Exploring the Independent and Combined Effects of Persistent Organic Pollutants and Hypoxia on Human Adipocyte Functions

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Abstract

Persistent organic pollutants (POPs) and adipose tissue hypoxia have been shown to independently affect adipocyte functions. The goals of this study were to (1) determine the effect of PCB-77, PCB-153, and DDE on the differentiation of human preadipocytes, and (2) investigate the cross-talk between PCB-77 and hypoxia in differentiated human adipocytes. First, human preadipocytes were exposed to PCB-77, PCB-153, or DDE during the entire 14-day differentiation period. We found no effect of low POP levels on lipid accumulation. Second, differentiated human adipocytes were exposed to a combination of PCB-77 and hypoxia. We demonstrated gene-specific cross-talk between PCB-77 and hypoxia, showing an additive effect of PCB-77 on VEGF, MCP-1, and adiponectin, as well as an inhibition of PCB-77-induced expression of CYP1A1 by hypoxia. This work has expanded our understanding of the role of POPs and hypoxia in differentiated human adipocytes.

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Chapter 1

Introduction

1.1 General overview

Obesity, or excess adiposity, is a risk factor for serious metabolic problems including type 2 diabetes (Kahn, Hull, & Utzschneider, 2006), cardiovascular diseases (Van Gaal, Mertens, & De Block, 2006), and some types of cancer (van Kruijsdijk, van der Wall, & Visseren, 2009), among numerous others. As the prevalence of obesity continues to rise, increasing attention is being directed towards understanding the role of adipose tissue, particularly since adipose tissue is now recognized not only as a site for energy storage but also as a dynamic endocrine organ (Poulos, Hausman, & Hausman, 2010). This newly acquired knowledge has expanded our understanding of the association between excess fat mass and related metabolic disorders. More specifically, research has shown that high levels of adiposity are often associated with a state of chronic, low-grade inflammation (Maury & Brichard, 2010). In fact, inflammation is frequently identified as a main factor linking obesity with other metabolic disorders, although the factors leading to the inflammatory response itself still remain unclear. This thesis focuses on two potential factors, persistent organic pollutants (POPs) and adipose tissue hypoxia, involved in the inflammatory response of human adipocytes.

The first factor is a group of man-made organic compounds called persistent organic pollutants. POPs contaminate our environment and contribute to adverse health effects in living organism. We are exposed to POPs mainly through the ingestion of food products, at which point they bioaccumulate in lipid-rich sites in the body due to their lipophilic properties (Liem,

Fürst, & Rappe, 2000; Schafer & Kegley, 2002). As the largest site for lipid storage in the body, adipose tissue consequently acts as the main reservoir for POP storage (Müllerová & Kopecký, 2007). Many POPs are known endocrine disruptors but the health risks associated with chronic POP exposure are still unclear. It has been suggested that POPs may act as obesogens, meaning that exposure would increase the differentiation of human preadipocytes to form new adipocytes (Tang-Péronard, Andersen, Jensen, & Heitmann, 2011). To date, there are no studies examining the effect of POPs on preadipocyte differentiation using a human cell model. In addition, it is unclear how differentiated human adipocytes storing POPs contribute to inflammation in the obese state.

The second factor is adipose tissue hypoxia, a feature more recently identified and suggested to characterize obese adipose tissue. Local hypoxic patches are proposed to develop in adipose tissue due to inadequate oxygen diffusion to hypertrophic adipocytes (Trayhurn & Wood, 2004). As a response to oxygen deprivation, hypoxic adipocytes are thought to release greater levels of inflammatory adipokines. Rodent studies clearly show reduced partial oxygen pressure in obese adipose tissue (Hosogai et al., 2007; Ye et al., 2007), however the existence of adipose tissue hypoxia in humans is still controversial (Goossens et al., 2011; Hodson, Humphreys, Karpe, & Frayn, 2013; Pasarica et al., 2009). Nonetheless, *in vitro* and animal studies support a role for hypoxia in inflammation development during obesity (Wood, Stezhka, & Trayhurn, 2011; Ye et al., 2007).

POPs and hypoxia are potentially harmful for the cell and can therefore activate specific signaling pathways, thus allowing the cell to respond appropriately. More precisely, some cytotoxic responses to POPs are mediated through the aryl hydrocarbon receptor (AhR) (Denison & Nagy, 2003), while hypoxic responses are determined primarily by the hypoxia-inducible

factor-1 alpha (HIF-1α) (Semenza, 2012). Both AhR and HIF-1α are nuclear transcription factors that act by dimerizing with a common sub-unit, the aryl hydrocarbon receptor nuclear translocator (Arnt). As a result of this overlap, we might expect the simultaneous exposure of adipocytes to POPs and hypoxia to elicit a different response than the one observed from exposure to each factor independently. To our knowledge, the cross-talk between the AhR and HIF-1 pathways in human adipocytes has not yet been established. Exploring this cross-talk is the primary objective of this thesis.

This thesis is organized as a series of articles. Following the general introduction, a review article accepted for publication in *Obesity Reviews* is included as the literature review section in the first chapter (see Appendix I for letter of acceptance). The second chapter is a detailed description of the methods used to collect and analyse data. The third chapter is Manuscript 1, which explored the effect of POPs (PCB-77, PCB-153, and DDE) on human preadipocyte differentiation. The fourth chapter is Manuscript 2, which investigated the interaction between a selected POP, PCB-77, and hypoxia in the inflammatory response of differentiated human adipocytes. Finally, the fifth chapter is a general discussion that highlights the main findings, strengths, and limitations of the thesis, as well as areas for future research.

1.2 Abbreviations

AhR – Aryl hydrocarbon receptor

Arnt – Aryl hydrocarbon receptor nuclear translocator

BMI – Body mass index

CYP1A1 – Cytochrome P450, family 1, member A1

DDE – Dichlorodiphenyldichloroethylene

ELISA – Enzyme-linked immunosorbant assay

HIF-1 – Hypoxia inducible factor-1

IL-6 – Inteuleukin-6

MCP-1 – Monocyte chemotactic protein-1

PCB-153 – 2, 2', 4, 4', 5, 5'-Hexachlorobiphenyl

PCB-77 – 3, 3', 4, 4'-Tetrachlorobiphenyl

POP – Persistent organic pollutant

qPCR – Quantitative polymerase chain reaction

RNA – Ribonucleic acid

RT – Reverse transcription

TNF-α – Tumor necrosis factor-alpha

VEGF – Vascular endothelial growth factor

1.3 Literature review – Persistent organic pollutants meet adipose tissue hypoxia: Does cross-talk contribute to inflammation during obesity?

The following manuscript was accepted for publication in the peer-reviewed journal *Obesity Reviews* and conforms to the submission guidelines of this journal.

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Potential conflicts of interest

None to declare

1.1.1 Abstract

Lipophilic persistent organic pollutants (POPs) accumulate in lipid-rich tissues such as human adipose tissue. This is particularly problematic in individuals with an excessive adipose tissue mass, a physiological state that may be additionally characterized by local adipose tissue hypoxia. Hypoxic patches occur when oxygen diffusion is insufficient to reach all the hypertrophic adipocytes in the tissue. POPs and hypoxia independently contribute to the development of adipose tissue-specific and systemic inflammation often associated with obesity. Inflammation is induced by increased pro-inflammatory mediators such as tumor necrosis factor-alpha, interleukin-6, and monocyte chemotactic protein-1, as well as reduced adiponectin release, an anti-inflammatory and insulin-sensitizing adipokine. The aryl hydrocarbon receptor (AhR) mediates the cellular response to some pollutants, while hypoxia responses occur through the oxygen-sensitive transcription factor hypoxia-inducible factor (HIF)-1. There is some overlap between the two cell signaling pathways since both require a common sub-unit called the aryl hydrocarbon receptor nuclear translocator (Arnt). As such, it is unclear how adipocytes respond to simultaneous POP and hypoxia exposure. This brief review explores the independent contribution of POPs and adipose tissue hypoxia as factors underlying the inflammatory response from adipocytes during obesity. It also highlights that the combined effect of POPs and hypoxia through the AhR and HIF-1 signaling pathways remains to be tested.

1.1.2 Obesity and adipose tissue inflammation: The basics, revisited

Obesity has become a leading worldwide health burden by increasing the risk of developing related health disorders such as type 2 diabetes (1), cardiovascular disease (2), and some types of cancer (3), among others. Understanding the link between the excess adipose tissue defining obesity and the development of metabolic diseases has been increased by our expanding knowledge of the dynamic energy storage and endocrine functions of adipose tissue. Specialized cells called adipocytes are responsible for lipid storage and are the major component of adipose tissue in terms of weight, while pre-adipocytes, endothelial cells, and macrophages make up the remaining fraction (4). Through the secretion of adipose-specific protein factors called adipokines, adipocytes play a major role in the regulation of fuel utilization, glucose metabolism, immune function, appetite, and other physiological processes (5). A recent article by Lehr and colleagues has expanded the list of adipokines secreted from human adipocytes to include a total of 263 proteins (6).

A shift towards the secretion of primarily pro-inflammatory adipokines is observed during obesity. In fact, adipokine release is correlated with adipocyte volume, although this association is lost for anti-inflammatory adipokines such as adiponectin and IL-1ra after correcting for cell surface (7). Tumor necrosis factor-alpha (TNF-α) is a major contributor to the inflammatory process and, in addition to its receptor (soluble tumor necrosis factor receptor-2, sTNFR-2), is positively correlated with adipocyte volume (8). TNF-α exposure induces an up-regulation of pro-inflammatory adipokines interleukin-6 (IL-6), TNF-α itself, and monocyte chemotactic protein-1 (MCP-1) gene expression in differentiated adipocytes (9). IL-6 contributes to the development of insulin resistance (10), while MCP-1 promotes the localization and accumulation

of macrophages in adipose tissue (11). There is also evidence that a high number of macrophages can stimulate the conversion of pre-adipocytes to macrophages (12). This further amplifies adipose tissue inflammation since macrophages are the major producers of pro-inflammatory cytokines and chemokines from adipose tissue in the obese state (11).

Inflammation is among a number of factors associated with the growth of adipose tissue during obesity (Figure 1). The normal biological function of inflammation is to act as a protective mechanism. In fact, it has beneficial effects even during obesity that include promoting adipose tissue remodeling to support growth, clearing dead cells, increasing energy expenditure, and limiting food intake (13). However, inflammation can become pathological and lead to metabolic complications when it persists. In the case of obesity, the beneficial effects may be outweighed by the harmful effects like promoting insulin resistance and endothelial dysfunction, which can progress to type 2 diabetes and atherosclerosis, respectively. The underlying factors that trigger chronic inflammatory responses from adipose tissue are still not clear, although potential factors have been identified. The following sections will describe the current knowledge surrounding two emerging factors potentially contributing to inflammation during obesity: persistent organic pollutants (POPs) and adipose tissue hypoxia. The final section will explore the possibility of POP and hypoxia interactions with emphasis on the overlapping cellular signaling pathways.

1.1.3 Inflammation: the role of POPs

Persistent organic pollutants accumulate in human adipose tissue and are correlated with obesity-related health issues

Over the past decades, interest in the role of environmental pollutants on human health has been growing. Under the broad umbrella of environmental pollutants, persistent organic pollutants (POPs) are organic, man-made compounds derived primarily from industrial activities. These synthetic chemicals are or were used as pesticides, plasticizers, flame retardants, and surfactants, among others (14). POPs can be further classified into groups based on chemical structure, namely organochlorine (OC) pesticides, polychlorinated biphenyls (PCBs), dioxins, polybrominated flame retardants (BFRs), and other compounds (15). POP mixtures contaminate the air, soil, and water; however, humans are exposed to POPs primarily through food sources, especially fatty foods of animal origin such as milk, meat, and fish (16, 17). Since POPs are not readily metabolized or excreted, their levels increase with the food chain levels, a process called biomagnification (14). Furthermore, with increasing climate change, POPs that had deposited in the Arctic due to cold temperatures are beginning to revolatilize. In fact, POP levels in Arctic air over the past two decades have increased and continue to rise (18), indicating the relevance of studies focused on POP toxicity even though a large number have since been banned (14).

Adipose tissue, the most important lipid-storage site in the body, acts as a reservoir for a large fraction of these lipophilic contaminants, a process called bioaccumulation. Recently, Bourez and colleagues have shown that PCB bioaccumulation in adipocytes is determined by adipocyte triglyceride content and lipophilicity of the PCB congener (19, 20). Additionally, as mentioned previously POPs are highly resistant to environmental and biological degradation, thus they biomagnify in the food chain (21). Therefore, once stored in the body, POPs remain stable for years and can contribute to chronic adverse health effects. An increasing number of epidemiological studies show an association between POP levels and the prevalence of the metabolic syndrome (22) and some metabolic disorders such as insulin resistance (23), type 2

diabetes (24–31), and cardiovascular diseases (32). Furthermore, Pelletier and colleagues have observed strong positive correlations between plasma organochlorine concentrations and BMI and fat mass, indicating that obese individuals have greater organochlorine levels relative to plasma lipids than lean individuals (33). These results are supported by Kim et al., who similarly demonstrate that the total body burden of POPs relative to fat mass is 2 to 3 times more elevated in obese compared to lean individuals (34). These results are supported by many studies showing that individual and combinations of serum POPs are correlated with different measures of adiposity including BMI, fat mass, and waist circumference (35–40).

Most epidemiological studies employ plasma POP levels in their analyses. This is justified since strong positive correlations between plasma organochlorine concentrations and subcutaneous abdominal and femoral adipose tissue have been observed (33, 34). In other words, adipose tissue POP levels reflect those measured in the plasma. POPs have been shown to modulate in a positive and negative manner the determinants of adipose tissue growth. These determinants are adipocyte hypertrophy (increase in adipocyte size) and hyperplasia (increase in adipocyte number), although hyperplasia is only observed up to 20 years of age in humans (41). Indeed, the insecticide 1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane (p,p'-DDT) has been found to increase adipocyte differentiation in a concentration-dependent manner (42), while organochlorines PCB-77, PCB-153, and DDE increase human pre-adipocyte proliferation *in vitro* (43). Furthermore, the biocide tributyltin enhances lipid accumulation and aP2 expression, a marker of adipocyte differentiation (44), and low PCB-77 concentrations induce adipocyte differentiation in 3T3-L1 adipocytes, as well as body weight gain in mice (45). In contrast, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) exposure has been reported to inhibit adipocyte differentiation (46). Overall, POP toxicity is modulated by adipose tissue (47) and the effect of

POPs is likely dependent on the level of exposure, as well as the mechanism leading to toxic effects.

Aryl hydrocarbon receptor responds to POPs by up-regulating xenobiotic-metabolizing enzymes and plays a role in inflammation

POPs are known endocrine disruptors and can interfere with adipocyte endocrine function. The cellular mechanisms responsible for the toxic effects of POPs have not yet been fully elucidated; however evidence indicates a certain role for the aryl hydrocarbon receptor (AhR). AhR is a ligand-activated transcription factor involved in detoxification and immune response (48). Some POPs are known AhR agonists, namely the most potent 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), which has been assigned a toxic equivalency factor (TEF) of 1. Other congeners acting via the same mechanism are referred to as 'dioxin-like' and are assigned a TEF relative to that of TCDD based on available *in vitro* and *in vivo* studies (49). These include dioxin-like co-planar PCBs such as PCB-77 with an assigned TEF of 0.0001, meaning its effects are less toxic than those of TCDD (17). As depicted in Figure 2a, the dioxin or dioxin-like compounds first enter the cell and bind to AhR. This complex then translocates to the nucleus and dimerizes with a second sub-unit, the aryl hydrocarbon receptor nuclear translocator (Arnt) (50). The newly formed complex identifies and binds to xenobiotic response elements (XRE) and induces the expression of xenobiotic-metabolizing enzymes such as those from the cytochrome P (CYP) family (50). There also exists a non-genomic pathway through which ligand-activated AhR can elicit inflammatory responses, reviewed in detail by Matsumura (51).

