 secretion by PCB126 is consistent with previous studies conducted in cultured adipocytes (Gourronc et al., 2018; Klingelhutz et al., 2018). Interestingly, there is evidence that both IL-6 and IL-8 inductions by PCB126 and TCDD are dependent on AhR signaling (Gourronc et al., 2018; Guarnieri et al., 2020; Hollingshead et al., 2008; Matsumura & Vogel, 2006); however, neither adipokine appeared repressed under hypoxia in our experiments. These observations suggest that the PCB126 induction of IL-6 and IL-8 by AhR may be independent of AhR/ARNT dimerization in adipocytes. In this regard, it is increasingly well recognized that AhR can activate signaling pathways independently of ARNT dimerization (a non-classical mechanism of action); one such pathway is NF-κB (Denison et al., 2011; Larigot et al., 2018), an important inflammation modulator and a regulator of IL-6 and likely IL-8 expression (Georganas et al., 2000). Based on our observations, it is most likely that the increases in IL-6 and IL-8 secretion by PCB126 even under hypoxia could occur through such an ARNT-independent AhR signaling pathway, as previously demonstrated (P. H. Chen et al., 2012; Vogel & Matsumura, 2009).

An interesting observation regarding IL-6 and IL-8 is that ACF led to an overall 40% increase in IL-6 secretion, and to a 25% increase in IL-8 secretion. This effect of ACF was unexpected and cannot be easily explained. To our knowledge, the effect of ACF on inflammatory markers has never been studied per se, but some works suggest that ACF could have both anti-inflammatory (Priyanka et al., 2014) and inflammatory (DNA-disrupting) actions (Pepin et al., 2017). Although they are unexplained, these observations strongly support the fact that the robust increase in IL-6 secretion under hypoxia is not likely to be a result of HIF-1α activation.

We observed that adiponectin secretion was reduced under 3% O_2 but was not affected by PCB126 exposure (**Figure 4**). The reduction in adiponectin secretion under hypoxia is consistent with previous studies (Alexandra Grosfeld et al., 2002; Jiang et al., 2013; I. S. Wood et al., 2011; Ye et al., 2007). in line with our observations, Jiang *et al.* reported that 5 µM ACF restores adiponectin secretion in vitro in 3T3-L1 exposed to the chemical hypoxia mimetic $CoCl_2$, and also demonstrated that HIF-1α is responsible for the decrease in adiponectin secretion under hypoxia by affecting SOCS3-STAT3 signaling (Jiang et al., 2013). Interestingly, they reported—similarly to our findings—that 5 µM ACF does not fully restore adiponectin secretion in 3T3-L1 under chemical hypoxia. This suggests that although HIF-1α may be an important regulator of adiponectin expression, other factors may limit adiponectin production in conditions in which HIF-1α is activated. Contrary to hypoxia, PCB126 exposure had no effect on adiponectin secretion in our experiments. Previous studies of the effect of PCB126 on adiponectin secretion are conflicting. In vitro, Klingelhutz *et al.* (2018) and Gadupudi *et al.* (2018) both showed that exposing preadipocytes to 10 µM PCB126 during proliferation inhibits their differentiation into mature adipocytes and decreases adiponectin secretion up to 2 weeks after PCB126 treatment. On the other hand, Caron *et al.* (2020) observed an increase in adiponectin secretion in insulin-resistant 3T3-L1 treated for 24 h with PCB126 at doses of 10 and 100 nM. It has also been shown that a single PCB126 intraperitoneal injection (5 µmol/kg) in rats significantly increased serum adiponectin levels after 28 d (Gadupudi et al., 2015). Taken together, these observations are difficult to reconcile but may suggest that the effect of PCB126 on adiponectin may depend on the PCB dose as well as the timing and duration of exposure.

2. Limitations and Strengths

The main limitations of the current study include the use of a single POP at supraphysiological doses, although these are commonly used, and the fact that both the hypoxia and PCB126 exposures were performed acutely. In living humans, adipocytes are exposed to a cocktail of different POPs as well as hypoxia for widely varying durations, which may exert different effects on adipocyte metabolism. Furthermore, in living organisms, adipocyte metabolism can possibly be altered indirectly through the effects exerted by POPs or hypoxia on other tissues. These limitations call for caution when extrapolating findings from our in vitro study to in vivo conditions. One strength of the current study lies on the fact that we used a range of O_2 tensions:

a commonly considered normoxic condition, although it is sometimes referred to as hyperoxia (21% O_2); a mild hypoxic condition, though it is sometimes considered a physiological normoxic condition (10% O_2); and a clearly hypoxic condition (3% O_2) (Hodson, 2014).

CONCLUSIONS

The present study supports the theories that hypoxia and xenobiotic signaling pathways interfere in human adipocytes, and that this interference is likely explained by HIF-1α preventing the dimerization of AhR with ARNT, which may partially repress the xenobiotic transcriptional response. However, our results showed that PCB126 increased IL-6 and IL-8 secretion, an effect that is not repressed by hypoxia. This observation suggests that the PCB126 induction of IL-6 and IL-8 by AhR may be independent of AhR/ARNT dimerization, and that AhR can activate non-classical signaling pathways such as NF-κB. Therefore, the long-term co-exposure of adipocytes to hypoxia and POPs could accelerate the establishment of a pro-inflammatory state in the adipose tissue, a widely acknowledged crucial step in the development of obesity-related metabolic complications.

Author Contributions

Z.E.A., J.-F.M. and P.I. conceived and designed the experiments. Z.E.A. and J.-F.M. performed the experiments. Z.E.A., J.-F.M. and P.I. analyzed the data. Z.E.A., J.-F.M. and P.I. interpreted the data. All authors have read and agreed to the published version of the manuscript.

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3.3 THESIS ARTICLE 3

Human Preadipocytes Differentiated under Hypoxia following PCB126 Exposure during Proliferation: Effects on Differentiation, Glucose Uptake and Adipokine Profile

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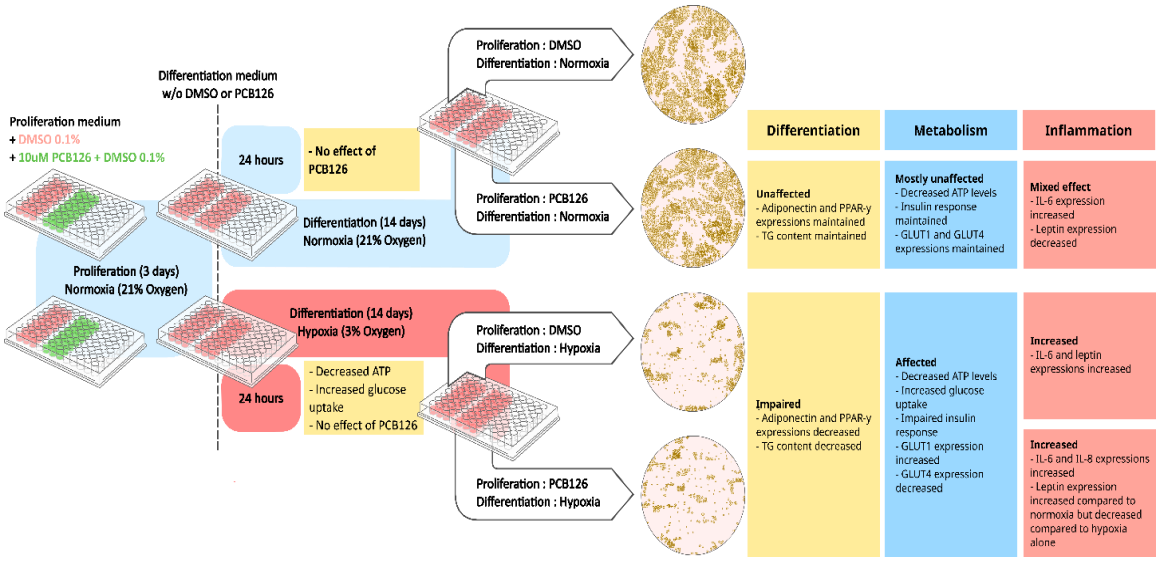
ABSTRACT

Persistent organic pollutants (POPs) accumulation and hypoxia are two factors proposed to adversely alter adipose tissue functions in the context of excess adiposity. Studies have shown that preadipocytes exposure to dioxin and dioxin-like POPs have the greatest deleterious impact on rodent and immortalized human preadipocyte differentiation, but evidence on human preadipocytes is lacking. Additionally, hypoxia is known to strongly interfere with the dioxin-response pathway. Therefore, we tested the effects of pre-differentiation polychlorinated biphenyl (PCB)126 exposure at 10 µM for 3 days and subsequent differentiation under hypoxia on human subcutaneous adipocytes (hSA) differentiation, glucose uptake and expression of selected metabolism- and inflammation-related genes. Pre-differentiation PCB126 exposure lowered the adenosine triphosphate (ATP) content, glucose uptake and leptin expression of mature adipocytes but had limited effects on differentiation under normoxia (21% O_2). Under hypoxia (3% O_2), preadipocytes ability to differentiate was significantly reduced as reflected by significant decreased lipid accumulation and downregulation of key adipocyte genes such as peroxisome proliferator-activated receptor gamma (PPARγ) and adiponectin. Hypoxia increased glucose uptake and glucose transporter 1 (GLUT1) expression but abolished the adipocytes insulin response and GLUT4 expression. The expression of pro-inflammatory adipokine interleukin-6 (IL-6) was slightly increased by both PCB126 and hypoxia, while IL-8 expression was significantly increased only following the PCB126-hypoxia sequence. These observations suggest

that PCB126 does not affect human preadipocyte differentiation, but does affect the subsequent adipocytes population, as reflected by lower ATP levels and absolute glucose uptake. On the other hand, PCB126 and hypoxia exert additive effects on adipose tissue inflammation, an important player in the development of chronic diseases such as type 2 diabetes and cardiovascular diseases.

Keywords: hypoxia; polychlorinated biphenyl; inflammatory adipokines; human differentiated adipocytes; AhR; ARNT.

Number of figures: 10



INTRODUCTION

Proper adipose tissue maintenance through proliferation and differentiation of preadipocytes is essential for whole-body energy homeostasis (M.-J. Lee et al., 2010; K. Sun et al., 2011). The accumulation of lipophilic persistent organic pollutants (POPs) and hypoxia are two factors that are recognized to adversely alter adipose tissue metabolism and to contribute to the development of obesity-related metabolic complications (E. Dirinck et al., 2011; Myre & Imbeault, 2014; Trayhurn, 2014).

POPs are synthetic compounds that consist of many groups of halogenated compounds, such as polychlorinated biphenyls (PCBs). POPs, which bioaccumulate in the food chain and resist biodegradation, are commonly referred to as “metabolic disruptors” (Y. Lee et al., 2017; Merrill et al., 2013). Traditionally, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and dioxin-like PCBs

have been reported to exhibit their toxicity by activating the aryl-hydrocarbon receptor (AhR), a transcription factor that regulates the expression of genes containing a xenobiotic response element (XRE) in their promoter (Denison et al., 2011). TCDD and dioxin-like PCBs have been shown to alter the acquisition of adipocyte phenotype (also called "adipocyte differentiation") and adipocyte metabolism (Nadal et al., 2017; Shan et al., 2020). For instance, it has been reported that 3T3-L1 cells and immortalized human preadipocytes (Normal Pre-Adipocytes, NPADs) exposure to dioxin and dioxin-like PCB126 prior to differentiation impaired subsequent adipocyte differentiation (C. Chen et al., 1997; Gadupudi et al., 2015; Phillips et al., 1995). In NPADS, the impairment of adipocyte differentiation by PCB126 has later been associated with inflammation, specifically the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells activation (NF-κB) (Gourronc et al., 2018).

Hypoxia, another important metabolic disruptor, is known to profoundly affect adipocyte functions (Netzer et al., 2015; Trayhurn, 2013). For instance, acute exposure (24 h) to low oxygen tension is well known to decrease peroxisome proliferator-activated receptor γ (PPARγ) expression and to increase leptin secretion in differentiated human adipocytes (Amine et al., 2022; A Grosfeld et al., 2002; B. Wang et al., 2007, 2008). Acute hypoxia exposure also impacts the adipocyte's lipid storage and mobilization functions by reducing the activity of the lipoprotein lipase while increasing basal intracellular lipolysis (Mahat et al., 2021; O'Rourke et al., 2013). Moreover, exposure to acute hypoxia has been shown to increase GLUT1 facilitative glucose transporter gene and protein levels, glucose transport (B. Wang et al., 2007; I. S. Wood et al., 2007) as well as expression and release of inflammation-related adipokines (Amine et al., 2022; Famulla et al., 2012; I. S. Wood et al., 2011) in human differentiated adipocytes. These cellular responses to hypoxia are mostly exerted via the genomic action of the hypoxia inducible factor 1a (HIF-1α), a transcription factor that is stabilized and activated when O_2 levels drop below 4–5% (Q. Lin et al., 2006; Mazzatti et al., 2012; Sheng et al., 2013; Weiszenstein et al., 2016).