There are an increasing number of studies interested in the role of AhR in adipose tissue inflammation. Arsenescu et al. showed that exposure to an AhR antagonist abolished the inflammatory effects of the AhR ligand PCB-77 in murine adipocytes. Furthermore, endothelial cells exposed to co-planar PCB congeners 77, 126, and 169 increased inflammatory signaling pathway NF-κB DNA binding activity, as well as the expression of an adhesion molecule involved in endothelial cell function called vascular cell adhesion molecule 1 (VCAM-1). This could not be observed in *AhR*-KO mice, showing that the increase in VCAM-1 could be dependent on AhR (52). Studies performed in human multipotent adipose-derived stem cells indicate that genes involved in inflammation, immune response, and metabolism were among the most affected following treatment with TCDD, co-planar PCB-126, and/or non-coplanar PCB-153 (53). The introduction of AhR-antagonist α-naphthoflavone reduced the effects of TCDD and PCB-126, both of which are AhR ligands. In the same study, mice treated with TCDD had increased levels of pro-inflammatory adipokines and AhR activated gene expression, which was abolished in *AhR*-KO mice. Furthermore, TCDD-treated mice had greater macrophage infiltration, characteristic of obese adipose tissue (53). The activation of the AhR pathway in adipose tissue in the obese state has not yet been determined in humans *in vivo*; therefore, it is difficult to predict its contribution to inflammation during obesity in humans. Nonetheless, it appears as though POPs act through AhR to stimulate a pro-inflammatory state within adipose tissue.

1.1.4 Inflammation: the link with O_2 tension

Adipose tissue hypoxia: a matter of debate

As illustrated in Figure 1, adipose tissue expansion requires adipocyte hypertrophy, along with a concurrent expansion of vasculature to support the growth process. Trayhurn and colleagues first suggested that, during obesity, the distance that oxygen must diffuse to supply all adipose tissue cells with adequate oxygen can reach up to 140-180μm, which exceeds the diffusion limit of approximately 100μm (54–56) (Figure 3). As a result, hypoxic patches are created within adipose tissue when its expansion is greater than the ability for vasculature development to keep up. Adipose tissue hypoxia has been observed in obese rodents by indirect methods including immunohistochemistry using pimonidazole as an exogenous marker and increased local lactate production as an endogenous marker, as well as adipose tissue hypoperfusion (55). A second study in rodents also observed adipose tissue hypoxia using indirect hypoxia probes and a group of hypoxia-response genes (57). Additionally, they employed direct measurements of interstitial partial oxygen pressure using an oxygen meter, finding a 70% decrease PO_2 in adipose tissue of obese mice (57). In humans, adipose tissue oxygen levels were quantified by the insertion of a polarographic Clark electrode in the subcutaneous adipose tissue of lean and obese individuals. Significantly lower oxygen levels were observed in the obese group compared to those of the lean group (58). In contrast, a separate study by Goossens and colleagues showed no difference in adipose tissue oxygenation in lean and obese individuals using a microdialysis method (59). In fact, the obese group showed adipose tissue hyperoxia, which the authors suggest is due to a lower adipose tissue oxygen consumption (59). Since there is considerable overlap between the pO_2 values for lean and obese subjects in both studies, the presence of adipose tissue hypoxia in humans is still controversial.

Moreover, it has been well documented that adipose tissue blood flow (ATBF) is compromised during obesity. McQuaid and colleagues clearly show a reduced basal level of

ATBF in obese *vs* lean men. Plus, a spike in ATBF is observed after each meal in lean men, while there is no response in abdominally obese men (60). Limited ATBF was proposed to contribute to adipose tissue hypoxia by decreasing oxygen supply to the tissue. Indeed, O_2 delivery to adipose tissue was reduced in obesity. However the authors suggest that the adipose tissue is glycolytic but not hypoxic due to a low consumption of oxygen (61). It is possible that oxygen supply varies during stages of adipose tissue expansion and obesity progression.

HIF-1 mediates cellular responses to hypoxia by regulating metabolism, angiogenesis, and vascular remodeling

Oxygen is highly important for mammalian cell survival, thus multiple processes exist to maintain oxygen homeostasis. At the cellular level, the hypoxia-inducible factor (HIF)-1 acts as the master oxygen sensor and mediates cellular responses to chronic hypoxia (62). The HIF-1 transcription factor is activated upon the heterodimerization of two sub-units, O_2-sensitive HIF-1α and constitutively expressed aryl hydrocarbon receptor nuclear translocator (Arnt or HIF-1β), also required for AhR activation as previously stated. Figure 2b shows that under normoxia, HIF-1α is hydroxylated by prolyl hydroxylase-domain proteins (PHD) leading to its degradation by the proteasome (62, 63). In this process, oxygen acts as the limiting factor for PHD activity (63). Under low oxygen levels, HIF-1α is not hydroxylated and remains stable, allowing it to translocate to the nucleus where it dimerizes with Arnt and binds to hypoxia-response elements (HRE) to promote transcription. HIF-1 is involved in gene expression of over 60 known genes with functions ranging from glucose metabolism, angiogenesis, and cell death (63, 64).

HIF-1α mRNA can be detected in human subcutaneous adipose tissue (scAT) and its levels are increased in obese *vs* lean women (65). Moreover, surgery-induced weight loss leads to

decreased HIF-1α expression in the scAT of the same women. Interestingly, gene expression analyses show that the HIF-1α over-expression occurs in macrophages but not isolated adipocytes (65). Plus, a separate study found no difference in HIF-1α mRNA between obese and lean individuals (66). It seems as though HIF-1α protein and not mRNA may be a more appropriate indicator of hypoxia (67). Even so, there is evidence that other obesity-related factors beside hypoxia can stabilize the HIF-1α protein such as adipogenesis and insulin (68). Nonetheless, HIF-1α expression is also positively correlated with BMI in adipocyte progenitor cells, plus adipocyte progenitor cells show greater proliferation when incubated at 1% O_2 compared to normoxia (20% O_2) (69). As pointed out by the authors Maumus and colleagues, the microenvironment surrounding the progenitor cells has a great impact on their proliferation; this includes the secretion of adipokines by surrounding cells as well as adipose tissue hypoxia.

When faced with a hypoxic microenvironment, classic hypoxic-response genes are activated such as those involved in metabolic processes. For example, glucose uptake is increased due to an HIF-1 mediated up-regulation of GLUT-1 receptors (70, 71). Pyruvate dehydrogenase kinase is up-regulated by HIF-1 and serves to shut down the citric acid cycle by deactivating pyruvate dehydrogenase, the enzyme responsible for converting pyruvate to acetyl CoA (72, 73). Thus, a switch to anaerobic glycolysis occurs and the main ATP source becomes lactate production via lactate dehydrogenase (74).

Another set of genes activated by hypoxia are those involved in angiogenesis (the formation of new blood vessels from pre-existing ones) (75), and vascular remodeling (changes to the blood vessel structure) (76). Vascular endothelial growth factor (VEGF) promotes angiogenesis (70) and matrix metalloproteinases (MMPs) are vascular remodeling proteins (77); both are up-regulated in an attempt to re-structure the vasculature within expanding tissue. Also, studies in

murine adipocytes show an HIF-1-mediated increase in apelin, an adipokine involved in aspects of cardiovascular function including angiogenesis, when exposed to 1% O_2 or chemical HIF-1 activators (78). Overall, hypoxia can have a profound impact on adipose tissue structure and function through HIF-1 activation.

Hypoxia leads to inflammatory responses from adipocytes: *in vitro* and *in vivo* evidence

Hypoxia is a hallmark feature of numerous inflammatory disorders including inflammatory bowel disease (79) and rheumatoid arthritis (80). As mentioned previously, hypoxia may be involved in the development of low-grade inflammation during obesity. *In vitro*, human adipocytes exposed to varying degrees of hypoxia demonstrate a pro-inflammatory state by increasing IL-6 secretion, as well as decreasing anti-inflammatory adiponectin (71). Also, HIF-1 is known to up-regulate leptin gene expression, a pro-inflammatory adipokine with many other functions (81). Inflammatory responses to hypoxia are not limited to cell culture studies. Adipose tissue inflammation and reduced adiponectin levels are observed in mice with obesity-induced adipose tissue hypoxia (57). Adipose tissue hypoxia also initiates other responses that may indirectly lead to inflammation. More specifically, hypoxia has been shown to block insulin-stimulated glucose uptake, decrease fatty acid uptake, and stimulate lipolysis in murine adipocytes and in rodents (82). Furthermore, hypoxia was found to induce cell death through both necrosis and apoptosis in a time-dependent manner (82). Cell death is increased in obese mice and humans, which leads to a pronounced macrophage infiltration. Macrophages mainly surround necrotic adipocytes where they fuse to form giant multinucleated cells that activate inflammatory processes and clean up cell debris and lipid droplets (83). As inflammation itself is known to invoke metabolic and vascular changes, the stimulation of inflammation by hypoxia creates a cycle that promotes disease progression if the inflammatory signal is not attenuated.

Establishing a role for hypoxia and HIF-1 in obesity is a current research challenge. If there are in fact hypoxic patches in adipose tissue, is it enough to activate HIF-1 and consequently lead to gene activation? It seems likely based on the current knowledge, although further investigation is required.

1.1.5 POPs meet hypoxia: 'Arnt' as a common factor

Cellular responses are the product of a network of interacting factors

The microenvironment surrounding a cell is constantly being monitored to ensure appropriate cellular responses. Multiple signals occur at once and activate various signaling pathways within the cells. The resulting responses are often not the result of a single gene or protein, but a complex network regulated to elicit proper responses. Deciphering these networks is crucial in understanding normal biological function and disease states (84). As such, network analysis is becoming an increasingly useful tool in molecular biology of diseases.

Cross-talk exists between AhR and HIF-1α signaling pathways in various *in vitro* and *in vivo* models

Previous sections have provided evidence that POPs and adipose tissue hypoxia could independently contribute to the inflammatory response of adipocytes during obesity. There also exists an overlap between the xenobiotic and hypoxic cellular signaling pathways. Both AhR and HIF-1α are members of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of transcription factors and require Arnt to form an active complex. Taking this into account, we might expect the simultaneous stress of POPs and local hypoxia in adipose tissue to result in different patterns of gene expression than those which would be observed with each stressor

acting alone. Studies using several different cell lines and animal models have concluded that cross-talk between AhR and HIF-1 does occur but the results are not always in agreement. The term 'cross-talk' is often employed but rarely defined. Here, cross-talk refers to one pathway's agonist also affecting a separate pathway because both pathways share molecular targets. Cross-talk between systems has the potential to increase or decrease the response of a single stressor acting on a single pathway (85). To date, we are unaware of any studies regarding the interaction of POPs and hypoxia in inflammation development in adipocytes. It is unknown if AhR ligands affect HIF-1 or if hypoxia affects AhR activation in murine or human adipocytes. This section aims to outline the main findings of available studies regarding AhR and HIF-1 cross-talk.

It was first hypothesized that cross-talk occurs because AhR and HIF-1α compete for a limiting factor, in this case Arnt. In fact, studies in mouse (Hepa-1), rat (H4IIE), and human (HepG2) hepatoma cell lines found that AhR and HIF-1α are not in competition for Arnt, as it was present in excess in the nucleus with only a small fraction of the entire pool being used for gene expression (86). Although it is unclear if all Arnt proteins in this pool are in fact available for dimerization, the rapid and constant degradation of AhR and HIF-1α proteins following the induction of gene expression liberate Arnt for further action.

Some studies have observed an inhibitory effect of hypoxia on the AhR pathway. Studies in fish cells exposed to hypoxia (1% O_2) and an AhR agonist (BaP, PCB-126, or BkF) demonstrate an inhibitory effect of hypoxia on AhR signaling, as measured by an AhR reporter gene. Upon over-expressing Arnt, AhR signaling was restored. There was also a minor effect of AhR agonists on HIF-1 signaling, which was not influenced by Arnt over-expression (87). This suggests a unidirectional cross-talk and may be explained by greater affinity between Arnt and

HIF-1α (87). A separate study identified an additive effect of hypoxia and dioxin on Epo mRNA expression, later on identifying xenobiotic response elements in addition to hypoxia response elements in the Epo promoter region (88). Also, hypoxia was found to decrease AhR-mediated CYP1A1 gene expression, as well as AhR mRNA levels in human pulmonary microvascular endothelial cells (89) and human lung carcinoma cells (90).

In contrast, other studies have found that AhR agonists could interfere with hypoxia signaling. A study in a species of teleost fish found that prior exposure to PCB-77, a known AhR agonist, prevented the hypoxia-induced increase in glycolysis equilibrium enzyme activity, suggesting that exposure to AhR-activating environmental pollutants can affect the tolerance to hypoxia (91). Contrarily, orange-spotted grouper fish showed increased hypoxic response when treated with BaP, an AhR agonist found in cigarette smoke, compared with hypoxia alone, while the AhR pathway remained unaffected by hypoxia (92). Pertaining to mammalian systems, Seifert and colleagues showed that combined TCDD and hypoxia exposure reduced HIF-1α stabilization (93). Also, a mice model with induced hindleg ischemia was used to test the regulation of angiogenesis in the presence of an AhR agonist. AhR-null mice showed increased HIF-1α levels and HIF-1α-Arnt DNA binding activity leading to a greater angiogenic response (VEGF expression) as compared to wild type mice after exposure to BaP (94). Furthermore, mouse hepatoma cells treated with TCDD reduced hypoxia-induced carbonic anhydrase IX (CA IX) induction (95). In all, it is clear that the cross-talk responses between xenobiotic and hypoxic exposure are inconsistent between studies.

Experimental limitations prevent the application of cross-talk findings to human adipocytes

To date, many cross-talk studies have been conducted using fish models. However, due to fundamental differences existing between fish and mammals (87), the results of these studies cannot be extrapolated to human adipocytes. Even within mammalian cell models, differing responses are observed. A study conducted in human breast carcinoma and human hepatocyte cell lines showed differences in AhR responsiveness leading to varied ability of hypoxia to interfere with AhR signaling, highlighting the importance of choosing the appropriate cell/animal model (93). AhR: HIF-1α: Arnt ratios may differ between species or cell lines and can impact cross-talk (96). As pointed out in many studies, both the AhR and HIF-1α transcription factors share not only a main sub-unit (Arnt) but also many co-activators (87, 88, 94). Their involvement in the cross-talk process is currently undetermined but these co-factors may significantly impact gene regulation during POP and hypoxic stress. Additionally, the regulatory regions of AhR- or HIF-1-induced genes has been found to contribute to cross-talk (97, 98). Other factors possibly explaining the different results obtained between studies include the level or administration technique of hypoxia, the AhR agonist used, and the use of a reporter gene versus an endogenous gene (87).

1.1.6 Implications and Perspective

In summary, it is clear that many factors contribute to the development of inflammation during adipose tissue expansion. The studies mentioned here point to some form of cross-talk between POP- and hypoxia-initiated inflammatory signaling pathways. However, a large diversity of cellular and animal models has been employed and this interaction has yet to be tested with human adipocytes. It is difficult to predict the inflammatory response of adipocytes

to a combination of POPs and hypoxia: Will there be an inhibitory effect? An additive or synergistic effect? Will these interactions be mediated through AhR and HIF-1 pathways? Additionally, this brings up many questions regarding human physiology: Are individuals with a greater POP burden more or less likely to develop and/or respond to hypoxia within adipose tissue? Can the measure of POP burden and adipose tissue oxygenation predict inflammation and other metabolic risks? Many outstanding questions exist and require further study. Understanding these types of interactions can allow us to better predict the response to multiple stressors and identify pertinent biomarkers to assess the severity of inflammation within adipose tissue in individuals with obesity. It is noteworthy to mention that responses to POPs and hypoxia are normally adaptive and only become pathological if the signals persist for extended periods of time, as is the case with an overexpansion of fat mass observed during obesity.

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1.1.8 Figures

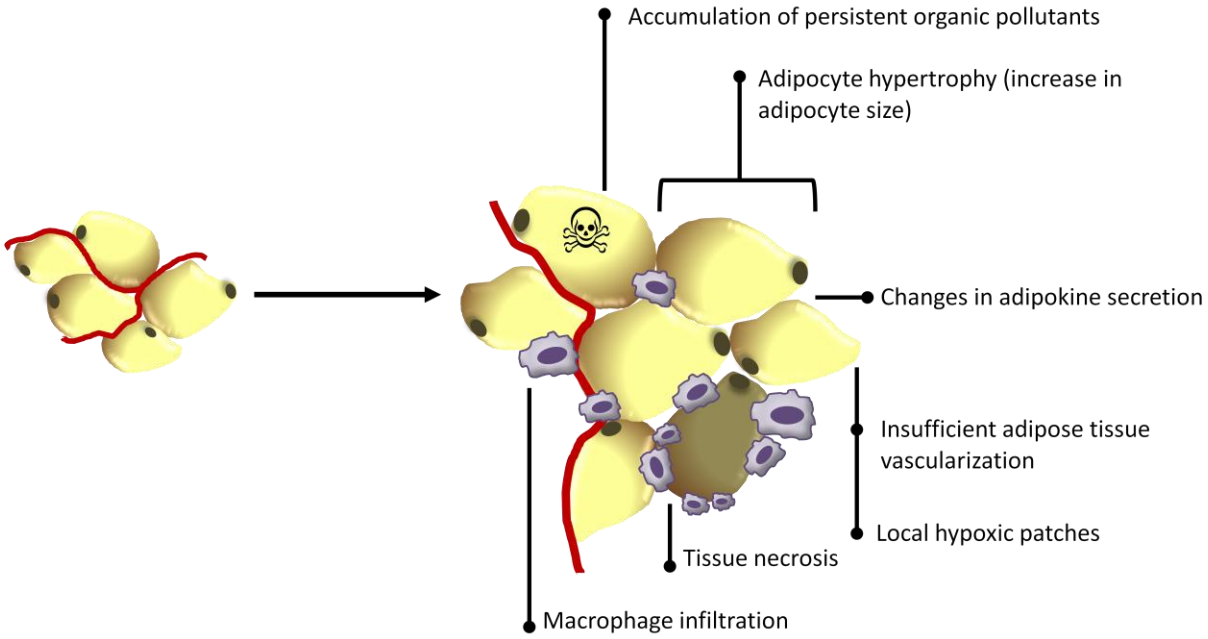

Figure 1: Overview of the changes that occur during adipose tissue growth

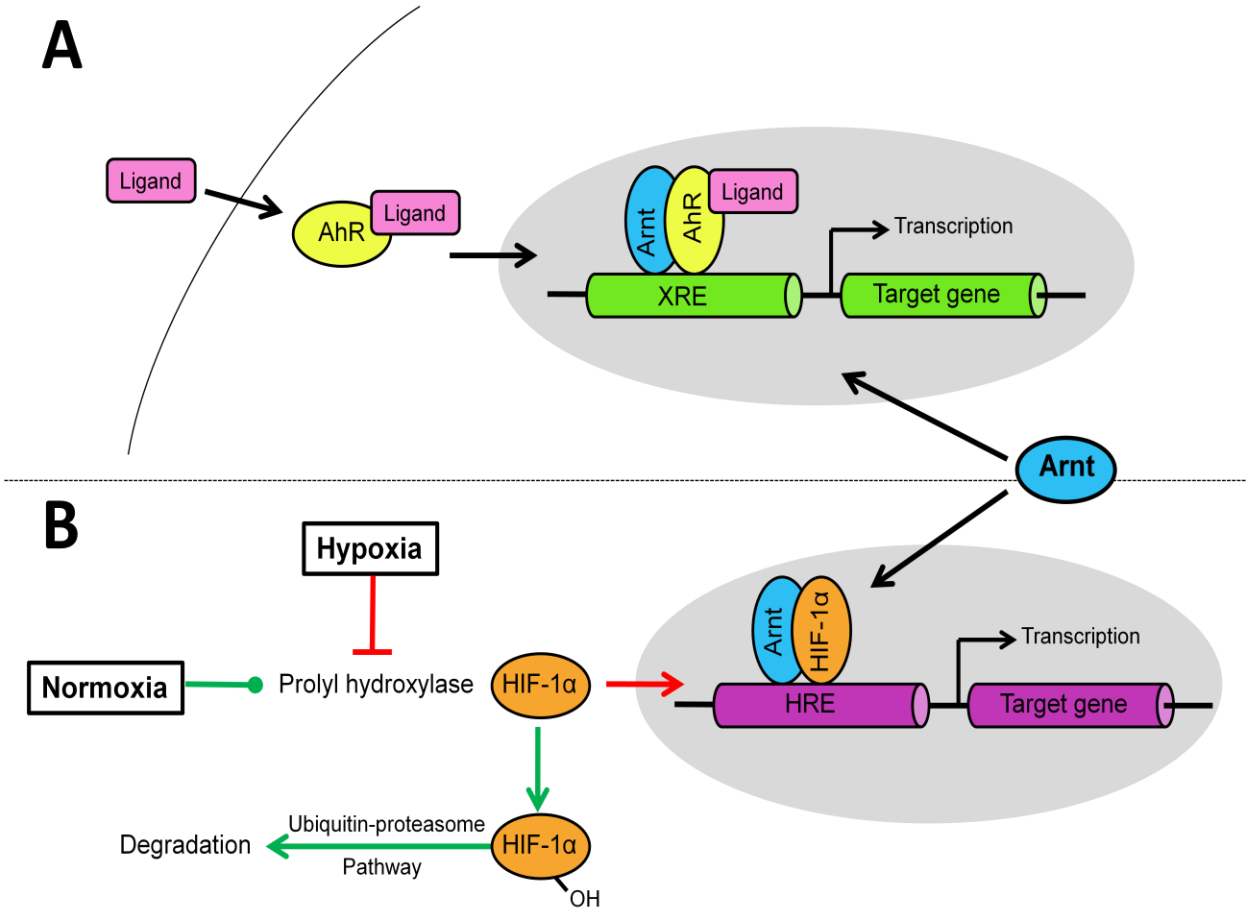

Figure 2: Xenobiotic and hypoxic signaling pathways

A) AhR/Arnt mechanism: AhR ligands such as the dioxin TCDD and the coplanar PCB-77 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 normoxic conditions, prolyl hydroxylases use oxygen to hydroxylate HIF-1α, which initiates the ubiquitin-proteasomal pathway and eventually leads to HIF-1α degradation. Under hypoxic conditions, prolyl hydroxylase is unable to hydroxylate HIF-1α. Thus, stable HIF-1α translocates to the nucleus, dimerizes with Arnt and activates gene transcription by binding to hypoxia-response elements (HRE). Abbreviations: HIF-1α: hypoxia-inducible factor 1 alpha; Arnt: aryl hydrocarbon receptor nuclear translocator; AhR: aryl hydrocarbon receptor.

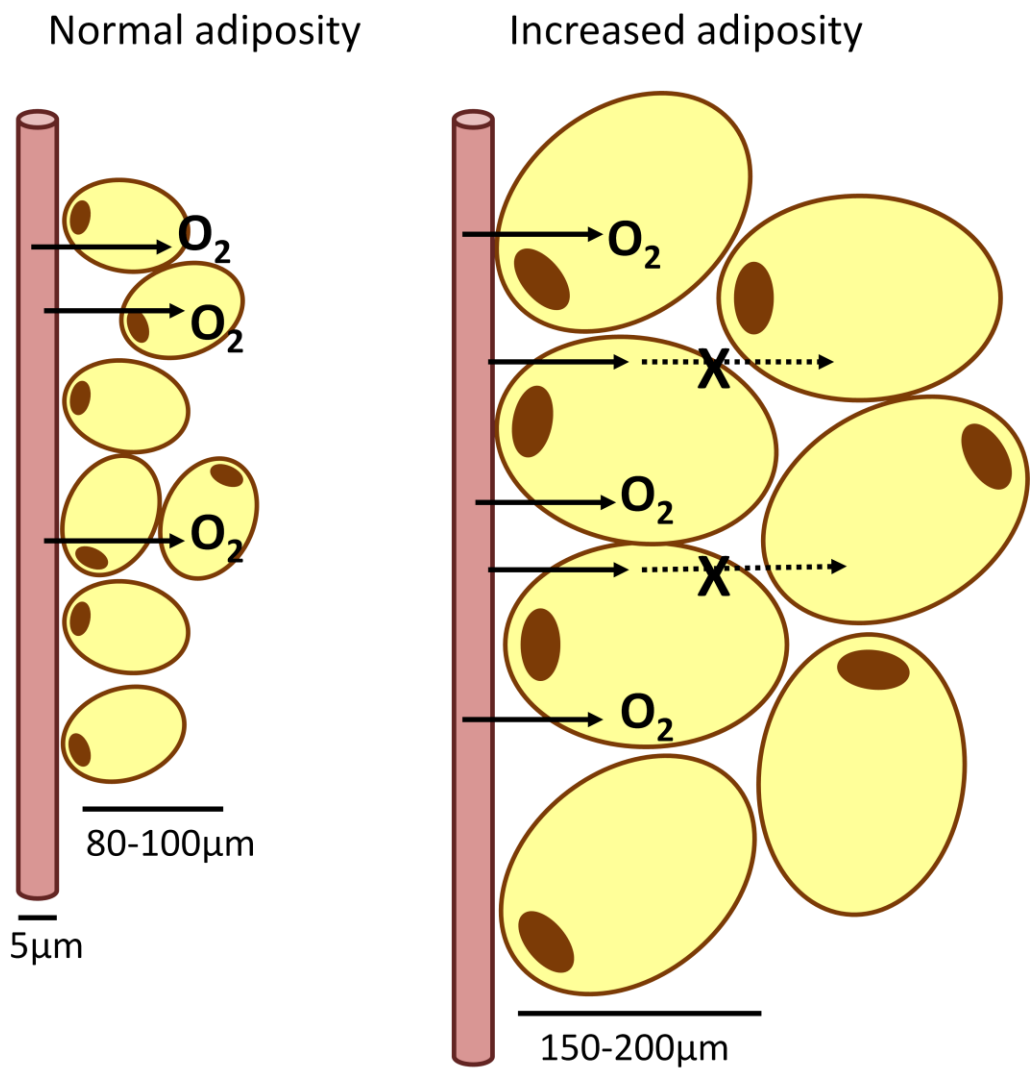

Figure 3: Oxygen diffusion in adipose tissue

A) Normal adiposity: Oxygen diffusion gradient (~100µm) is sufficient to supply all cells with adequate oxygen.

B) High adiposity: Oxygen diffusion gradient does not allow the supply of oxygen to all cells, creating hypoxic patches within the adipose tissue.

1.4 Objectives and hypothesis

This study investigated **(1)** the role of select POPs on preadipocyte differentiation and **(2)** the interaction between an AhR-activating POP and hypoxia to elicit inflammatory response from adipocytes. Specific objectives and hypotheses for each study are as follows:

Manuscript 1 – Low levels of select persistent organic pollutants do not affect human preadipocyte differentiation

This study's goal was to determine the effect of selected prevalent POPs on human subcutaneous preadipocyte differentiation. Based on previous studies performed with coplanar POPs on murine adipocyte cell lines, we expected that a lower concentration (3.4μM) of PCB-77, a coplanar PCB acting as an agonist of AhR, PCB-153, a non-coplanar PCB, or DDE, a pesticide, would significantly increase the lipid accumulation in differentiated adipocytes compared to the control condition, while a higher concentration (34μM) would significantly decrease the lipid accumulation. Furthermore, we anticipated that the addition of an AhR antagonist would counteract the effects of the AhR ligand PCB-77 but not PCB-153 or DDE.

Manuscript 2 – PCB-77 and hypoxia cross-talk in differentiated human adipocytes: Role in inflammation

This was the primary study, which aimed to determine the changes in inflammatory adipokine gene expression and secretion of differentiated human subcutaneous adipocytes exposed to a combination of PCB-77 (3.4μM or 340μM) and hypoxia (2% O_2 or 8% O_2).

Quantification of mRNA (gene expression) and protein (secreted proteins) levels was determined for leptin, vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), tumor necrosis factors-alpha (TNF-α), and adiponectin. Gene expression was also determined for CYP1A1 and AhR. We expected to confirm the decrease in adiponectin and increase in MCP-1 previously observed during PCB-77-only exposure in murine adipocytes. We also expected to confirm the decrease in adiponectin and increase in leptin, VEGF, and IL-6 observed in human adipocytes following hypoxia-only exposure. Building on these results, we anticipated that the combined exposure to PCB-77 and hypoxia would elicit additive inflammatory effects.

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Chapter 2

Methods

2.1 Experimental design

Study 1: Human preadipocytes were exposed to a concentration of 3.4µM or 34µM of PCB-77, PCB-153, or DDE with or without α-naphthoflavone during the entire 14 day differentiation period. Next, the accumulation of lipids was quantified using a triglyceride assay.

Study 2: Differentiated human adipocytes were exposed to a combination of PCB-77 (3 levels: vehicle, 3.4µM, and 340µM) and hypoxia (3 levels: 21% O_2, 8% O_2, and 2% O_2) for 24 hrs. Inflammatory adipokines levels were quantified in the cell culture media for leptin, VEGF, IL-6, TNF-α, MCP-1, and adiponectin by ELISA. Inflammatory gene expression for leptin, VEGF, IL-6, TNF-α, MCP-1, and adiponectin was quantified by qPCR conducted on RNA isolated from the adipocytes. The gene expression for CYP1A1 and AhR was also quantified by qPCR.

2.2 Human subcutaneous preadipocyte culture

Obtaining cells and lot composition

Cryopreserved subcutaneous preadipocytes were purchased from ZenBio Inc. (Research Triangle Park, NC, USA). Upon arrival, cells were immediately stored in liquid nitrogen and cell culture media was stored at -20°C. All experiments were performed using cells from the

same lot and following the manufacturers protocol (ZenBio, Inc., 2010). See section **2.8 Tables and Figures**, Table 1, for lot characteristics.