Interestingly, hypoxia and dioxins signaling both exploit the aryl hydrocarbon receptor nuclear translocator (ARNT) transcription factor, which can be recruited by both AhR and HIF-1α thus establishing a significant foundation for a possible crosstalk between these two signaling pathways (Myre & Imbeault, 2014; Vorrink & Domann, 2014). We recently showed in differentiated human adipocytes that 24 h of hypoxia and PCB126 co-exposure resulted in an

increase in vascular endothelial growth factor-A (VEGFA) expression, a traditional marker of HIF-1α activation, and in a strong decrease in the transcription of CYP1A1, a detoxifying enzyme member of the cytochrome P450 superfamily and a traditional marker of AhR activation (Amine et al., 2022). In the same study, we reported that exposure to PCB126 or hypoxia alone or in combination increases IL-6 secretion in a dose dependent manner, which suggests that long-term co-exposure of adipocytes to hypoxia and POPs could favor the establishment of a pro-inflammatory state in the adipose tissue.Considering the previously reported effects of dioxin-like PCBs and hypoxia on adipocytes, we exposed proliferating human preadipocytes to PCB126 and then differentiated them under hypoxia to determine how the combination of both stressors would affect adipocyte differentiation, glucose uptake and key inflammatory markers.

METHODS

1. Cell Culture

Pooled human subcutaneous preadipocytes from donors with a body mass index over 30 kg/m^2 (SP-F-3) were obtained from Zenbio (Durham, NC, USA) and cultured according to the manufacturer's instructions. Cells suspended in Zenbio's PM-1 culture media were seeded in culture-treated 96-well plates at a density of at least 40,000 cells/cm^2. Cells were then placed in a HeraCell 150iO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA) incubator maintained at 100% humidity and 5% CO_2. After 3 days of proliferation, medium was replaced by Zenbio DM-2 to induce differentiation. After 7 days of differentiation, the medium was partially replaced with Zenbio AM-1 in which cells remained until day 14 after induction.

2. PCB126 and Hypoxia Exposure

During proliferation, which was carried for 3 days, preadipocytes were exposed to either 10 µM PCB126 in dimethyl sulfoxide (DMSO) or DMSO alone (final DMSO concentration 0.1% in both conditions). Media were not changed during the proliferation phase. A concentration of 10 µM PCB126 was used because it has the greatest detrimental effect on the differentiation capacity of NPAD cells without inducing selective cytotoxicity (Gadupudi et al., 2015) and it is well tolerated by differentiated human preadipocytes (Amine et al., 2022). After 3 days of proliferation, media were switched to DM-2 containing no DMSO nor PCB126 to induce differentiation. At the same time, hypoxia exposure was initiated and carried out for

14 days in a HeraCell 150iO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA) maintained at 3% O_2 with medical nitrogen and 5% CO_2. During the differentiation phase, media were partially changed every other day to avoid acidification of the culture media by lactate accumulation under hypoxia. This procedure was also carried out for cells cultured under normoxia.

3. Cell Expansion and Viability

Cell viability was assessed daily by visual examination using a Zeiss Axiovert 40 °C light microscope (Carl Zeiss, Göttingen, Germany) to detect anomalies such as cell detachment from culture ware. Twenty-four hours and 14 days following induction, cells expansion/viability was assessed by bioluminescent ATP quantification using CellTiterGlo 2 reagent from Promega (Madison, WI, USA). Because ATP content was affected by PCB126 and hypoxia, ATP measurements were refined by measuring the ADP/ATP ratio using the MAK135 assay kit from Millipore-Sigma (Oakville, ON, Canada). Lactate dehydrogenase (LDH) activity, another indicator of cell apoptosis and necrosis, was measured in culture media after 14 days of differentiation using the CyQUANT LDH cytotoxicity assay (Life Technologies Inc., Burlington, ON, Canada).

4. Glucose Uptake

Glucose uptake was measured using Promega bioluminescent GlucoseUptakeGlo assay based on 2-deoxyglucose (2-DG) uptake according to manufacturer instructions. Cells were first glucose-starved by washing 3 times with Zenbio basal medium (BM-1) containing no glucose followed by incubation for 1.5 h in glucose-free BM-1. Insulin was added to relevant wells to a final concentration of 100 nM 30 min before glucose uptake assessment. BM-1 was removed from wells and cells were incubated 12 min with 1 mM 2-DG in PBS. The GLUT-specific inhibitor Glutor (Reckzeh & Waldmann, 2019) was used at a final concentration of 1 µM to confirm GLUT-specific glucose uptake. On average, Glutor decreased glucose uptake by 90% under normoxia and by 85% under hypoxia. Glucose uptake was normalized to cellular ATP to account for the possible effects of treatments on cell expansion and/or energy metabolism. ATP measurements considered for this adjustment came from Glutor wells because 2-DG uptake is known to deplete cellular ATP as 2-DG is phosphorylated but not further metabolized (Chandramouli & Carter, 1977).

5. TG Content

TG concentrations were measured in glucose uptake cell lysate using the Wako L-type Triglyceride M (FUJIFILM Wako Chemicals U.S.A. Corporation, Richmond, VA, USA) assay adapted for microplates.

6. Gene Expression

Cells were rapidly washed 3 times with ice-cold PBS and lysed using Qiagen RLT buffer containing 1% Beta-mercaptoethanol. Lysates were spun on Qiagen QiaShredder columns and lysates were kept at −80 °C until further processed. Total ARN was extracted using Qiagen RNeasy mini kit and concentrated using Qiagen MinElute clean up kit when necessary. Complementary DNA (cDNA) was obtained using the Qiagen Quantitect Reverse transcription kit and the maximum volume of RNA template allowed by the kit (12 µL) on a T-personal Combi thermocycler (Biometra, Gottingen, Germany) and real-time quantitative polymerase chain reaction (RT-qPCR) was conducted using Quantitect primers (Qiagen, Germantown, MD, USA) and MBI EVOlution EvaGreen rtPCR mix (Montreal Biotech Inc., Dorval, QC, Canada) on a RG-3000 Rotor-Gene (Corbett Research Ltd., Mortlake, Australia). The Quantitect primers used had the following Qiagen catalog numbers: Adiponectin QT00014091; Beta-actin QT00095431; CYP1A1 QT00012341; GLUT1 QT00068957; GLUT4 QT00097902; IL-6 QT00083720; IL-8 QT00000322; Leptin QT00030261; PPAR-gamma QT00029841; VEGFA QT01682072. Melting curve analyses were performed to ensure that amplification yielded single products. Amplification curves were analyzed using the Rotor-Gene Q Series software version 2.2.3. Amplification efficiency was determined for each individual amplification reaction using the comparative quantitation feature of the Q software, which is based on the fluorescence increase over 4 cycles following “take-off” of the fluorescence signal. The “take-off” point corresponds to the point at which the second derivative of the amplification curve reaches 20% of its maximum (determined automatically by the Rotor-Gene Q software). Average amplification factors were very stable within and across genes (averaging 1.7 to 1.8). Fluorescence thresholds for *CT* determination were determined for each gene in the first experiments and arbitrarily assigned the value corresponding

to half the average fluorescence at take-off cycle. Fold-changes were calculated using the formula (Pfaffl, 2001):

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

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

7. Statistical Analyses

Data were analyzed by linear mixed modeling using restricted maximum likelihood with "proliferation" (PCB126 or DMSO during proliferation) and "differentiation" (21% or 3% O_2 during differentiation) as main effects and "experiment" as cluster variable. Intercept was set as random across experiments to account for correlation between measurements within individual experiment. Three independent experiments were conducted, and each combination of treatment was tested in duplicate (2 wells) within each experiment. We included individual data from replicate wells in the statistical design because replicate wells per experiment were minimal and because we believe within-experiment replicates provide valuable information on the robustness of the biological effect. For clarity's sake, purely technical replicates (*i.e.*, duplicate measurements from a same well for a given variable) were averaged and contributed unique datum to the statistical model. For gene expression, statistical analyses were conducted on ΔΔCT values and for glucose uptake and triglyceride content, analyses were conducted on raw luminescence values or ratios of raw luminescence values. Significant interactions were further investigated by post hoc multiple comparison analyses using Bonferroni correction. Figures represent main effects as averaged percent of control condition (proliferation in DMSO and differentiation under normoxia) within each experiment to better illustrate treatment effects by excluding part of the inter-experiment variability. Analyses were performed using the Jamovi statistical software version 2.2.5 with the GAMJL module version 2.6.6.

RESULTS

1. Effects of Treatments on Cell Viability and Growth

The daily visual examination of the cells showed no obvious anomaly such as reduced cell proliferation or cell detachment from culture ware in response to treatments throughout the experiment duration (17 days total) (Figure 1). After 72 h of proliferation, cellular ATP was slightly lower (−7%, $p = 0.009$) in PCB126-exposed cells but the ADP/ATP ratio and media lactate dehydrogenase (LDH) activity were similar between PCB126- and DMSO-exposed cells ($p = 0.981$ and 0.248), suggesting that PCB126 had only a minor effect on preadipocytes proliferation. After 24 h of differentiation, ATP content was no more affected by PCB126 exposure while a 20% decrease in ATP ($p = 0.003$) (Figure 2) and a minor increase in ADP/ATP ratio (0.247 vs. 0.224, $p = 0.004$) were observed in cells exposed to hypoxia. LDH activity

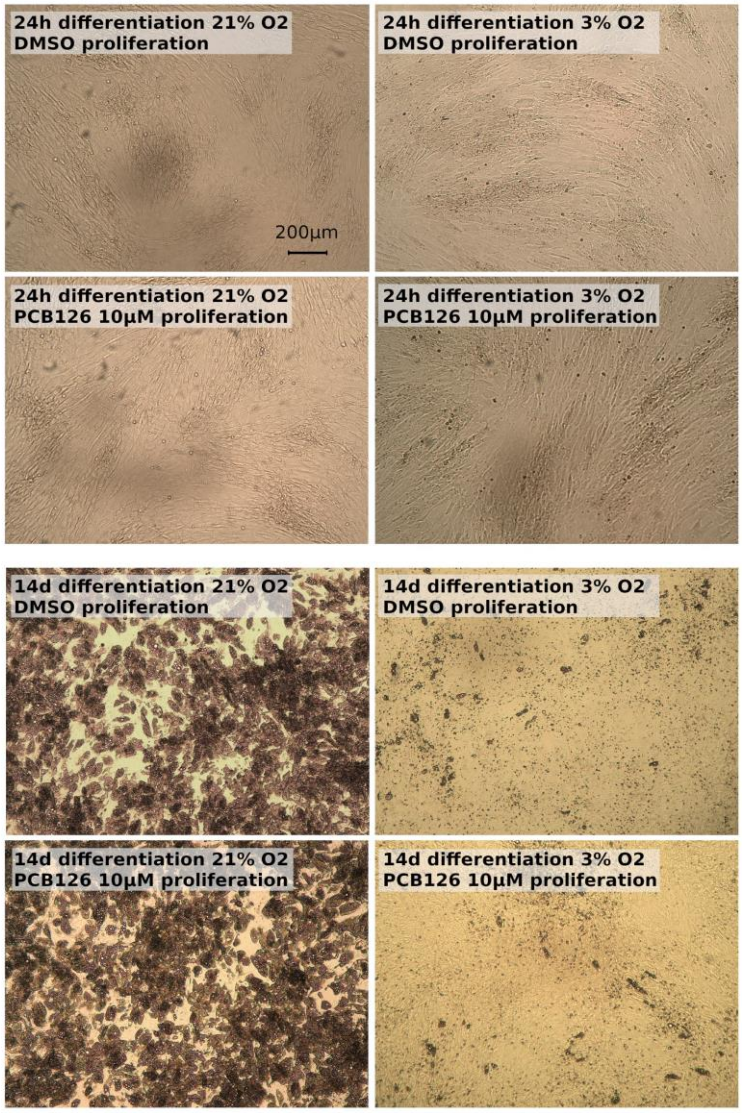


Figure 1. Differentiating preadipocytes exposed to either DMSO (vector) or 10 µM PCB126 during proliferation (3 days). The top 4 pictures were taken after the first 24 h of differentiation under either 21% or 3% O2. Bottom 4 pictures were taken after 14 days of differentiation under either 21% or 3% O2. All pictures were taken using a light microscope at 5× magnification.

in the media was not performed at that time. After 14 days of differentiation, both PCB126 and hypoxia treatments were associated with a significant ~30% decrease in ATP content (main effect of PCB126 $p = 0.014$, main effect of hypoxia $p = 0.02$) (Figure 2). Media LDH activity was not different between cells exposed to PCB126 or DMSO at that time, but media LDH activity (Figure 2) and ADP/ATP ratio were, in average, reduced by ~50% in cells exposed to hypoxia (main effect of hypoxia $p < 0.001$ for both variables).