Plating cryopreserved subcutaneous preadipocytes

According to the ZenBio protocol, preadipocytes were removed from liquid nitrogen and thawed in a water bath set at 37°C. Next, cells were transferred to a sterile centrifuge tube containing 10 mL of Preadipocyte Medium (PM-1) and centrifuged at 1,200 rpm (282xg) at 20°C for 5 minutes. Upon aspiration of supernatant, the cells were resuspended in PM-1 and were added to each well of a 96-well (Study 1) or a 6-well (Study 2) sterile cell culture plate (Corning Life Sciences, Corning, NY, USA). The plates were placed in a sterile, humidified incubator at 37°C with 5% CO_2 for 24 – 48 hrs until cells reached confluence (Fig. 4a). Full confluence is reached when the entire surface of the well is covered with cells.

Preadipocyte differentiation – Study 1

Upon reaching full confluence, the entire medium was aspirated and replaced with 150µl/well of Adipocyte Differentiation Medium (DM-2) containing DMSO as a vehicle, or selected POPs. For the preparation of the media, see section **2.3 POP treatment preparation and hypoxia incubation**. Following a 7-day incubation period, 90µl/well was removed and replaced with 120µl/well of Adipocyte Maintenance Medium (AM-1) also containing the treatment.

Preadipocyte differentiation – Study 2

Upon reaching full confluence, the entire medium was aspirated and 3mL of Adipocyte Differentiation Medium (DM-2) was added to each well. Plates were then incubated at 37°C

with 5% CO_2 for 7 days. After 7 days (Fig. 4b), 1.8 mL of DM-2 media was removed and replaced with 2.4 mL of Adipocyte Medium (AM-1). The incubation conditions remained the same. At the end of the 14-day period, an accumulation of large lipid droplets in the cytoplasm was observed, indicating that the preadipocytes had differentiated into mature adipocytes (Fig.4c).

2.3 POP treatment preparation and hypoxia incubation

3, 3', 4, 4'-Tetrachlorobiphenyl (PCB-77), 2, 2', 4, 4', 5, 5'-Hexachlorobiphenyl (PCB-153) and dichlorodiphenyldichloroethylene (DDE) were purchased from Ultra Scientific (North Kingstown, Rhode Island, USA). Stock solutions (0.01M) were made by dissolving PCB-77, PCB-153, and DDE in an organic solvent (DMSO, Sigma Aldrich, Oakville, ON, CA) and vortexing for a few minutes. The stock solutions were diluted using cell culture media (DM-2 or AM-1) to obtain the desired final concentration of the pollutant (DMSO as a vehicle, 3.4μM, 34μM, or 340μM). Similar solutions were prepared with the addition of 20μM α-naphthoflavone (Sigma Aldrich, Oakville, ON, CA), an AhR antagonist.

Study 1 was conducted in a 96-well plate, with each experimental condition performed in 4 wells. Fig. 5 shows the plate layout and specific experimental conditions. POPs were maintained in the culture media during the entire 14-day differentiation period.

Study 2 was conducted in 6-well plates, with each experimental condition performed in triplicate. Fig. 6 shows the plate layout and specific experimental conditions. Plates exposed to 21% O_2 were placed in a sterile, humidified CO_2 incubator at 37°C with 5% CO_2 for 24 hrs. Plates exposed to 2% O_2 and 8% O_2 were placed in a specialized hypoxia incubator (HERAcell,

Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with 5% CO_2 for 24 hrs. Decreased oxygen levels were attained by replacing oxygen with nitrogen in the incubator.

2.4 Adipogenesis quantification

The Triglyceride Assay Kit (Cat# TG-1-NC, ZenBio Inc., 2010) was used to quantify cellular lipid accumulation in Study 1. First, the media was removed and the cells were washed with a Wash Buffer. Then, the cells were incubated with a Lysis Buffer to allow the release of intra-cellular lipids. The lysates were incubated for 2 hrs at 37°C with a reagent to hydrolyze the triglyceride molecules, releasing fatty acids and glycerol. In this time, glycerol standards ranging from 3.125μM to 200μM were prepared. Finally, the samples and standards were added to a 96-well plate with a reagent to generate a color detectible at 540nm. The glycerol concentration for each sample was determined by subtracting the blank reading and using the equation for the standard curve. The standard curve was generated by placing a line of best fit through the data points of the standard wells, with the absorbance on the y-axis and glycerol concentration on the x-axis.

2.5 Adipokine quantification by ELISA

Enzyme-linked immunosorbent assays (ELISA) were performed to quantify adipokine levels in the culture media. All kits were obtained commercially (Quantikine ELISA, R&D Systems, Minneapolis, MN, USA) and performed following the manufacturer's protocol. Briefly, standards were prepared and samples were diluted with calibrator diluents. The standards and samples were added in duplicate to each well of a 96-well plate pre-coated with adipokine-specific antibodies and incubated for 2 hrs. Following several washes, an enzyme-

linked adipokine-specific conjugate was added to each well and the plate was incubated for 1-2 hrs. The wells were washed again and a substrate solution was added, initiating the development of color in proportion to the amount of adipokine initially bound to the antibody in the well. Following a 20-30 min incubation period, a stop solution was added to each well. Finally, the absorbance at 450nm was measured to determine the intensity of color in each well. A measure at 540nm was used to correct for optical imperfections in the plate.

A standard curve was generated by placing a line of best fit through the data points of the standard wells, with the absorbance on the y-axis and adipokine concentration on the x-axis. The concentration for each sample was determined by subtracting the blank reading and using the equation for the standard curve. Finally, the concentration values were multiplied by the initial dilution factor.

2.6 RNA extraction and gene quantification by qPCR

RNA extraction

The cell culture media was removed and stored at -80°C until further analysis. Following the instructions for the RNeasy RNA extraction kit (Qiagen), cell lysis buffer and ethanol was added to each well and mixed by pipetting. The lysate was added to spin columns placed in a 2mL tubes. Following centrifugation for 15s at 8000 x g, the flow-through was discarded and the RNA bound to the membrane was washed with several buffers supplied in the kit. Finally, the RNA was eluted using 30-50µl of RNase-free water. A volume of 2µl was used to determine RNA yield by measuring the absorbance at 260nm using a BioTek Synergy HT instrument

(BioTek Instruments Inc., Winooski, VT, USA). RNA samples were stored at -80°C until needed.

Reverse transcription

The reverse transcription reaction allows the synthesis of complimentary DNA (cDNA) from RNA thanks to the activity of a reverse transcriptase enzyme. This was accomplished using the QuantiTect Reverse Transcription kit (Qiagen). Briefly, 1µg RNA from each sample was incubated for 2 mins at 42°C with a gDNA Wipeout buffer. Then, a master mix containing the reverse transcriptase enzyme, buffer, and primer mix was added and incubated at 42°C for 15mins, followed by 95°C for 3 mins to deactivate the reaction. cDNA samples were stored at -80°C until further analysis.

Quantitative polymerase chain reaction (qPCR)

Quantitative polymerase chain reactions (qPCR) were performed following the protocol outlined by the EVOlution 5 x EvaGreen qPCR Mix (Montreal Biotech Inc., Montreal, QB, CA). Each reaction, performed in duplicate, consisted of 25ng cDNA template, gene-specific primers (QuantiTect Primer Assays, Qiagen), and EvaGreen master mix. An initial denaturation step of 95°C for 10 mins activated the polymerase and was followed by 40 cycles of denaturation (95°C for 10s), annealing (60°C for 15s), and elongation (72°C for 15s). EvaGreen is a fluorescent dye that is specifically incorporated into double stranded DNA (dsDNA) and can therefore be used as a detection probe. As the template cDNA is amplified during the qPCR run, the proportion of fluorescence is directly associated with the amount of EvaGreen that is bound to dsDNA.

Data analysis

Cycle threshold (Ct) values were calculated and normalized to the expression of the β-actin gene. The Ct value indicates the number of cycles required for the fluorescence of the qPCR to exceed the threshold level, i.e. the background levels of fluorescence. β-actin is a commonly used housekeeping gene whose expression does not fluctuate between experimental and control conditions. The Ct values were obtained by calculating the average of technical replicates and then $\Delta Ct_{treated}$ and $\Delta Ct_{control}$ were calculated as follows:

$$\Delta Ct_{treated} = Ct\ (\text{gene of interest\_treated}) - Ct\ (\text{normalizer\_treated})$$

$$\Delta Ct_{control} = Ct\ (\text{gene of interest\_control}) - Ct\ (\text{normalizer\_control})$$

The average of replicate ΔCt (biological replicates) was taken and the ΔΔCt was calculated:

$$\Delta\Delta Ct = \Delta Ct_{treated} - \Delta Ct_{control}$$

Finally, the fold change between the control and experimental conditions was determined:

$$\text{Fold change} = 2^{(-\Delta\Delta Ct)}$$

For example, to compare the gene expression for adiponectin between condition 2 (treated) and condition 1 (control):

$$\Delta\Delta Ct = \Delta Ct\ (\text{Adiponectin}_{cond2} - \beta\text{-actin}_{cond2}) - \Delta Ct\ (\text{Adiponectin}_{cond1} - \beta\text{-actin}_{cond1})$$

2.7 Statistical analysis

All data are expressed as a mean with standard error (±). For Study 1, a two-way ANOVA with the non-repeated factors of "POP concentration" (3 levels: DMSO, 3.4µM, and 34µM) and "AhR antagonist" (2 levels: with and without) was used to analyze the dependent variable of lipid accumulation. For Study 2, a two-way ANOVA with the non-repeated factors of "PCB-77 concentration" (3 levels: DMSO, 3.4µM, and 340µM) and "oxygen tension" (3 levels: 2%, 8%, and 21%) were used to analyze the dependent variables of (1) leptin, VEGF, IL-6, TNF-α, MCP-1, and adiponectin protein levels in the cell culture media and (2) leptin, VEGF, IL-6, TNF-α, MCP-1, adiponectin, AhR, and CYP1A1 gene expression levels. In the case of significant findings, post-hoc comparisons with Bonferroni corrections were performed using independent t-tests. The significance level was set at $p<0.05$. All statistical analyses were performed using the statistical software package SPSS 22.0 for Windows (SPSS Inc. Chicago, Illinois, USA).

2.8 Tables and Figures

Table 1: Lot composition characteristics of cryopreserved preadipocytes

Lot #	L041102
Number of donor(s) in lot	1
Gender of donor(s)	Male
Age	31
BMI	31.52
Smoker	Unknown
Ethnicity	Unknown
Location	Abdomen
Medication	None

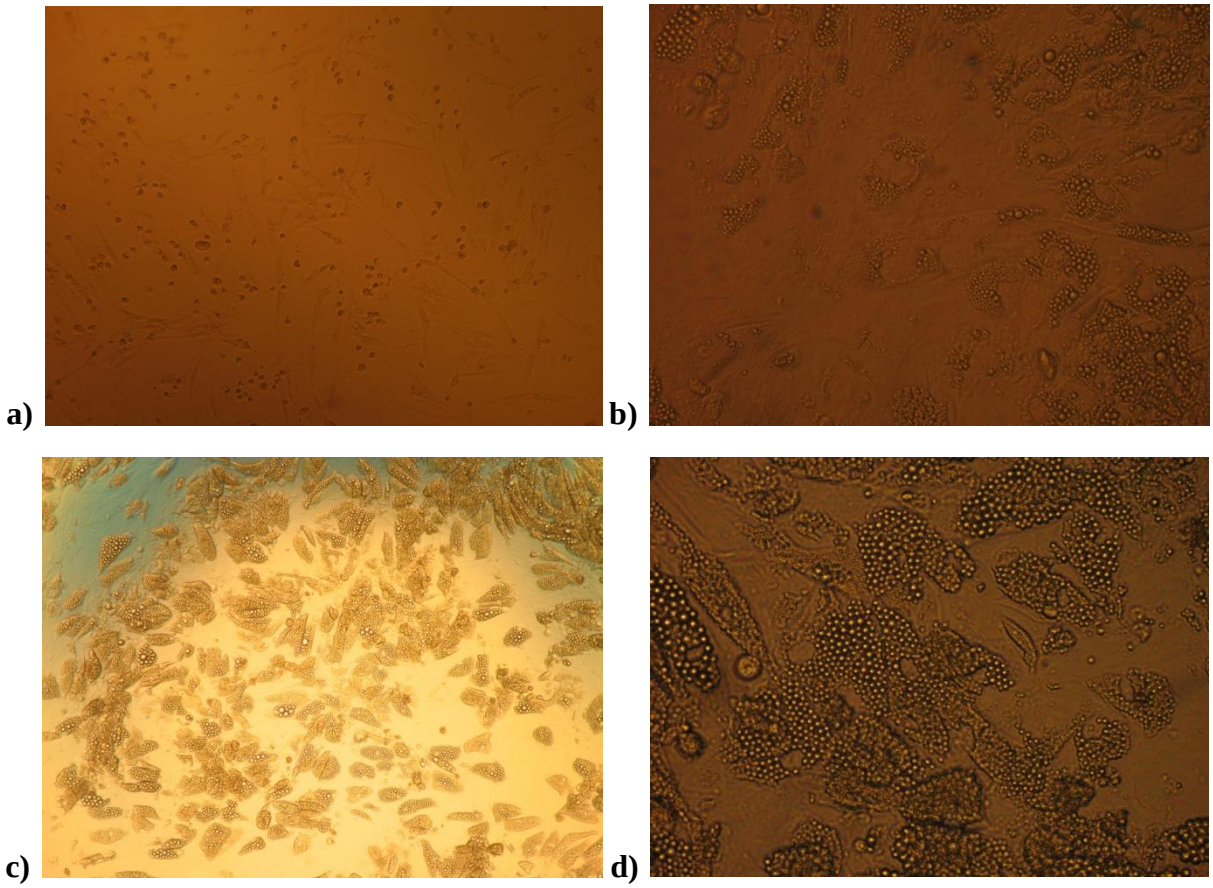

Figure 4: Photographs of unstained human adipocytes during differentiation

a) Confluent preadipocytes 24 – 48 hrs after plating (20X magnification)

b) Adipocytes 7 days after inducing differentiation (20X magnification)

c) Mature adipocytes 14 days after inducing differentiation (20X magnification)

d) Mature adipocytes 14 days after inducing differentiation (40X magnification)

	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO		PCB 77 3.4µM	PCB 77 34µM			PCB 153 3.4µM	PCB 153 34µM			DDE 3.4µM	DDE 34µM
B	DMSO		PCB 77 3.4µM	PCB 77 34µM			PCB 153 3.4µM	PCB 153 34µM			DDE 3.4µM	DDE 34µM
C	DMSO		PCB 77 3.4µM	PCB 77 34µM			PCB 153 3.4µM	PCB 153 34µM			DDE 3.4µM	DDE 34µM
D	DMSO		PCB 77 3.4µM	PCB 77 34µM			PCB 153 3.4µM	PCB 153 34µM			DDE 3.4µM	DDE 34µM
E	DMSO + α-NF		+ α-NF	+ α-NF			+ α-NF	+ α-NF			+ α-NF	+ α-NF
F	DMSO + α-NF		+ α-NF	+ α-NF			+ α-NF	+ α-NF			+ α-NF	+ α-NF
G	DMSO + α-NF		+ α-NF	+ α-NF			+ α-NF	+ α-NF			+ α-NF	+ α-NF
H	DMSO + α-NF		+ α-NF	+ α-NF			+ α-NF	+ α-NF			+ α-NF	+ α-NF

Figure 5: Plate layout and experimental conditions for Study 1

Preadipocytes were exposed to the specified POP during the 14-day differentiation period, at which point cells were lysed and lipid accumulation was assessed. A 96-well plate was employed and each experimental condition was performed in quadruplicate.

Abbreviations: α-NF: alpha-naphthoflavone

21% O_2 DMSO	21% O_2 DMSO	21% O_2 DMSO	21% O_2 340 µM PCB77	21% O_2 340 µM PCB77	21% O_2 340 µM PCB77
21% O_2 3.4 µM PCB77	21% O_2 3.4 µM PCB77	21% O_2 3.4 µM PCB77			
8% O_2 DMSO	8% O_2 DMSO	8% O_2 DMSO	8% O_2 340 µM PCB77	8% O_2 340 µM PCB77	8% O_2 340 µM PCB77
8% O_2 3.4 µM PCB77	8% O_2 3.4 µM PCB77	8% O_2 3.4 µM PCB77			
2% O_2 DMSO	2% O_2 DMSO	2% O_2 DMSO	2% O_2 340 µM PCB77	2% O_2 340 µM PCB77	2% O_2 340 µM PCB77
2% O_2 3.4 µM PCB77	2% O_2 3.4 µM PCB77	2% O_2 3.4 µM PCB77			

Figure 6: Plate layout and experimental conditions for Study 2

Differentiated adipocytes were exposed to a combination of PCB-77 and hypoxia for 24hrs, as indicated. Six-well plates were employed and each experimental condition was performed in triplicate

Chapter 3

Results

Manuscript 1 – Low levels of select persistent organic pollutants do not affect human preadipocyte differentiation

This manuscript conforms to the specifications for submission to the peer-reviewed journal *Molecular and Cellular Biochemistry*.

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3.1 Abstract

With the prevalence of obesity on the rise, any factor affecting adipose tissue development or function is under investigation. This includes persistent organic pollutants (POPs), a group of lipophilic environmental contaminants that bioaccumulate in adipose tissue. We aimed to determine the effect of select POPs on human preadipocyte differentiation. We also tried to determine the role of AhR, a ligand-activated transcription factor responsible for the effect of some POPs. As such, we exposed human preadipocytes to 3.4µM or 34µM of PCB-77, a coplanar PCB acting as an agonist of AhR, as well as the non-coplanar PCB-153 and the pesticide DDE, during the entire 14-day differentiation period. In order to test the involvement of AhR, we employed the antagonist alpha-naphthoflavone. We found a concentration dependent effect of PCB-77 exposure on triglyceride accumulation, where 34µM PCB-77 inhibited triglyceride accumulation and 3.4µM had no effect. There was also no effect of PCB-153 or DDE on triglyceride accumulation. The AhR antagonist alpha-naphthoflavone significantly inhibited triglyceride accumulation. These findings suggest that individual POPs do not promote the formation of new adipocytes from existing preadipocytes.

Keywords

PCB-77; PCB-153; DDE; Human preadipocytes; Adipogenesis; α-naphthoflavone

3.2 Introduction

Persistent organic pollutants (POPs) are man-made, carbon-based chemicals that primarily originate from industrial activities and consequently contaminate our environment. Humans are exposed to POPs through various sources including air, soil, and water. Food however is the primary source of exposure, especially lipid-rich foods of animal origin such as milk, meat, and fish [1, 2]. Since most POPs are highly lipophilic and resistant to environmental and biological degradation [3, 4], they are stored in human adipose tissue, the largest site for lipid storage in the body, where they remain stored for years [5]. This storage of POPs in adipose tissue is often considered a protective mechanism since it limits the systemic exposure to toxic lipophilic compounds [6]. However, POPs are known endocrine disruptors that have been shown to directly affect adipose tissue itself, which could result in adverse health effects. With the rise in the prevalence of obesity and obesity-related co-morbidities, determining the effect of POPs on adipose tissue, and in particular on adipogenesis, has become a current research challenge.

Two major events make up the process of adipogenesis, otherwise known as the formation of new mature adipocytes. The first step is determination, where mesenchymal stem cells become preadipocytes. While these two types of cells are morphologically identical, preadipocytes are committed to the adipocyte lineage and therefore cannot become any other type of cell [7]. Preadipocytes do however retain the ability to replicate/proliferate [8]. The second major step in adipogenesis is terminal differentiation, where preadipocytes undergo a cascade of events and become terminally-differentiated adipocytes [7]. At this stage, adipocytes express adipocyte-specific genes encoding adipogenic proteins that allow the cells to synthesize and accumulate lipids. A variety of factors are required to induce differentiation including

several transcription factors and coactivators, along with specific hormonal, nutritional, and neuronal signals [8]. It remains to be determined if POPs can stimulate or inhibit human preadipocyte differentiation.

A recent systematic review indicated that exposure to dichlorodiphenyldichloroethylene (DDE), a metabolite of the insecticide dichlorodiphenyltrichloroethane (DDT), was almost always associated with increased body weight in humans [9]. In addition, separate cell culture studies found that DDT itself and the biocide tributyltin increase lipid accumulation and adipocyte-specific genes in murine adipocytes [10, 11]. Furthermore, we have previously determined that some organochlorines, namely 2, 2', 4, 4', 5, 5'-hexachlorobiphenyl (PCB-153) and DDE, increase the proliferation of human preadipocytes in culture [12].

In contrast, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic dioxin, has been shown to inhibit murine preadipocyte differentiation, as indicated by a reduction in lipid accumulation and adipocyte-specific gene expression [13]. TCDD is also known to cause the wasting syndrome, where the individuals exposed show largely reduced body weight, among other symptoms [4, 14]. The toxic effects of TCDD exposure are primarily mediated through the ligand-activated transcription factor aryl hydrocarbon receptor (AhR). AhR is the main cellular sensor for many exogenous chemicals, including some POPs. Activated AhR regulates the expression of many detoxification genes and its activity has even been shown to inhibit adipogenesis [15], yet its role in POP-induced regulation of adipogenesis remains unclear. Contrarily, multiple studies have reported that lower doses of AhR-activating chemicals may have an obesogenic effect [16]. For instance, a study in murine adipocytes shows that exposure to the AhR ligand 3, 3', 4, 4'-tetrachlorobiphenyl (PCB-77) increases preadipocyte differentiation [17].

In all, these studies suggest a role for POPs in preadipocyte differentiation that is likely dose-dependent and regulated by AhR. We expect that lower POP levels, which better reflect actual physiological levels in human tissues, would promote the differentiation of preadipocytes. Building on previous findings, the goal of this study was to determine the concentration-dependent effect of three persistent organic pollutants, PCB-77, PCB-153, and DDE, on the differentiation of human preadipocytes to adipocytes. We also sought to determine if any effects were AhR-mediated by employing a commonly used AhR antagonist, α-naphthoflavone.

3.3 Materials and Methods

Treatment preparation

3, 3', 4, 4'-Tetrachlorobiphenyl (PCB-77), 2, 2', 4, 4', 5, 5'-Hexachlorobiphenyl (PCB-153), and dichlorodiphenyldichloroethylene (DDE) were purchased from Ultra Scientific (North Kingstown, Rhode Island, USA). Stock solutions were prepared by dissolving each POP in the organic solvent dimethyl sulfoxide (DMSO). The PCBs and DDE were diluted with Differentiation Medium (DM-2, ZenBio Inc.) or Adipocyte Maintenance Medium (AM-1, ZenBio Inc.) to obtain a final concentration of 3.4μM or 34μM. Similar solutions were prepared with the addition of 20μM α-naphthoflavone (α-NF, Sigma Aldrich, Oakville, ON, CA), an AhR antagonist.

Human preadipocyte differentiation and POP exposure

Human subcutaneous preadipocytes were purchased from Zen-Bio Inc. (Research Triangle Park, North Carolina, USA). Preadipocytes were plated with Preadipocyte Medium (PM-1, Zen-Bio Inc.) at a cell density of 40 000 cells/cm^2 in a 96-well plate (Corning Life Sciences, Corning, NY, USA), following the manufacturer's instructions. The plates were

placed in a sterile, humidified incubator at 37°C with 5% CO_2 for 24 – 48 hrs until cells reached full confluence.

To test the effect of POPs on preadipocyte differentiation, the PM-1 was aspirated and replaced with DM-2 containing DMSO (control condition, 0.3% v/v) or the pollutants (PCB-77, PCB-153, or DDE) at 3.4μM or 34μM. To determine the role of AhR in POP-induced regulation of differentiation, preadipocyte were exposed to 20 μM α-NF for 30 mins before adding the POPs, as described in [17]. After 7 days, the media was replaced with Adipocyte Maintenance Medium (AM-1) also containing the pollutants with or without α-NF. As such, the experimental treatment was maintained during the 14-day differentiation period. Each experimental condition was performed in quadruplicate.

Assessment of preadipocyte differentiation by lipid accumulation

Triglyceride accumulation was quantified using a Triglyceride Assay kit (Cat. # TG-1-NC, Zen-Bio Inc.). Briefly, cell culture media was removed and the cells were lysed, thus releasing the intracellular lipids (mainly triglycerides). Next, triglycerides were hydrolyzed, yielding 1M glycerol and fatty acids per 1M triglycerides. Finally, glycerol levels were quantified using a spectrophotometer plate reader to assess total triglyceride accumulation.

Statistical analysis

All data are expressed as a mean with standard error (±). A two-way ANOVA with the non-repeated factors of "POP concentration" (3 levels: DMSO, 3.4μM, and 34μM) and "AhR antagonist" (2 levels: with and without) was used to analyze the dependent variable of lipid accumulation. The significance level was set at $p<0.05$. In the case of significant findings, post-hoc comparisons with Bonferroni corrections were performed using independent t-tests. All

statistical analyses were performed using the statistical software package SPSS 21.0 for Windows (SPSS Inc. Chicago, Illinois, USA).

3.4 Results

We exposed human preadipocytes to two concentrations (3.4µM and 34µM) of PCB-77, PCB-153, and DDE with or without α-NF during a 14-day differentiation period. To assess the level of differentiation, we measured triglyceride accumulation for each experimental condition. As indicated in Fig. 1a, we found a significant effect of PCB-77 concentration on triglyceride accumulation ($p<0.0001$). Upon further analysis, we found no difference between 3.4µM PCB-77 and the control ($p=0.327$), while triglyceride levels were significantly lower at 34µM PCB-77 as compared with the control ($p<0.001$). In addition, as shown in Fig. 1b-c, we found no concentration effect for PCB-153 ($p=0.358$) and DDE ($p=0.139$) on triglyceride levels. Finally, for all POPs tested, the conditions 'with' α-NF showed significantly lower triglyceride levels than those 'without' α-NF ($p<0.001$).

3.5 Discussion

Over the past decades, there has been a growing interest in the role of environmental pollutants on human health. Persistent organic pollutants are a group of environmental contaminants known to bioaccumulate in adipose tissue and show endocrine-disruptive properties [3, 6, 18, 19]. Our study aimed to determine the effect of select POPs on human preadipocyte differentiation, as measured by triglyceride accumulation. Our lowest concentration (3.4µM) was chosen because it reflects the levels of PCBs found in human serum [20] and it allowed us to compare our findings to other studies using similar concentrations [12, 17, 20, 21]. More specifically, 3.4µM reflects the levels of PCBs found in humans that were

exposed to high PCB levels through their work or upon accidental acute exposure/ingestion [22, 23]. Plasma PCB levels commonly reported in humans vary from 1 to 50 nM according to the country and especially the dietary habits of the population [24–27]. Finally, a higher concentration (34μM) was chosen to determine the concentration effect.

We found no change in triglyceride accumulation between preadipocytes exposed to the lowest concentration of PCB-77, PCB-153, and DDE, as compared to the control. Similarly, studies in murine preadipocytes showed no increase of differentiation for cells exposed to PCB-153 and only a modest increase in differentiation for cells exposed to PCB-77 [17]. While murine cells are a useful model to predict cellular processes in humans, fundamental differences between the two cell lines can lead to different physiological responses. For instance, the media composition and time necessary to induce differentiation is different for murine and human cells [28]. Furthermore, there are inter-species differences in CYP-mediated drug metabolism and murine cells show a greater metabolic response to toxic compounds even when normalized with weight [29]. These differences can explain why we have found no effect of 3.4μM PCB-77 on human preadipocyte differentiation while Arsenescu's study found a small but significant increase [17].

Unlike PCB-153 and DDE, PCB-77 is a known AhR ligand. Since AhR activation [30], as well as the AhR ligand TCDD [13], inhibit adipogenesis, it is not surprising that an elevated concentration of PCB-77 inhibited differentiation in our study. Based on these results, we hypothesize that a concentration greater than 3.4μM is necessary to activate the AhR pathway and decrease human preadipocyte differentiation *in vitro*.

To determine if a compound acts by activating AhR, many studies have used an antagonist named α-naphthoflavone (α-NF) that acts by binding to AhR to prevent its activity [13, 17, 20, 30–33]. As such, we employed α-NF to test the role of AhR in POP-induced changes in preadipocyte differentiation. Unexpectedly, we observed a decrease in triglyceride accumulation with α-NF treatment both alone or with POPs. Since AhR has been shown to inhibit differentiation of murine adipocytes [15], we expected that blocking AhR activity with α-NF would lead to cellular triglyceride levels comparable to the control condition. Contrary to these expectations, α-NF inhibited triglyceride accumulation, as shown in Fig. 1a-c. We then identified a recent study showing that α-NF independently inhibits murine preadipocyte differentiation [34]. Previous studies also found that α-NF can act as both an agonist and antagonist to AhR, depending on the concentration [35]. These findings suggest that the use of α-NF as an AhR-specific antagonist may not be recommended in every situation. A better approach would be to employ gene silencing techniques to directly knockdown AhR gene expression.

To conclude, our results suggest that exposure to a low dose such as 3.4uM of PCB-77, PCB-153, or DDE alone does not promote human preadipocyte differentiation. However, in this study preadipocytes were exposed to individuals POPs, which may have negligible effects in comparison to the POP mixtures found *in vivo* in adipose tissue. Since little is known about the effect of POP combinations, future studies should look at the effect of multiple POP exposures on human preadipocyte differentiation. We also recommend the use of a different AhR antagonist in future studies, as α-NF shows other effects besides blocking AhR, such as inhibiting preadipocyte differentiation [34]. Nonetheless, determining the role of POPs in obesity remains relevant since they are involved in the disruption of other adipocyte functions,

such as inducing inflammation, cell death, and insulin-resistance [17, 21, 32]. Moreover, since POPs are found ubiquitously in the environment and in human tissues, determining the exact impact of POP on human health remains an area of investigation.