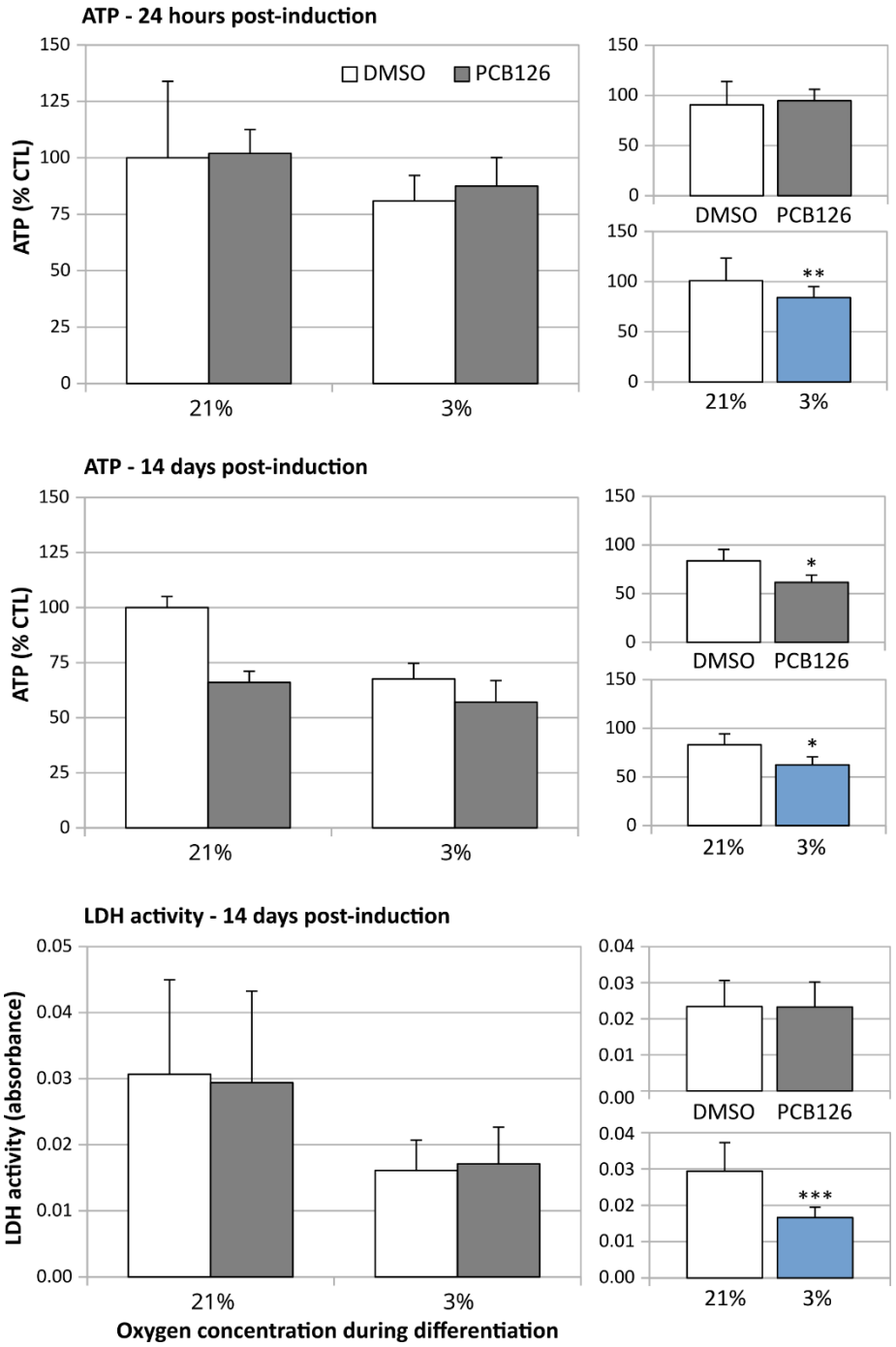


Figure 2. Effect of 10 µM PCB126 and hypoxia on cellular ATP content and media LDH activity. Top panels: Cell ATP content relative to control condition (DMSO, normoxia) 24 h following induction. Mid panels: Cell ATP content relative to control condition after 14 days of differentiation. Bottom panels: Media LDH activity measured after 14 days of differentiation. Main panels (left-hand side) illustrate the complete model and minor panels on the right-hand side summarize the separate main effects of PCB126 (upper minor panel) and hypoxia (lower minor panel). Main effect of PCB126 or hypoxia at * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$. Results are expressed as mean ± SEM of three separate experiments for each treatment group.

2. Gene Expression

VEGFA expression was measured as an indicator of the induction of HIF signaling by hypoxia. VEGFA expression was increased two-fold after 14 days of hypoxia (main effect of hypoxia $p < 0.001$) (**Figure 3**) and this effect was not altered by PCB126 exposure during proliferation. In contrast, there was a significant PCB126 × hypoxia interaction ($p < 0.001$) for CYP1A1 expression, a gene reflecting the induction of AhR signalling by PCB126. Specifically, following the initial PCB126 exposure, CYP1A1 expression was significantly increased after 14 days of PCB126-free normoxia or hypoxia treatment, this effect being greater upon normoxia (100-fold vs. 3-fold) (**Figure 3**). Adipose specific genes were affected differently by hypoxia and PCB126 pre-exposure. PPARγ and adiponectin expression were significantly suppressed under hypoxia by 5- and 100-fold, respectively (main effect of hypoxia $p < 0.001$ in both cases) (**Figure 4**). PPARγ and adiponectin transcript levels were unaffected by PCB126 pre-exposure. In contrast, leptin expression was significantly increased by hypoxia, but was lowered by pre-differentiation PCB126 exposure. The leptin-lowering effect of PCB126 was significantly attenuated by hypoxia (PCB126 x hypoxia interaction $p = 0.003$) (**Figure 4**).

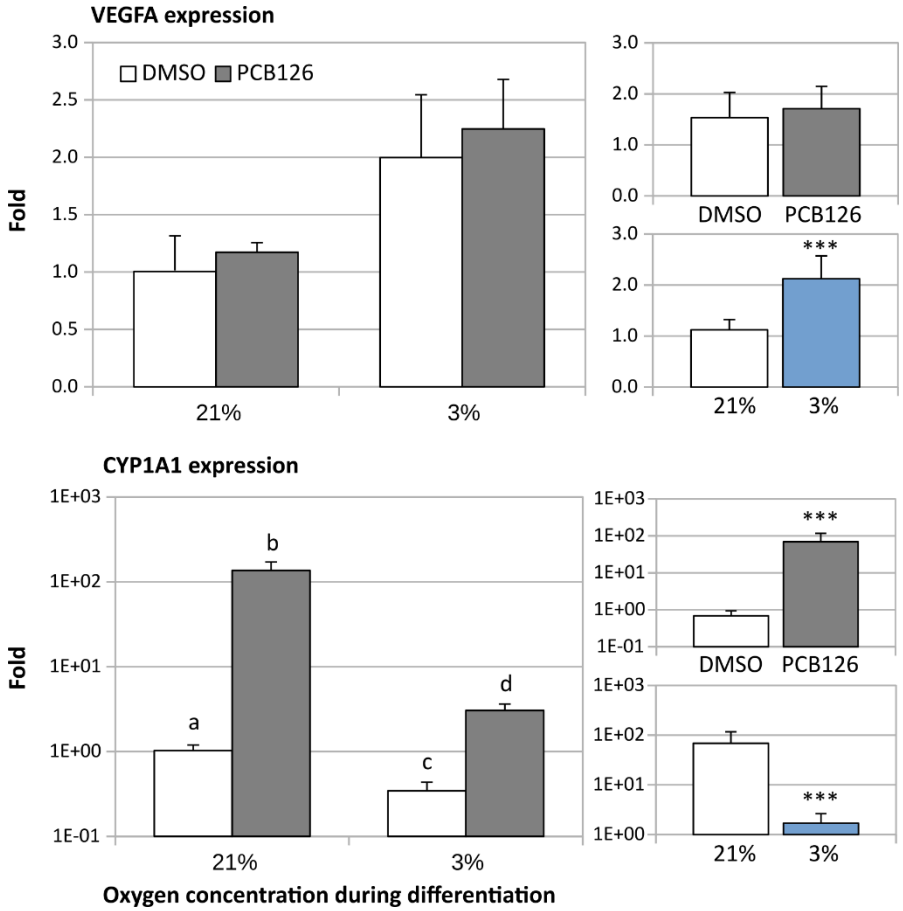


Figure 3. Effect of PCB126 and hypoxia on VEGFA (top panels) and CYP1A1 (bottom panels) expression in human adipocytes. Preadipocytes were treated with DMSO (vector) or 10 µM PCB126 for 3 days (pre-differentiation exposure), then PCB126 was removed from media and differentiation was induced. At this point, cells were subjected to 21 or 3% O_2 for 14 days. VEGFA and CYP1A1 gene expression was measured using RT-qPCR. Main panels (left-hand side) illustrate the complete model and minor panels on the right-hand side summarize the separate main effects of PCB126 (upper minor panel) and hypoxia (lower minor panel). Bars not sharing a common letter are statistically different at $p < 0.001$. Main effect of PCB126 or hypoxia at *** $p < 0.001$. Results are expressed as mean ± SEM of three separate experiments for each treatment group.