Acknowledgements

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3.7 Figures

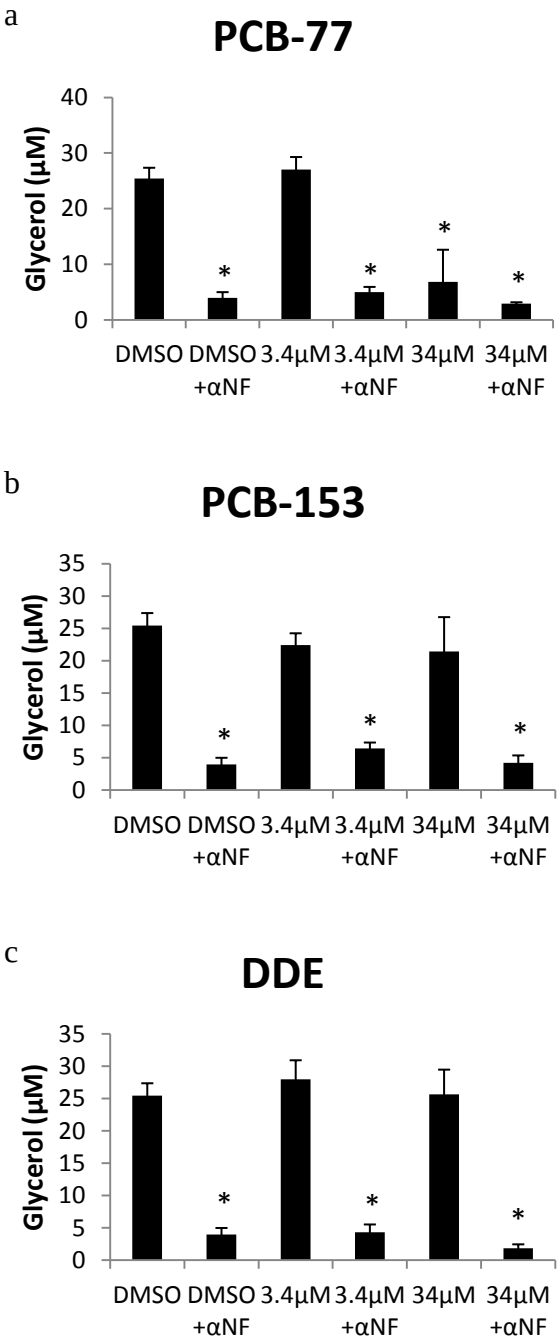

Figure 7: **Fig 1 Triglyceride accumulation in differentiated human adipocytes exposed to PCB-77, PCB-153, and DDE**

Human preadipocytes were exposed to two concentrations of coplanar PCB-77 (a), non-coplanar PCB-153 (b), and insecticide DDE (c) with and without α-NF for 14 days. Following the incubation period, cells were lysed and triglyceride accumulation was determined by measuring glycerol. Data are expressed as mean ± standard error (n=4), with significance set at *p<0.05

Chapter 4

Results

Manuscript 2 – PCB-77 and hypoxia cross-talk in human adipocytes: Role in inflammation

This manuscript conforms to the specifications for submission to the peer-reviewed journal *Molecular and Cellular Endocrinology*.

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4.1 Abstract

Persistent organic pollutants and adipose tissue hypoxia have been shown to independently stimulate inflammatory responses from adipocytes. Some pro-inflammatory responses are mediated by the aryl hydrocarbon receptor (AhR) and the hypoxia-inducible factor (HIF)-1 signaling pathways, which show areas of overlap such as the use of a common dimerization partner. The goal of this study was to examine the cross-talk between AhR and HIF-1 in the inflammatory response from differentiated human adipocytes. We found that PCB-77 had an additive effect on hypoxia at 8% O_2, but not 2% O_2, for VEGF, MCP-1, and adiponectin protein levels. Furthermore, the PCB-77-induced increase in CYP1A1 gene expression was inhibited by hypoxia. These findings indicate that POP and hypoxia cross-talk in human adipocytes occurs in a gene-specific manner.

Highlights

- As previously reported, hypoxia increases leptin, VEGF, and IL-6, and decreases MCP-1 and adiponectin, protein levels in a dose-dependent manner
- PCB-77 has additive effects on hypoxia-induced changes in VEGF, MCP-1, and adiponectin levels in the culture media
- Hypoxia inhibited PCB-77-induced increases in CYP1A1 gene expression
- PCB-77 and hypoxia cross-talk in differentiated human adipocytes is limited to certain genes

Keywords

Inflammatory adipokines; Xenobiotics; Hypoxia; AhR; HIF-1

4.2 Introduction

Obesity, or excess adiposity, is often associated with a state of chronic, low-grade inflammation. The contribution of adipose tissue to this inflammatory state has received much attention since adipocytes in the obese state increase the secretion of pro-inflammatory factors such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and monocyte chemotactic protein-1 (MCP-1), while also decreasing the major anti-inflammatory adipokine adiponectin (Park et al., 2005). Higher levels of circulating inflammatory proteins are thought to contribute to the pathogenesis of obesity-related metabolic disorders such as insulin resistance and endothelial cell dysfunction. Several factors have been hypothesized to stimulate the inflammatory response from adipocytes, including persistent organic pollutants (POPs) and adipose tissue hypoxia.

POPs are environmental contaminants that bioaccumulate in lipid-rich tissues due to their lipophilic properties (Müllerová and Kopecký, 2007). The combination of bioaccumulation and resistance to degradation leads to a biomagnification of POPs with the levels of the food chain, thus POPs can be detected in the plasma and adipose tissue of most living organisms, including humans (Pelletier et al., 2003). Stored POPs act as endocrine disruptors and have been suggested to promote inflammatory responses by activating specific cellular signaling pathways. In particular, the response to some POPs such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 3, 3', 4, 4'-tetrachlorobiphenyl (PCB-77) are mediated by the aryl hydrocarbon receptor (AhR) (Wilson and Safe, 1998). These POPs act as exogenous ligands for the cytosolic protein AhR, which then translocates to the nucleus to dimerize with a second factor called aryl hydrocarbon receptor nuclear translocator (Arnt). The AhR-Arnt complex promotes gene expression by

binding to genes with xenobiotic response elements (XREs) found in their regulatory region, such as CYP1A1, a phase I drug-metabolizing enzyme (Mimura and Fujii-Kuriyama, 2003).

Adipose tissue hypoxia is another factor possibly involved in adipose tissue inflammation. The concept of adipose tissue hypoxia was first proposed in a review article where the authors suggest that as adipocytes become hypertrophic during obesity, the development of vasculature is insufficient to provide oxygen to all the hypertrophic cells within the tissue, leading to the creation of hypoxic patches (Trayhurn and Wood, 2004). This has been well characterized in obese rodents (Hosogai et al., 2007; Ye et al., 2007). However, results from human studies are contradictory. One group found reduced oxygen levels in obese adipose tissue compared to a lean group (Pasarica et al., 2009), while others found no change (Goossens et al., 2011; Hodson et al., 2013). Nonetheless, differentiated human adipocytes in culture show a dose-dependent response to hypoxia (Wood et al., 2011). The majority of cellular responses to hypoxia are regulated by the hypoxia inducible factor (HIF)-1. Similar to AhR, HIF-1 is a transcription factor composed of two subunits, the oxygen-dependent HIF-1α and constitutively expressed Arnt. HIF-1α-Arnt complexes drive hypoxia-induced gene expression by binding to hypoxia response elements (HREs) (Semenza, 2001). In excess of 100 genes are directly regulated by HIF-1, including adipocyte-specific leptin and angiogenesis-promoting vascular endothelial growth factor (VEGF) (Semenza, 2000).

AhR and HIF-1α belong to the basic Helix–Loop–Helix/Periodic, AhR nuclear translocator, Single-minded (bHLH/PAS) family of proteins, which are responsible for monitoring many aspects of the micro-environment including toxic chemicals and oxygen decreases (Lindén et al., 2010). Consequently, AhR and HIF-1α share a common subunit, Arnt. Due to the overlapping cellular signaling pathways, we recently proposed that POP and hypoxia

cross-talk may promote inflammation in adipocytes (Myre & Imbeault, 2013). Here, cross-talk refers to one pathway's agonist also affecting a separate pathway due to shared molecular targets. To date, it is unknown if the simultaneous exposure of human adipocytes to an AhR ligand and hypoxia will lead to a different response than by activating each pathway independently.

The objectives of this study were to confirm previous findings showing an increase in inflammatory marker secretion from differentiated adipocytes exposed to PCB-77 and hypoxia independently. We then aimed to build on these findings to assess the impact of simultaneous PCB-77 and hypoxia exposure. This was the first study to our knowledge looking at this interaction in differentiated human adipocytes. We quantified leptin, VEGF, IL-6, MCP-1, TNF-α, and adiponectin protein and gene expression levels, as well as CYP1A1 and AhR gene expression. We hypothesized that there would be an additive effect of PCB-77 and hypoxia in adipokine protein and gene expression, as well as CYP1A1 and AhR gene expression.

4.3 Materials and Methods

4.3.1 Human subcutaneous preadipocyte differentiation

Human subcutaneous preadipocytes obtained commercially (ZenBio Inc., Research Triangle Park, NC, USA) were plated in Preadipocyte Medium (PM-1, ZenBio Inc.) at a cell density of 40 000 cells/cm^2 in a 6 well plate (Corning Life Science, Corning, NY, USA). The plates were placed in a sterile, humidified incubator at 37°C with 5% CO_2 for 24 – 48 hrs until cells reached full confluence. Next, the PM-1 was aspirated and replaced with Differentiation Medium (DM-2, ZenBio Inc.) containing a PPAR-γ agonist to induce differentiation. After 7 days in DM-2, a portion of DM-2 was replaced by Adipocyte Maintenance Medium (AM-1,

ZenBio Inc.). At 14 days post-induction, multiple lipids droplets could be observed in the cytoplasm, at which point the cells were considered to be mature adipocytes.

4.3.2 Differentiated adipocyte exposure to PCB-77 and hypoxia

3, 3', 4, 4'-Tetrachlorobiphenyl (PCB-77, Ultra Scientific) was dissolved in DMSO to make a 0.01M PCB-77 stock solution. The stock solution was diluted with AM-1 to reach the desired final concentrations (3.4μM or 340μM). Mature adipocytes (14 days post-induction of differentiation) were exposed to a combination of PCB-77 (3.4μM or 340μM) and hypoxia (2% or 8%). The low oxygen tensions were achieved using a specialized CO_2 incubator (HERAcell, Thermo Fisher Scientific) by replacing oxygen with nitrogen. The control plates contained DMSO as a vehicle and were incubated at 21% O_2. In all, the experimental exposure lasted 24 hrs and each experimental condition was tested in triplicate.

4.3.3 Adipokine quantification by ELISA

Cell culture media was removed prior to RNA extraction and stored at -80°C until further analysis. Adipokine concentrations were measured in the recuperated culture media by ELISA (R&D Systems, Minneapolis, MN, USA). The assays were performed in 96-well plates following the manufacturer's protocol. Assay sensitivities, or minimum detectible doses, were as follows: leptin, 7.8 pg/ml; VEGF, 5.0 pg/ml; IL-6, 0.70 pg/ml; MCP-1, 1.7 pg/ml; TNF-α, 1.6 pg/ml; and adiponectin, 0.246 ng/ml. Due to low levels of secreted proteins at every oxygen level, we believe that 340μM PCB-77 had cytotoxic effects. Previous *in vitro* studies have employed concentrations no greater than 68μM of PCB-77 (Arsenescu et al., 2008; Chapados et al., 2012; Hennig et al., 2002; Majkova et al., 2009; Wang et al., 2010). As such, the conditions

containing 340μM PCB-77 were excluded from the statistical analyses. Also, we were unable to detect TNF-α for all experimental conditions, probably due to a lack of sensitivity of the kit.

4.3.4 RNA extraction and quantification by qPCR

Cell lysis and total RNA extraction was performed with the commercially available RNeasy kit (Qiagen), following the manufacturer's protocol. Extracted RNA was then reverse transcribed to create a cDNA library using the QuantiTect Reverse Transcription kit (Qiagen).

Quantitative polymerase chain reactions (qPCR) were performed for each gene of interest using 25ng of cDNA template and QuantiTect Primer Assays (Qiagen) as gene-specific primers. EvaGreen technology was used as a detection probe (Montreal Biotech Inc., Quebec, Canada). An initial denaturation step of 95°C for 10mins activated the polymerase and was followed by 40 cycles of denaturation (95°C for 10s), annealing (60°C for 15s), and elongation (72°C for 15s). The Ct values obtained were normalized to the expression of the β-actin gene and the ΔΔCt method was used to determine fold change between each experimental condition and the control.

4.3.5 Statistical analysis

All data are expressed as a mean with standard error (±). A two-way ANOVA with the non-repeated factors of "PCB-77 concentration" (2 levels: DMSO and 3.4μM) and "Oxygen concentration" (3 levels: 2%, 8%, and 21%) were used to analyze the dependent variables of leptin, VEGF, IL-6, MCP-1, and adiponectin protein levels in cell culture media, as well as leptin, VEGF, IL-6, MCP-1, CYP1A1, and AhR gene expression levels. In the case of significant findings, post-hoc comparisons with Bonferroni corrections were performed using independent t-tests. The significance level was set at $p<0.05$. All statistical analyses were

performed using the statistical software package SPSS 21.0 for Windows (SPSS Inc. Chicago, Illinois, USA).

4.4 Results

We found no PCB*Oxygen interaction for leptin protein levels ($p=0.955$, Fig. 1a) or gene expression ($p=0.369$, Fig. 2a). We identified a main effect of oxygen level on leptin protein and gene expression levels ($p<0.0001$), showing that leptin levels were increased with hypoxia, as expected. There was no main effect of PCB-77 on either the protein or gene expression for leptin.

A PCB*Oxygen interaction was found for VEGF protein levels ($p=0.009$, Fig. 1b) and gene expression ($p=0.003$, Fig. 2b). In the case of the protein levels, the interaction indicated that PCB-77 had an additive effect on VEGF levels at 8% O_2 but not at 2% or 21% O_2. In contrast, the interaction for the gene expression indicated that PCB-77 had an inhibitory effect on VEGF expression at 2% O_2 but not at 8% or 21% O_2.

As with leptin, there was no PCB*Oxygen interaction for IL-6 protein ($p=0.634$, Fig. 1c) or gene expression ($p=0.057$, Fig. 2c). Il-6 protein levels were increased with hypoxia, as shown by the main effect of oxygen ($p=0.00013$), while there no effect of PCB-77 ($p=0.761$). Similarly, IL-6 gene expression was increased with hypoxia ($p<0.0001$), but not with PCB-77 ($p=0.167$).

Despite missing data, we found a PCB*Oxygen interaction for MCP-1 protein levels ($p=0.006$, Fig. 1d), showing that PCB-77 inhibited MCP-1 production at 8% O_2, but not at 2% O_2. A main effect of oxygen showed that MCP-1 levels were decreased with hypoxia ($p=0.003$),

whereas no main effect of PCB-77 was identified (p=0.061). There was no interaction (p=0.573, Fig.2d) or main effects for oxygen (p=0.208) or PCB-77 (0.181) at the gene level for MCP-1.

A PCB*Oxygen interaction for was found for adiponectin protein levels (p=0.001, Fig. 1e), meaning that the addition of PCB-77 additively decreased protein levels at 8% O_2 but not at 2% O_2. Furthermore, adiponectin levels were independently decreased with oxygen (p=0.0001) and PCB-77 (p=0.008).

We found a significant PCB*Oxygen interaction for CYP1A1 expression (p=0.001, Fig. 3a). That is to say that PCB-77 increased CYP1A1 gene expression at 21% O_2 with PCB-77 compared to the DMSO control, however this increase was attenuated with hypoxia at both 8% and 2% O_2. Consequently, main effects for oxygen (p=0.002) and PCB-77 (p=0.003) were identified. Finally, despite the significant changes in CYP1A1, there was no interaction (p=0.762) or main effects of hypoxia (p=0.338) or PCB-77 (p=0.826) for AhR gene expression (Fig. 3b).

4.5 Discussion

Without appropriate cellular responses, exposure to toxic environmental contaminants and oxygen deprivation could compromise the survival of the cell. Fortunately, specific signaling pathways are activated in response to stressful stimuli. However, these pathways do not exist in isolation and in some cases common factors are required for multiple pathways, which can potentially lead to cross-talk or interaction between signaling pathways. The objective of this study was to examine the interaction between PCB-77 and hypoxia in the inflammatory response from differentiated human adipocytes.

First, we measured leptin and VEGF, two genes known to be directed regulated by HIF-1. Leptin is secreted almost exclusively from adipocytes and its levels reflect the total lipid content in the body, i.e. is increased with greater adiposity (Considine et al., 1996; Kim et al., 2011). Leptin is a major endocrine factor with diverse physiological functions including the regulation of energy homeostasis and appetite, reproduction, angiogenesis, and immune function, among others (Frühbeck et al., 2001). As an HIF-1-inducible gene, it is not surprising that we and others have found leptin to be increased with hypoxia (Grosfeld et al., 2002; Kanda et al., 2006; Wood et al., 2011). We found no additional effect of PCB-77 on leptin secretion or gene expression (Fig. 1a and 2a), suggesting that PCB-77-activated pathways do not interfere with leptin expression.

VEGF plays a major role in angiogenesis, a process of creating new blood vessels from existing ones, which promotes the development of vasculature required for adipose tissue growth (Sung et al., 2013). As a classic HIF-1-stimulated gene, VEGF is often employed as an indicator of hypoxia response in cross-talk studies. As previously demonstrated, we show that hypoxia increases VEGF protein secretion from differentiated human adipocytes (Kanda et al., 2006; Wood et al., 2011). We found that PCB-77 was able to further increase VEGF protein levels at 8% (Fig. 1b), which suggests that PCB-77 has an additive effect to hypoxia in the regulation of VEGF. In fact, there may even be a role for AhR in the induction of vasculature development (Stevens et al., 2009). A separate study found an additive induction of the erythropoietin (Epo) gene in response to combined AhR-activating TCDD and hypoxia (Chan et al., 1999). Further analysis identified XREs in addition to HREs in the Epo regulatory regions. We do not expect this to be the case for VEGF since PCB-77 exposure under normoxia did not increase VEGF protein or gene expression levels; however this remains to be tested.

In comparison to the protein levels of VEGF, we found a reduction in gene expression at 2% O_2 with PCB-77 compared to 2% O_2 with DMSO (Fig. 2b). Contrary to the additive effect of PCB-77 on VEGF proteins, these results suggest that PCB-77 can inhibit hypoxia signaling and lead to a decreased angiogenic response. Similarly, mice with induced hindleg ischemia treated with AhR ligand benzo[a]pyrene (BaP) inhibited the hypoxia-induced upregulation of VEGF, which consequently lead to an inhibition of angiogenesis (Ichihara et al., 2007). The differences in VEGF protein and gene expression levels are not completely surprising. Indeed, recent reports indicate that in general, weak association exist between gene transcripts and protein levels (Vogel and Marcotte, 2013). Due to the close relationship between adipogenesis and angiogenesis, further studies clarifying the factors that can potentially alter these processes are necessary.

We also measured IL-6, MCP-1, and TNF-α, pro-inflammatory adipocyte-derived cytokines that are increased with obesity but are not directly regulated by HIF-1 (Park et al., 2005). These proteins are better known as cytokines since they are not only released from adipocytes but mainly immune cells. First, IL-6 strongly stimulates lipolysis and impairs insulin signaling (Frühbeck et al., 2001). IL-6 was increased in a dose-dependent manner with hypoxia, as previously reported (Kanda et al., 2006; Wood et al., 2011). Despite findings showing that PCB-77 treatment increases IL-6 secretion from human umbilical vascular endothelial cells (HUVEC) (Wang et al., 2010) and hepatocytes (Hennig et al., 2002), we found no additive effect of PCB-77 on IL-6 protein or gene expression from differentiated human adipocytes. However, it appears that PCB-77 may have an additive effect on IL-6 gene expression at 8%O_2 (Fig. 2c, $p=0.057$), although this was not statistically significant.

Next, MCP-1 is a chemotactic protein that is released to attract macrophages to a specific area (Kanda et al., 2006). Although MCP-1 is released by adipocytes, the primary source of MCP-1 is the secretion from macrophages and endothelial cells (Kanda et al., 2006). PCB-77 has been shown to up-regulate MCP-1 in endothelial cells (Majkova et al., 2009), but we saw no main effect of PCB-77 in differentiated adipocytes (Fig. 1d and 2d). In contrast, we did observe a reduction in MCP-1 with hypoxia, as previously reported (Famulla et al., 2012). Despite missing data, we found that MCP-1 protein release is reduced at 8% O_2 with PCB-77, whereas this additive reduction is absent at 2% O_2.

Another adipocyte-specific adipokine is adiponectin and in comparison to many other adipokines its levels are decreased in the obese state. Due to its anti-inflammatory and insulin-sensitizing effects, reductions in adiponectin are suggested to contribute to the pathogenesis of obesity-related comorbidities, including inflammation, insulin resistance, and atherosclerosis (Ukkola and Santaniemi, 2002). We have confirmed previous findings showing that PCB-77 exposure leads to a decrease in adiponectin protein secretion by adipocytes (Arsenescu et al., 2008). We also show that hypoxia independently reduces adiponectin protein levels in human adipocytes, which is supported by several other studies (Grosfeld et al., 2002; Kanda et al., 2006; Wood et al., 2011; Ye et al., 2007). Furthermore, we observed that PCB-77 had an additive effect on adiponectin decreases at 8% O_2, but not at 2% O_2 (Fig. 1e).

Unlike the previously discussed adipokines known to be regulated by hypoxia, CYP1A1 is a drug-metabolizing protein upregulated by exogenous chemicals via AhR (Mimura and Fujii-Kuriyama, 2003). It acts by biotransforming potentially toxic chemical to increase their water solubility and eventually their excretion (Stevens et al., 2009). Although CYP1A1 is mainly expressed in the liver, Ellero and colleagues have described its induction in human white adipose

tissue (Ellero et al., 2010). We quantified the gene expression for CYP1A1 to test the impact of hypoxia on a gene directly regulated by AhR. Hypoxia alone had no effect on CYP1A1, while PCB-77 exposure in normoxia increased CYP1A1, as expected. The combination of hypoxia with PCB-77 exposure inhibited the PCB-77-induced increase in CYP1A1 (Fig. 3a). This is in accordance with other studies in mammalian cell lines (Allen et al., 2005; Chan et al., 1999; Schults et al., 2010; Zhang and Walker, 2007). Hypoxia inhibition of CYP1A1 has been found to be HIF-1-independent in hepatocytes (Allen et al., 2005), however the direct role of HIF-1 has yet to be determined in adipocytes. It should be mentioned that others have found no effect (Pollenz et al., 1999; Seifert et al., 2008) and even additive effects (Frericks et al., 2008) of hypoxia on CYP1A1 induction.

In summary, we have found that the cross-talk between PCB-77 and hypoxia in differentiated human adipocytes differs between adipokines. Lee and colleagues have also showed additive and competitive forms of cross-talk between genes regulated by dioxin and hypoxia signaling pathways; these include genes with various functions such as coagulation, cell proliferation, fatty acid metabolism, and others (Lee et al., 2006). Mechanisms other than competition for Arnt, such as shared co-activators, are likely involved in the cross-talk (Chan et al., 1999; Fleming et al., 2009; Ichihara et al., 2007; Nie et al., 2001). Other experimental factors influencing the cross-talk include the choice of AhR ligand and the technique for hypoxia induction. The choice of AhR ligand is crucial since activation time varies widely between ligands, their molecular targets differ, and some lead to ligand metabolism whereas others are not degraded (Stevens et al., 2009).

Furthermore, despite numerous cross-talk studies, this is the first study performed in differentiated human adipocytes. Therefore, it is also difficult to compare our results with other

studies since results are inconsistent between species and cell-types. Seifert and colleagues have reported differences in AhR responsiveness following hypoxia treatment between cell types (Seifert et al., 2008). Plus, Nie and colleagues suggest that differences in AhR: HIF-1α: Arnt ratios between cell lines may alter the outcome of cross-talk (Nie et al., 2001). However, these ratios in human adipocytes are currently unknown. Further research is needed to determine the detailed mechanisms involved in POP and hypoxia cross-talk.

To conclude, the cross-talk between AhR and HIF-1 may influence the metabolism of POPs in individuals with increased adiposity further characterized by adipose tissue hypoxia, as indicated by the inhibition of CYP1A1 expression by hypoxia. Also, a larger POP burden may play a role in the angiogenic process necessary for adipose tissue growth. Our findings also suggest that POPs and hypoxia act in an additive manner to regulate adipokines involved in inflammation during obesity. Clearly, a complex and intricate network of signals is involved in the inflammatory response of adipocytes in the obese state.

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4.7 Figures

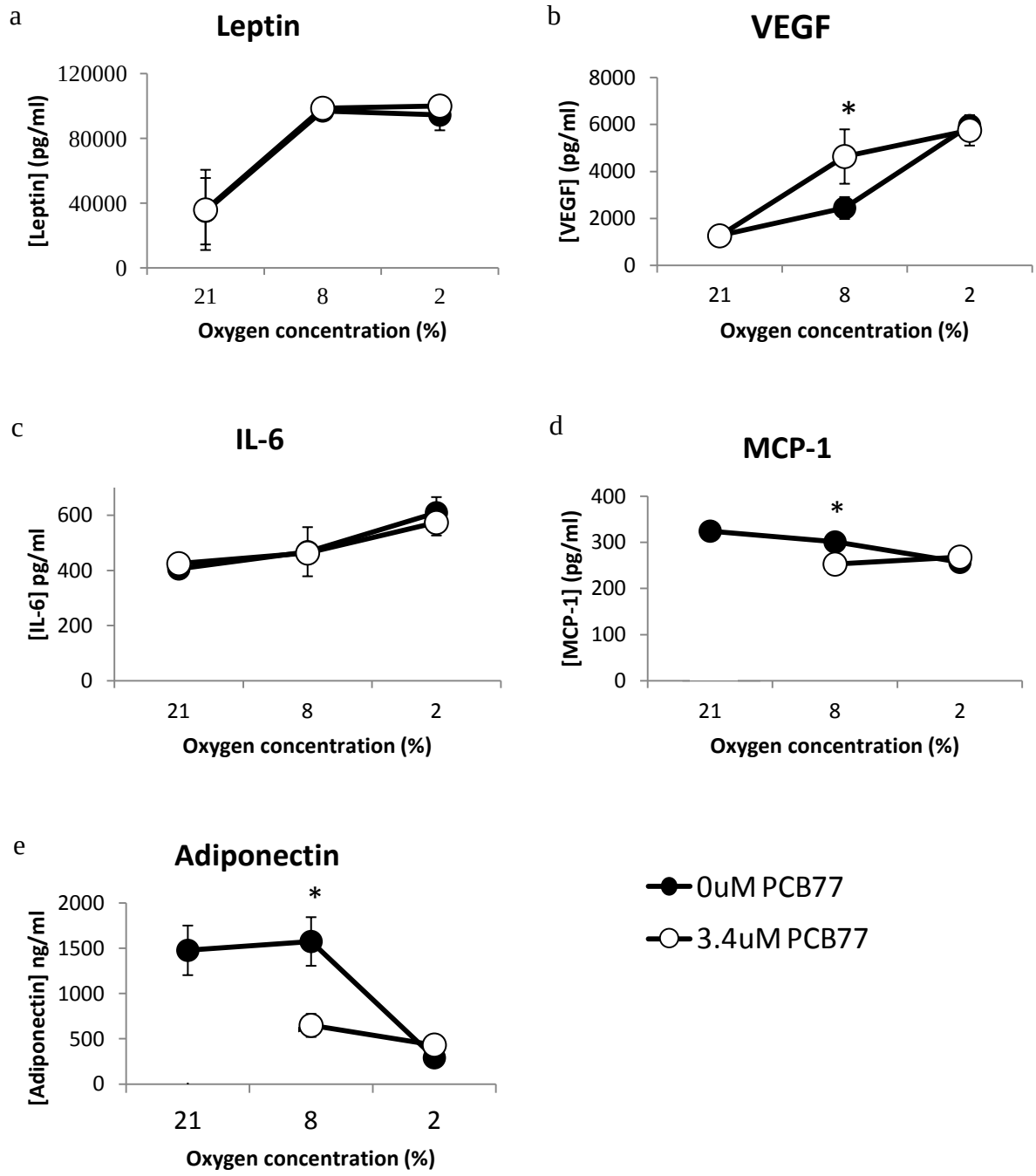

Figure 8: **Fig. 1 Adipokine protein levels following PCB-77 and hypoxia exposure**

(a) Leptin, (b) VEGF, (c) IL-6, (d) MCP-1, and (e) adiponectin. Values are mean ± SEM (n=3).