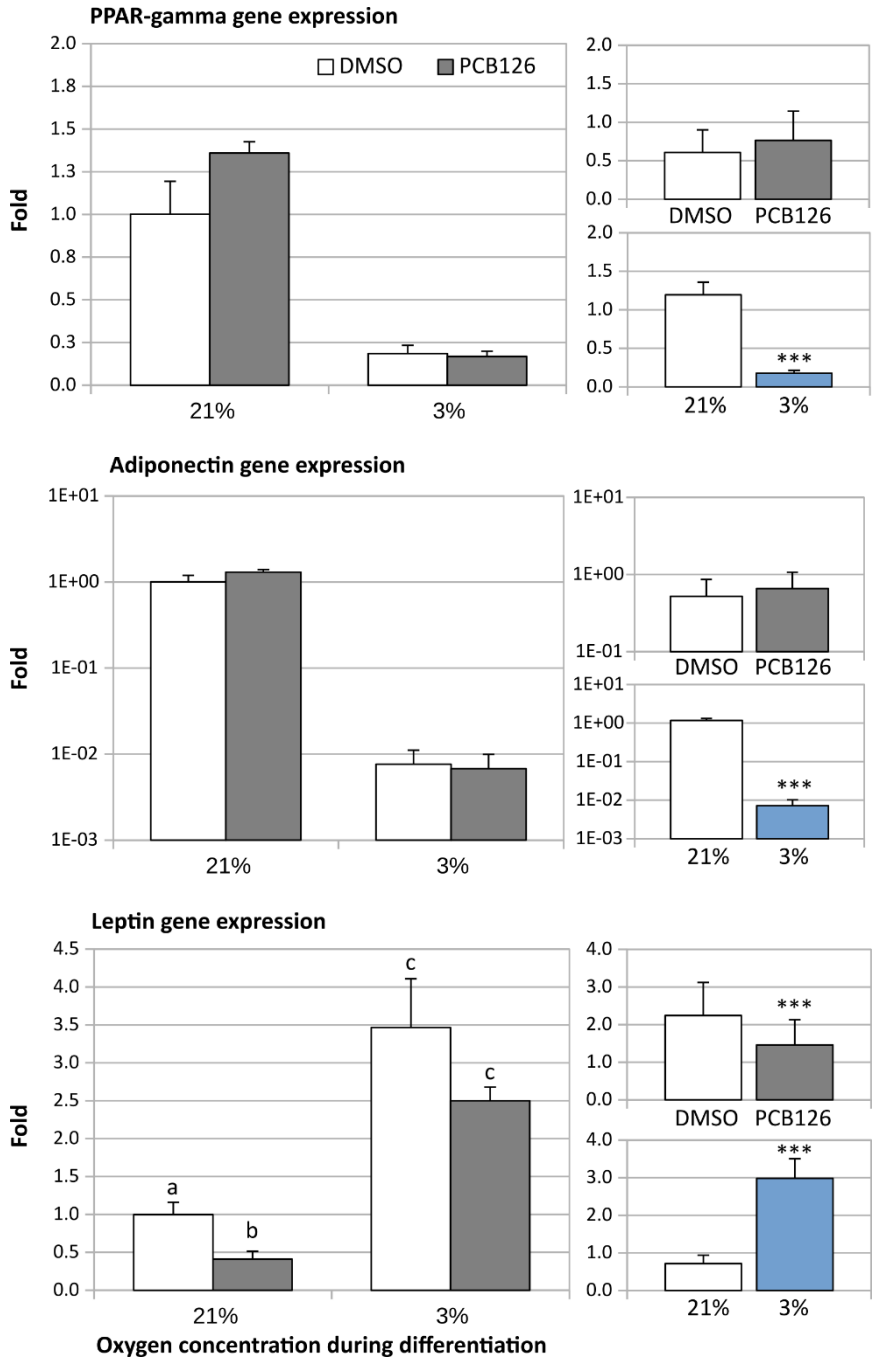


Figure 4. Effect of PCB126 and hypoxia on PPARγ (top panels), adiponectin (middle panels) and leptin (bottom panels) expression in human adipocytes. Preadipocytes were treated with DMSO (vector) or 10 µM PCB126 for 3 days (pre-differentiation exposure), then PCB126 was removed from media and differentiation was induced. At this point, cells were subjected to 21 or 3% O_2 for 14 days. Gene expression of PPARγ, adiponectin and leptin was measured using RT-qPCR. Main panels (left-hand side) illustrate the complete model and minor panels on the right-hand side summarize the separate main effects of PCB126 (upper minor panel) and hypoxia (lower minor panel). Bars not sharing a common letter are statistically different at $p < 0.001$. Main effect of PCB126 or hypoxia at *** $p < 0.001$. Results are expressed as mean ± SEM of three separate experiments for each treatment group.

The expression of pro-inflammatory adipokine IL-6 was slightly but significantly increased by both PCB126 exposure during proliferation and hypoxia exposure during differentiation (p = 0.009 and p = 0.006, respectively) (**Figure 5**). IL-8 expression, on the other hand, was significantly increased by PCB126 exposure only in cells subsequently exposed to hypoxia during differentiation (PCB126 × hypoxia interaction p = 0.013) (**Figure 5**).

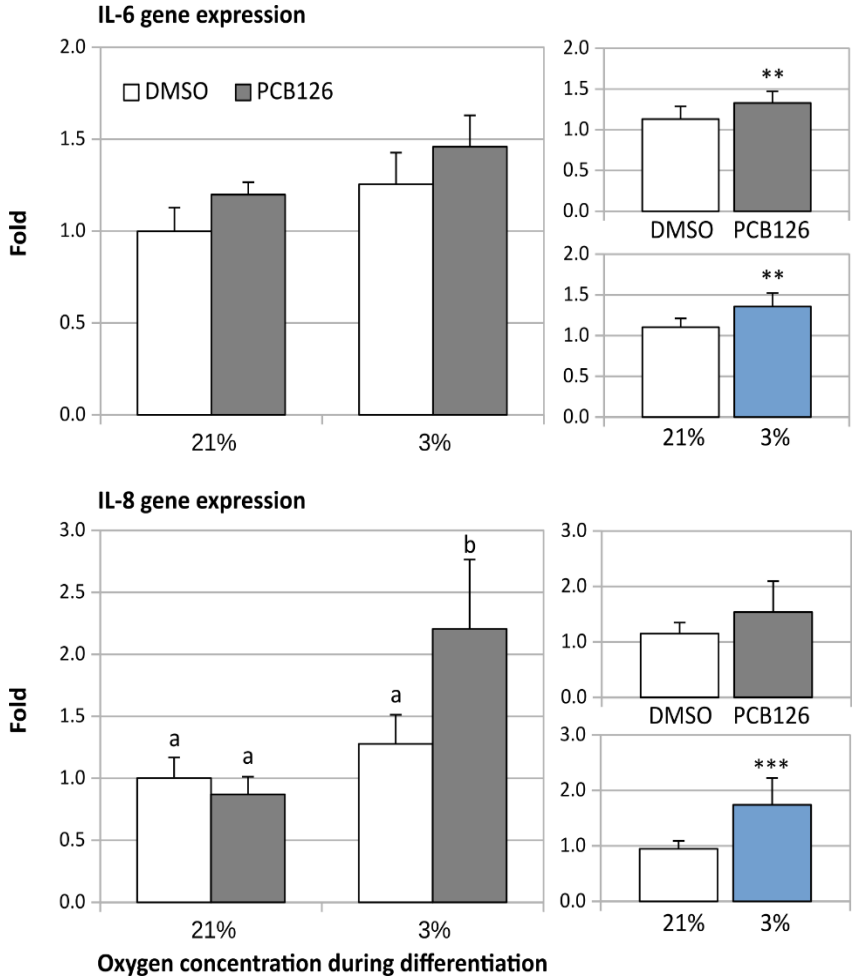


Figure 5. Effect of PCB126 and hypoxia on Il-6 (top panels) and IL-8 (bottom panels) expression in human adipocytes. Preadipocytes were treated with DMSO (vector) or 10 µM PCB126 for 3 days (pre-differentiation exposure), then PCB126 was removed from media and differentiation was induced. At this point, cells were subjected to 21 or 3% O_2 for 14 days. Gene expression of IL-6 and IL-8 was measured using RT-qPCR. Main panels (left-hand side) illustrate the complete model and minor panels on the right-hand side summarize the separate main effects of PCB126 (upper minor panel) and hypoxia (lower minor panel). Bars not sharing a common letter are statistically different at $p < 0.05$. Main effect of PCB126 or hypoxia at ** $p < 0.01$ or *** $p < 0.001$. Results are expressed as mean ± SEM of three separate experiments for each treatment group.

3. TG Content and Glucose Uptake

Adipocyte TG content decreased by more than 80% following 14 days of hypoxia exposure (main effect of hypoxia $p < 0.001$) (**Figure 6**). Interestingly, when TG content was normalized to ATP content, hypoxia was still associated with a reduction in TG content, but PCB126 exposure during proliferation tended to be associated with greater TG content, especially under normoxia (PCB126 × hypoxia interaction $p = 0.06$) (**Figure 6**).

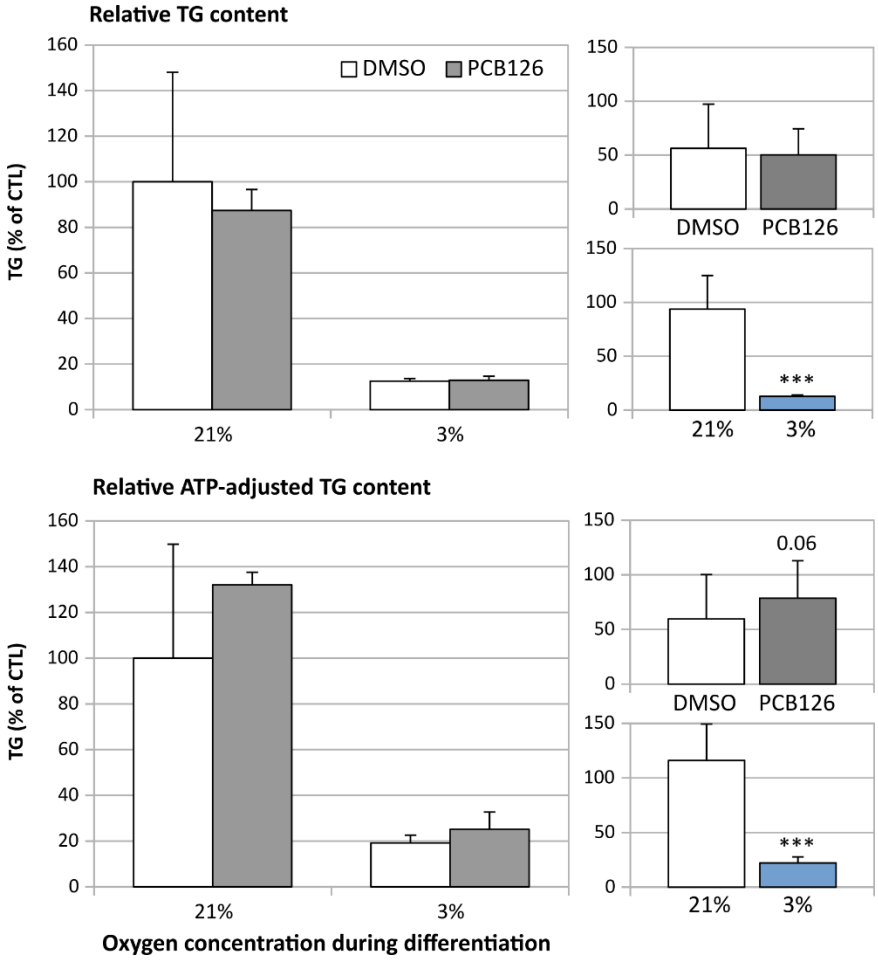


Figure 6. Relative TG content of human differentiated preadipocytes exposed to 10 µM PCB126 or DMSO for 3 days during proliferation followed by differentiation for 14 days under either 21% or 3% O_2. Top panels illustrate relative TG content compared to DMSO/normoxia. Bottom panels illustrate TG content normalized to ATP, relative to DMSO/normoxia. Main panels (left-hand side) illustrate the complete model and minor panels on the right-hand side summarize the separate main effects of PCB126 (upper minor panel) and hypoxia (lower minor panel). Main effect of PCB126 or hypoxia at *** $p < 0.001$. Results are expressed as mean ± SEM of three separate experiments for each treatment group.

After 24 h of differentiation, basal glucose uptake (either unadjusted or adjusted for ATP content) was increased by ~50% under hypoxia (main effect of hypoxia $p < 0.001$), regardless of

PCB126 exposure during proliferation (**Figure 7**). After 14 days of differentiation, basal glucose uptake unadjusted for ATP was still increased in response to hypoxia but was significantly reduced in cells exposed to PCB126 during proliferation (main effects of hypoxia and PCB126 both $p < 0.001$) (**Figure 8**). However, when glucose uptake was adjusted for cell ATP content, only the effect of hypoxia remained significant (main effect of hypoxia $p < 0.001$, main effect of PCB126 $p = 0.610$) (**Figure 8**). Glucose uptake stimulation by insulin was only assessed at day 14 and was virtually completely abolished by differentiation under hypoxia while being unaffected by PCB126 exposure during proliferation (main effect of hypoxia $p < 0.001$) (**Figure 9**). The expression of glucose transporters GLUT1, the main transporter responsible for basal glucose uptake, and GLUT4, responsible for the increase in glucose transport in response to insulin, were consistent with the ATP-adjusted glucose uptake rates. PCB126 had no effect on either GLUT1 or GLUT4 expression, while hypoxia induced a two-fold increase in GLUT1 expression and virtually abolished GLUT4 expression (main effect of hypoxia for both gene $p < 0.001$) (**Figure 10**).