* Significantly different between DMSO and 3.4μM PCB-77 at a given oxygen level ($p<0.05$)

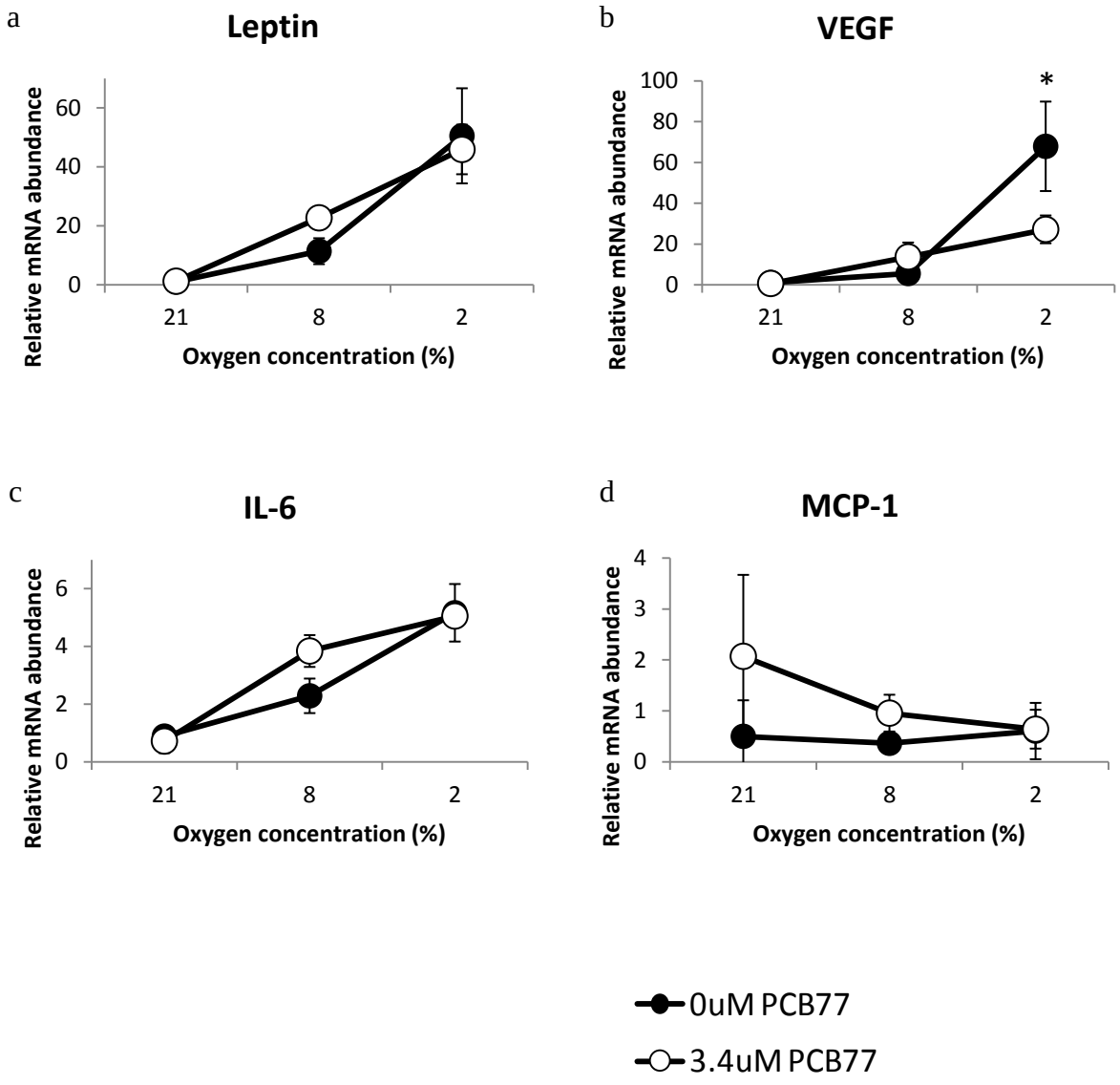

Figure 9: **Fig. 2 Adipokine gene expression levels following PCB-77 and hypoxia exposure** (a) Leptin, (b) VEGF, (c) IL-6, (d) MCP-1. Values are mean ± SEM (n=3). * Significantly different between DMSO and 3.4μM PCB-77 at a given oxygen level ($p<0.05$)

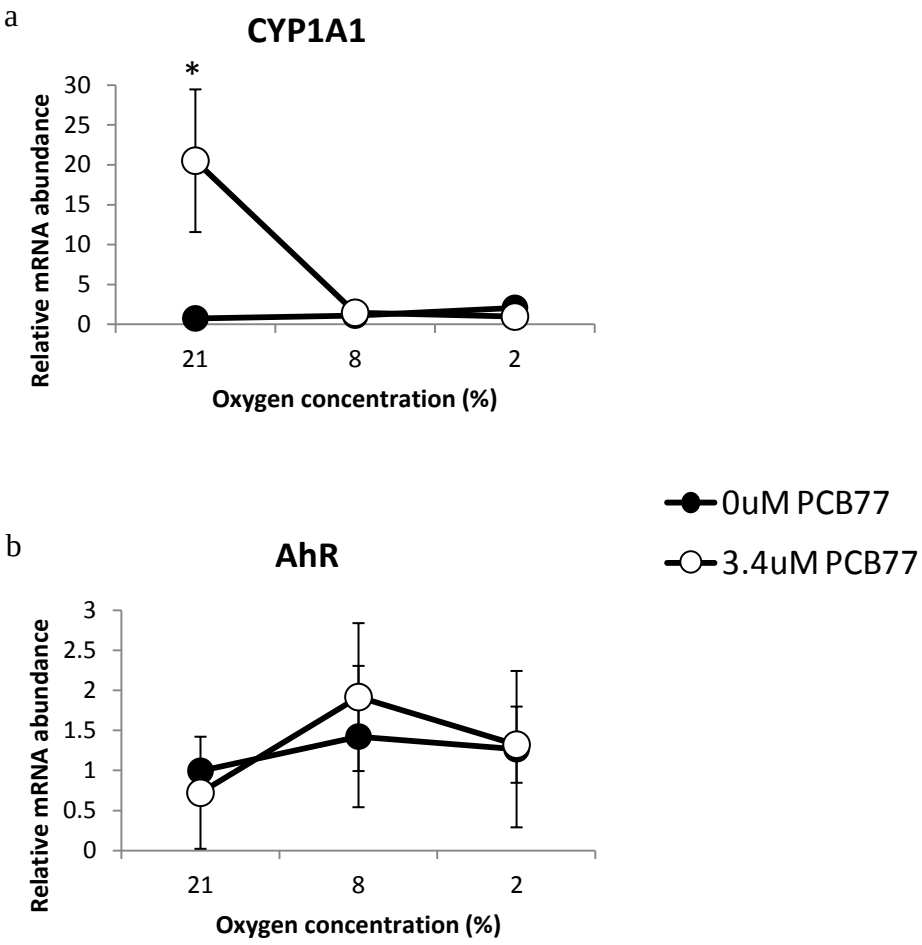

Figure 10: **Fig. 3 Gene expression levels for CYP1A1 and AhR following PCB-77 and hypoxia exposure**

(a) CYP1A1 and (b) AhR. Values are mean ± SEM (n=3). * Significantly different between DMSO and 3.4μM PCB-77 at a given oxygen level ($p<0.05$)

Chapter 5

General discussion

5.1 Key findings

The accumulation of POPs in adipose tissue has been linked to measures of obesity including BMI, waist circumference, and percent body fat (Elobeid, Padilla, Brock, Ruden, & Allison, 2010; Lee et al., 2011; Roos et al., 2012). This is true for lower levels of POPs that fail to elicit obvious acute toxic effects, such as the levels resulting from everyday environmental exposure (Arsenescu, Arsenescu, King, Swanson, & Cassis, 2008; Elobeid et al., 2010; Tang-Péronard, Andersen, Jensen, & Heitmann, 2011). Furthermore, several *in vitro* studies have demonstrated a role for various POPs in the formation of new adipocytes (Inadera & Shimomura, 2005; Moreno-Aliaga & Matsumura, 2002; Newbold, 2010; Sargis, Johnson, Choudhury, & Brady, 2010). However, Study 1 was the first to assess preadipocyte differentiation with exposure to POPs in human primary cells.

We found that a low concentration (3.4μM) of PCB-77 elicited no change in triglyceride accumulation, while a higher concentration (34μM) inhibited differentiation. In comparison, exposure to PCB-153 and DDE at both concentrations had no effect on preadipocyte differentiation. We know that PCB-77 acts as an AhR ligand, while PCB-153 and DDE do not act via this pathway. We hypothesized that the PCB-77-mediated inhibition was due to AhR, which is also known to inhibit preadipocyte differentiation.

In addition, we found that the AhR antagonist α-naphthoflavone (α-NF), employed to test the role of AhR in POP-mediated effects on differentiation, inhibited differentiation alone or with the PCBs or DDE. As a result of the discovery that it produces AhR-independent effects, such as inhibiting differentiation (He et al., 2013), we recommended that it should not be employed as an AhR-specific antagonist in future studies.

In all, these findings suggest that PCB-77, PCB-153, and DDE exposure alone do not increase preadipocyte differentiation in human cells. Nonetheless, although these POPs may not be directly involved in the differentiation process of preadipocytes, they have been shown to disrupt adipocyte function in other ways, for instance by promoting inflammation, as shown in Study 2.

In a recent review, we proposed that cellular cross-talk between AhR-activating POPs and adipose tissue hypoxia may occur in the obese state, leading to greater inflammatory responses (Myre & Imbeault, 2013). Study 2 was the first to determine the inflammatory response of differentiated human adipocytes exposed to a combination of the AhR ligand PCB-77 and hypoxia. Following a 24hr treatment period, we measured the protein and gene expression levels of key adipokines involved in inflammation, namely leptin, VEGF, IL-6, MCP-1, TNF-α, and adiponectin. Furthermore, we measured the gene expression level for CYP1A1 and AhR, involved in the response to exogenous toxic compounds. We found that PCB-77 had an additive effect on VEGF, MCP-1, and adiponectin protein levels at 8% O_2 but not at 2% O_2. Furthermore, hypoxia inhibited the PCB-77-induced increase in CYP1A1 mRNA. To our knowledge, this is the first study exploring the interaction of multiple factors on human adipocyte inflammation. In all, our findings suggest that the interaction between PCB-77 and hypoxia in adipocytes is dependent on the gene or protein and the level of hypoxia.

5.2 Strengths

Our studies were conducted *in vitro* using human subcutaneous preadipocyte primary cell culture. *In vitro* techniques have previously been validated as biologically relevant models to predict *in vivo* processes (Bérubé, Prytherch, Job, & Hughes, 2010; Cross & Bayliss, 2000). The primary cells used in our studies are asexual diploid cells that are obtained directly from an individual (see Chapter 2, Table 1). Although there is a possibility of variability between donors, it has been found that in general, their response reflects the actual response in the body (Bérubé et al., 2010). Within the field of adipocyte biology, *in vitro* preadipocyte and adipocyte models have been invaluable in studying the processes of adipogenesis, adipokine secretion, lipid metabolism, and gene/protein expression, among others (Poulos, Dodson, & Hausman, 2010). The specific use of primary human preadipocytes in our studies has the advantage to reflect the human *in vivo* context better than murine (e.g. 3T3-L1, 3T3-F442A) cell lines. Additionally, using an *in vitro* technique allowed us to control the growth conditions, thus eliminating many confounding variables that exist *in vivo*, for instance differences in POP burdens and genetic backgrounds between individuals, among others. Therefore, we were able to isolate pollutant-specific and oxygen-specific responses at a cellular level.

5.3 Limitations

Despite the many advantages associated with the use of primary cell culture, this method is subject to certain limitations. For instance, two-dimensional cell culture does not encompass the three dimensional complexity of multi-cellular organisms (Bérubé et al., 2010; Grimm, 2004). However, in our studies this can be considered an advantage because it provided a controlled setting to study precise cellular responses (Grimm, 2004). It remains to be determined

if adipocytes respond differently when surrounded by other cells types or in a living organism. Furthermore, our studies only focused on the responses from adipocytes, yet adipose tissue is composed of several other cell types including preadipocytes, endothelial cells, and macrophages. It would be interesting to determine their response to the same experimental conditions. Nonetheless, in our case human primary preadipocyte culture was a cost-effective, relevant model to address our research questions.

Another limitation is the experimental exposure to individual pollutants rather than mixtures containing multiple pollutants. While in reality humans are exposed to a combination of pollutants, we wanted to isolate the individual effect of each pollutant in order to determine specific mechanism involved in the cellular response. As such, the results obtained may not reflect those observed *in vivo*, but they are indicative of the individual toxicity and mode of action for each pollutant.

The final limitation discussed here is that our experimental design did not allow for true biological replicates. As discussed by Lazic, this is a common problem in cellular biology experiments since oftentimes the cells employed are all obtained from the same person (Lazic, 2010). We attempted to overcome this limitation by performing each experimental condition in three or four cell culture wells. However, we realize that this is an inadequate solution because each well underwent the same conditions, such as cell culture media, incubator conditions, etc. We did however confirm some results found in other studies, which strengthens the validity of these findings. For example, our results confirm the changes in adipokine secretion from human adipocytes exposure to various oxygen levels, as determined by Wood et al. (Wood, Stezhka, & Trayhurn, 2011). In the future, researchers should attempt to include biological replicates, i.e., samples from multiple subjects, in the experimental design of their cell culture studies.

5.4 Future research

To expand the findings of Study 1, future research should examine the effect of pollutant mixtures, since this would better reflect the *in vivo* POP burden. Also, while we only assessed lipid accumulation as an indicator of differentiation, it would also be of interest to quantify the levels of adipocyte-specific genes such as peroxisome proliferator activator receptor gamma (PPAR-γ) and CCAAT/enhancer-binding proteins (C/EBPs), which are the main transcription factors responsible for differentiation (Rosen & MacDougald, 2006), as well as lipoprotein lipase (LPL), adipocyte lipid-binding protein (aP2), and cluster of differentiation (CD36), which are involved in lipid metabolism and increased in adipocytes (Arsenescu et al., 2008).

To build on the results found in Study 2, future research should continue to explore the cellular responses to factors promoting inflammation, especially since many aspects of POP-hypoxia cross-talk remain unclear. The amount and proportion of AhR, HIF-1α, and Arnt proteins in adipocytes should be determined. Also, a better understanding of the direct role of AhR and HIF-1α are necessary. To isolate the specific role of these transcription factors, we suggest the use of silencing RNA (siRNA), a recently developed tool for gene silencing. This technique consists of introducing a short RNA molecule into the cells that selectively targets and destroys specific mRNA transcripts. Furthermore, we should seek to understand how the cells comprising the stoma-vascular fraction of adipose tissue respond to POP and hypoxia exposure. This is relevant since these cells make up over half of adipose tissue and are highly involved in its inflammatory state.

There are also numerous avenues of research that remain unexplored with regards to the impact of POPs, hypoxia, and their interaction on human adipose tissue. For instance, there is likely a role of endoplasmic reticulum stress via markers of the unfolded protein response (UPR)

(Attie & Scherer, 2009), a pathway activated in human adipose tissue and positively correlated with levels of adiposity (Sharma et al., 2008). Similarly, there may be a level of impaired mitochondrial function resulting from POP and hypoxia exposure, which has been shown to lead to a greater production of reactive oxygen species and a decrease in adiponectin secretion in adipocytes (de Ferranti & Mozaffarian, 2008). The effect of POPs and hypoxia on endoplasmic reticulum and mitochondrial function has yet to be determined.

5.5 Conclusion

Our work with differentiated human adipocytes has expanded our understanding of the role of POPs and hypoxia in adipocyte functions. More specifically, our results show that human preadipocyte differentiation is not affected by low levels of select POPs. We also report that POPs and hypoxia interact to regulate the protein and gene expression levels of some adipokines, namely VEGF, MCP-1, and adiponectin, as well as the gene expression for CYP1A1. While our work is a first step in understanding the underlying mechanisms involved in POP and hypoxia cross-talk, many unanswered questions remain and should be addressed in future studies. It is also important to remember that these types of responses are adaptive and often only lead to adverse health problems following chronic exposure.

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Appendix

Appendix I: Letter of acceptance for publication of review article in the journal *Obesity Reviews*

From: David York, Chief Editor

To: Maxine Myre

Subject: Obesity Reviews - Decision on Manuscript ID OBR-06-13-1628.R1

04-Aug-2013

Dear Ms. Myre,

Thank you for revising your review entitled "Persistent organic pollutants meet adipose tissue hypoxia: Does cross-talk contribute to inflammation during obesity?" and for taking the reviewers' criticism and advice into consideration. The review is now accepted for publication in Obesity Reviews and will be forwarded to the publisher for inclusion in a coming issue.

[...]

Thank you for submitting your work to Obesity Reviews.

Yours sincerely,

Prof. David York
Editor-in-Chief, Obesity Reviews