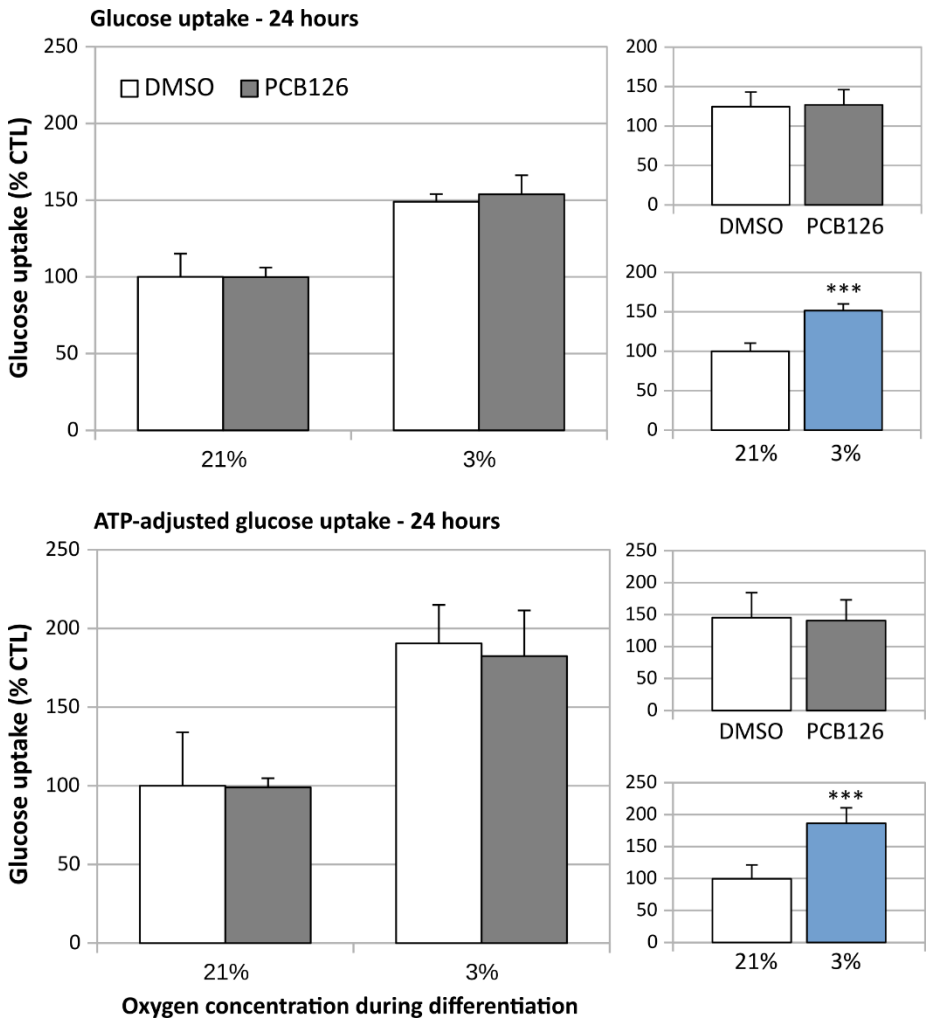


Figure 7. Early effect of PCB126 and hypoxia on basal glucose uptake in human adipocytes. Preadipocytes were treated with DMSO (vector) or 10 µM PCB126 for 3 days (pre-differentiation exposure), then PCB126 was removed from media and differentiation was induced. At this point, cells were subjected to 21 or 3% O_2 for 24 h, after which glucose uptake was assessed. Main panels (left-hand side) illustrate the complete model and minor panels on the right-hand side summarize the separate main effects of PCB126 (upper minor panel) and hypoxia (lower minor panel). Main effect of PCB126 or hypoxia at *** $p < 0.001$. Results are expressed as mean ± SEM of three separate experiments for each treatment group.

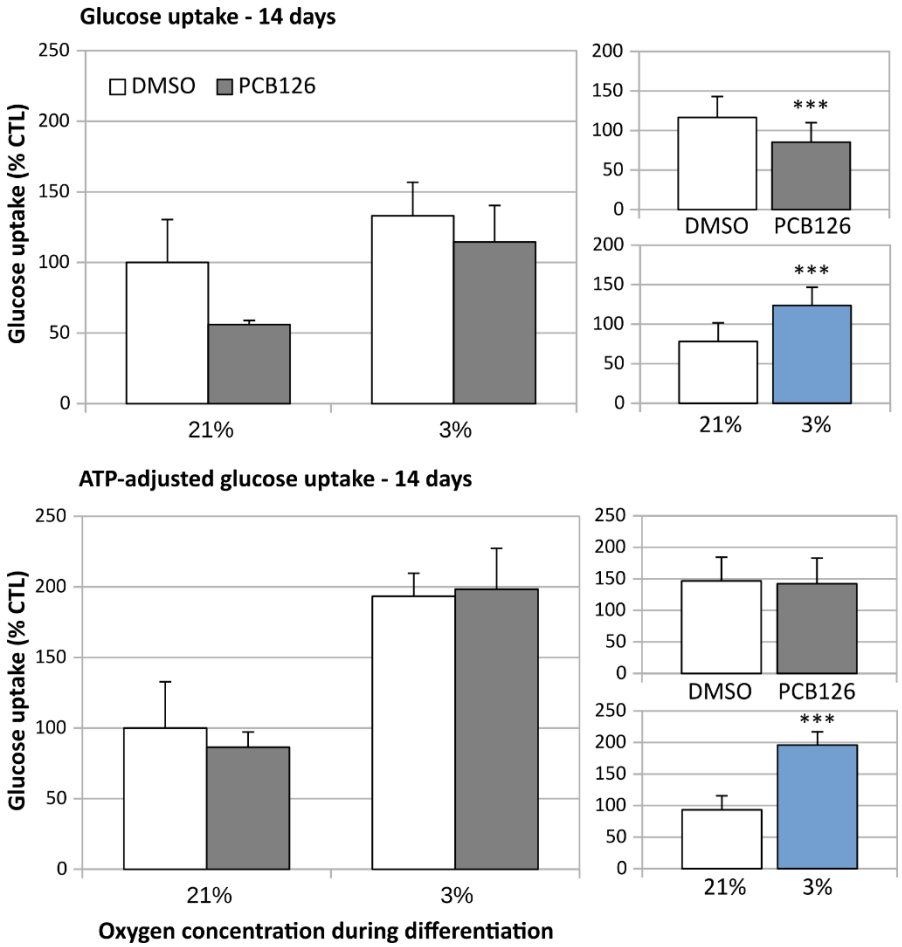


Figure 8. Effect of PCB126 and hypoxia on basal glucose uptake in human differentiated preadipocytes on day 14. Preadipocytes were treated with DMSO (vector) or 10 µM PCB126 for 3 days (pre-differentiation exposure), then PCB126 was removed from media and differentiation was induced. At this point, cells were subjected to 21 or 3% O_2 for 14 days. Glucose uptake was assessed at the end of the 14-day differentiation period. Main panels (left-hand side) illustrate the complete model and minor panels on the right-hand side summarize the separate main effects of PCB126 (upper minor panel) and hypoxia (lower minor panel). Main effect of PCB126 or hypoxia at *** $p < 0.001$. Results are expressed as mean ± SEM of three separate experiments for each treatment group.

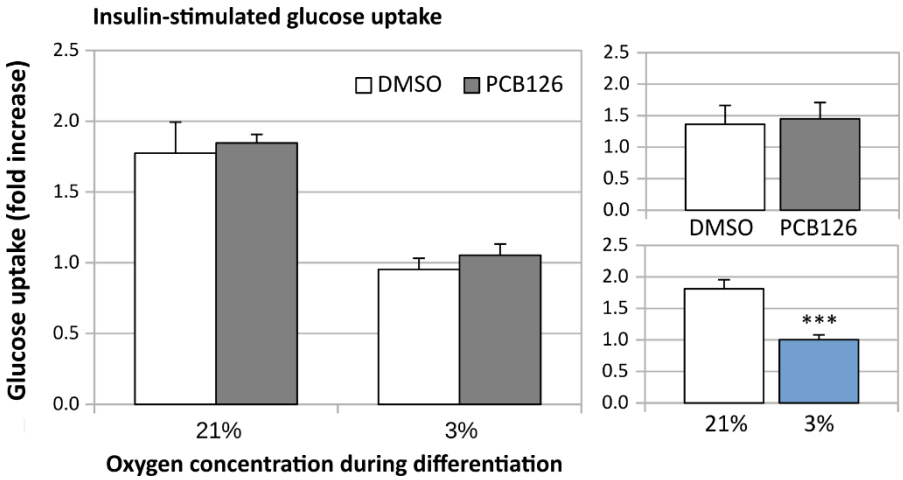


Figure 9. Effect of PCB126 and hypoxia on insulin-stimulated glucose uptake in human adipocytes. Preadipocytes were treated with DMSO (vector) or 10 µM PCB126 for 3 days (pre-differentiation exposure), then PCB126 was removed from media and differentiation was induced. At this point, cells were subjected to 21 or 3% O_2 for 14 days and measurement was done on day 14 post induction. Main panels (left-hand side) illustrate the complete model and minor panels on the right-hand side summarize the separate main effects of PCB126 (upper minor panel) and hypoxia (lower minor panel). Main effect of PCB126 or hypoxia at *** $p < 0.001$. Results are expressed as mean ± SEM of three separate experiments for each treatment group.

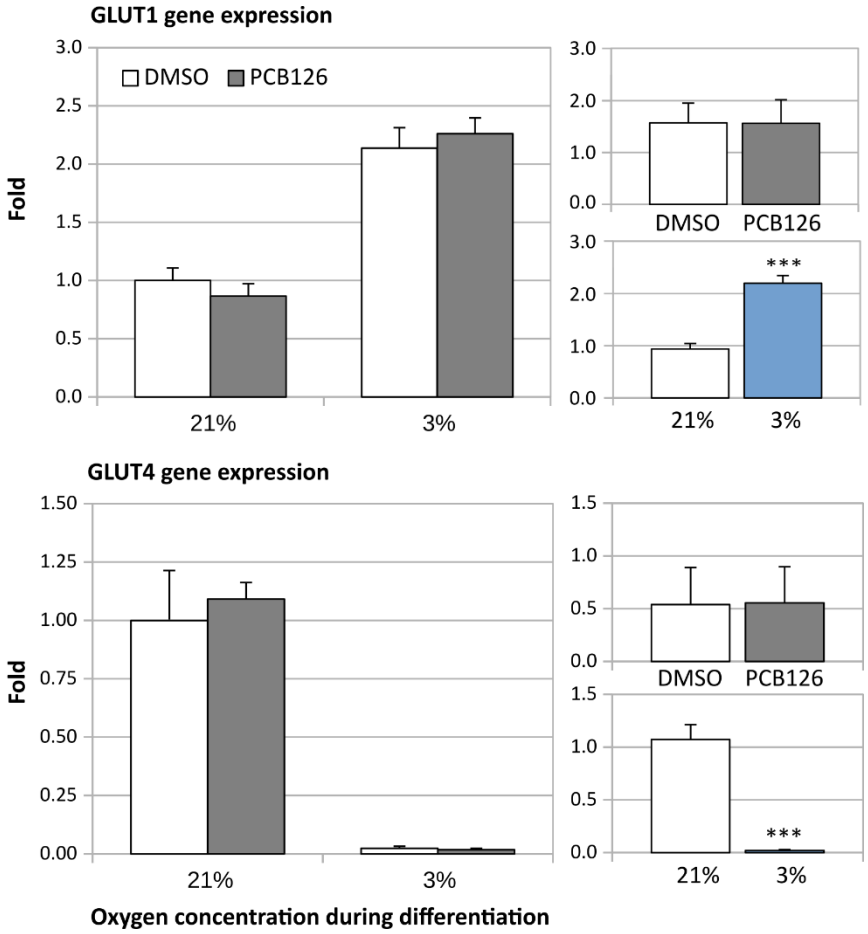


Figure 10. Effect of PCB126 and hypoxia on GLUT1 (top panel) and GLUT4 (bottom panel) expression in human adipocytes. Preadipocytes were treated with DMSO (vector) or 10 µM PCB126 for 3 days (pre-differentiation exposure), then PCB126 was removed from media and differentiation was induced. At this point, cells were subjected to 21 or 3% O_2 for 14 days. Main panels (left-hand side) illustrate the complete model and minor panels on the right-hand side summarize the separate main effects of PCB126 (upper minor panel) and hypoxia (lower minor panel). Main effect of PCB126 or hypoxia at *** $p < 0.001$. Results are expressed as mean ± SEM of three separate experiments for each treatment group.

DISCUSSION

A growing body of evidence shows that hypoxia and POP accumulation in adipose tissue, two features observed when adipose tissue mass excessively expands, cause endocrine and metabolic alterations (Y. Lee et al., 2017; Merrill et al., 2013; Nadal et al., 2017; Netzer et al., 2015; Shan et al., 2020; Trayhurn, 2013). Several studies suggested that AhR activation through exposure to chemical insults such as TCDD or PCBs induces inflammation in adipose tissue (Arsenescu et al., 2008; Baker et al., 2015; M. J. Kim et al., 2012; J. H. Lee et al., 2010), and impairs adipocyte differentiation (Barouki et al., 2015; Loiola et al., 2016; Merrill et al., 2013). However, these studies were conducted almost exclusively in guineapigs and rodents. Also, when the effects of organic pollutants are assessed in vitro, exposure is almost always conducted throughout or at the end of the differentiation phase. However, in the case of the murine 3T3-L1 adipogenic line, the inhibition of adipocyte differentiation occurs exclusively when cells are exposed to TCDD before induction of differentiation (C. Chen et al., 1997; Phillips et al., 1995). Work on a recently developed human adipogenic immortalized cell line (NPAD cells) indicated that PCB126 exposure during proliferation also impairs differentiation in these adipocyte precursors (Gadupudi et al., 2015). Nonetheless, this effect was not easily reproduced in human mesenchymal stem cells using standard culture conditions (Behan-Bush et al., 2023). Since the effect of early dioxin-like organic pollutants exposure on the differentiation capacity of adipocyte precursor seems highly variable among cell lines, we investigated whether PCB126 exposure prevents the differentiation of human subcutaneous preadipocytes. Given our recent observation indicating that hypoxia strongly inhibits dioxin signaling, we also sought to examine how chronic hypoxia following early PCB126 exposure could affect adipocyte differentiation. Our results show that pre-differentiation exposure to PCB126 had no significant effect on adipocyte differentiation and glucose uptake in hSA. Instead, after 14 days of differentiation, the main effect of early PCB126 exposure was a minor decrease in cellular ATP content and significant modulation in the expression of adipokines and adipose specific genes. Conversely, differentiation under continuous hypoxia strongly suppressed adipocyte differentiation, profoundly altered the expression of adipokines and adipose-specific genes and exacerbated the PCB126-induced inflammatory response.

1. Effects of PCB126 Exposure on Human Preadipocytes under Normoxia

CYP1A1 is a membrane-bounded hemoproteins part of the cytochrome P450 (CYP) enzyme family that plays a key role in the detoxification of xenobiotics (Manikandan & Nagini, 2018; Zanger & Schwab, 2013). CYP1A1 induction is mostly transcriptional, and this mainly occurs by the AhR mechanism (Manikandan & Nagini, 2018; Zanger & Schwab, 2013). We and others have previously shown that CYP1A1 expression was significantly induced by PCBs exposure in hSA (Amine et al., 2022; Ellero et al., 2010; Gadupudi et al., 2015). In the current study, we found that CYP1A1 expression is still significantly induced (100-fold) in mature adipocyte obtained from preadipocytes that were exposed to PCB126 14 days earlier. The persistence of high CYP1A1 transcript levels in mature adipocytes following PCB126 exposure during preadipocyte proliferation has already been reported in NPAD cells (Gadupudi et al., 2015). Together, the induction of CYP1A1 expression indicates that human preadipocytes are sensitive to xenobiotics compounds that activate the AhR signaling pathway.

In the present study, pre-differentiation exposure to PCB126 had no obvious consequence on adipocyte differentiation under normoxia, as demonstrated by the absence of alteration in PPARγ and adiponectin expressions as well as in TG content. This finding differs from the previous study, showing that exposure of NPAD cells to PCB126 resulted in significant reduction in their ability to fully differentiate into adipocytes (Gadupudi et al., 2015). This divergence could be attributed to several factors. First, in both studies, cells were exposed to PCB126 until confluence was reached, but this amounted to 3 days in the present study, compared to 6 days in the study of Gadupudi *et al.* (2015). Since PCBs have been shown to have different long term deleterious effects, such as DNA adducts formation, a longer incubation in the presence of PCB126 may have altered the cell cycle and the ability of NPAD cells to differentiate (Dungen et al., 2017; D. Liu et al., 2015). Second, the examination of the data reported by Gadupudi *et al.* suggests that NPAD cells seem to proliferate in a significant manner after confluence is reached (Gadupudi et al., 2015). NPAD cells could, therefore, be resistant to enter quiescence, which would be consistent with the seemingly low differentiation rates (25% in control conditions) that are concomitantly reported (Gadupudi et al., 2015). This, in turn, suggests that NPAD cells may be natively less efficient at differentiating into adipocyte, making them, perhaps, more sensitive to disrupting factors such as dioxin-like PCBs. Also, a more recent report by the same research group shows that treating human

adipose mesenchymal/stromal cells in a similar fashion with Aroclor, a POP mixture containing PCB126 among others, does not impair their differentiation into adipocytes unless fetal bovine serum is almost completely removed from the culture media (Behan-Bush et al., 2023). This latter observation further supports the fact that different cell lines respond differently to POP exposure, which makes our observation that human subcutaneous preadipocytes behave differently from NPAD or 3T3-L1 cells less surprising.

Importantly, the inhibitory effect of PCB126 on NPAD differentiation reported by Gourronc *et al.* was accompanied by an inflammatory response characterized by a strong upregulation of IL-8 expression (Gourronc et al., 2018). We and others showed previously that acute exposure to PCB126 increases the production of IL-8 in differentiated human adipocytes (Amine et al., 2022) and human multipotent adipose-derived stem cells (M. J. Kim et al., 2012). However, in the present study, the PCB126-normoxia sequence did not affect IL-8 expression, but instead led to a slight but significant increase in IL-6 expression, as previously observed in human monocytes or endothelial cells by others (D. Liu et al., 2015; C. Wang et al., 2019). While it cannot be excluded that IL-8 expression may have been transiently increased at any time point during proliferation or differentiation, this eventuality had no apparent impact on the capacity of human subcutaneous preadipocyte to differentiate into adipocytes under normoxic conditions.

Interestingly, pre-differentiation exposure to PCB126 significantly decreased leptin expression under normoxia. In our previous work (Amine et al., 2022), differentiated adipocytes acutely exposed to PCB126 for 48 h had no significant change in leptin secretion. Lower serum leptin levels were reported in the Yusho victims highly exposed to PCBs and polychlorinated dibenzofurans (PCDF) (Uchi et al., 2013), but this effect was not observed in rats exposed to 50 g/kg of TCDD (Lindén et al., 2005). How dioxin-like pollutants affect leptin secretion is currently poorly understood. Nonetheless, the current study provides preliminary evidence that human preadipocyte exposed to PCB126 appears to yield mature adipocytes that produce less leptin. Given the strong and positive association with leptin levels and body adiposity (Considine et al., 1996; Maffei et al., 1995), whether dioxin exposure during adipose tissue development could, in the long term, alter leptin levels and modulate the regulation of energy balance in humans remains to be explored.

Basal glucose uptake unadjusted for ATP was not affected by PCB126 after 24 h of differentiation but was significantly lower after 14 days under normoxia. The absolute glucose uptake under insulin stimulation was also lower in cells that were exposed to PCB126); however, when expressed as fold increase relative to basal glucose uptake, the insulin response appeared not affected by PCB126 exposure. In vivo and in vitro studies have linked dioxin and dioxin-like PCBs to impaired glucose uptake and glucose intolerance, but these studies were all conducted in guineapigs, rodents or rodent cell lines (Baker et al., 2015; Enan et al., 1992; Loiola et al., 2016; Olsen et al., 1994), which may behave differently in response to dioxin and other organic pollutants. The present study, therefore, suggests that preadipocyte exposure to PCB126 may produce populations of mature adipocytes displaying global reduced glucose uptake.

It is important to note that cells' ATP content, although initially unaltered in cells exposed to PCB126, was significantly reduced 14 days post exposure. Interestingly, when glucose uptake on day 14 is normalized to ATP content, the reduction in glucose uptake induced by PCB126 disappears. The reason behind the decrease in cellular ATP is not obvious since neither media LDH activity nor ADP/ATP ratio provide evidence of decreased cell viability in response to PCB126 exposure. It is, however, interesting to note that ATP correction makes glucose uptake measurements fall in line with the observation that GLUT1 and GLUT4 expressions (the main basal and insulin-stimulated glucose transporters) are not altered by PCB126. We believe that the concordance of these two relative measurements (glucose uptake relative to ATP and GLUTs relative to B-actin) is not trivial and, combined with the rest of our observations, suggest that human preadipocytes exposed to PCB126 produce normally differentiating adipocytes, but with a limited capacity for glucose uptake that could be attributable to lower ATP levels. Our study unfortunately provides no explanation for the delayed decrease in cellular ATP following PCB126 exposure other than that it does not appear to be apoptosis- or necrosis-related, based on media LDH activity and the ADP/ATP ratio. Dioxin-like pollutants have been shown to produce DNA adducts in the long term, which can lead to cell cycle arrest (Nebert & Dalton, 2006). It is, therefore, possible that PCB126/Ahr may induce slight impairments in human preadipocyte proliferation that may not produce noticeable effects after a few doubling times (*i.e.*, 4 days) but may become significant after several (*i.e.*, after 17 days) (Gadupudi et al., 2015). However, the visual examination of the cultures (**Figure 1**) did not support a substantial difference in cell number

between cultures initially exposed to DMSO and those exposed to PCB126. Another possible explanation for the decrease in ATP levels observed in cells exposed to PCB126 before differentiation could reside in the fact that POPs have been shown to impair mitochondrial oxygen consumption and ATP production, especially in the longer term, in vivo and in vitro (Chehade et al., 2022; S. A. Kim et al., 2022; Rainey et al., 2017). While further investigations are required to explain the observed delayed decrease in ATP levels 14 days following PCB126 exposure, media LDH activity, cellular ADP/ATP ratio and visual examination strongly suggest that the cells' viability was not compromised. The apparent relationship between the depleted ATP content and glucose uptake warrants further investigations.

2. Effects of Hypoxia on Differentiating Human Preadipocytes

The chronic exposure to hypoxia during differentiation was used to simulate the environment of hypoxic patches that may develop during the excessive development of adipose tissue mass (Trayhurn, 2013). VEGFA expression was increased significantly in adipocytes differentiated under hypoxia, indicating activation of the hypoxia response pathway persisting after 14 days. Cellular ATP levels were lower after both 24 h and 14 days of hypoxia. However, both media LDH activity and the ADP/ATP ratio were also lower after 14 days of hypoxia, suggesting that depressed ATP levels may not reflect increased apoptosis or necrosis, but could instead indicate a state of cellular hypometabolism and alterations in adenosine metabolism that has been proposed to occur under hypoxia (C. Gu & Jun, 2018). A rapid decrease in cellular ATP unrelated to increase in ADP or cellular apoptosis/necrosis has also been reported in primary culture rat hepatocytes exposed to hypoxia (K. Hayashi et al., 1997). Such a mechanism could be critical for cell survival under hypoxia by preventing oxidative stress that could arise from ADP-stimulation of oxidative phosphorylation under hypoxic conditions. To the best of our knowledge, this effect of hypoxia on adipocyte ATP content has not been reported before, and further studies will be required to better understand the mechanisms underlying this effect and how it may impact adipocyte function.

In the present study, hypoxia caused a clear visual reduction in adipocyte TG droplets size. This is consistent with our previous observation on human preadipocytes differentiated under chronic severe hypoxia and could be explained by a strong reduction in the expression of key adipogenic genes such as carbohydrate response element binding protein (ChREBP), acetyl-CoA

carboxylase (ACC), fatty acid synthase (FASN), diacylglycerol acyl transferase 1 and 2 (DGAT1 and DGAT2) (Mahat et al., 2021). In the present study, the reduction in lipid droplets was accompanied by a strong repression of PPARγ expression, indicating that continuous hypoxia greatly represses adipocyte differentiation. These results are in line with other studies that utilized prolonged hypoxia exposure after differentiation induction (Hashimoto et al., 2013; Weiszenstein et al., 2016). The decrease in adiponectin expression and increased in leptin and Il-6 expression under hypoxia are also consistent with previous observations (Amine et al., 2022; Chaiban et al., 2008). These alterations in lipid accumulation and adipokine profile further confirm that chronic severe hypoxia results in an impairment of differentiation in cultured human adipocytes.

Glucose uptake also behaved as expected in response to hypoxia exposure. After both 24 h and 14 days of exposure, a strong increase in basal glucose uptake was observed, either adjusted or not for ATP content concomitant to an increase in GLUT1 expression, as observed by Wood *et al.* (2007). However, the fact that glucose uptake remained significantly increased under hypoxia following ATP-adjustment is interesting and may reflect a shift in substrate partitioning toward glucose utilization and greater contribution of anaerobic glycolysis to energy metabolism under hypoxia. Finally, also as expected, hypoxia completely prevented insulin from promoting glucose uptake in adipocyte, which was accompanied by a strong repression of GLUT4 expression, as previously observed (A Grosfeld et al., 2002).

3. Interaction between Hypoxia and PCB126 on Differentiating Preadipocytes

We previously showed that hypoxia represses but does not completely abolish the induction of the dioxin response in human differentiated adipocytes acutely exposed to PCB126 (Amine et al., 2022). Consistently, in the present study, most of the effects of PCB126 exposure, notably the decrease in leptin expression, were similarly obscured by 14 days of hypoxia. However, much like in our previous report, the induction of CYP1A1 by early exposure to PCB126 was strongly attenuated but still significantly present after 14 days of hypoxia. Together, these results confirm that hypoxia strongly interferes with, but does not completely abolish, the xenobiotic sensing pathway in adipocytes so that the combination of both factors could lead to additive effects. As such, IL-8 expression was not increased by PCB126 exposure followed by normoxia but was significantly increased following the PCB126-hypoxia sequence (PCB126 × hypoxia $p = 0.013$).

PCB126 is increasingly well known to induce IL-8 expression in the short term (hours to days) in adipocyte cell lines, although this effect could be only transient (Gadupudi et al., 2015; Gourronc et al., 2018). Hypoxia, on the other hand, has been shown to acutely induce IL-8 expression in several cell types in culture (lungs, ovarian cancer, macrophages) but, to the best of our knowledge, this has not been reported in human or rodent adipocytes. The present study seems to confirm that, even in the long term, hypoxia does not induce IL-8 expression in hSA. Therefore, a likely explanation for the increase in IL-8 expression by the PCB126-hypoxia combination is that continuous hypoxia may have allowed PCB126 to persistently stimulate IL-8 expression after several days of PCB-free culture. One way hypoxia could have achieved this is by preventing adipogenesis and impairing the "safe" deposit of remaining lipophilic PCB126 in lipid droplets, the major sink for adipose dioxin since adipocyte do not express CYP1A2, which is the protein responsible for the preferential sequestration of dioxins in organs such as the liver. Another contributing factor could be that remaining PCB126 molecules may have been more slowly metabolized and detoxified due to the strong repression of CYP1A1 expression by hypoxia. However, experimental data demonstrated that human cytochrome P450 does not metabolize PCB126 (Yamazaki et al., 2011), which, if accurate, would make this later explanation unlikely. Regardless, while the interaction between acute and long-term hypoxia requires confirmation and the mechanisms responsible for this interaction need to be elucidated, the present observation suggest that hypoxia could potentiate the inflammatory response induce by dioxin-like pollutants. This is especially relevant knowing that (1) humans are exposed to dioxin-like pollutants at early age (breast milk), (2) that the proportion of individuals living with obesity, a condition increasingly recognized as inducing adipose tissue hypoxia, continue to rise worldwide and (3) that adipose tissue inflammation is increasingly recognized as one of the initiating factors in the metabolic cascade leading to obesity-related complications.

4. Study Limitations

Some limitations and strengths of this study warrants discussion. First, the use of a single POP precludes extrapolation of our results to the more complex mixture of POPs preadipocytes/adipocytes are exposed to during their lifetime. Also, the single concentration of PCB126 (10 µM) used to maximize its effect on preadipocytes differentiation, despite being well tolerated by hSA, is likely falling off the physiologically range of total intracellular PCB levels

observed in human adipose tissue, as reported by Bourez *et al.* (2012). Another limitation lies in the use of a single fixed hypoxia condition consisting of 3% O_2. Despite the fact that 3% O_2 is well recognized as being hypoxic in adipose tissue (Trayhurn, 2013), this organ is likely exposed to fluctuations in O_2 tensions of varying magnitudes, which may exert different effects on adipocyte metabolism. Hence, caution is warranted when extrapolating findings from our in vitro study to in vivo conditions.

CONCLUSIONS

The present study suggests that human subcutaneous preadipocytes exposed to PCB126 differentiate normally into adipocytes, but the resulting adipocyte population is characterized by lower ATP content, absolute glucose uptake and leptin expression. Exposure to hypoxia, on the other hand, exerts a much greater repression on adipocyte differentiation and lipid storage compared to PCB126 exposure during proliferation. Finally, our observations suggest that hypoxia and early PCB126 exposure have additive effects in terms of inflammatory response, a key feature of the development of chronic diseases such type 2 diabetes and cardiovascular diseases.

Author Contributions

Z.E.A., J.-F.M. and P.I. conceived and designed the experiments. Z.E.A. and J.-F.M. performed the experiments. Z.E.A., J.-F.M. and P.I. analyzed the data. Z.E.A., J.-F.M. and P.I. interpreted the data. Graphical abstract by J.-F.M. All authors edited, revised, and approved the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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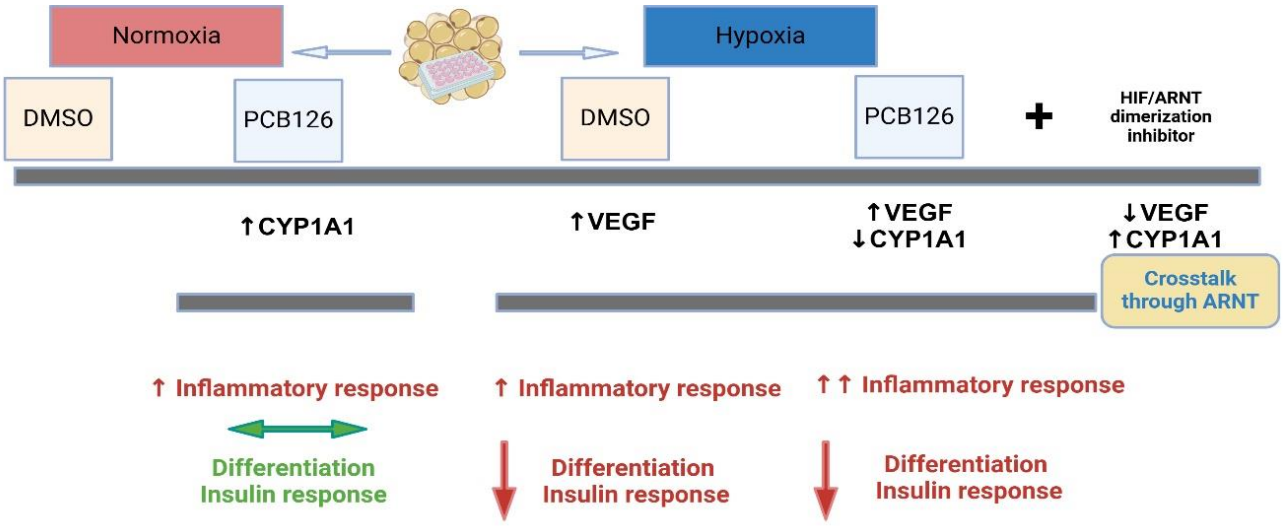
CHAPTER 4 - THESIS DISCUSSION

4.1 SUMMARY OF FINDINGS

Obesity, a prevalent metabolic disorder in the Western Hemisphere, arises from complex interactions among genetic, environmental, and behavioral factors (Blüher, 2019). Emerging evidence suggests a potential link between exposure to environmental chemicals, such as dioxins and DLCs, and obesity, particularly during critical developmental periods (Cock et al., 2014; E. Dirinck et al., 2011; Ghosh et al., 2014; Nadal et al., 2017). Lipophilic dioxin-like PCBs, notably PCB126, accumulate primarily in adipose tissue, posing significant health risks to both humans and wildlife (Jackson et al., 2017; Regnier & Sargis, 2014). The accumulation of these PCBs is influenced by triglyceride content in adipocytes, a feature associated with hypertrophied adipocytes and a pro-inflammatory hypoxic environment (Bourez et al., 2013; Trayhurn, 2014). Since the xenobiotic and the hypoxia sensing pathways share a common factor, this thesis aims to explore their crosstalk in hSA, especially in the context of PCB126/hypoxia exposure. The hypothesis suggests that such an interconnection may exacerbate adverse effects on adipocyte metabolism and differentiation when co-exposed to both stressors.

Graphical abstract of the main findings



The first article provides a comprehensive narrative review emphasizing the role of white adipose tissue (WAT) beyond energy storage, particularly its involvement in obesity-related metabolic disorders driven by hypoxia. Oxygen levels in WAT are crucial for its physiological functions, with hypoxia implicated in adipose tissue expansion, inflammation, and altered cellular responses. Short-term hypoxia triggers adaptive responses in adipose tissue, including angiogenesis and inflammation regulation, whereas chronic hypoxia in obesity leads to adverse alterations, such as oxidative stress and chronic inflammation (Foti & Brunetti, 2017; Trayhurn, 2014; R. Wang et al., 2022). With excessive adiposity, chronic hypoxia influences adipocyte metabolism, affecting glucose uptake, insulin sensitivity, lipid mobilization, and adipogenesis (Lempesis et al., 2020; Trayhurn, 2013). Additionally, adipose tissue serves as a reservoir for lipophilic contaminants, exposing adipocytes to PCBs like PCB126, which may exacerbate hypoxia-induced effects.

The subsequent original study (Article 2) examined the interaction between AhR and hypoxia sensing pathways on hSA simultaneously exposed to PCB126 and hypoxia. The key finding of this first study was that hypoxia inhibits PCB126-induced CYP1A1 expression through, at least partly, ARNT in a dose-dependent manner. The results also show that this crosstalk is unidirectional, with hypoxia signaling prevailing over xenobiotic signaling at every dose of PCB126 tested. However, PCB126 increased inflammatory cytokine secretion independently of hypoxia, potentially compromising adaptive responses to hypoxia.

Further investigations (Article 3) explored the impact of prolonged hypoxia and PCB126 pre-differentiation exposure on adipocyte metabolism and differentiation. Prolonged hypoxia reduces adipocyte differentiation and insulin sensitivity, exacerbating inflammation and impairing lipid storage. It is widely acknowledged that dysfunction in adipose tissue plays a central role in the development of comorbidities and chronic diseases associated with obesity, including type 2 diabetes mellitus and CVD. In obesity, hypertrophic adipose tissue exhibits a diminished capacity to buffer lipids effectively. This leads to the accumulation of lipids in crucial metabolic organs such as the liver and skeletal muscle, a phenomenon known as ectopic fat storage, which is closely linked to insulin resistance. In line with this paradigm, our results showed that prolonged hypoxia significantly reduced the ability of human subcutaneous preadipocytes to differentiate, with significant decrease in lipid accumulation and downregulation of key adipocyte genes such as

PPARγ and adiponectin while increasing leptin expression. Also, hypoxia increased glucose uptake and GLUT1 expression but decreased GLUT4 expression which resulted in an abolished insulin response (*i.e.*, decreased insulin sensitivity). Regarding the expression of pro-inflammatory adipokine, PCB126 and hypoxia exerted additive effects on adipose tissue inflammation. IL-6 was slightly increased by both PCB126 and hypoxia, and IL-8 expression was significantly increased following the PCB126-hypoxia sequence.

In article 3, the effects of hypoxia and PCB126 on mature adipocytes ATP content were also investigated. ATP serves as a fundamental metabolite that is crucial for cell growth and intracellular concentration of ATP is dictated by factors such as its utilization and production. The ATP biosynthesis rate is associated with cell growth and metabolic activities. *In vitro* studies that examined ATP dynamics in association to metabolism, cell growth, and cell cycle of single-cell found that lower growth rate is correlated with higher ATP fluctuations (W.-H. Lin & Jacobs-Wagner, 2022). In our experiment, lower ATP levels along with lower glucose uptake were observed in 14-days full differentiated adipocytes that had been exposed to PCB126 during proliferation. The reduced ATP content could be due to a slightly lower cell growth rate applied over many cell cycles, resulting in a reduced mass of the adipocyte population. If that was the case, then PCB126 bioaccumulating in adipose tissue could affect adipocytes proliferation rate with prolonged exposure and compromise normal adipose tissue expansion. In a study that examined the association between chronic exposure to low-dose POPs and mitochondrial function, assessed by oxygen consumption rate (*i.e.* estimate the ability of mitochondria to synthesize ATP and perform its functions), the results from both human and *in vitro* studies align, indicating a potential harmful impact of chronic exposure to low-dose POPs on humans, particularly by disrupting mitochondrial oxidative phosphorylation. This dysfunction in mitochondria might be the key mechanism underlying recent evidence linking low-dose POPs to several chronic human diseases (S. A. Kim et al., 2022). More investigation is needed to explore the effect of PCB126 exposure on mitochondrial function of adipocytes.

Collectively, these findings underscore the complex interplay between environmental pollutants like PCB126, hypoxia, and adipocyte metabolism, highlighting the importance of studying these interactions in human adipocytes. Such research contributes to our understanding

of mechanisms possibly contributing to obesity-related metabolic disorders, including the development of chronic diseases like type 2 diabetes and cardiovascular diseases.

4.2 LIMITATIONS

The two original studies of the thesis are the first to assess the crosstalk of PCB126 and hypoxia sensing pathways in human adipocytes. These studies were performed *in vitro* and on homogenous cell populations constituted of a single cell type. Because of this, extrapolation to the *in vivo* condition requires caution since adipose tissue is composed of adipocytes at several developmental stages with various TG content beside other cell types such as macrophages and endothelial cells, which may respond differently to PCB126 and even possibly affect how adipocytes may respond to PCB126. Also, the use of more physiologically relevant models, especially those that more closely mimic the human body (such as 3D co-culture models) could help investigate the effects of POPs exposure on adipose tissue in a more realistic way; since the traditional 2D models we used can at the same time significantly overestimate or underestimate the effects of pollutants exposure depending on the endpoint being monitored (Marques dos Santos et al., 2022). Moreover, the cell line, the donor, and the window of exposure all affect the responses to PCBs (Leijs et al., 2019; Marques dos Santos et al., 2022; Morin et al., 2022). This highlights the importance to use human adipocytes from well characterized donors and possibly investigate several exposure modalities at different stages of the cell cycle to better appreciate and evaluate the global effect of PCB126 on human adipose tissue.

Another limitation to note relates to the use of one single dioxin-like PCB congener, PCB126, which is the dioxin-like PCB with the highest toxicity secondary to TCDD. Although PCB126 is the main contributor to dioxin-like PCBs contamination (Chirollo et al., 2018), humans are exposed environmentally to a cocktail of toxins, which may exerts different effects than compared to PCB126 alone. Also, PCB126 could potentially accumulate at important levels at any differentiation stage *in vitro* proportionally to TG content (Bourez et al., 2012), making them subject to potential metabolic perturbations. Nevertheless, the various doses of PCB126 used in our studies (ranging from 0.1, 1, 10 μM) were all above normal physiological doses (Alawi & Al-Tameemi, 2016; W. H. Park et al., 2017). The use of physiologically irrelevant concentrations complicates the inference of our observation to adipose tissue.

Our results showed that pre-differentiation and post-differentiation exposure to PCB126 sustained a high degree of AhR activation (increase in CYP1A1 expression). AhR activation is currently accepted to be responsible for most of the effects exerted by PCB126 (Morin et al., 2022). Although our *in vitro* study showed no significant effect of PCB126 on adipocytes metabolism or differentiation; the estimated biological half-life of most chlorinated congeners is relatively long (11.5 years) (Ritter et al., 2011) and could thus alter signaling pathways *in vivo* over a longer period of time exerting more deleterious effect with prolonged activation of AhR (Leijs et al., 2019).

With regards to hypoxia, we used various doses of oxygen levels: 21%, 10% and 3% O_2, which are commonly used in *in vitro* studies to respectively simulate control normoxic conditions as well as mild and severe hypoxia. Also, we used acute and prolonged hypoxia exposure. The physiological oxygen levels observed in adipose tissue ranges between 6-8 % with small differences between individuals with normal or high obesity (Trayhurn, 2013). The modality of hypoxia exposure used in our *in vitro* studies may not reflect PO_2 levels experienced by adipocytes in developing hypoxic patches nor the duration of hypoxic events that may occur in adipose tissue of individuals living with obesity. These technical limitations, as was the case for our results regarding the effects of PCB126 on adipocyte metabolism, prevent the direct transfer of our observations regarding the effects of hypoxia on human adipocytes in culture to actual human adipose tissue.

PERSPECTIVES

The narrative review and two experimental studies presented in this thesis aimed to elucidate the interplay between hypoxia and environmental pollutants in human adipocytes, which serve as the primary reservoir for lipophilic pollutants. In the first study, our focus was on investigating the role of ARNT, the factor expected to be responsible for this interaction. We observed that hypoxia, mediated by HIF/ARNT, superseded the dioxin-induced pathway by sequestering ARNT and subsequently reducing CYP1A1 expression. Despite this, the inflammatory effect induced by dioxin was still detected and evident through increased IL-8 secretion, suggesting the presence of an inflammatory signaling pathway that may still involve AhR but would be ARNT-independent. Further studies are therefore warranted to determine the

nature of the signaling pathway that triggers inflammation in response to PCB126 independently of ARNT.

Beyond AhR possible role in inflammation, other particularities of AhR and the xenobiotic response deserve further examination. AhR is known to modulate gene expression by interacting with various partners and is involved in a multitude of physiological and pathological processes, including immunity, hormone signaling, and cell growth (Larigot et al., 2018; Puga et al., 2009). For example, studies in rodents exposed perinatally to dioxin-like PCB126 have shown increased visceral fat pad weights observed in adulthood, with the effect being more pronounced in females.(Rashid et al., 2013). Such responses were not observed with non-dioxin-like PCB153 (van Esterik et al., 2015). These observations suggest that the xenobiotic response, AhR signaling specifically, may be affected by sexual metabolic dimorphisms. As such, AhR has been proposed to interact with the estrogen receptor (ER) signaling pathways in energy homeostasis regulation (Le Magueresse-Battistoni, 2020). Since estrogen plays a protective role in adipose tissue, modulating adipocyte function and mitigating inflammation and fibrosis, investigating the interaction between dioxins and estrogen signaling in adipose tissue during obesity presents a compelling avenue for further research (Bjune et al., 2022).

Interestingly, Cyclin-dependent kinase inhibitors (p27kip1), a known AhR/ARNT target gene, is involved in G1-phase passage (Vondráček et al., 2005) where AhR can regulate cell proliferation and survival either negatively or positively (Jiuheng Yin et al., 2016). Notably, p27kip1 was also found to be upregulated by hypoxia via an ARNT dependent pathway (G. Wang et al., 2003). Investigating the physiological function of AhR in cell proliferation adipocytes and its interaction with hypoxia would be of interest. Moreover, the inhibitory role of hypoxia exposure on AhR signaling was confirmed in our results. Hence, more specific AhR target genes and additional readouts for metabolic endpoints warrant investigation, considering that AhR has been shown to play an important role in lipid metabolism, glucose metabolism and cell proliferation (S. Kim et al., 2009; Lu et al., 2020; Sayed et al., 2022).

Another aspect related to PCB exposure that will deserve further investigation is related to the consequences of long term CYP450 enzymes induction. Coplanar PCBs, such as PCB126 which possess a high affinity for AhR, induce CYP1A1 which may be a source of oxidative stress

(J. Liu et al., 2020). Although dioxin-like PCBs have been shown to induce CYP450 enzymes to onset detoxification, the role of PCB metabolites (products of PCB detoxification) in inducing oxidative stress has received less attention. Recent studies are starting to address the role of CYP450 enzymes in generating metabolites that may contribute to oxidative stress development (Gao et al., 2023; J. Liu et al., 2020; Shan et al., 2020). In our second study, we observed no significant effect of PCB126 on adipocyte differentiation but noted a significant prolonged increase in CYP1A1 expression following pre-differentiation exposure to PCB126. This sustained activation of CYP1A1 may have deleterious effects on adipocytes, leading to decreased metabolism and glucose uptake due to mitochondrial dysfunction on the long term (S. A. Kim et al., 2022). Such effect was difficult to examine *in vitro* due to the relatively short lifespan of cells in culture. Investigating the effects of CYP1A1 activation in adipocytes in the long-course presents an intriguing area for further exploration especially that CYP1A1 enzyme can contribute to, but also protect against oxidative stress (Stading et al., 2020).

The impact of POPs on adipocyte metabolism and differentiation as endocrine disrupter has been extensively studied. Another important aspect of adipose tissue physiology that may warrant further research related to the effects of environmental pollutants and hypoxia concerns the well-known fact that different adipose depots, namely the subcutaneous and the visceral depots, display very different metabolic behaviors (Badimon & Cubedo, 2017). Therefore, POPs may not exert the same effects on visceral adipose tissue as they do on subcutaneous adipose tissue. Visceral adipose tissue, due to its proximity to internal organs and rapid expansion, is metabolically active and releases higher amounts of pro-inflammatory cytokines compared to subcutaneous adipose tissue (Tchkonia et al., 2013). This increased metabolic activity makes visceral adipose tissue more prone to inflammation when developing areas of hypoxia compared to subcutaneous adipose tissue (Ali et al., 2021; O'Rourke et al., 2013; Petrangeli et al., 2016). Moreover, inflammation, insulin resistance, and the release of pro-inflammatory cytokines are associated with visceral adipose tissue and contribute to the complications of obesity (Badimon & Cubedo, 2017; Lessard & Tchernof, 2012; Villaret et al., 2010). However, the specific effects of POPs on visceral adipose tissue compared to subcutaneous adipose tissue may vary, suggesting that visceral adipose tissue may be more susceptible to certain POPs induced metabolic disturbances and inflammatory responses. Therefore, it would be valuable for experimental studies

to investigate whether the effects of POPs/hypoxia on adipose tissue metabolism and inflammation differ between visceral and subcutaneous adipose tissue. This could provide insights into the potential differential impact of such stressors on different adipose tissue depots and their contributions to metabolic dysfunction and obesity-related complications.

In our two studies, we employed various doses of a single PCB congener, PCB126. However, it's important to note that individual's exposure to different concentrations of PCBs can vary. Arsenescu *et al.*, (2008) showed that different concentrations of PCB77 exerted different responses in 3T3-L1, where lower concentration of PCB77 caused increase in differentiation while higher ones inhibited cell differentiation. Alpha naphthoflavone (α-NF), an AhR inhibitor, abolished the adipocyte differentiation induction effect of low concentration of PCB77 which indicates the involvement of AhR pathway. However, low doses of TCDD (TEF=1) did not cause significant increase in 3T3-L1 differentiation (Arsenescu et al., 2008), suggesting different responses in the cells to various dioxin congeners and doses with different affinity for AhR and toxicity strength. A model receptor action study on PLHC-1 cells showed that only 1–2% of the AHR molecules need to be occupied by high-intrinsic efficacy agonists, like PCB77, PCB126 and TCDD, for 50% CYP1A1 induction (Hestermann et al., 2000). Since PCB77 and PCB126 are main constituents of Aroclor mixture 1254 which is a high contaminant of old school buildings in the USA (Behan-Bush et al., 2023) and different doses of same or different congeners show different responses, it would be relevant to study the effect of different doses of these dioxin-like PCBs on human adipocyte ability to differentiate and on their adipokine profile.

A final idea that could be explored in future research relates to the profound effect prolonged hypoxia has on adipocyte differentiation and metabolism under post-induction exposure. It would be of great interest to investigate if pre-differentiation exposure to hypoxia exerts the same effect on hSA, since pre-adipocytes are also exposed to hypoxia in the context of obesity.

4.3 THESIS CONCLUSIONS

The main goal of this thesis was to gain new insight into the crosstalk of AhR and HIF-1α induced pathway and their regulation of cellular responses in the context of PCB126 exposure in

different oxygen environments. Eventually, our studies have given insight into the interconnection of the AhR and hypoxia signaling pathways through ARNT, providing valuable information on general mechanisms of action of PCB126 on both human adipocytes and preadipocytes with different time window of exposure. We also gained a better understanding of the inhibitory mechanism of hypoxia on PCB126 induced AhR regulated responses (CYP1A1). Furthermore, long-term exposure to hypoxia exhibited a more deleterious effect on adipocyte ability to differentiate and its inflammatory profile while AhR induced CYP1A1 activation showed no significant effect on adipocyte differentiation. Nevertheless, pre-differentiation exposure to PCB126 had inflammatory effect and it was additive under prolonged hypoxia despite significant inhibition of CYP1A1, suggesting the involvement of AhR/ARNT independent mechanisms in inflammation induction. Together, these results contributed to further our understanding of the detrimental biological effects of PCB126 on hSA and the interconnection of the dioxin and hypoxia signaling pathways in the context of obesity.

CHAPTER 5 - REFERENCES

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